Roles of the Cytoskeletal Proteins Desmin, Titin and Nebulin in Muscle

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Introduction

Myofibrils are the contractile elements and primary cytoskeletal structures in skeletal muscle cells [see Goll et al. (1984) and Robson et al. (1980, 1981, 1984) for recent reviews]. As listed in Table 1, the myofibrillar/cytoskeletal structures are composed of approximately 12 to 14 significant proteins (Goll et al., 1984). And the list may grow to as many as 20 or more (Greaser et al., 1981; Starr and Offer, 1982; Evans et al., 1982; Evans and Robson, 1983, Pardo et al., 1983; Yates and Greaser, 1983) if several additional minor, poorly characterized proteins clearly are shown to be bona fide myofibrillar/cytoskeletal components in skeletal muscle cells (e.g., gelsolin, vinculin, gamma-actinin, eu-actinin, F-protein, I-protein, Z-nin and synemin). We recently have focused some of our efforts on three of the more recently discovered proteins listed in Table 1; namely, desmin (O'Shea et al., 1979, 1981; Huiatt et al., 1980; Robson et al., 1980, 1981, 1982, 1984; Richardson et al., 1981; Rathbun et al., 1982; LaSalle et al., 1983a), titin (Wang et al., 1979; LaSalle et al., 1983b; Lusby et al., 1983; Wang and Ramirez-Mitchell, 1983) and nebulin (Wang and Williamson, 1980; Wang, 1981; Ridpath et al., 1982, 1984; Huiatt et al., 1983).

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

Proteins	*Primary Myofibrillar/ Cytoskeletal Location*	*Approx. % of Myofibril*	*Approx. MW (No. Subunits)*
Myosin	Thick filament	45	521,000* (6)
C-Protein	Thick filament	2	140,000 (1)
Myomesin (M-protein)	M-line	3	170,000 (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)
β-Actinin	Thin filament-Free end	< 1	71,000 (2)
Vinculin	Membrane-Z-line actin attachment plaques (costameres)	< 1	130,000 (1)
α-Actinin	Z-line	2	206,000 (2)
Filamin	Z-line periphery	< 1	500,000 (2)
→Desmin	Z-line/10-nm filaments	< 1	55,000 (1)
→Titin (Connectin)	Longitudinal sarcomeric filaments?	10	1,000,000 (1)
→Nebulin	N_2-line/I-band/Attached to titin filaments?	5	500,000 (1)

*Arrived at using a value of 223,000 for the molecular weight of each myosin heavy chain (Yates and Greaser, 1983).
→Proteins discussed herein.

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These three proteins in particular are important to meat scientists and muscle biochemists because they seemingly have roles related to maintenance of muscle integrity. We briefly will review and describe for each of these three proteins the following: 1) known characteristics of the protein,

including some recent findings concerning the protein from our laboratory; 2) the possible role(s) of the protein in skeletal muscle and 3) postmortem implications of the protein to meat quality. For a more exhaustive review, see Robson et al. (1984).

Desmin

Known Characteristics and Recent Studies of Desmin

It is now clear that, in addition to the about 6-nm diameter micro (actin)-filaments and the about 25-nm diameter microtubules, nearly all vertebrate cells contain a third, less characterized class of cytoplasmic filaments with diameters of about 10 nm [refer to Lazarides (1982), Osborn and Weber (1982) and Osborn et al. (1982) for recent reviews]. The 10-nm filaments also are commonly called "intermediate filaments" because their diameter is between the diameters of the 6-nm actin filaments and the 14- to 16-nm myosin filaments. The name "intermediate-sized" was introduced by Ishikawa et al. (1968), who saw these 10-nm filaments in developing muscle cells in culture. The 10-nm filaments from different cell types share some structural and chemical properties, but there now is general agreement that there are at least five major subclasses of 10-nm filaments composed of distinct proteins in different cells (Table 2). Some cells, such as certain vascular smooth muscle cells, which contain both desmin and vimentin (Quinlan and Franke, 1982; Schmid et al., 1982), and many cells grown in vitro, which contain the intermediate filament protein typical of their derivation plus vimentin, coexpress vimentin and one other type of 10-nm filament protein (Osborn and Weber, 1982). The proteins composing the 10-nm filaments are all relatively insoluble in solvents that are routinely used to dissolve other cellular filaments (e.g., actin filaments, microtubules and myosin filaments when present). As might be expected, the proteins composing the different types of 10-nm filaments contain considerable homology in primary structure (Geisler and Weber, 1982; Geisler et al., 1983).

