Biochemical and Structural Properties of Titin, Nebulin and Intermediate Filaments in Muscle

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Introduction

In a "generic" vertebrate somatic cell, microfilaments (actin filaments), microtubules and intermediate filaments (IFs) are the three major cytoskeletal protein filament systems (Robson, 1989). The primary cytoskeletal structures of skeletal muscle cells, however, are the very long myofibrils, which literally pack the inside of the cell, and are responsible for contraction in the living cell and for most of the desirable qualities of muscle used as food (Robson et al., 1980, 1981, 1984; Parrish and Lusby, 1983; Goll et al., 1975, 1984). Thus, in what many of us feel is obviously the "most beautiful" cell in the animal kingdom, the myofibrils can be considered as "representing," or taking the place of, the microfilaments of non-muscle cells. The other two cytoskeletal filament systems (microtubules and IFs) are present in smaller amounts than in most other cell types, and are dwarfed by the myofibrils. In Table 1 are listed some of the significant myofibrillar/cytoskeletal proteins of the mature skeletal muscle cell. Although this list seems long, many additional important proteins, albeit often present in small amounts, could have been included.

As will become evident in this paper, the Z-line is a key structure of the myofibrils and of the overall skeletal muscle cell cytoskeleton. Three categories of Z-line proteins exist in the striated muscle cell (Table 2). The *first* category, considered *"integral"* (within the interior of striated muscle Z-lines) Z-line proteins, includes α-actinin (Robson et al., 1970; Schollmeyer et al., 1973; Endo and Masaki, 1982; Yamaguchi et al., 1978, 1983a,b, 1985; Goll et al., 1991a), Cap Z (Casella et al., 1987, 1989; Caldwell et al., 1989) (note that β-actinin is now considered equivalent to Cap Z protein; Maruyama et al., 1990) and zeugmatin (Maher et al., 1985; Colley et al., 1990). Z-nin, a 300-400 kDa protein (Suzuki et al., 1981), has also been identified as an integral Z-line protein (Suzuki et al. 1985), but it now seems possible that Z-nin is a proteolytic fragment of a larger protein such as nebulin or titin (see Furst et al., 1988). Two other rather incompletely characterized proteins, namely Z-protein (Ohashi and Maruyama, 1989) and tensin (Wilkins et al., 1986; Davis et al., 1991), also may belong in the integral Z-line category based upon their published properties, but this must yet be carefully documented. The *second* category of Z-line proteins, located at the periphery ("peripheral" Z-line proteins) of the myofibrillar Z-lines or in the intermyofibrillar spaces between Z-lines of adjacent myofibrils, includes a number of additional cytoskeletal proteins such as desmin (O'Shea et al., 1979, 1981; Richardson et al., 1981; Tokuyasu et al., 1983; Yagyu et al., 1990a,b; M.M. Bilak et al., 1991a,c), synemin (Granger and Lazarides, 1980; S.R. Bilak et al., 1989, 1990; Bright et al., 1991; M.M. Bilak et al., 1991a,c), filamin (Bechtel, 1979; Gomer and Lazarides, 1981), plectin (Wiche et al., 1983; Foisner and Wiche, 1991), spectrin (Craig and Pardo, 1983; Nelson and Lazarides, 1983), ankyrin (goblin) (Nelson and Lazarides, 1984) and vinculin (Evans et al., 1984; Terracio et al., 1990). A *third* rather "special" category of Z-line proteins includes ones that we often do not usually think of as Z-line proteins at all, but which may be considered so because they (or a portion of them) are partly located within the Z-line structure. This category includes actin, perhaps tropomyosin and troponin, and the two giant proteins of the myofibril, titin and nebulin. Certainly, the "barbed" ends of actin filaments penetrate the interior of the Z-lines. In fact, F-actin comprises the *longitudinal* filamentous backbone of the structure and can be considered a major protein component of Z-lines (Yamaguchi et al., 1978, 1983a, 1985). It is not yet clear, however, as to whether *any* of the other two major thin filament proteins, tropomyosin and troponin, also penetrate (instead of stopping at the edge) the interior of the Z-line structure (Yamaguchi et al., 1983b; Trombitas et al., 1990). This is because immunolocalization studies are dependent upon penetration of antibodies, and the integral domain of the Z-line conceivably may not always permit their entry, or possibly epitopes on tropomyosin and troponin (if the proteins are present) are masked. As will be described later, the largest portion of each titin and nebulin molecule is not located in the Z-line structure, but one end (probably the C-terminus) of each molecule evidently extends into the Z-line structure (Furst et al., 1988; Wang and Wright, 1988). As a result, these two proteins may be very important in Z-line structure and in the alterations that occur in this key structure postmortem.

We will describe four proteins, the two giant proteins, titin and nebulin, and two IF proteins, desmin and synemin, because we believe these four proteins play important cytoskeletal roles in maintenance of muscle cell and overall

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Table 1. Properties of Some Significant Myofibrillar/Cytoskeletal Proteins in Mammalian Skeletal Muscle.

Protein	*Primary Myofibrillar/ Cytoskeletal Location*	*Approx. % of Myofibril*	*Approx. MW (# of Subunits)*
Myosin	Thick filament	45	520,000 (6)
C-Protein	Thick filament	2	140,000 (1)
Myomesin	M-line	1	185,000 (1)
M-Protein	M-line	2	165,000 (1)
Creatine Kinase	M-line	<1	84,000 (2)
Actin	Thin filament	20	42,000 (1)
Tropomyosin	Thin filament	5	66,000 (2)
Troponin	Thin filament	5	69,000 (3)
*Titin (Connectin)	Longitudinal sarcomeric filaments (M- to Z-line)	10	2,800,000 (1)
*Nebulin	Parallels thin filaments to Z-line	4	800,000 (1)
α-Actinin	Z-line (integral)	2	204,000 (2)
Cap Z	Z-line (integral)	<1	66,000 (2)
Zeugmatin	Z-line (integral)	<1	2,000,000 (2)
*Desmin	Intermediate filaments at Z-line (peripheral)	<1	212,000 (4)
*Synemin	Intermediate filaments at Z-line (peripheral)	<1	460,000 (2)

*Proteins discussed herein.

