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Identification of intermediate filaments and their proteins in mature mammalian striated muscle

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Identification of intermediate filaments and their proteins in mature mammalian striated muscle

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GENERAL INTRODUCTION

Overall Muscle Structure and Myofibrillar Proteins

Three types of muscles can be identified in vertebrate animals, namely skeletal, cardiac, and smooth muscles. The first two types also are called striated muscles. When viewed by either light or electron microscopy, a skeletal muscle fiber (cell) contains hundreds of long myofibrils (each ~1 to 2 μm in diameter). These myofibrils exhibit alternating dark and light bands along their long axis, which are in register with those of adjacent myofibrils. In the middle of each light band, called the I-band, is a transverse, very dark band or line called the Z-line. Each myofibril contains hundreds of structural repeating units, called sarcomeres, which are each defined by the distance (2.3-2.5 μm in rest-length muscle) between two successive Z-lines [Huxley, 1953, 1963; Huxley and Niedergerke, 1954].

When viewed at high magnification by electron microscopy, the dark middle band of each sarcomere, called the A-band, is composed of longitudinally running thick filaments (~1.5 μm in length by ~15 nm in diameter) and the free ends of thin filaments that enter the A-band from each nearby Z-line. The thick filaments are comprised primarily of the protein myosin, which is known to function as the enzyme responsible for converting chemical energy into mechanical energy during muscle contraction [for reviews, see Squire, 1986; Huxley, 1988]. A rather translucent region, called the H-zone, can be seen in
the middle of the A-bands of relaxed myofibrils. The H-zone represents the area where actin filaments running from the two adjacent Z-lines into the A-band do not meet and are absent. Another structure, called the pseudo-H-zone, also resides in the middle of the A-band and is the area defined by the absence of myosin cross-bridges in the central part of thick filaments. In longitudinal sections viewed by electron microscopy, a transverse structure called the M-line or M-band, which consists of three to five bands (depending on the species), can be seen in the exact center of the A-band [Knappeis and Carlsen, 1968; Luther and Squire, 1978]. The M-line, in turn, is composed of M-bridges, M-filaments, and secondary M-bridges [Luther and Squire, 1978]. In cross sections, the M-bridges connect all adjacent thick filaments into a hexagonal network [Knappes and Carlsen, 1968; Trinick and Lowey, 1977; Luther and Squire, 1978]. At the middle of each M-bridge, a small thickening can sometimes be observed [Luther and Squire, 1978; Carlsson and Thornell, 1987], which may be due to M-filaments that run parallel to the thick filaments and that connect adjacent M-bridges. The secondary M-bridges may, in turn, connect adjacent M-filaments.

At least three proteins have been localized in the M-line region [Grove et al., 1984]. The muscle form of creatine kinase (two subunits of ~42 kD each) is thought to be a major constituent of at least some of the M-bridges [Turner et al., 1973; Walliman et al., 1983]. The exact locations of the other two
proteins remain rather uncertain, but M-protein (one subunit of ~165 kD) may comprise the M-filaments [Woodhead and Lowey, 1982], and myomesin (one subunit of 185 kD) may make up some of the M-bridges [Grove et al., 1985]. Although it is generally assumed that the M-line structure holds and aligns the thick filaments in lateral register, in vitro studies suggest very weak or no interaction among M-line proteins and myosin [Woodhead and Lowey, 1983].

Some of the other proteins associated with thick filaments of vertebrate striated muscle cells include C-protein (one subunit of ~140 kD) [Offer et al., 1973; Starr et al., 1985; Bennett et al., 1986], X-protein [Starr et al., 1980], 86K protein [Bahler et al., 1985], H-Protein (one subunit of ~74 kD) [Starr and Offer, 1983; Yamamoto, 1984, 1988], and skelemin (two subunits of ~220 and 200 kD) [Price, 1987]. X-protein, H-protein, and 86K protein are very similar to C-protein. The exact function of each of these proteins remains unclear. For instance, it is still controversial as to whether any of these thick filament-associated proteins affect thick filament assembly [Yamamoto, 1988; for a recent review, see Epstein and Fischman, 1991]. It has been suggested by Price [1987] that skelemin may act to link the M-lines of adjacent myofibrils. Interestingly, recent gene sequencing data revealed that C-protein belongs to the immunoglobulin superfamily [Einheber and Fischman, 1990], and that nearly all proteins thought to bind to thick filaments (e.g., titin, C-protein, 86K protein, skelemin, myosin light chain kinase, and adenylic deaminase) share some...
sequence homology [Epstein and Fischman, 1991].

Actin filaments make up the backbone of thin filaments (~1 μm in length by 6-8 nm in diameter) in the I-band, are each anchored at one end to the Z-line (called the barbed end), and each interdigitates with thick filaments in the A-band at its free end (called the pointed end) [for a review, see Squire, 1986]. Actin can exist in two inter-convertible forms called globular actin or G-actin (monomers) and filamentous actin or F-actin, a polymer of G-actins [for review, see Pollard and Cooper, 1986]. Each G-actin monomer (~42 kD) contains a bound divalent cation (Ca or Mg), and an ATP. Because each G-actin has asymmetry and the G-actins align in the same orientation during polymerization, F-actin also exhibits polarity. The barbed end (corresponds to the Z-line end in situ) is the preferred "growing" end during polymerization in vitro. [Pollard and Cooper, 1986].

At least two proteins are located along the long axis of the actin filaments. A tropomyosin strand lies in each of the two long grooves of the F-actin helix and binds cooperatively to F-actin [for a review, see Payne and Rudnick, 1989]. The tropomyosin molecule consists of two, 100% alpha-helical subunits (each ~32 kD) that are wound together to form a coiled-coil structure. In the grooves of the thin filaments, the long tropomyosin molecules exhibit head-to-tail interaction, and form the long strands that run the length of the thin filament. Each tropomyosin molecule binds to seven actin monomers along the
thin filament [Hitchcock-DeGregori and Varnell, 1990], and also helps stabilize F-actin structure [Moore et al., 1970]. To each tropomyosin, in turn, is bound one molecule of the protein, troponin. The tropomyosin and troponin complex functions as the calcium regulatory site of the myofibrils in vertebrate striated muscle cells [for a review, see Zot and Potter, 1987]. After nerve stimulation of a muscle fiber and release of bound calcium from the sarcoplasmic reticulum, the calcium is bound by one of the three troponin subunits, troponin-C (~18 kD) [Greaser and Gergely, 1971; Zot et al., 1987; Satyshur et al., 1988]. This initiates a series of conformational changes in the thin filament that result in tropomyosin movement in the F-actin grooves via a complex mechanism. In resting muscle cells, troponin-I (~21 kD), another subunit of troponin, is bound to actin. The conformational changes that occur within the troponin complex, after troponin-C binds calcium, releases troponin-I from actin and simultaneously overcomes the inhibitory effect of troponin-I. In turn, this signal is sent to tropomyosin via the third subunit of troponin, troponin-T (~31 kD). This series of events, in which the tropomyosin strands move in the F-actin grooves, remove what is sometimes called the "steric blockage" of actin-myosin interaction [Zot and Potter, 1987], and permit contraction of the myofibrils, muscle cell, and muscle.