In the early to mid-1970's, we were examining Z-line-enriched fractions from skeletal muscle and the analogous dense body-enriched fractions from smooth muscle [see Robson et al. (1981) for a more thorough description of events/observations that led us to desmin]. A small amount of a previously undescribed protein of $M_r = 55{,}000$ was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) upon analysis of the skeletal muscle fractions. A much larger amount of the protein was present in the smooth muscle (gizzard) fractions. By preparing polyclonal antibodies to electrophoretically purified preparations of the smooth muscle protein, we (Schollmeyer et al., 1976) were able to demonstrate that the 55,000-dalton protein was present in the rather prominent 10-nm filaments that surround and connect dense bodies in smooth muscle cells. The protein later was named "desmin" by Lazarides and Hubbard (1976) and "skeletin" by Small and Sobieszek (1977). We (Huiatt et al., 1980) subsequently devised a scheme that routinely resulted in highly purified preparations of smooth muscle desmin in good yield. In the procedure, we first took advantage of desmin's marked insolubility and prepared muscle fractions enriched in the protein, then solubilized the desmin in a denaturing solvent (i.e., urea) and purified the protein by chromatography in the presence of urea (Huiatt et al., 1980).

Table 2. Five Classes of 10-nm (Intermediate) Filaments*

Name	*Major Cell Type*	*Subunit MW of Major Protein by SDS-PAGE*
Keratin (tono) filaments	Epithelial and epithelial-derived	~47-68 K
Neurofilaments	Neurones	200, 150, 68 K
Glial filaments	Glial	54 K
Vimentin filaments	Mesenchymal and mesenchymally-derived (e.g., fibroblasts) and most vascular smooth muscle	58 K
Desmin filaments	Differentiated muscle (except) some vascular smooth)	55 K

*Based largely upon polypeptide analysis by one- and two-dimensional gel electrophoresis and immunological cross-reactivity.

Desmin also has been clearly identified in differentiated striated (skeletal and cardiac) muscle cells [reviewed in Robson et al. (1980, 1981, 1984), O'Shea et al. (1981), Richardson et al. (1981), Lazarides (1982) and Price and Sanger (1983)] by immunofluorescence studies using antibodies to smooth muscle desmin that cross-react with the striated muscle protein and by two-dimensional (isoelectric focusing followed by SDS-PAGE) gel electrophoretic studies. By incorporating what we had learned in our studies of smooth muscle desmin (Huiatt et al., 1980), we were able to devise procedures for isolating in a highly purified state the small amount (desmin constitutes only about 0.35% of the protein in typical myofibrillar preparations) of desmin present

Table 3. Desmin: Major Characteristics, Role in Muscle and Postmortem Implications

Major Characteristics

1. Desmin is a myofibrillar/cytoskeletal protein (M_r = 55,000) that is rather insoluble. It is present in skeletal, cardiac and most smooth muscle cells of vertebrates.
2. Desmin was successfully purified from mammalian skeletal muscle by O'Shea and colleagues (1979) and shown to self-assemble into synthetic 10-nm diameter filaments (O'Shea et al., 1981).
3. Immunoelectron microscope localization studies demonstrate that desmin comprises a set of 10-nm diameter filaments that encircle the Z-line and radiate out perpendicularly to the myofibrillar axis to ensnare and connect adjacent myofibrils.

Role in Muscle

1. Desmin appears to play an important structural role in connecting the myofibrils and in maintaining overall integrity and organization of the skeletal muscle cell.

Postmortem Implications

1. Desmin is degraded in postmortem muscle at about the same rate as is the highly susceptible troponin-T.
2. Purified desmin or desmin in intact myofibrils is rapidly broken down by proteases, including CAF.
3. Because of its role in muscle cells, alterations in desmin would be expected to disrupt muscle cell integrity, especially transversal (cross-links) elements, and to improve meat tenderness and quality.

in mammalian skeletal muscle [see O'Shea et al. (1981) for detailed protocol].