muscle integrity. As a result, these proteins and their filamentous forms are of interest to muscle biochemists, molecular biologists, and microscopists in terms of organization and proper function of living cells, and to meat scientists in terms of their importance in maximization of meat quality. Because of the rapidly growing interest in titin and nebulin among both basic and applied muscle scientists, we will describe these two proteins in a little more detail than the other two proteins. For each protein, we will first summarize its major biochemical and structural characteristics, then indicate its possible role(s) in living muscle cells, and finally describe its importance in postmortem muscle. It is worth remembering that until fairly recently (1975-1980), we did not know that these four proteins existed, and many current biochemistry textbooks have still not included these proteins in chapters on muscle and/or the cell cytoskeleton. For reviews encompassing many of the early studies (up to about 1983) on titin, nebulin and desmin, see Robson and Huiatt (1983) and Robson et al. (1984). A considerable amount of exciting information has been developed for each of these proteins in just the past two to three years. Following the descriptions of these four proteins, we will finish by describing how all of these proteins "meet" at the Z-line.

Table 2. Three Categories of Z-line Proteins.

Integral	*Peripheral*	*Special*
α-Actinin	Desmin	Actin
Cap Z	Synemin	Tropomyosin?
Zeugmatin	Filamin	Troponin?
Z-nin?	Plectin	Titin
Z-protein	Spectrin	Nebulin
Tensin?	Ankyrin	
	Vinculin	

Titin

Major Characteristics of Titin

Many of us "grew up" with the relatively simple thick- and thin-filament model of the sarcomere, and of contraction hypotheses based upon this two-filament system (Huxley and Hanson, 1954). Although there were many suggestions over the years for the possible presence of other filaments in the sarcomere (e.g., see discussions and references on S-filaments, gap filaments, C-filaments, T-filaments, and core filaments in Wang et al., 1979; Wang, 1985; Trinick, 1991), these observations/reports were largely dismissed as artifacts. This approximately 30-year period of relative quiet began to change in the late 1970's.

A little over ten years ago, Kuan Wang and associates at the University of Texas-Austin (Wang et al., 1979) discovered three incredibly large polypeptides (initially called bands 1, 2 and 3 in increasing order of migration) in striated muscle by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by utilizing low percentage acrylamide, high porosity gels. The largest two polypeptides, which migrate as a closely spaced doublet (denoted T_1 and T_2 herein, with T_2 migrating slightly faster than T_1), were collectively named titin. Because of its extremely large size (T_1 = ~2,800 kDa; T_2 = ~2,400 kDa) Kurzban and Wang, 1988), Wang and associates (Wang et al., 1979; Wang, 1982) were able to isolate titin directly from chicken skeletal muscle myofibrils by gel filtration chromatography. Because titin also was highly

insoluble, solubilization and chromatographic purification of titin had to be conducted in the presence of protein denaturing solvents. Before titin was discovered, Maruyama and associates (Maruyama et al., 1976, 1977) had isolated a very insoluble, elastic-like protein from muscle and called it "connectin." One of the many proteins present in the early connectin preparations turned out later to be titin (Maruyama et al., 1981; Maruyama, 1986), and the names titin and connectin are sometimes used interchangeably. We will use the name titin herein. Kimura and Maruyama (1989) have reported that they can prepare α-connectin (supposedly equivalent to intact titin, T_1,) with somewhat less harsh treatment than used by Wang (1982) for preparation of T_1, but it is not absolutely certain that they have isolated the *entire* molecule (see discussion in Trinick, 1991).

Although highly organized sarcomeres are only found in skeletal and cardiac muscle cells, smooth muscle or nonmuscle isoforms of many of the myofibrillar proteins are found in other cell types. Titin, however, appears unique to striated muscle cells (Wang et al., 1979; Hu et al., 1986; Locker and Wild, 1986; Trinick, 1991) where, in the case of skeletal muscle, it comprises approximately 8% to 10% of total myofibrillar protein (Wang et al., 1979, 1984; Wang, 1982; Trinick et al., 1984). Thus, titin, the largest protein ever discovered, is the third most abundant myofibrillar/cytoskeletal protein in these cells (Table 1).

Much of what is known about the properties of the titin molecule has resulted from the finding that the slightly degraded form of titin, T_2 (also referred to by some as β-connectin), is soluble in high salt, non-denaturing solvents and, thus, can be purified in a "native" form (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984). In our laboratory (J. Engelhardt, R. Robson, and T. Huiatt, unpublished observations) we have found it quite easy to prepare highly purified T_2 by utilizing hydrophobic chromatography. Native titin (T_2) is an extremely asymmetric molecule, with a very thin (~3-4 nm diameter) long (~900 nm) tail attached to a globular head (Nave et al., 1989). The early immunolocalization studies on titin were complex, but suggested that titin may be present throughout much of the sarcomere's length (Wang et al., 1979; LaSalle et al., 1983; Maruyama et al., 1985). Although the exact arrangement of titin in the sarcomere remains unclear, the use of monoclonal antibodies that recognize specific epitopes along the long titin molecule in immunoelection microscopy clearly have shown that titin spans from the M-line region to the Z-line (Furst et al., 1988, 1989a; for a summary of myofibrillar decoration patterns obtained with 14 monoclonal antibodies to titin, see Fig. 4 in Furst et al., 1989a). Current models (e.g., Fig. 10 in Wang and Wright, 1988; Fig. 1 in Fulton and Isaacs, 1991; Fig. 1 in Trinick, 1991) of titin arrangement within the sarcomere indicate that two titin molecules can span the entire length of the sarcomere, with one running from the M-line to one of the Z-lines and one running from the M-line to the other Z-line. However, other arrangements such as a single bipolar molecule with central symmetry at the M-line that spans the entire length of the sarcomere (Model B in Furst et al., 1988); single titin molecules that run from Z-line to Z-line but with opposite polarity; or a single titin molecule that runs from Z-line to Z-line, but which is composed of two anti-parallel polypeptide subunits, cannot yet be unequivocally ruled out. In part, the one titin per half-sarcomere model is preferred because of the reported length (~1 μm) of the T_2 molecules (Nave et al., 1989). Based upon: 1) the number of amino acid residues per titin polypeptide molecule (~25,000), 2) that titin contains a high amount of β-structure (Maruyama et al., 1986) and 3) the distance traversed per residue in this extended conformation (~3.475 Å), a single titin molecule conceivably could be extended up to a length of 8-9 μm (Hainfeld et al., 1988), which is over three times the rest length of a sarcomere! Use of specific monoclonal antibodies and immunoelectron microscopy have also shown that the end of titin with the globular head is positioned at the M-line (Nave et al., 1989), and the end of the long tail of titin is at the Z-line (Furst et al., 1988, 1989a). The part cleaved off intact titin, T_1, and, thus missing in T_2, is the Z-line end of the titin molecule (Furst et al., 1988). The Z-line end of titin also has been reported to represent the carboxy-terminal end of the molecule (S.-M. Wang et al., 1991), but this must be further documented.