In addition to the well known thick and thin filament system of the myofibrils, there have been many reports indicating the presence of an
additional filamentous network(s). For instance, Locker and Leet [1975] proposed the presence of an elastic filamentous system that may somehow give structural support to the thick and thin filament system. They saw fine filaments (~2-6 nm in diameter) in the gap created between the ends of the thick and thin filaments when muscle was super-stretched, and called them "gap" filaments. Several years later, Wang et al. [1979] discovered a high molecular weight protein named titin, which immunolocalization studies suggested was located primarily at the A-I junctions, but at other locations in the sarcomere as well. Subsequently, it has been shown that titin has a very large molecular mass (~2,800 kD) [Kurzban and Wang, 1988], that it is a very long molecule (~1 μm) [Wang et al., 1984; Nave et al., 1989], and that a single titin molecule stretches from near the M-line all the way to the Z-line [Itoh et al., 1988; Furst et al., 1988, 1989]. Thus, it is now thought that titin filaments represent an elastic filamentous network in the sarcomere [Wang, 1985; Funatsu et al., 1990; for a recent review, see Fulton and Isaacs, 1991]. Maruyama and associates [Maruyama, 1976; Maruyama et al., 1977] isolated a very insoluble, elastic-like protein from muscle and called it "connectin". One of the proteins in the early connectin preparations turned out to be titin [Maruyama et al., 1981], and the name connectin is sometimes used interchangeably with titin.

In their original titin publication, Wang et al. [1979] also described an
additional high molecular weight protein. It was subsequently given the name "nebulin" [Wang, 1981]. Early immunofluorescence labeling studies suggested that nebulin was predominantly located at the transverse N₂ line near the Z-line [Wang and Williamson, 1980]. For awhile, it was thought that nebulin was attached to [Wang and Williamson, 1980; Wang, 1981], or part of [Wang, 1985; Horowits et al., 1986], the elastic titin filaments. More recent studies, however, have shown that nebulin may be closely associated with actin filaments in the l-band, and not associated with the elastic titin filaments [Wang and Wright, 1988; Pierobon-Bormioli et al., 1989; Maruyama et al., 1989]. Thus, nebulin actually may form a "fourth" filament system of skeletal muscle sarcomeres, and may extend from the Z-line to approximately the free ends of the actin thin filaments [Wang and Wright, 1988]. Nave et al. [1990] have reported that nebulin binds to alpha-actinin (an integral Z-line protein) in vitro. And, Jin and Wang [1991] have shown that cloned nebulin fragments bind to F-actin. Based upon these latter results and examination of nebulin's primary structure [Jin and Wang, 1991; Lebeit et al., 1991], it seems quite possible that nebulin may serve as a giant actin-binding template or ruler for the thin filaments in skeletal muscle cells. Certainly the protein, with a molecular mass of ~600-900 kD [Wang and Wright, 1988; Hu et al., 1989], is large enough to extend the entire distance of the thin filament. Interestingly, nebulin has not been identified in cardiac or smooth muscle, and it remains unclear as to
whether or not a counterpart of the protein is present in either muscle [Furst et al., 1988; Wang and Wright, 1988].

The transverse Z-lines represent structures where thin filaments from opposing sarcomeres and, thus, of opposite polarity are linked. In longitudinal section, the width of the Z-lines varies depending on degree of overlap between the antipolar thin filaments of adjacent sarcomeres in the Z-line (e.g., from ~30 nm for narrow Z-lines of fish and amphibians to ~100 nm for wide Z-lines of mammalian cardiac muscle) [Yamaguchi et al., 1983, 1985]. The Z-line model of Yamaguchi et al. [1985] explains in structural detail the variations in Z-line width. The model is based on a pair of Z-filaments, which they called a Z-unit. The Z-filaments in the Z-unit are linked near their centers at a 90° angle, and form bridges between neighboring antipolar thin filaments. A square lattice of four Z-filament pairs, termed the Z-line unit, defines the geometrical position of the thin filaments as they enter the Z-line domain in a square lattice. Wider Z-lines have additional sets of Z-line units. The exact three-dimensional structure of the vertebrate Z-line remains to be agreed upon, but popular models include those described by Yamaguchi et al. [1985], Goldstein et al. [1990], and Luther [1991].

Major proteins located in the Z-lines are alpha-actinin, which is thought to make up the Z-filaments [Yamaguchi et al., 1983, 1985], and actin [Yamaguchi et al., 1978]. Alpha-actinin has a molecular mass of about 200 kD, and
contains two subunits (~100 kD each) arranged in anti-parallel fashion [Suzuki et al., 1976; Arakawa et al., 1985; Wallraff et al., 1986; Goll et al., 1991]. Based on its in vitro cross-linking ability [Goll et al., 1972] and its re-binding location when added to Z-line-extracted fibrils [Stromer and Goll, 1972], it was suggested many years ago that alpha-actinin may serve to cross-link thin filaments from adjacent sarcomeres. Recent gene sequencing data for alpha-actinin revealed that each alpha-actinin polypeptide can be divided into three domains: an N-terminal actin-binding domain, a middle domain containing four internal "spectrin-like" repeats, and a C-terminal domain containing two EF-hand-like calcium-binding regions [Noegel et al., 1987; Baron et al., 1987; Arimura et al., 1988]. The middle domain is thought to be the portion of the alpha-actinin subunit that binds to another alpha-actinin subunit, in an anti-parallel fashion, and, thus, responsible for forming the native, dimeric alpha-actinin molecule [Wallraff et al., 1986: Baron et al., 1987]. With an actin binding domain positioned at each end of the molecule, it is easy to envision how alpha-actinin can cross-link two actin filaments.