A summary of major characteristics of desmin is given in Table 3. The purified mammalian desmin appears as two variants (O'Shea et al., 1981), which differ only in degree of post-translational phosphorylation (Steinert et al., 1982), when examined by two-dimensional gel electrophoresis. Ultraviolet circular dichroism spectra of purified porcine skeletal muscle desmin obtained after renaturation of the protein demonstrate that the protein contains considerable (about 46%) alpha-helical content (Rathbun et al., 1982). Perhaps the most important property of the purified desmin is its inherent ability to form filaments in vitro (O'Shea et al., 1981; Rathbun, 1982; Rathbun et al., 1982; Robson et al., 1984) that appear as long, flexible strands with a diameter of approximately 10 nm; i.e., similar to native 10-nm filaments. Thus, it seems almost certain that desmin is the major component of skeletal muscle cell 10-nm filaments. We currently are determining the nature of the assembly process (Ip et al., 1983; Pang et al., 1983). Our results presently suggest that an about 2- to 2.5-nm diameter protofilament, which consists of a dimer of a normal two-stranded coiled-coil (i.e., a tetrameric organization consisting of four 55,000-dalton desmin subunits), is the building block used for formation of the macromolecular 10-nm filaments. The protofilament units aggregate in an unknown fashion into short 10-nm diameter filaments and then grow into long (over 1 μm) 10-nm filaments, again by an unknown mechanism.

Immunofluorescence studies have shown that desmin is located at or near the Z-lines of developed skeletal muscle cells (Lazarides and Hubbard, 1976; Franke et al., 1980; Richardson et al., 1981; LaSalle et al., 1983a). The resolution of the light microscope, however, is not sufficient to tell if the desmin is present as the aggregated form (10-nm filament) or to determine how the desmin is arranged at the Z-line. We have answered these questions, at least in part, by immunoelectron microscope localization studies of desmin in myofibrils of chicken skeletal muscle cells (Richardson et al., 1981) and of porcine and bovine skeletal muscle cells (LaSalle et al., 1983a). These studies indicate that desmin is present in a filamentous form (about 9-11 nm diameter) at the periphery of each myofibrillar Z-line (i.e., in a collar-like arrangement). The 10-nm desmin filaments span the gaps between Z-lines of adjacent myofibrils. In support of our immunoelectron localization results, we also have recently identified filaments with diameters of approximately 10 nm connecting adjacent myofibrils at their Z-line levels in negatively stained preparations of "on-grid" potassium iodide-extracted skeletal muscle myofibrils (Schreiner, 1982; Schreiner, P. J., Robson, R. M. and Stromer, M.H., manuscript in preparation).

Role of Desmin in Muscle

The results from the immunoelectron localization studies and the "on-grid" electron microscope studies, when taken together with the results showing that highly purified skeletal muscle desmin will self-assemble into 10-nm filaments under conditions similar to those present in a muscle cell, strongly suggest that the role of desmin in skeletal muscle cells is to constitute the 10-nm filaments that connect all the myofibrils inside the cell into the overall cytoskeleton (Table 3). Thus, the desmin filaments should contribute in a major way to the internal integrity of the muscle cell and to an entire muscle. The beautiful striated appearance of striated muscle, muscle cells and myofibrils is likely due in part to the presence and properties of desmin. Just how important desmin is in myofibril assembly, however, remains unclear.

Postmortem Implications of Desmin

Because of desmin's important role of linking and tying myofibrils together in the cytoskeleton, it is likely that any degradation of the protein and its aggregated form (10-nm filaments) would be responsible for some of the postmortem physical changes in muscle [see Goll et al. (1977) and Robson et al. (1980, 1981)]. We have examined the integrity of the desmin polypeptide in bovine skeletal muscle stored postmortem. Our results (Robson et al., 1980, 1981, 1982, 1984) have shown clearly that desmin is degraded in postmortem bovine muscle. Storage of bovine skeletal muscle under aseptic conditions for only one day at 15°C results in a loss of about 10% to 25% of 55,000-dalton desmin [see results of SDS-PAGE in Robson et al. (1984)]. After seven days of storage at 15°C, nearly all intact desmin is gone. The rate of decrease in content of intact desmin parallels the rate of decrease in content of the very proteolytically susceptible troponin-T, which is known to be degraded in muscle postmortem (Olson et al., 1977).