The titin epitopes located in the I-band move away from both the M- and Z-lines as sarcomere length increases, indicating that this part of the titin molecule is highly elastic (Furst et al., 1988, 1989a; Itoh et al., 1988; Maruyama et al., 1989) and forms an elastic connection between the end of a thick filament and the Z-line (Itoh et al., 1988; K. Wang et al., 1991). In contrast, those epitopes located in the A-band do not move relative to the M-line with change in sarcomere length (Furst et al., 1988, 1989a), indicating that this part of the titin molecule in situ is inextensible, presumably because this part of the titin molecule is firmly attached to the thick filaments (Whiting et al., 1989). However, even these epitopes may move if the sarcomere is markedly stretched, as shown by Pierobon-Bormioli et al. (1989). The part of the titin molecule present in the sarcomeric A-band may be an integral part of the thick filament (Trinick, 1991). Trinick and associates (Whiting et al., 1989; Trinick, 1991) have suggested that there are probably six (or possibly 12) titin molecules per thick filament if one considers the sarcomere in three dimensions (i.e., how titin is arranged *around* the outside of the circular thick filament), based upon: 1) the position of titin on the outside of the thick filament (see Locker, 1987, for an interesting model), 2) the bipolar nature of the thick filament, 3) the number of myosin molecules per thick filament, 4) the three-fold rotational symmetry of the thick filament, and 5) the amount of titin in the skeletal muscle myofibril.

A great deal more about titin will become known as its complete primary structure is elucidated. The small amount of cDNA-derived amino acid sequence reported to date (Labeit et al, 1990) already has resulted in interesting findings. Two classes of motifs (domains), each approximately 100 residues long, have been identified. Class I motifs are similar to type III fibronectin, and class II motifs are similar to the C2 group of immunoglobins. Both motifs were earlier thought to be present only in extracellular proteins. Also quite intriguing is the finding that other proteins, which also bind to thick (myosin) filaments, such as C-protein, an 86 kDa protein, and skelemin, also contain these class-I and -II motifs, although the motifs are arranged somewhat differently along the sequence of these latter proteins (see discussion in Labeit et al., 1990; Ayme-Southgate et al., 1991; Epstein and

Fischman, 1991; Trinick, 1991). Comparisons of data from primary sequence, antibody cross-reactivity, and other biochemical procedures have also shown the relatedness of titin to several very large (albeit smaller than vertebrate titin) proteins present in invertebrate muscles, such as mini-titin (Nave and Weber, 1990; Nave et al., 1991), twitchin (Benian et al., 1989), and projectin (Hu et al., 1990; Ayme-Southgate et al., 1991; Vigoreaux et al., 1991). As pointed out by Trinick (1991), it is possible that all these invertebrate proteins, which have high molecular masses (~600-1, 100 kDa), will turn out to be essentially the same protein or mini-titin homologue. Although these invertebrate proteins may seem far removed from the interests of meat scientists, because of the relative ease of genetic manipulation of the titin-related proteins in some of these experimental systems, important functional implications for vertebrate muscle titin should result from their study.

Possible Roles of Titin in Muscle

The focus of this paper is on proteins in developed muscle cells, but many investigators (e.g., Furst et al., 1989b; Handel et al., 1989; Huiatt et al., 1989; Isaacs et al., 1989; Mikhail et al., 1990) also are interested in the properties and function of titin in developing skeletal muscle cells. Many details remain to be elucidated, but evidence from both in-vitro and in-vivo studies suggests that titin plays an important morphogenetic scaffolding role in sarcomeric organization (myofibrillogenesis) early in development (Table 3) (for a recent review, see Fulton and Isaacs, 1991). In the mature myofibril, titin forms a third longitudinally-oriented filament system that may provide sarcomeric alignment i.e., keep myosin filaments in register and centered within the sarcomere (Horowits et al., 1986, 1989; Horowits and Podolsky, 1987). It is also quite possible that titin helps determine or regulate the length of the thick filament (Whiting et al., 1989). Titin, thus, appears to play a major role in maintaining overall structural integrity of the sarcomeres and, as a result, of the myofibrils and entire muscle cell.

The part of the titin molecule present in the I-band may provide an elastic element of the sarcomere and muscle. By measuring the resting tension-sarcomere length curves of six rabbit skeletal muscles that express three slightly different size classes of titin isoform, coupled with immunoelectron microscope studies of an epitope in the extensible part of the titin molecule, K. Wang et al. (1991) have provided direct evidence for titin's participation in genesis of resting tension.

Table 3. Titin—Major Characteristics, Possible Roles in Muscle, and Importance in Postmortem Muscle.

Major Characteristics

1. Titin is a rather insoluble, giant myofibrillar protein ($M_r \simeq 2.8 \times 10^6$) present in skeletal and cardiac muscle cells of vertebrates and invertebrates. It was discovered by Dr. Kuan Wang and colleagues in 1979. Titin is sometimes called connectin.
2. Titin comprises about 8% to 10% of total myofibrillar protein in vertebrate skeletal muscle.
3. Intact skeletal muscle titin (T_1) is isolated and purified in the presence of denaturing solvents. A slightly degraded, "native" form of skeletal muscle titin (T_2) can be isolated and purified in the absence of denaturing solvents, and observed by electron microscopy. Titin is a very long (>1 μm) molecule, with a globular head and a very long thin tail.
4. A titin molecule spans one half the width of a sarcomere, i.e., from the M-line to the Z-line and, thus, forms a third filament within the myofibril.
5. The part of titin located within the sarcomeric A-band, where it appears bound to the outside of the thick filament shaft, is relatively inelastic. The part of titin located within the sarcomeric I-band is "elastic".