Granger and Lazarides [1978] introduced a unique myofibrillar extraction procedure to produce honeycomb-like structures consisting of many adjacent transverse myofibrillar Z-lines, called Z-sheets. Using antibodies against some of the Z-line proteins and immunofluorescence microscopy, they separated the Z-line proteins into two different categories, namely "integral" and "peripheral"
Z-line proteins. The integral Z-line proteins were considered as being "inside" the Z-line (within the cross-sectional myofibrillar area at the Z-line) where actin filaments are inserted. The peripheral Z-line proteins were considered as being located at the periphery of the myofibrillar Z-line in a "collar-like" arrangement. Some of the peripheral Z-line proteins they [Gomer and Lazarides, 1981] identified include filamin (2 subunits of ~500 kD each) [Wang et al., 1975] which cross-links F-actin in vitro, desmin, a 53 kD subunit of intermediate filaments (IFs) [Lazarides and Hubbard, 1976; Schollmeyer et al., 1976; Huiatt et al., 1980], and synemin, a 230 kD intermediate filament-associated protein [Granger and Lazarides, 1980; Bilak et al., 1990]. Other proteins often included as peripheral Z-line proteins are spectrin [Craig and Pardo, 1983; Nelson and Lazarides, 1983], ankyrin [Nelson and Lazarides, 1984], and vinculin [Terracio et al., 1990].

Although alpha-actinin [Schollmeyer et al., 1976; Endo and Masaki, 1982; Yamaguchi et al., 1985] and those actin molecules inserted into the interior of the Z-line [Yamaguchi et al., 1985] are considered the major integral Z-line proteins, several others, often present in very small amount, also are considered as such. One of these is Cap Z [Casella et al., 1986, 1987], a heterodimer containing 33 and 31 kD subunits (from cDNA-derived sequences) [Casella et al., 1989; Caldwell et al., 1989], which acts as a barbed-end capping protein of actin filaments in vitro. Another is zeugmatin, a high molecular mass protein
having two doublet polypeptides over 500 kD as determined by sodium
dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Maher et al.,
1985]. Others, for which evidence is minimal, include Z-protein (~55 kD)
[Ohashi and Maruyama, 1989] and eu-actinin (42 kD) [Kuroda et al., 1981]. Z-
nin, a 300-400 kD protein, has been shown by immunolocalization procedures
to be located in the interior of Z-lines [Suzuki et al., 1985], but Z-nin is released
from myofibrils by the proteinase calpain [Suzuki et al., 1981] and it seems
likely that it is a proteolytic fragment of a larger Z-line protein such as titin
[Furst et al., 1988]. Although by far the largest part of the titin [Furst et al.,
1988] and nebulin [Wang and Wright, 1988] molecules are not located in the Z-
line structure, the C-terminal domains of both titin [Wang et al., 1991] and
nebulin [Jin et al., 1990] are now thought to be inserted into the Z-line. As a
result, at least parts of each of these giant myofibrillar proteins can be
considered as integral Z-line proteins.

Like skeletal muscle, mammalian cardiac muscle also is striated, but it has
much shorter cells in comparison to skeletal muscle cells. The cardiac myofibril
contains a similar sarcomeric structure to that of a skeletal muscle sarcomere
[Sommer and Waugh, 1978; Forbes and Sperelakis, 1984]. One of the
structures unique to cardiac muscle cells is a structure called the intercalated
disk, which occurs at the junction between the ends of two cardiac muscle
cells. Cardiac muscle is not under voluntary control, and intercalated disks
serve as electrical coupling sites between cells so that neighboring cells
contract in unison. In a sense, a part of the intercalated disk structure also
takes the place of the terminal myofibrillar Z-line in cardiac muscle cells, i.e., it
also serves as an actin-attachment site [Yamaguchi et al., 1988]. Three types
of specialized junctions (parts) of intercalated disk structure include: (1)
attachment plaque or fascia adherens (actin attachment site), (2) desmosome or
macula adherens (intermediate filament attachment site, and a mechanical
coupling site between cells), and (3) gap junction or nexus (electrical coupling
site) [Severs, 1985]. Immunolocalization studies have shown that vinculin
[Geiger et al., 1980; Tokuyasu et al., 1981; for a review, see Otto, 1990],
alpha-actinin [Tokuyasu et al., 1981], filamin [Koteliansky et al., 1986], and
zeugmatin [Maher et al., 1985] are components of the fascia adherens,
whereas several other proteins, including the desmoplakins [Mueller and Franke,
1983] and desmin [Kartenbeck et al., 1983; Tokuyasu et al., 1983b], are
located in or attached to the macula adherens.

Mammalian smooth muscle can be found primarily in the walls of the
digestive, respiratory, and urogenital tracts, and of the blood vessels. As in the
case of cardiac muscle, smooth muscle is not under voluntary control. The
smooth muscle cells are generally small, elongated, or spindle-shaped
[Burnstock, 1970]. The thick and thin filaments are not arranged so that a
banded sarcomeric appearance exists [for review, see Somlyo et al., 1984], but
the contractile apparatus of the cell may be more well-ordered than is usually thought [Kargacin et al., 1989]. The myofibrillar protein composition of smooth muscle cells is somewhat different from that of striated muscle cells. The thick filaments of smooth muscle contain myosin, but not C-protein or M-proteins. There also is no bare zone in the middle of the smooth muscle thick filament [Craig and Megerman, 1977; Cooke et al., 1989]. Thin filaments are much longer (up to ~4.5 μm) than those of skeletal muscle [Draeger et al., 1990; Small et al., 1990], and contain the proteins caldesmon and calponin, but lack troponin [for a review, see Trybus, 1991]. The smooth muscle cell does not have Z-line structures, but rather an analog of the Z-line called the cytoplasmic dense body [Ashton et al., 1975]. The exact protein composition of the dense body is unclear [for a review, see Bagby, 1986], but the dense bodies clearly contain alpha-actinin [Schollmeyer et al., 1976], actin [Ashton et al., 1975], and zeugmatin [Maher et al., 1985], as do the Z-lines of striated muscle.

IFs are very abundant in smooth muscle cells [Small and Sobieszek, 1977; Huiatt et al., 1980; Stromer and Bendayan, 1988, 1990]. Uehara and his collaborators [1971] originally identified IFs in smooth muscle cells of the digestive system. They observed that IFs appeared to be associated with dense bodies, and proposed that the IFs may play a role in organization of dense bodies. A few years later, individuals in our research group were examining dense body-enriched fractions from smooth muscle [reviewed in Robson et al.,]
and observed by SDS-PAGE analysis the presence of a considerable amount of a previously undescribed protein (~55 kD). Using polyclonal antibodies to electrophoretically-purified preparations of this "new" protein, these investigators demonstrated for the first time that this protein was present in the rather prominent 10-nm diameter filaments (IFs) that surround and connect some, or all, of the dense bodies in smooth muscle cells of the digestive system [Schollmeyer et al., 1976]. This protein was later named desmin by Lazarides and Hubbard [1976] and skeletin by Small and Sobieszek [1977]. In smooth muscle cells of the digestive system, desmin is clearly the major IF protein present [Huiatt et al., 1980; Garfield and Somlyo, 1985; Somlyo and Franzini-Armstrong, 1985]. In addition to the role(s) of IFs in linking elements of the contractile apparatus, Stromer and Bendayan [1990] recently reported association of desmin-IFs with both the nuclei (in juxtanuclear region) and mitochondria in gizzard smooth muscle cells. They suggested that the IFs may be involved in positioning these subcellular organelles within the cell [for a general review of the role of IFs in connecting cellular structures, see Goldman et al., 1985, 1986].