Ten-nanometer filaments and their constituent proteins, in

general, are extremely susceptible to a variety of proteolytic enzymes. We have shown (O'Shea et al., 1979) that skeletal muscle desmin is an excellent substrate for purified CAF, the endogenous Ca^{2+}-activated protease previously characterized in our laboratory (Dayton et al., 1975, 1976). Studies from several laboratories including ours [reviewed in Goll et al. (1977), Robson et al. (1980, 1981, 1984)] have shown that one of the major structural changes that occurs in skeletal muscle during postmortem storage involves the Z-line and Z-line associated structures and proteins. There also is marked alteration and weakening postmortem of intermyofibrillar links at the Z-line levels (Davey and Gilbert, 1969; Davey et al., 1976; Young et al., 1980-81). In a recent review article, Goll et al. (1983) have summarized the evidence that activity of CAF, or activities of a combination of proteases including CAF, are responsible for these Z-line associated changes postmortem.

In view of desmin's role in muscle and its susceptibility to degradation postmortem, it seems likely that desmin plays a key role in the postmortem physical changes in muscle, such as the increase in meat tenderness. It also has been suggested (Honikel et al., 1981) that postmortem transversal alterations in cross-links between myofilaments influence water-holding capacity of bovine muscle. As a result, changes in proteins such as desmin that are responsible for muscle integrity also may be important in characteristics of processed meats. A summary of the postmortem implications of desmin is given in Table 3.

Titin

Known Characteristics and Recent Studies of Titin

Wang and associates (Wang et al., 1979) recently identified three new, very large polypeptides in striated muscle samples by SDS-PAGE. The largest two ($M_r \doteq 1 \times 10^6$) migrated as a closely spaced doublet and together were given the name "titin." Because of its unusually large size, Wang and associates (Wang et al., 1979; Wang, 1982) were able to purify titin directly from chicken skeletal muscle myofibrils by gel permeation chromatography in the presence of denaturing solvent. Wang et al. (1979) found that titin (doublet band) and the third high molecular weight protein ($M_r \doteq 5 \times 10^5$; later given the name of "nebulin") were major components of myofibril fractions, together constituting a rather surprising approximately 15% of total protein.

Before titin was discovered, Maruyama and associates (Maruyama et al., 1976; 1977a, b) isolated a very insoluble, elastic-like protein from muscle and gave it the name of "connectin." The exact composition of their connectin preparations is unknown, but it is evident that they were highly heterogeneous and probably contained differing amounts of several proteins, possibly including titin, nebulin, residual amounts of myosin, desmin and actin, and even some connective tissue proteins. By SDS-PAGE analysis, it was demonstrated that connectin preparations contained some polypeptides larger than those of the myosin heavy chains (King and Kurth, 1980). Maruyama et al. (1981a) have recently shown, by using the procedures of Wang et al. (1979) on their connectin fractions, that some of the high molecular weight polypeptides (a doublet of $M_r \doteq 1 \times 10^6$) of typical connectin preparations are titin. We prefer to use the name titin rather than connectin because titin refers specifically to the doublet bands observed by SDS-PAGE, whereas connectin, unless purified by procedures used to make titin, also contains differing amounts of several other polypeptides.

Immunofluorescence localization patterns obtained on skeletal muscle myofibrils using polyclonal antibodies to titin or to the high molecular weight doublet proteins of connectin are variable, complex and hard to interpret (Wang et al., 1979; Maruyama et al., 1981a; Ohashi et al., 1981; LaSalle et al., 1983b), but show that titin is a component of the myofibrillar/cytoskeletal apparatus and that titin is present in much of the sarcomere. In particular, there seems to be intense labeling near the A-band/I-band junction. It has been suggested that titin may constitute a set of rather thin, longitudinally running elastic filaments that might span from Z-line to Z-line within a sarcomere (Wang and Ramirez-Mitchell, 1979, 1981, 1983). Roughly similar suggestions also have been made for connectin (dos Remedios and Gilmour, 1978; Toyoda and Maruyama, 1978; Locker and Daines, 1980). Many of these latter electron microscope studies on connectin, however, utilized potassium iodide-extracted residues. Wang and Ramirez-Mitchell (1983) have recently shown that titin translocates to the edge of residual Z-structures during similar potassium iodide extraction experiments. And we have confirmed this latter observation (LaSalle et al., 1983b). Thus, other investigators may have misidentified residual filaments (possibly including some 10-nm filaments) and net-like structures between Z-structures of thick and thin filament depleted sarcomeres as connectin (titin).