Possible Roles in Muscle

1. In developing muscle, titin may play a role as part of a morphogenetic scaffolding during sarcomeric organization.
2. In the mature myofibril, titin forms a third filament system that provides sarcomeric alignment (e.g., keeps myosin filaments in register, possibly regulates the length of the thick filaments), and helps maintain overall structural integrity of the sarcomeres, myofibrils and muscle cells.
3. The part of the titin molecule/filament *not* associated with the A-band (thick filaments) may provide an elastic (provide recoil?) element of the sarcomeres, and accounts for the "gap filaments" observed in overstretched bovine skeletal muscle several years ago by Dr. Ronald Locker and colleagues.

Importance in Postmortem Muscle

1. Titin is the third most abundant myofibrillar protein and plays a significant cytoskeletal role in determining the degree of integrity (strength) of myofibrils, muscle cells and muscle tissue (i.e., titin is the *only* myofibrillar protein that is present from Z-line to Z-line).
2. Titin is degraded postmortem by endogenous proteinases, presumably including the calpains.
3. Degree of titin degradation seems to parallel measures of fresh meat quality (e.g., tenderness).
4. Postmortem alterations in titin and titin extractability appear to be associated with increased beef and pork muscle water-holding capacity.

They also suggest that skeletal muscle cells may modulate stiffness/elasticity coordinately by selectively expressing specific size isoforms of titin (see references listed and discussion in K. Wang et al., 1991, for other work supporting an important role for titin in muscle elasticity). It also is now apparent that the "gap filaments" described in the longitudinal gaps between thick and thin filaments in over-stretched bovine skeletal muscle several years ago by Dr. Ronald Locker and associates in New Zealand (Locker and Leet, 1975, 1976a), which raised great interest among meat scientists, but was largely ignored by many fundamental muscle scientists (see discussions in Locker et al., 1977; Locker, 1982, 1984), are composed of titin (LaSalle et al., 1983).

In view of titin's position throughout the sarcomere, it is conceivable that titin plays a direct role in contractility. Titin (and nebulin) undergoes phosphorylation/dephosphorylation in vivo, but the functional significance of this finding remains unknown (Somerville and Wang, 1988). Among many yet-to-be discovered, exciting properties of titin are how the titin molecules (filaments) traverse the distance from the ends of the thick filaments to the Z-line (see Funatsu et al., 1990, for an interesting approach to this problem) and how (and to what) the end of the titin molecule is bound at the Z-line. The latter discovery may have far-reaching implications to the use of muscle as food.

Importance of Titin in Postmortem Muscle

A great deal of interest is now being focused on titin with regard to its function in meat and meat products (Table 3). The giant protein is present in significant amount and is the only protein that is found throughout the myofibrillar sarcomere. Much of the overall integrity (strength) of myofibrils and muscle likely is due to titin. In contrast to myosin and actin, which are not degraded under most normal postmortem storage conditions (see Goll et al., 1991b), titin is degraded postmortem (Takahashi and Saito, 1979; Lusby et al., 1983; Paterson and Parrish, 1987; Bandman and Zdanis, 1988; Anderson and Parrish, 1989; Boles et al., 1991; Fritz and Greaser, 1991; Huff et al., 1991). Endogenous muscle proteinases, especially the calpains, are normally considered responsible for degradation of a selective few of the myofibrillar/cytoskeletal proteins postmortem (Goll et al., 1983, 1991b; Koohmaraie et al., 1991); and Zeece et al. (1986) have reported that myofibrillar titin is a substrate for calpain. Although a great deal of experimentation is yet needed to demonstrate the role of titin in meat quality, the degree of titin degradation seems to parallel measures of fresh meat quality, such as beef tenderness (Paterson and Parrish, 1986, 1987; Anderson and Parrish, 1989; Huff et al., 1991). Postmortem alterations in titin and titin extractability also appear to be associated with increased muscle water-holding capacity (Paterson et al., 1988; Boles et al., 1991).

Nebulin

Major Characteristics of Nebulin

In their original publication describing the discovery of titin by SDS-PAGE analysis of vertebrate skeletal muscle myofibrils, Wang et al. (1979) also described a third, extremely large polypeptide (initially described as≅ 500-600 kDa), referred to as "band 3." Like intact titin (T_1), nebulin also was highly insoluble and was subsequently solubilized and chromatographically purified in the presence of denaturing solvents (Wang and Williamson, 1980). Wang (1981) shortly thereafter named the protein "nebulin" in honor of its purported immunolocalization to one of the nebulous N-lines of muscle structure. Structures called N-lines occasionally were described in some very early electron microscope studies of skeletal muscle (e.g., Pease and Baker, 1949), but N-lines have not been consistently and reproducibly observed by electron microscopy. The perplexing, elusive N_2-line, when present, was described in electron micrographs as a broad dark line that crosses the I-band, with one line parallel to and on either side of the Z-line (Page, 1968; Franzini-Armstrong, 1970). Locker and Wild (1984a) went even further and subdivided and described "four" N_2-lines running parallel to and on each side of the Z-line, but most investigators have not subdivided the N_2-line. When the sarcomere length changes, the N_2-line varies its position but maintains the same proportional distance between the M-line and Z-line (Franzini-Armstrong, 1970; Locker and Leet, 1976b). Because antibodies to nebulin tended to coincide with position of the N_2-lines, it initially was suggested that nebulin (band 3) was a component of this structure and that nebulin was somehow attached to the elastic, longitudinally-running titin filaments (Wang and Williamson, 1980; Wang, 1981). From about 1980 to 1983-4, nebulin continued to be considered a possible component of the mysterious N_2-line of skeletal muscle myofibrils (Wang and Williamson, 1980; Robson and Huiatt, 1983; Locker, 1984; Locker and Wild, 1984a). From about 1984 to 1988, it was suggested that nebulin might even comprise the ~N_2-line to Z-line part of the longitudinal set of elastic, titin-containing filaments (Wang, 1985).