In contrast to smooth muscle cells in the digestive tract, vascular smooth muscle cells show heterogeneity in the IF protein expressed [Frank and Warren, 1981; Osborn et al., 1981]. Osborn and her collaborators [1981] presented morphological data indicating two populations of vascular smooth muscle cells
that relate location and function of the vascular system to the type(s) of IF proteins expressed. They saw strong desmin staining in their immuno-fluorescence studies in cells of "muscular" vessels (e.g., veins, arteries, and arterioles), and strong vimentin (a protein very homologous to desmin) [Robson, 1989], but weak desmin staining, in cells of "elastic" vessels such as the aorta. Thus, they proposed that desmin expression may coincide with muscle cells exhibiting a more contractile function. Some cells clearly contain only vimentin, some only desmin, and some both proteins [Schmid et al., 1982]. The existence of these two proteins in many vascular smooth muscle cells was further supported by immunoelectron microscopy data [Fujimoto et al., 1987], and it now is generally agreed that desmin and vimentin are differentially distributed in various regions of the vascular bed [Osborn et al., 1987].

Desmin in vascular smooth muscle cells increases in amount during hypertrophy [Berner et al., 1981]. In contrast, desmin decreases in amount in human vascular smooth muscle cells undergoing the atherosclerotic process [Osborn et al., 1987], and during aging [Nikkari et al., 1990]. The significance of these findings, however, is not clear.

Structure and Biochemistry of Intermediate Filaments

The name "intermediate-sized" filaments was introduced by Ishikawa et al. [1968], who observed filaments with a diameter (~10 nm) intermediate to
those of thick (~15 nm) and thin (~7 nm) filaments in the sarcoplasm of
developing skeletal muscle cells cultured from chicken embryonic muscle. The
names ten-nm filaments and IPs are used interchangeably in the scientific
literature. It now is accepted that IPs represent one of the three (the other two
being microfilaments and microtubules) major cytoskeletal filamentous systems
present in virtually all vertebrate cells [for reviews, see Steinert and Roop,
1988; Robson, 1989].

Based upon electrophoretic and immunofluorescence studies, cytoplasmic
IFs and IF proteins have been classically divided into five major subclasses on
the basis of their tissue-restricted (or specific) distribution [Lazarides, 1980;
Geisler and Weber, 1982]. These are the cytokeratin filaments, neurofilaments,
glial filaments, vimentin (primarily in mesenchymally-derived nonmuscle cells
like fibroblasts), and desmin filaments (in differentiated striated muscle cells and
smooth muscle cells of the digestive and urogenital tracts). However, it also is
now well known [e.g., Franke et al., 1979; Granger and Lazarides, 1979;
Fujimoto et al., 1987; Sugi, 1990] that many cells express more than one type
of IF protein. As previously discussed, some cells, such as certain vascular
smooth muscle cells that contain both desmin and vimentin [Schmid et al.,
1982], and many cells grown in vitro, which contain the IF protein typical of
their derivation plus vimentin, co-express vimentin and their other characteristic
type of IF protein [Lazarides, 1982; Steinert and Roop, 1988]. A sixth major
subclass (noncytoplasmic, however) comprised of the lamins, which form a meshwork of IFs lining the nucleoplasmic surface of the inner nuclear membrane of eukaryotic somatic cells, more recently has been added to the IF list [for reviews, see Franke, 1987; Gerace and Burke, 1988; Robson, 1989].

The presence of more than one type of IF protein in a cell has limited the usefulness of the classification scheme based on tissue of origin. Indeed, cells with as many as three and four types of IF protein have been described [Gustafsson et al., 1989]. All major IF proteins share some similarity in amino acid sequence. Based upon analysis and comparison of the primary structure (from conventional protein sequencing techniques and from cDNA-derived sequences) of the classes of IF proteins, a newer classification scheme has emerged [for reviews, see Steinert and Roop, 1988; Robson, 1989]. These types are: type I, the acidic keratins of epithelial cell IFs; type II, the neutral-basic keratins of epithelial cell IFs; type III, desmin, vimentin, glial fibrillary acidic protein (GFAP, the protein of glial cells and astrocytes), and peripherin (a recently identified IF protein in peripheral neurons) [Leonard et al., 1988; Parysek et al., 1988]; type IV, the major triplet polypeptides (NF-L, NF-M, NF-H) of neurofilaments; and type V, the nuclear lamins [Franke, 1987; Gerace and Burke, 1988; Moir et al., 1991]. Recent analyses obtained by molecular biology techniques suggest that the family of IF proteins will grow (e.g., an IF protein called nestin has been identified in stem cells of the central nervous
system, and it may represent a new type VI IF [Lendahl et al., 1990; Steinert and Llem, 1990].

In general, IF proteins contain three major, structurally distinct domains including: (1) a short, basic, highly protease-sensitive, non-helical, amino-terminal headpiece domain, (2) a long (~48 nm) rod-like domain of approximately 310 amino acids (~38 kD), which is very high in alpha-helical secondary structure, and (3) a non-alpha-helical, acidic, carboxy-terminal tailpiece domain that differs considerably in size among the types of IF proteins [Geisler and Weber, 1982; Geisler et al., 1983; Steinert and Roop, 1988; Pieper et al., 1989]. The N- and C-terminal end domains exhibit high sequence variability among the types of IF protein. The rod domain exhibits higher sequence homology than the end domains when IF proteins are compared, and this homology in the rod domain is quite high when proteins within a given type are compared (e.g., GFAP, vimentin, and desmin, three type III proteins).

All IF proteins are fairly similar in some general properties, although there are small differences even among specific IF proteins within a subclass (e.g., insolubility) [Steinert et al., 1981; Hartzer, 1984]. The IFs, as a group, are relatively insoluble in physiological-like buffers, especially in comparison to other cytoskeletal filamentous systems such as microfilaments, microtubules, and myosin filaments [Lazarides, 1980]. As a result, IF proteins generally must be solubilized and purified in denaturing solvents [Huiatt et al., 1980; O’Shea et
al., 1981; Robson and Huiatt, 1983]. Once purified, most of the IF proteins can be renatured by dialysis against low ionic strength, slightly alkaline buffers [Huiatt et al., 1980].