Many reports have appeared in the literature over the past 30 years that have suggested the possible existence of a set of additional (to thick and thin) longitudinally running filaments in vertebrate muscle sarcomeres. In general, the existence of such filaments has not been proved to the satisfaction of most scientists [e.g., see Ullrick et al. (1977)]. One of the more intriguing proposals for a set of filaments is the "gap filament" proposal of Locker and Leet (1976a). Based upon their electron microscope studies, most of which have utilized over-stretched bovine sternomandibularis muscle, Locker and associates (Locker and Leet, 1975, 1976a, b; Locker et al., 1977; Locker and Daines, 1980; Locker, 1982; Locker and Wild, 1982) have proposed a model in which each gap filament forms a core within, and links through the Z-line, two thick filaments in adjacent sarcomeres. As a result of their model, gap filaments should be most easily seen in over-stretched (thick and thin filaments pulled completely apart so that no overlap exists) muscle samples in which a gap appears in the region between the ends of thin and thick filaments. Few scientists seem to have accepted their model outright, but the discovery of titin (i.e., a candidate for such a new putative set of filaments) has spurred new interest in it. At a minimum, it seems likely that the model (Locker and Leet, 1976a) will have to be modified (e.g., labeling of at least some of the A-band with titin antibodies seems inconsistent with titin being a "core" inside thick filaments). Nevertheless, the importance of the model perhaps lies in stimulation of further studies (Locker, 1982).

Because nondegraded titin is virtually insoluble in

nondenaturing solvents, little is yet known about the protein's physical chemic properties other than its amino acid composition (Maruy na et al., 1981a; Gruen et al., 1982; Lusby et al., 1983). Be use of its incredible size and poor solubility properties, it h been natural to suspect that titin (or high molecular weight doublet bands of connectin) is a product (from one or several different smaller proteins) of post-translational cross-linking reactions. Some evidence for this contention has been reported by Maruyama and associates (Maruyama et al., 1977a; Fujii et al., 1978; Fujii and Kurosu. 1979; Kimura et al., 1979; Fujii and Maruyama, 1982). Others have not supported this view (Robins and Rucklidge, 1980). And, in a recent seemingly rather thorough study concerning the presence or absence of cross-links in the high molecular weight components of connectin (titin), Gruen et al. (1982) found no evidence of cross-links and suggested that titin is synthesized as the intact polypeptide. If so, titin may be the largest polypeptide synthesized in nature.

Wang and Ramirez-Mitchell (1982), studying the morphology of titin molecules, have seen electron microscope images of rotary and unidirectional shadowed titin preparations that indicate titin is a very long (400 to 700 nm; c.f. myosin is only 160 nm long) molecule. They also observed what appear to be somewhat regular, periodic structural domains along the titin molecule. A summary of the major characteristics of titin is given in Table 4.

Table 4. Titin: Major Characteristics, Possible Roles in Muscle and Postmortem Implications

Major Characteristics

1. Titin is a highly insoluble, very high molecular weight myofibrillar protein ($M_r \doteq 1 \times 10^6$) present in both skeletal and cardiac muscle cells of vertebrates and invertebrates. It was discovered by Dr. Kuan Wang and associates at the University of Texas at Austin (Wang et al., 1979).
2. Titin accounts for ~ 10% of total myofibrillar proteins.
3. Titin is evidently identical to the high molecular weight components (doublet band) present in Maruyama's connectin preparations.
4. Antibody localization and selective extraction studies suggest that titin is a major structural protein of the sarcomere, but that it is not exclusively either a component of thin (actin) or thick (myosin) filaments. Titin may comprise a set of very thin, longitudinally running "elastic" filaments in the sarcomere.