In 1988, two papers (Furst et al., 1988; Wang and Wright, 1988) appeared that substantially altered our understanding of nebulin's location in the skeletal muscle myofibrillar sarcomere. By using a battery of monoclonal antibodies, some of which recognized several different specific epitopes of titin, and others which recognized several specific different epitopes of nebulin, Furst et al. (1988) demonstrated by immunoelectron microscopy that titin was located throughout the sarcomere (from Z-line to Z-line), including the N_2-line to Z-line region, i.e., they suggested that the elastic-like filaments consisted entirely of titin, and that nebulin was *not* part of this set of longitudinally-running filaments. Wang and Wright (1988) used immunoelectron microscopy with monospecific antibody to nebulin that labeled six pairs of transverse stripes spaced at 0.1 to 1.0 μm distances from the Z-line within each sarcomere. The epitopes labeled by this polyclonal antibody maintained a constant distance from the Z-line, *irrespective* of sarcomere length. As a result, a very different picture of nebulin has evolved, and nebulin is now considered to be a very long (~1 μm) *inextensible* molecule (filament) that runs in parallel, and in close association with the thin (actin) filaments of the sarcomere. Maruyama et al. (1989) have subsequently reported that the binding sites for antibodies to nebulin do not move in the I-band in response to change in sarcomere length. Pierobon-Bormioli et al. (1989) also have presented immunoelectron microscope evidence that nebulin is not a constituent of the titin filament.

Exactly why nebulin was initially observed and described as an N_2-line component is still not entirely clear. Many antibodies prepared to nebulin do have a tendency to recognize epitopes in the N_2-line region (Furst et al., 1988), indicating that this part of the nebulin molecule is highly antigenic. For those interested in the N_2-line, both Wang and Wright (1988) and Furst et al. (1988) discuss this enigmatic structure. It seems possible that N_2-lines may be artifactually induced during either handling of the tissue before fixation, or during the fixation process. As a result, fragmented or damaged titin (and/or nebulin) may translocate and accumulate in this region of the I-band (Wang and Wright, 1988). While our understanding of nebulin is increasing, our understanding of the N-lines remains rather nebulous.

Although it was originally reported that nebulin is a component of both skeletal and cardiac muscle (Wang and Williamson, 1980; Wang, 1985), it is now generally agreed that nebulin is *not* a component of cardiac muscle cells (Hu et al., 1986; Locker and Wild, 1986; Furst et al., 1988; Wang and Wright, 1988). Among various skeletal muscles of the rabbit, at least two nebulin size isoforms have been identified (Wang and Wright, 1988), but the significance of this finding is not yet evident. Recent estimates of the size of nebulin suggest molecular masses between about 600 and 900 kDa (Hu et al., 1986, 1989; Locker and Wild, 1986; Stedman et al., 1988; Wang and Wright, 1988; Jin and Wang, 1991), depending upon the specific muscle, species and developmental stage. Nebulin comprises approximately 3% to 4% of total myofibrillar protein in adult skeletal muscle (Wang, 1982).

Nave et al. (1990) have reported that nebulin binds to α-actinin in vitro. And cloned nebulin fragments bind to F-actin in vitro (Jin and Wang, 1991). Although the exact position of nebulin's amino-terminal end is not precisely known, it is near the distal (free) end of the thin (actin) filament (Wang and Wright, 1988; Jin et al., 1990), which also suggests that its carboxy-terminal end is anchored at the Z-line. Molecular cloning and sequencing studies on nebulin cDNA have revealed some very interesting repeating motifs (domains) in nebulin's primary structure. One of these is an approximately 35 amino acid residue module that is repeated over 200 times along most of the long nebulin molecule. Another, higher order repeat or module of about 240 amino acid residues, consisting of seven of the ~35-residue modules, also can be identified that appears to match the three-dimensional contour of the thin filament (i.e., the seven-actin thin filament periodicity). These workers (Jin and Wang, 1991) also have found that the ~35 amino acid residue motifs are very basic (pI≅10). Labeit et al. (1991) also have reported some partial cDNA-derived amino sequence for nebulin, and have substantiated the report of Jin and Wang (1991). Labeit et al. (1991) predict that the ~35 amino acid motifs are likely to be largely α-helical and to interact with both actin and tropomyosin in thin filaments. Putting together the in-vitro findings of Nave et al. (1990) and Jin and Wang (1991) on nebulin's interactions with other proteins suggests that nebulin's repeating domains can bind to F-actin and, perhaps via its C-terminal domain, can bind to α-actinin in the Z-line (Table 4). The exact three-dimensional arrangement of nebulin in the skeletal muscle sarcomere remains to be elucidated. Based upon the amount of nebulin present and the number of thin (actin) filaments in the sarcomere, there may be about three to four nebulin molecules (filaments?) per thin filament (Wang and Wright, 1988). However, the amount of nebulin present is not precisely known, and one easily can envision models in which a nebulin filament contains more than one long polypeptide chain per molecule (e.g., a two-chained molecule with the subunits arranged in parallel, etc.) Based upon the screw symmetry of the thin filament, two filaments with two nebulin molecules per filament seems a likely possibility (Labeit et al., 1991).

Possible Roles of Nebulin in Muscle

Because our focus primarily is on mature muscle, we have not discussed nebulin's properties in developing muscle cells. However, in view of nebulin's close relationship with thin (actin) filaments, and its appearance at about the same time as the thin filaments are properly arranged in the I-bands of developing sarcomeres (Huiatt et al., 1989), it seems reasonable to suggest that nebulin has an important role in organization of the thin filaments during myofibrillogenesis (Table 4).

Based upon: 1) the location of the long nebulin molecule in parallel with the thin filament, 2) the interaction demonstrated in vitro between cloned nebulin fragments and F-actin (Jin and Wang, 1991), and 3) the extremely interesting sequence motifs recently identified, both Jin and Wang (1991) and Labeit et al. (1991) recently have proposed that nebulin might act as a template/ruler for assembly (i.e., regulate thin filament length) and/or scaffold for stability of thin filaments in skeletal muscle sarcomeres. Labeit et al. (1991) have presented some evidence suggesting that the size differences existing among nebulin molecules from different species are correlated with slightly differing lengths of thin filaments in the respective species (e.g., beef nebulin is a bit larger than rabbit nebulin, and thin filaments also are a bit longer in beef than in rabbit skeletal muscle).