Another important characteristic shared by IF proteins is their ability to assemble into 10-nm diameter filaments in vitro that are very similar to those present in cells in vivo [e.g., see Huiatt et al., 1980; Geisler and Weber, 1981]. All type III proteins can self-assemble into synthetic IFs individually [Steinert and Roop, 1988]. In the case of the keratins, an exact one-to-one ratio of the type I and type II keratins are necessary for assembly into IFs [Hatzfeld and Weber, 1990a; Steinert, 1990]. In the case of the type IV IFs, NF-L can assemble into long IFs, but NF-M and NF-H generally only form IFs when mixed with NF-L [Geisler and Weber, 1981; Liem and Hutchinson, 1982].

Many investigators have examined the assembly process of IFs [for reviews, see Steinert and Roop, 1988; Aebi et al., 1988]. The major steps involved and the identity of assembly intermediates are still very active areas of research, but major intermediates are generally believed to include the IF polypeptide, dimer, tetramer, octamer, short IFs, and long IFs [Ip et al., 1985a,b; Aebi et al., 1988]. The first step involves formation of a dimer. The rod domains of each of the two polypeptides form a coiled-coil structure [for a review, see Robson, 1989]. The two polypeptide chains in the dimer are in parallel and in exact register [Parry et al., 1985]. The next step in assembly is lateral association of
two coiled-coil dimers to form a tetramer (represents the predominant stable intermediate) [Geisler and Weber, 1982; Pang et al., 1983], which also is called a protofilament (~2-3 nm diameter by 48 nm long) [Stromer et al., 1981; Ip et al., 1985a,b]. The geometric arrangement of the two dimers in the tetramer remains unclear. It is likely that the two dimers are arranged in an anti-parallel fashion [Geisler et al., 1985; for a review, see Steinert and Roop, 1988], although not all agree [e.g., Birkenberger and Ip., 1990], and the possibility of structural polymorphism exists [Aebi et al., 1988; Stewart et al., 1989; Birkenberger and Ip, 1990; Potschka et al., 1990]. Whether the dimers are in register [Ip et al., 1985a,b; Hisanaga et al., 1990a] or staggered [Stewart et al., 1989; Potschka et al., 1990] also remains equivocal. Details regarding formation of the octamer and the short IF must await unequivocal determination of the structure of the tetramer. Growth of short IFs into long IFs presumably involves addition of tetramers (or octamers) to both ends of the short IFs because IFs (at least in vitro) are thought by many investigators to exhibit no polarity (i.e., both ends of an IF are identical because the tetramer, or the octamer, exhibits no polarity) [Geisler et al., 1985; Hisanaga et al., 1990a].

Nearly all of the N-terminal domain is necessary for IF assembly [Traub and Vorgias, 1983; Kaufmann et al., 1985; Albers and Fuchs, 1989; Quinlan et al., 1989; Ratts et al., 1990]. The C-terminal tail domain is usually not considered necessary for IF assembly [Kaufmann et al., 1985; Hatzfeld and Weber,
1990b), although a particular recombinant derivative of GFAP lacking the entire tail domain failed to form IFs [Quinlan et al., 1989]. In the fully-assembled IF, both the head- and the tail-domains evidently are arranged at the surface of the filament. The N-terminal domain, for instance, is available to proteinases [Traub and Vorgias, 1983; Perides et al., 1987], protein kinases, and phosphatases [Inagaki et al., 1989a]. The degree to which the C-terminal tail domain is exposed at the surface of IFs remains unclear. Part of it is accessible to proteinases [Kaufmann et al., 1985]; however, part of the tail, at least in the case of desmin, is unaccessible to antibodies [Birkenberger and Ip, 1990].

Two of the areas of IF research that have resulted in exciting findings within the past four years have been the role of phosphorylation in regulation of IF assembly/disassembly and the possibility of vectorial assembly of IFs in cells [for review, see Robson, 1989]. Many electron microscope and immunocytochemical studies [for a review, see Goldman et al., 1986] have suggested that IFs may associate with and somehow link several cellular structures. Georgatos and Marchesi [1985] examined the interaction of vimentin IFs with the plasma membrane of human erythrocytes and reported that the subplasmalemmal protein ankyrin was the IF binding site. This group of scientists [Georgatos et al., 1985] also found that the ankyrin binds to the N-terminal head domain of vimentin, indicating that IFs bind to the cell membrane in an end-on fashion. Subsequently, Georgatos and Blobel [1987a] identified a
second binding site for vimentin at the nuclear membrane. By utilizing proteolytic fragments of vimentin, they also found that the binding of vimentin to the nuclear membranes was mediated via vimentin's C-terminal tail domain. Thus, they proposed an interesting model for receptor-regulated or -modulated assembly in which IFs somehow originate at the nuclear envelope, course through the cell, and terminate at the plasma membrane. This now is generally referred to as vectorial assembly of IFs in higher eukaryotic cells. A very surprising result from these workers [Georgatos and Blobel, 1987b] was their finding that the binding site for the C-terminal domain of vimentin at the nuclear envelope was lamin B, a polypeptide of the nuclear lamina that lines the nucleoplasmic side of the inner nuclear membrane. If this result is proven, then IFs should only interact with lamin B in vivo by entering through the nuclear pores. As described previously, the nuclear lamins are now known to represent a class of IFs, so the possibility that a cytoplasmic IF protein can interact with a nuclear IF protein seems plausible. These results suggest that apolar IFs somehow acquire functional polarity in cells [Georgatos et al., 1987]. It must, however, be emphasized that the apolar nature of IFs has been determined in vitro, but not in vivo, and that not all investigators [e.g., Birkenberger and Ip, 1990] even agree with the conclusion that all IFs in vitro are apolar.

By using in vivo expression of mutant keratin cDNA in an epithelial cell line (PtK2) to produce a type I keratin in which the N-terminal head domain or the
C-terminal tail domain was deleted, Albers and Fuchs [1989] have proposed a model whereby initiation of IF assembly occurs at distinct sites on the nuclear envelope and the IFs then grow from the nucleus into the cytoplasm. They also suggested that in these transfected epidermal cells, the desmosomal junctions at the plasma membrane might function in an analogous fashion (i.e., as cappers) to that previously shown for ankyrin in cells with type III IFs [Georgatos et al., 1987]. This suggestion by Albers and Fuchs [1989] was strengthened by the work of Cartaud et al. [1990], who found that a 140 kD desmosomal protein shares epitope(s) with lamin B and that this 140 kD protein binds to vimentin in an in vitro assay. Although the studies of Albers and Fuchs [1989] and those of others [Vikstrom et al., 1989] support the concept of vectorial assembly, not all scientists are in agreement. By using regulated expression of mouse vimentin cDNA in human cell lines that have, or do not have, a pre-existing vimentin IF network and by monitoring the appearance of the mouse vimentin, Sarria et al. [1990] have concluded that assembly of IFs occurs throughout the cytoplasm. Clearly, the concept of vectorial IF assembly remains open to further study.