Possible Roles in Muscle

1. Serve as "elastic" elements of the sarcomere.
2. Help maintain myosin filaments in longitudinal register in the A-band and/or regulate insertion of thin filaments into distal ends of the A-band.
3. Help maintain longitudinal structural integrity of the myofibrils and muscle cell.

Postmortem Implications

1. Titin is degraded in postmortem muscle.
2. Titin in myofibrils is rapidly broken down by proteases, including CAF.
3. Because titin seemingly has an important cytoskeletal role, degradation of titin by endogenous proteolytic enzymes may be involved in postmortem decrease in muscle cell integrity and improvement in meat tenderness and quality, but exact relationships are not yet known.

Possible Roles of Titin in Muscle

The precise roles of titin in muscle are unknown. It seems noteworthy that 1) titin is present as the third most abundant protein in the myofibrillar/cytoskeletal apparatus and 2) that the titin molecule is so huge ($M_r \doteq$ one million; 0.4 μm or more in length)! It does seem possible that titin, or possibly its aggregated form, serves as some sort of elastic element in the sarcomere, perhaps helping to keep myosin filaments in proper longitudinal register in the A-band and/or helping actin filaments insert into distal ends of the A-band. Perhaps most crucial to determining the role of titin is the clear establishment of the presence (or absence) of an additional set of longitudinally running filaments *inside* the sarcomere. If present, then clear evidence is needed to show a direct relationship between titin and the filaments (i.e., gap filaments or something similar to them). If such evidence is found, then it seems that titin's major role and responsibility may be to provide a significant part of myofibrillar and muscle cell integrity. We recently (LaSalle et al., 1983b; manuscript in preparation) have conducted immunoelectron microscope localization studies with polyclonal antibodies to titin on bovine sternomandibularis and longissimus dorsi muscles that had been over-stretched by the method of Locker and Leet (1975). Electron-microscope examination of immunoperoxidase labeled, over-stretched muscle showed the electron-dense reaction product primarily had labeled a set of approximately 3- to 5-nm diameter ("gap"?) filaments of unknown nature that span the region or gap between A-bands and I-bands. Reaction product also was present in the outer approximately one-half of the I-bands and the outer edge of the A-bands. Immunofluorescence results obtained on cryostat sections of over-stretched bovine muscle samples also showed intense titin staining in the gap regions (LaSalle et al., 1983b; manuscript in preparation). Our results, although difficult to interpret, do suggest that titin constitutes some form of longitudinal structure (filament, net?) within the sarcomere.

Postmortem Implications of Titin

It is clear from the work of Wang and Ramirez-Mitchell (1983) that titin is easily degraded during its preparation. Lusby et al. (1983) in our laboratory recently have shown that titin also is degraded in bovine muscle postmortem. In myofibrils from at-death muscle samples, the titin migrated as the closely spaced doublet when examined by SDS-PAGE. With storage, the top band of the doublet gradually disappeared, then the lower band (evidently a breakdown product of the upper band) disappeared. The rate of disappearance was accelerated with increase in temperature of storage. Those working with connectin or with the putative gap filaments likewise have suggested that they are prone to breakdown postmortem (Davey and Graafhuis, 1976; Maruyama et al., 1977a; Takahashi and Saito, 1979; King and Kurth, 1980; Young et al., 1980-81). Locker and his colleagues (Locker et al., 1977; Locker, 1982; Locker and

Wild, 1982) maintain that degradation of gap filaments is responsible for increasing tenderness postmortem. The latter suggestion remains controversial. King and colleagues (King et al., 1981; King and Harris, 1982), for instance, have reported that connectin is broken down or degraded in meat during cooking and, thus, that connectin [or gap filaments if composed of connectin (titin)] may not be of much importance in tenderness.

We have examined the effect of purified CAF on titin in bovine skeletal muscle myofibrils and have found that titin is rapidly broken down to smaller polypeptides by CAF (Zeece et al., 1983). Our results do not agree with those of Maruyama et al. (1981b) who found that the high molecular weight doublet (titin) of connectin in chicken skeletal muscle myofibrils was not degraded by CAF. Our results, however, seem in agreement with results of Wang and Ramirez-Mitchell (1983) that show that addition of EDTA greatly reduces proteolysis of titin (and nebulin) that occurs during routine glycerination of myofibrils.