It is interesting to speculate upon whether one should consider nebulin as comprising a *fourth* (in addition to thin filaments, thick filaments, and titin filaments) filamentous system in the sarcomere (see Wang and Wright, 1988; Nave et al., 1990), or whether nebulin is so intimately associated with the thin filament that we should simply consider it as *part of* the thin filament (Table 4). The latter possibility may help explain why it has been difficult to clearly identify additional filaments (more than can be accounted for by the thin actin filaments) in the I-band region (e.g., Ullrick et al., 1977), although the nebulin filaments also may simply be sufficiently thin (<2 nm) to render their identification in cross sections a difficult task (see discussion in Wang and Wright, 1988; Labeit et al., 1991).

As we have indicated in Table 4, it also seems reasonable to propose that nebulin helps anchor the thin filament firmly to the Z-line structure. No protein other than nebulin has been shown to bind to both α-actinin and F-actin, although obviously more evidence for this role is needed.

Importance of Nebulin in Postmortem Muscle

As described in Table 4, nebulin is rapidly degraded in postmortem muscle. Several reports (e.g., see Lusby et al., 1983; Locker and Wild, 1984b; Paterson and Parrish, 1987;

Table 4. Nebulin—Major Characteristics, Possible Roles in Muscle and Importance in Postmortem Muscle.

Major Characteristics

1. Nebulin is a very insoluble, very high molecular weight myofibrillar protein ($M_r \simeq 6$ to 9×10^5) present in the skeletal (not cardiac or smooth) muscle cells of vertebrates where it accounts for about 3-4% of total myofibrillar protein. It was discovered by Dr. Kuan Wang and colleagues in 1980.
2. Nebulin is apparently a very elongated (~1 μm) molecule and constitutes a set (the fourth sarcomeric set?) of *inextensible* longitudinal filaments running closely in parallel with the thin actin filaments. Nebulin's N-terminus is near the distal (free) end of the thin filament and its C-terminus is located at the Z-line.
3. Results of partial primary sequence studies of nebulin reveal repeating motifs (e.g., an ~35 aa residue = very basic, pI ≃ 10, module repeated >200 times) that seemingly matches the 3-D contour of actin in the thin filament.
4. In-vitro studies indicate that nebulin's repeating domains can bind to F-actin *and* perhaps via its C-terminal domain, to α-actinin (Z-line).

Possible Roles in Muscle

1. In developing muscle cells, nebulin may play an important role in organization of the thin filaments during myofibrillogenesis.
2. In the mature sarcomere, nebulin may continue to act as a template for assembly (i.e., regulate thin filament length) and/or scaffold for stability of the thin filaments. Nebulin might even comprise part of the thin filament structure!!!
3. Nebulin may help link/anchor the thin filament firmly to the Z-line structure.

Importance in Postmortem Muscle

1. Nebulin is rapidly degraded postmortem, even faster than is titin. This degradation may trigger subsequent postmortem alterations in the myofibril.
2. The degradation of nebulin that occurs postmortem is presumably due to endogenous proteinases, including the calpains, but this must be carefully documented.
3. Because nebulin is a long, fibrous, structural protein of the sarcomere, and may anchor thin filaments to Z-lines, nebulin's demise postmortem may decrease overall cytoskeletal integrity of the myofibrils, muscle cells and muscle tissue. These exact relationships, however, remain nebulous.

Anderson and Parrish 1989; Boles et al., 1991; Fritz and Greaser, 1991) have shown this to date. As one of the earliest detected changes in the myofibrillar proteins postmortem, it seems possible that alterations in nebulin may, in turn, trigger subsequent alterations in the myofibril (e.g., in titin; see discussion in Fritz and Greaser, 1991). Although we generally assume that the postmortem degradation of nebulin is due to endogenous proteinases, including the calpains, this must be carefully documented. In view of nebulin's purported role(s) with the thin filaments of skeletal muscle, it seems reasonable to suggest that this protein may be quite important in modulating integrity of muscle postmortem (Table 4).

Desmin

Major Characteristics of Desmin

Intermediate-sized filaments (IFs), approximately 10 nm in diameter, represent one of the three major cytoskeletal protein filament systems of most vertebrate cells (Robson, 1989; Stewart, 1990). New members of the IF protein family continue to be discovered (e.g., Lendahl et al., 1990), but the family can be divided by origin into six rather well-described groups (Steinert and Roop, 1988; Robson, 1989). Five of these are cytoplasmic IFs and include the keratins (epithelial cells), desmin (sarcomeric muscle, visceral and some vascular smooth muscle cells), vimentin (mesenchymal cells such as fibroblasts), glial fibrillary acidic protein (glia and astrocytes), and neurofilament triplet polypeptides (neurones). The sixth group surprisingly consists of the nuclear lamins, which compose the nuclear lamina just inside the nuclear envelope of nucleated cells. Although a great deal of progress has been made in understanding the properties of the individual proteins and of their cellular, aggregated forms (IFs), we are somewhat naive when it comes to understanding their specific function(s). Although we may seemingly indicate otherwise herein, this also is the case for desmin, the major IF protein in developed striated muscle cells. For years we (Robson et al., 1980, 1981, 1984) and others (Lazarides, 1982) often have ascribed a role in mechanical integration of cellular space and components in muscle cells to desmin IFs, but the unequivocal evidence for such a role has been difficult to obtain. In contrast to titin and nebulin, which form *longitudinally-running* filaments *within* the myofibrillar sarcomere, we believe the IFs are primarily *transversely-running* elements in mature skeletal muscle cells that link adjacent myofibrils together.

Many years ago in our laboratory, we became very interested in trying to understand something seemingly simple, i.e., why is striated muscle striated? This quest eventually led us to a protein with a molecular mass of about 55 kDa (for a description of the early observations that led us to IFs and

their major protein component, see Schollmeyer et al., 1976; Robson et al., 1981; Robson and Huiatt, 1983). The protein later was named desmin (Lazarides and Hubbard, 1976). As described in Table 5, desmin is a rather insoluble protein, although it is not as insoluble as intact titin, T_1, or nebulin. We succeeded in purifying desmin by urea-solubilization of the protein from IF-enriched muscle fractions and chromatography of the extract in the presence of urea (O'Shea et al., 1979, 1981; Huiatt et al., 1980).