Until the past three to four years, IFs have been considered as rather stable cytoskeletal structures [for a review, see Skalli and Goldman, 1991]. This notion largely has been abandoned as many studies [e.g., Lamb et al., 1989; Vikstrom et al., 1989; Angelides et al., 1989] have obtained evidence that IFs
are dynamic structures, undergoing considerable subunit incorporation, exchange, and organization in vivo. A major reason scientists previously viewed IFs as being rather static structures was that whenever IF proteins were studied under in vitro conditions mimicking those in vivo (~neutral pH, and ionic strength of ~0.18), over 99% of the protein was present as long IFs that seemingly showed no tendency to disassemble [e.g., Huiatt et al., 1980; Stromer et al., 1987]. This all changed when Inagaki et al. [1987] demonstrated in a purified system in vitro, that phosphorylation of synthetic vimentin IFs by protein kinase A induced depolymerization of the IFs into a soluble state (perhaps protofilaments). Prior to this report, about the only other regulatory mechanism hypothesized for disassembly of IFs in vivo involved proteolysis of IF proteins by the calpains [Nelson and Traub, 1983; Kaufmann et al., 1985]. Since the report by Inagaki et al. [1987], many studies [for reviews, see Inagaki et al., 1989a; Robson, 1989] have reported that many classes of IF proteins [even the lamins, Ward and Kirschner, 1990], are good substrates for protein kinases (especially protein kinases A and C) and protein phosphatases, have identified specific amino acid residues (those affecting state of assembly are in the N-terminal head domain) that are phosphorylated, and have shown that the phosphorylated state favors IF disassembly whereas the dephosphorylated state favors IF assembly. These studies have included ones conducted both in vitro [e.g., Geisler and Weber, 1988; Inagaki et al., 1988;
Ando et al., 1989; Kitamura et al., 1989] and in vivo [e.g., Evans, 1988; Lamb et al., 1989; Chou et al., 1990]. Although site-specific phosphorylation of IF proteins is gaining favor as a major mechanism in IF assembly/disassembly, considerable uncertainties remain concerning many details of the process, such as which protein kinases are involved, the most critical IF amino acid substrate residues involved, the number of residues that are phosphorylated, the degree of IF disassembly, and the identity of the disassembled or disordered state (e.g., protofilament, protofibrils, or small aggregates of bundled IFs) involved in the phosphorylation mechanism [Geisler et al., 1989; Kitamura et al., 1989; Lamb et al., 1989; Sihag and Nixon, 1989; Hisanaga et al., 1990b]. Likewise, other regulatory mechanisms governing assembly/disassembly of IFs also may be involved [Inagaki et al., 1989b].

The rest of this review will be concerned primarily with the type III IF proteins, in particular desmin, which is believed to be the major IF protein in mature striated muscle cells. It is known that myoblasts (proliferating and postmitotic) and very young myotubes express vimentin (another type III IF protein), that desmin is generally absent in proliferating myoblasts, is present in small amounts in postmitotic myoblasts, and that it is expressed in increasing amounts at about the onset of fusion [Bennett et al., 1979; Lazarides, 1980; Lazarides et al., 1982; Babai et al., 1990]. It remains somewhat controversial as to whether expression of vimentin eventually is turned completely off during
development of striated muscle cells. Most investigators suggest that there is no detectable vimentin in fully differentiated skeletal muscle cells [Bennett et al., 1979; Osborn et al., 1982; Tokuyasu et al., 1984, 1985a,b; Osinska and Lemanski, 1989] or in working myocardium of adult heart muscle cells [Price and Lazarides, 1983; Tokuyasu et al., 1985b; Kjorell et al., 1987]. However, there are some reports indicating the presence of a small amount of vimentin in fully-differentiated skeletal muscle cells [Granger and Lazarides, 1979; Lazarides et al., 1982; Price and Lazarides, 1983] and heart muscle cells [Osinska and Lemanski, 1989]. As indicated earlier in this review, desmin and vimentin are closely related to each other, as shown by their gene sequence data [Bloemendal et al., 1985]. Comparison of conventionally-derived amino acid sequences has shown very high homology between the two proteins, including ~93% identity in the conserved alpha-helical rod domain [Geisler and Weber, 1982]. It also has been shown that isolated desmin and vimentin can copolymerize in vitro [Steinert et al., 1981; Hartzer, 1984].

Intermediate Filaments and Their Relationship to Other Cytoskeletal Structures in Adult Striated Muscle Cells

Most studies on IFs in muscle cells have been conducted using adult and embryonic smooth muscle [for recent reviews, see Stromer, 1990; Chou, 1991] or embryonic or cultured cardiac and skeletal muscle cells [for a review, see
Price and Sanger, 1983]. This often has been done because of the abundance of the IF proteins in these tissues (smooth muscle), or because the interior of the cells appears less congested, i.e., they are not as packed with myofibrils (embryonic or cultured cells). Throughout most of the 1970's and early 1980's, much of what was learned concerning the presence and distribution of IFs and their subunit proteins in muscle cells was obtained by using immunofluorescence microscopy [Lazarides, 1980, 1982]. Based largely on the results of these studies conducted at the light microscope level, it often was assumed that IFs play an important role in holding adjacent myofibrils together in lateral register by somehow connecting their Z-lines. To obtain convincing proof that desmin was present in an IF form and, if so, to determine the exact location of IFs in muscle cells, studies at the electron microscope level were needed. In contrast to adult smooth muscle cells, however, it has been comparatively difficult to visualize desmin IFs in adult striated muscle cells by using electron microscopy. In fact, there have been relatively few reports of unambiguous identification of IFs in normal (non-diseased), mature, striated muscle cells. Although the major reasons for this difficulty are, firstly, that these cells are literally packed with contractile and membranous elements, leaving little space to permit visualization of other structures, and, secondly, that desmin is present in rather low amounts in striated muscle (e.g., desmin comprises about 0.16% of skeletal muscle protein) [O'Shea et al., 1981], a
third reason may be limitation of thin sectioning techniques, i.e., the sections lack enough thickness to include widely separated IF network(s).