A summary of postmortem implications of titin is shown in Table 4. Future studies on titin hold considerable promise of understanding muscle cell integrity and meat quality. But, clear establishment of the structural form and location of titin is needed before we can evaluate and perhaps modify titin's task in muscle and meat.

Nebulin

Known Characteristics and Recent Studies of Nebulin

Another recently discovered myofibrillar/cytoskeletal protein that may be involved in the maintenance of muscle cell integrity is nebulin. This protein was first identified via SDS-PAGE of vertebrate skeletal muscle myofibrils by Wang et al. (1979) as one of the three high molecular weight components of myofibrils and was initially referred to simply as "band 3" (the titin doublet constitutes bands 1 and 2). It is highly insoluble, like titin, and has a molecular weight of approximately $5x10^5$. The name "nebulin" was coined later (Wang, 1981). Nebulin has been purified from both rabbit psoas myofibrils (Wang and Williamson, 1980) and chicken breast myofibrils (Maruyama et al., 1981a; Ridpath et al., 1982, 1984). The initial steps in the purification are the same as that used for purification of titin; namely, extraction of myofibrils with a denaturing solvent, followed by gel filtration chromatography of the extract in the presence of a denaturing solvent. Nebulin can then be further purified by ion exchange chromatography or preparative SDS-PAGE (Wang, 1982). Polyclonal rabbit antibodies to nebulin do not cross-react with purified titin or other myofibrillar proteins (Wang and Williamson, 1980; Ridpath et al., 1982, 1984). Peptide mapping studies (Wang, 1981) and amino acid analysis (Maruyama et al., 1981a) also demonstrate that nebulin is distinct from titin. Because its insolubility in nondenaturing solvents and its high molecular weight make it difficult to work with, little else is currently known about the biochemical properties of nebulin.

Antibodies to nebulin have been used to localize the protein in myofibrils by using indirect immunofluorescence (Wang and Williamson, 1980; Ridpath et al., 1982, 1984). In myofibrils from both cardiac and skeletal muscles from a variety of vertebrate species, the anti-nebulin fluorescent staining appears as a pair of bands running across the I-band, perpendicular to the myofibril axis, with one band on either side of the Z-line. This location corresponds to the location of the N_2-line. The N_2-line has been identified in electron micrographs of muscle tissue as a broad (100-150 nm wide), dark line across the I-band parallel to the Z-line (Page, 1968; Franzini-Armstrong, 1970), which often appears as a row of bead-like thickenings between adjacent thin filaments. For reasons that are not entirely clear, this structure has not consistently been observed in ultrastructural studies of muscle. Thus, the structural organization and function of N_2-lines remain poorly understood. When sarcomere length changes, the position of the N_2-line also changes, such that it maintains the same proportional distance between the M-line and the Z-line (Page, 1968; Franzini-Armstrong, 1970; Locker and Leet, 1976b). This result suggests that N_2-lines are not rigidly attached to the thin filaments in the I-band. The position of the immunofluorescent staining with nebulin antibodies also changes with sarcomere length in the same manner (Wang and Williamson, 1980), indicating that nebulin is a component of this structure. A summary of nebulin's major characteristics is given in Table 5.

Possible Roles of Nebulin in Muscle

The exact function of nebulin and N_2-lines remains poorly understood. It is interesting to note that the position of the N_2-

Table 5. Nebulin: Major Characteristics, Possible Roles in Muscle and Postmortem Implications

Major Characteristics

1. Nebulin is a high molecular weight ($M_r \doteq 5 \times 10^5$), highly insoluble, myofibrillar protein. It was discovered by Dr. Kuan Wang and associates at the University of Texas at Austin (Wang et al., 1979; Wang and Williamson, 1980).
2. Nebulin is found in both skeletal and cardiac muscles of invertebrates and vertebrates. In vertebrate skeletal muscle, it constitutes ~ 5% of the total myofibrillar protein.
3. Immunofluorescence studies suggest that nebulin is located in the I-band of the myofibril at the level of the N_2-line.