One of the most interesting properties of purified desmin is that it has the ability to self-assemble into synthetic, 10-nm diameter, very long (>1-2μm) filaments that look like those in the original muscle cell (Huiatt et al., 1980; O'Shea et al., 1981). This assembly process occurs via several structural intermediates, including tetrameric protofilaments, octameric protofibrils, short, full-width IFs, and finally the elongated IFs (Ip et al., 1985; Chou et al., 1990).

Immunofluorescence localization studies indicated over ten years ago (see discussions in Richardson et al., 1981; Lazarides, 1982; Robson et al., 1981; Robson and Huiatt, 1983; Tokuyasu et al., 1983) that desmin was located primarily at or near the periphery of the myofibrillar Z-line of mature skeletal muscle cells, but the level of resolution in the light microscope studies left unanswered such questions as whether the desmin was present in a filamentous form (IFs), and how the desmin was precisely arranged with respect to the myofibrillar Z-lines. This was exacerbated by the difficulty of clearly identifying IFs in mature skeletal muscle (especially mammalian) cells by conventional transmission electron microscopy. We recently have returned to these questions and have systematically examined, by electron microscopy, samples of semitendinosus muscle removed at death from healthy adult porcine animals (Yagyu et al., 1990a; M.M. Bilak et al., 1991a,b). In all cells examined, myofibrils in most regions were too tightly packed to permit unambiguous identification of IFs. In nearly half the cells, however, small areas were found with increased intermyofibrillar space. In these areas, filaments with a diameter of approximately 10 nm were very frequently observed in the intermyofibrillar spaces and these filaments (IFs) connected adjacent myofibrils at their Z-line levels. Examination of stereo pairs indicated that these IFs surround, rather than penetrate, the integral myofibrillar Z-line domains. IFs also were identified that connected the myofibrillar Z-lines to other cellular structures including mitochondria, nuclei and the sarcolemma (M.M. Bilak et al., 1991b). Immunoelectron microscope localization studies have shown that these IFs contain desmin (Yagyu et al., 1990b; M.M. Bilak et al., 1991a,c).

Possible Roles of Desmin in Muscle

During differentiation and development of muscle cells, vimentin is replaced by desmin (S.R. Bilak et al., 1987). As described in Table 5, desmin IFs may help align and tie together adjacent myofibrils during myofibrillogenesis, but the evidence for this remains equivocal (c.f., Schultheiss et al., 1991; Tao and Ip, 1991). In the mature skeletal muscle cell, desmin IFs appear to play an important cytoskeletal role in connecting the myofibrils and, in turn, linking the myofibrils to subcellular organelles and the cell membrane. Thus, desmin IFs may play a significant role in maintaining overall integrity and organization of the skeletal muscle cell.

Synemin

Major Characteristics of Synemin

We are very interested in understanding exactly how IFs interact with the myofibrillar Z-lines. One possibility is that a cross-linking protein exists that can fulfill this role. About 10 years ago, Lazarides and associates (Granger and Lazarides, 1980; Sandoval et al., 1983) discovered the presence

Table 5. Desmin—Major Characteristics and Possible Roles in Muscle.

Major Characteristics

1. Desmin is one of the major types/isomers of proteins comprising 10-nm diameter intermediate filaments (IFs) that, in turn, are part of the cytoskeleton of virtually all animal cells.
2. Desmin is a rather insoluble, myofibrillar/cytoskeletal protein (M_r of subunit = 53,000) present in skeletal, cardiac and most smooth muscle cells of vertebrates.
3. Desmin was successfully purified from mammalian skeletal muscle by Robson and colleagues in 1979. The purified protein has the ability, via several structural intermediates, to self-assemble into synthetic, 10-nm diameter, very long (>1-2 μm) filaments.
4. Immunoelectron microscope localization studies indicate that desmin comprises a set of IFs that encircle the Z-line periphery and radiate out perpendicularly to the myofibril axis to ensnare and connect adjacent myofibrils. These studies suggest that the desmin IFs also link myofibrils to subcellular organelles, such as nuclei and mitochondria, and to the cell membrane skeleton.

Possible Roles in Muscle

1. In developing muscle cells, desmin IFs may help align and tie together adjacent myofibrils, but this remains to be proven.
2. In the developed muscle cell, desmin IFs appear to play an important cytoskeletal role in connecting the myofibrils and, in turn, tie or anchor the myofibrils to subcellular organelles and the cell membrane, i.e., desmin IFs may play a significant role in maintaining overall integrity and organization of the skeletal muscle cell.

Table 6. Synemin—Major Characteristics and Possible Roles in Muscle.

Major Characteristics

1. Synemin represents one of the types of intermediate filament-associated proteins (IFAPs). It is generally found together with desmin (sometimes with the desmin homologue, vimentin). Synemin was discovered by Lazarides and colleagues in avian smooth muscle in 1980, and a synemin homologue was identified in mammalian skeletal muscle by Robson and colleagues in 1989.
2. Synemin is a rather insoluble myofibrillar/cytoskeletal protein (M_r of subunit = 230,000) present in skeletal, cardiac and smooth muscle cells of vertebrates.
3. Synemin interacts with desmin in vitro, altering IF assembly and dynamics.
4. Immunofluorescence localization studies show that synemin is co-localized with desmin at the Z-line (periphery) of striated muscle myofibrils. Immunoelectron microscope studies indicate that synemin is attached to, or is part of, the desmin IFs that link myofibrils together, and myofibrils to other cellular structures.

Possible Roles in Muscle

1. Synemin may modulate assembly/disassembly of IFs in muscle cells.
2. Synemin may act as an IF cross-linking protein and/or play a significant cytoskeletal role in linking desmin IFs to other structures such as the myofibrillar Z-lines.

of a very small amount of a protein with a molecular mass of ≅230 kDa in avian smooth muscle, and named the protein "synemin". Because the protein copurified with desmin during initial purification steps, and was colocalized with desmin at the myofibrillar Z-line, synemin was considered an intermediate filament-associated protein (IFAP) (for reviews of IFAPs, see Steinert and Roop, 1988; Robson, 1989; Foisner and Wiche, 1991). Lazarides and associates (Price and Lazarides, 1983; Sandoval et al., 1983) were unable to identify a mammalian homologue.