More success has been achieved in studies involving identification of IFs in cardiac muscle than in skeletal muscle, presumably because of the slightly higher content of desmin in cardiac muscle (~1-2% of total protein) [Hartzer, 1984; Price, 1984] and because of a slightly expanded intermyofibrillar space in some areas of cardiac muscle cells, caused at least in part by the large numbers of intermyofibrillar mitochondria. The electron microscope studies by Ferrans and Roberts [1973] were perhaps the first that dealt with identification of IFs in adult, mammalian muscle cells of the working (as apposed to myocardial cells whose function involves conduction of the electrical impulse) myocardium. They observed IFs running at about right angles to the long axis of the myofibrils at the Z-lines, and near intercalated disks, nuclei, and the sarcolemma. They suggested that the IFs may interconnect adjacent myofibrils at their Z-lines and to the nucleus, and that IFs may help keep myofibrils in lateral register. This proposed cytoskeletal role for IFs in mature striated muscle cells came some three years before the major IF protein was given the name desmin, and that it was proposed that desmin had a cytoskeletal role [Lazarides and Hubbard, 1976]. Ferrans and Roberts [1973] also noted an increase in number of IFs in hypertrophied human myocardium.

In another early report of electron microscope studies, Behrendt [1977] also
identified filaments of about 10-nm in diameter in working myocardium of normal adult rats, and more of these filaments in adult rats that had been fed an anabolic steroid for a long period of time. He found that these IFs were connecting adjacent myofibrils at their Z-line levels, and that the IFs also appeared to attach myofibrils to transverse tubules, i.e. he also proposed that IFs may have a cytoskeletal role in cardiac muscle. Granger and Lazarides [1978] and Lazarides and Granger [1978] also suggested that IFs can serve to connect myofibrils to transverse tubules, but this suggestion was not supported by later studies [Richardson et al., 1981; Tokuyasu et al., 1983a, 1985b]. In an electron microscope study of ventricular myocardial cells of adult mice, Forbes and Sperelakis [1980] described IFs collected into bundles and oriented at right angles to the long axis of myofibrils at their Z-lines, and near desmosomes in the intercalated disk region of the cells.

In the first immunoelectron microscope (immuno-EM) localization study of adult striated muscle, including avian cardiac muscle, Richardson et al. [1981] identified filaments of about 10-nm in diameter that connected Z-lines of adjacent myofibrils and that were labeled by the desmin antibodies. This work was followed by similar studies of Tokuyasu and coworkers [Tokuyasu, 1983; Tokuyasu et al., 1983b, 1985b] using anti-desmin labeling of ultrathin frozen sections of chicken cardiac muscle. They observed desmin labeling between the Z-lines of adjacent myofibrils in the intermyofibrillar space and, when
mitochondria were present, they saw wavy or convoluted strands of desmin labeling that bridged the distance between Z-lines and nearby mitochondria. In comparison to similar studies they conducted on chicken skeletal muscle [Tokuyasu et al., 1983a], they saw a more pronounced three-dimensional network of IFs within the intermyofibrillar space of the cardiac muscle cells, with the IFs often splaying out from the Z-line in a wide pattern into the intermyofibrillar spaces [Tokuyasu et al., 1983b, 1985b]. Tokuyasu [1983] observed, in addition to the transversely-oriented networks that surround the periphery of Z-lines, longitudinally-oriented networks of IFs in the intermyofibrillar spaces in avian cardiac muscle cells that were either not present or as prominent in skeletal muscle cells [Tokuyasu et al., 1983a]. As a result, Tokuyasu [1983] proposed that the arrangement of the desmin IFs in avian skeletal and cardiac muscle cells may be considerably different from each other, and suggested that the desmin IFs may be involved in resisting stress during the repeated contraction/relaxation cycles of cardiac muscle. Tokuyasu [1983] also suggested that the IFs may attach myofibrils to nuclei, in agreement with earlier studies [Ferrans and Roberts, 1973; Forbes and Sperelakis, 1980]. In a more recent immuno-EM localization study on adult rat myocardium with antibodies to desmin and tubulin, Watkins et al. [1987] concluded that desmin IFs formed transverse connections between adjacent myofibrillar Z-lines, but they observed few longitudinally oriented desmin
filaments and few connections between these filaments and mitochondria, nuclei, or the sarcolemma. They suggested, instead, that microtubules, rather than desmin IFs, constituted the main cytoskeletal structure near the mitochondria, nuclei, and the sarcolemma.

In their early immunofluorescence studies on IFs, Lazarides and coworkers [Lazarides and Hubbard, 1976; Lazarides, 1978; Granger and Lazarides, 1978, 1979; Lazarides, 1980] proposed that, in addition to linking intracellular structures, IFs also may be involved in directly attaching actin filaments to Z-lines of cardiac and skeletal muscle and to the intercalated disks of cardiac muscle. Certainly in the case of the cardiac intercalated disks, conventional electron microscope studies [Ferrans and Roberts, 1973; Forbes and Sperelakis, 1980] and immuno-EM localization studies [Kartenbeck et al., 1983, Tokuyasu, 1983b, 1985b; Watkins et al., 1987; Sætersdal et al., 1989] have shown that this is not a role of desmin because desmin IFs are not attached at the fascia adherens, where the actin filaments are attached, or at nexuses (gap junctions), but are attached at the macula adherens (desmosomes) of the intercalated disks in an oblique or lateral fashion. Lack of clear morphological evidence for IFs in striated muscle cells also led to an early speculation that the IF protein, desmin, may be present in nonfilamentous form in these cells [Bennett et al., 1979; Lazarides et al., 1982]. The later electron microscope studies, however, indicated that, especially in cardiac muscle cells, desmin (at
least much of it) exists in a filamentous form [Richardson et al., 1981; Tokuyasu, 1983; Tokuyasu et al., 1983b, 1985b; Watkins et al., 1987; Sætersdal et al., 1989].

Although it has been relatively difficult to identify IFs in normal working myocardium, IFs have been much more easily observed in specialized conducting cells of the heart such as in the Purkinje fibers [Eriksson and Thornell, 1979; Thornell and Eriksson, 1981; Thornell et al., 1985], and in cardiomyopathies [Ferrans and Roberts, 1973; Stoeckel et al., 1981; Watkins et al., 1987].