Possible Roles in Muscle

1. Localization studies suggest that nebulin may be a structural component of the N_2-line. It may function in the organization of the changing three-dimensional lattice of the thin filaments in the I-band.
2. Nebulin may also be attached to longitudinal, elastic filaments (titin?) in the sarcomere.

Postmortem Implications

1. Electrophoretic studies suggest that nebulin is rapidly degraded postmortem, even more quickly than is titin.
2. Nebulin in myofibrils is highly susceptible to degradation by proteases, including CAF.
3. Degradation of nebulin by endogenous proteases may be involved in the postmortem decrease in muscle integrity, but exact relationships remain nebulous.

line corresponds roughly to the position in the I-band where the three-dimensional lattice of the thin filaments, as seen in cross section, changes from the square lattice found near the Z-line to the hexagonal lattice found near the A-I junction and in the A-band. Thus, the N_2-line may function in the maintenance of the geometry of the thin filaments (Franzini-Armstrong, 1970; Locker and Leet, 1976b; Traeger et al., 1983). Locker and Leet (1976b) also suggested that the N_2-line may be attached to a set of the longitudinal, elastic, "gap" filaments that supposedly link A-bands in adjacent sarcomeres (see earlier "titin" section). Although the existence of gap filaments remains controversial, it is possible that titin constitutes a set of some type of longitudinal, elastic filament and that nebulin and N_2-lines may interact with this set of elastic filaments, or even that nebulin constitutes a portion of these filaments in the N_2-line region (Wang, 1983).

In our laboratory, we have attempted to determine the function of nebulin by examining the possible role of this protein in the process of myofibril assembly in embryonic muscle cells. Primary cultures of embryonic chick skeletal muscle were used to examine the localization and rate of accumulation of nebulin during differentiation of the muscle cells (Ridpath et al., 1982, 1984; Ridpath, 1983). Indirect immunofluorescence using antibodies to nebulin demonstrated a progression from a diffuse staining pattern in early myogenic cells to a pattern similar to that seen in adult myofibrils in later cultures that contained myotubes with assembled myofibrils. No staining of either fibroblasts or presumptive myoblasts was seen in 24 h cultures. In 48 h cultures, a diffuse staining pattern was observed near the sarcolemmal edges of the myogenic cells. After 72 and 96 h in culture, the nebulin fluorescence appeared as distinct bands in the I-band region of the nascent myofibrils in myotubes. Quantitation of the rate of accumulation of nebulin by immunoprecipation of ^{35}S-methionine labeled cultures demonstrated an approximately three-fold increase in nebulin accumulation coincident with the period of greatest fusion activity. Both the correlation of the localization patterns with the pattern of myofibril assembly and the increase in nebulin accumulation after fusion, when other myofibrillar proteins are being synthesized and assembled into myofibrils, are consistent with the suggestion (Wang and Williamson, 1980) that nebulin may function in the organization of the I-band. However, considerably more work is needed on the biochemical properties and exact localization of nebulin, as well as on the structure of the N_2-line, before the function of this protein is completely understood. A summary of possible roles for nebulin in muscle is given in Table 5.

Postmortem Implications of Nebulin

Because nebulin, like titin and desmin, may have a role in the maintenance of muscle cell integrity, degradation of nebulin in postmortem muscle may be important in the structural changes that occur in postmortem muscle. It is clear that nebulin is extremely sensitive to proteolytic degradation (Wang and Williamson, 1980; Wang, 1981; Maruyama et al., 1981b; Zeece et al., 1983). Nebulin is especially sensitive to degradation by calcium activated proteases (Wang, 1981; Maruyama et al., 1981b; Zeece et al., 1983). Also, we have recently shown that N_2-lines are removed from myofibrils by treatment with CAF (Yamaguchi et al., 1983). Furthermore, work from our laboratory has shown that, in bovine longissimus muscle stored postmortem, nebulin is degraded at a rate even faster than that of titin (Lusby et al., 1982, 1983). Thus, it is tempting to speculate that degradation of nebulin is important in the postmortem changes in muscle tissue. The postmortem implications of nebulin are summarized in Table 5. It is clear from this summary that we are just beginning to understand the role of nebulin in both living and postmortem muscle.

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