We recently (S.R. Bilak et al., 1989, 1990; Bright et al., 1991) have isolated synemin and have identified a mammalian muscle homologue (Table 6.) Synemin is a rather insoluble protein and, like desmin, must be solubilized and purified in the presence of denaturing solvents (Sandoval et al., 1983; S.R. Bilak et al., 1991a). Once purified, synemin is very similar in its solubility characteristics to those of desmin or vimentin (Bilak et al., 1991a). Synemin interacts with desmin in vitro, and greatly alters desmin's ability to self-assemble into long IFs (Bilak et al., 1991b). Immunofluorescence microscope localization studies (M.M. Bilak, 1991a; S.R. Bilak et al., 1991a; Bright et al., 1991) show that synemin is colocalized with desmin at the periphery of the Z-lines of skeletal muscle myofibrils. Immunoelectron microscope studies (M.M. Bilak et al., 1991a,c) indicate that synemin is attached to, or can be considered part of, the desmin IFs that link myofibrils together, and the myofibrils to other cellular structures. We recently have found that synemin can interact in vitro with α-actinin (S.R. Bilak et al., 1991b).

Possible Roles of Synemin in Muscle

Based upon our finding that synemin can interact with desmin, and alter desmin assembly kinetics, we feel it reasonable to suggest that synemin modulates assembly/disassembly of IFs in muscle cells (Table 6.) Based upon synemin's location on the desmin IFs at the Z-line and on synemin's ability to interact in vitro with the Z-line protein α-actinin, we also propose that synemin may act as an IF/myofibril cross-linking protein.

Importance of Desmin/Synemin IFs in Postmortem Muscle

The desmin IFs are the *only* cytoskeletal elements discovered that link myofibrils together *and* the myofibrils to other structural entities in the muscle cell (Table 7). We demonstrated many years ago that purified desmin is an excellent substrate for calpain (O'Sheat et al., 1979). Likewise, the desmin associated with isolated myofibrils (Goll et al., 1991a) or muscle preparations in situ (Yamaguchi et al., 1983b, 1990) is quickly degraded by the calpains. Synemin is extremely susceptible to degradation during purification

Table 7. Importance of Desmin/Synemin Intermediate Filaments in Postmortem Muscle.

1. Desmin/synemin IFs link myofibrils, and myofibrils to the rest of the muscle cell; i.e., they are responsible for helping to maintain muscle cell integrity.
2. Purified desmin and the desmin associated with isolated myofibrils are rapidly degraded by proteinases, including the calpains. Synemin is probably degraded by the calpains, but this has not been proven.
3. Desmin is degraded in postmortem muscle at about the same rate as troponin-T. Synemin is degraded even much faster.
4. Because of their roles in skeletal muscle, alterations in desmin and synemin would be expected to disrupt muscle cell integrity, especially transversal elements (cross-links) at the Z-lines, and to alter meat quality (e.g., tenderness, water-holding capacity).

Table 8. What Does All This Have to Do with Meat? All Respectable Proteins Lead to the "Z-LINE".

A.	For about 20 to 25 years, evidence has accumulated that indicates the **Z-LINE** structure is a myofibrillar **HOT SPOT** in postmortem muscle, i.e., the **Z-LINES** are where the action is!
B.	Many postmortem studies show at least some degree of degradation of the **Z-LINE** structures.
C.	Calpains, which are endogenous muscle cell proteinases, remove the **Z-LINES** from myofibrils and release the **Z-LINE** protein α-actinin in a nondegraded form.
D.	Both titin and nebulin, the gigantic, longitudinally running proteins of the myofibril, are anchored at one end to the **Z-LINE**. At least nebulin, and perhaps titin, binds to α-actinin in the **Z-LINE**. Both proteins are degraded in muscle postmortem and both proteins are probably substrates of the calpains.
E.	Desmin/synemin IFs are attached to the periphery of the **Z-LINES**, both of these proteins are degraded in muscle postmortem, and desmin (and probably synemin) is a substrate of the endogenoses proteinases, the calpains.

(Sandoval et al., 1983; S.R. Bilak et al., 1991a), and is probably degraded by the calpains.

Desmin is degraded during postmortem storage of muscle at about the same rate as troponin-T (Robson et al., 1980, 1984), which is regarded as one of the better substrates for the calpains (Dayton et al., 1975). Others also have observed the degradation of desmin postmortem (Young et al., 1980-81; Hwan and Bandman, 1989; Koohmaraie et al., 1991). It is well known that there are distinct alterations and weakening of the intermyofibrillar links at the Z-line levels in postmortem muscle (Davey and Gilbert, 1969; Davey and Graafhuis, 1976; Davey et al., 1976). Interestingly, those astute observations were made before the transversely-running IFs were discovered. It is also likely that postmortem degradation of transversal crosslinks between myofibrils permit expansion of the muscle lattice and, thus, influence water-holding capacity of muscle (e.g., see Honikel et al., 1981).

What Does All This Have to Do with Meat?

For about 20 to 25 years, evidence has accumulated that points to the Z-line region as the myofibrillar/cytoskeletal element undergoing major changes postmortem (reviewed in Goll et al., 1975, 1977, 1983, 1991b; Robson et al., 1981, 1984). (Table 8). Calpains, which are endogenous muscle cell proteinases, rapidly remove Z-lines from myofibrils and release the Z-line protein α-actinin (Goll et al., 1991a). Both of the giant proteins of the myofibril, titin and nebulin, are anchored at one end to the Z-line. Nebulin, and perhaps titin, binds to α-actinin. Both of these gigantic longitudinally-dispersed proteins are degraded in muscle postmortem, and both are probable substrates for the calpains. The major transversal structural elements connecting myofibrils, the desmin/synemin IFs, are attached to the periphery of the Z-lines. Desmin and synemin are degraded in muscle postmortem, and desmin (and probably synemin) is an excellent substrate for the calpains. Finally, Goll et al. (1991b) have sagaciously concluded in these Proceedings that 90% or more of the tenderization occurring postmortem, with storage at 2° to 4° C, can be accounted for by the calpains.

Throughout the previous discussion, we have attempted to relate the properties of four important myofibrillar/cytoskeletal proteins to events that occur as muscle is converted into food. A great deal of careful study will be needed to elucidate precise relationships between the proteins and meat quality, and, more importantly, to control the conversion of muscle to food products.

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