As pointed out by several investigators [Bennett et al., 1979; Richardson et al., 1981; Lazarides et al., 1982], the unambiguous identification of IFs in normal adult skeletal muscle cells has been very difficult to achieve, even more so than in adult muscle cells of the working myocardium. Considering the large number of electron microscope studies done on this tissue, the reports including identification of IFs have been rare. In a very early report, Page [1969] described a network of filaments (diameter was not given) that encircled the myofibrils at their Z-line levels in a slow avian skeletal muscle (anterior latissimus dorsi), but saw none in the adjacent fast skeletal muscle (posterior latissimus dorsi). Price and Sanger [1979] observed the presence of filaments (about 10-nm in diameter) in the residue of chicken skeletal muscle myofibrils after they had used a myosin-extracting solution to remove thick filaments.
These filaments, however, connected successive Z-line remnants along and within the long axis of each myofibril rather than at right angles. They did not use antibody labeling or any other method to demonstrate that these remaining, longitudinally-oriented filaments were composed of an IF protein such as desmin. Thus, the filaments could also be explained as laterally-aggregated thin actin filaments, or remnants of the highly insoluble titin [Wang et al., 1979; Furst et al., 1988] and nebulin [Wang and Ramirez-Mitchell, 1988] filamentous systems. Wang and Ramirez-Mitchell [1983] used a somewhat similar approach by extracting most of the thick and thin filaments from rabbit skeletal muscle myofibrils with a KI solution, and then examining the remaining residues by electron microscopy. They saw one filamentous system that ran parallel to the long axis of myofibrils and that connected successive Z-lines within each myofibril remnant. These filaments, which were similar to those seen by Price and Sanger [1979], averaged about 10-nm in diameter, but were rather variable in diameter and appearance. Wang and Ramirez-Mitchell [1983] also observed filaments of about 10-nm in diameter that ran in a direction transverse to the myofibrillar axis and that seemingly connected Z-line remnants to Z-line remnants of adjacent myofibril residues and M-line remnants to M-line remnants of adjacent myofibril residues. It was not determined by other procedures whether any of these filaments were composed of IF proteins. In fact, their SDS-PAGE analysis of the myofibril residues showed primarily the presence of
titin, nebulin, myosin, actin, alpha-actinin, and only a very small amount of desmin [Wang and Ramirez-Mitchell, 1983]. It seems likely that the transverse filaments at the Z-line contained desmin, but those at the M-line, and perhaps many of the longitudinal filaments, may have been composed of non-IF proteins.

Nunzi and Franzini-Armstrong [1980] used a different approach to try to visualize IFs in amphibian skeletal muscle. They soaked frog skeletal and toadfish swimbladder muscles in hypotonic solution before, and during, fixation in order to increase the intermyofibrillar spaces, where they expected that the IFs may be found. In both muscles treated in this fashion, they identified, by conventional electron microscopy, two sets of filaments. One set was composed of long filaments that encircled the myofibrillar Z-lines and seemed to connect the Z-lines of adjacent myofibrils. The other set appeared as bundles of short filaments radiating from Z-lines and connecting the Z-lines to membranes of the sarcoplasmic reticulum and transverse tubular system. However, because no measurements were reported, and because membranous structures such as transverse tubules, sarcoplasmic reticulum, and mitochondria were probably poorly preserved due to the hypotonic condition they used, some of the structures identified as filaments may have been altered membranous structures present around the Z-line region of amphibian skeletal muscle myofibrils. It seems likely that the long filaments connecting Z-lines of adjacent
myofibrils were desmin IFs, but much more evidence would be necessary to
demonstrate that the set of filaments connecting Z-lines to nearby membranes
were composed of IF proteins.

Richardson and her collaborators [1981] were the first ones to use immuno-
EM to demonstrate localization of desmin in adult (avian) skeletal muscle cells. By using immunoperoxidase labeling on isolated myofibrils, they demonstrated
that the desmin was localized almost entirely in the intermyofibrillar areas
between the Z-lines of adjacent myofibrils. The reaction products followed an
approximately linear course in the peri-Z-line region, suggesting that the desmin
was present in the form of IFs, and that the IFs connect adjacent myofibrils at
their Z-lines. They also observed that the reaction products were more
noticeable when the section plane was obtained closer to the surface of the
myofibrils. From this observation, Richardson et al. [1981] suggested that the
desmin-IFs surround the myofibrils in avian skeletal muscle rather than
penetrate the integral Z-line domains. In the sections obtained at or very close
to the surface of the myofibrils, they also observed that the reaction products
were on, or immediately adjacent to, filaments that measured 9 to 12 nm in
diameter. This study provided the first real evidence that the transverse
filaments that had so rarely been seen connecting Z-lines of adjacent myofibrils
were composed of desmin and, thus, that the desmin in adult avian skeletal
muscle cells was actually present in the form of IFs. Somewhat later, Tokuyasu
et al. [1983a], using immunoferritin labeling of ultrathin frozen sections of intact adult avian skeletal muscle, showed that desmin labeling was confined to the intermyofibrillar spaces at the Z-line levels between adjacent myofibrils, in agreement with the study of Richardson et al. [1981]. Tokuyasu et al. [1983a; 1985b] also demonstrated connections between myofibrillar Z-lines and adjacent nuclei, mitochondria, and the sarcolemma, but not between the Z-lines and the transverse tubule system.

Pierobon-Bormioli [1981] was one of the first investigators who studied normal adult mammalian skeletal muscle (rat diaphragm) cells by using conventional electron microscopy with the purpose of trying to identify new cytoskeletal elements. She observed transverse filamentous structures that connected adjacent myofibrils at their Z-lines, and myofibrils to the sarcolemma at both Z- and M-lines. The filaments, however, were irregular in thickness and unmeasurable. It seems possible that at least some of the filaments or cables she described were, in fact, poorly fixed membranes [an interpretation also reached for the studies of Pierobon-Bormioli, 1981, by Kentish, 1983].

Another possible reason for the lack of clear identification of IPs in mammalian skeletal muscle cells is that they are arranged in a different fashion than in the avian system [see discussions in Tokuyasu, 1983; Watkins et al., 1987].

Although it has been extremely difficult to visualize IPs in non-diseased,
adult skeletal muscle cells, it has been much easier to observe cytoplasmic IFs in several muscle disease processes, including Duchenne muscular dystrophy [Thornell et al., 1980], distal myopathy [Edstrom et al., 1980], and other more poorly defined myopathies [Osborn and Goebel, 1983; Pellissier et al., 1989]. Transverse, as well as longitudinal IFs, have been reported to increase in such diseased conditions. In general, it is believed to be easier to identify IFs in diseased muscle fibers because there are more IFs present or perhaps because the intermyofibrillar spaces are enlarged. The underlying cause(s) of this increase in desmin is unclear, although various hypotheses, including incorrect turnover and/or synthesis of desmin [Edstrom et al., 1980], have been proposed. One very interesting study [Rappaport et al., 1988] has shown that in a familial myopathy there was an intra-sarcoplasmic accumulation of an electron-dense granulo-filamentous material next to the myofibrillar Z-lines. When examined by two-dimensional gel electrophoresis, these diseased muscle samples exhibited not only an increase in relative amount of desmin, but an increase in the number of desmin isovariants. The investigators [Rappaport et al., 1988] suggested that the extra isovariants were due to abnormal levels of post-translational desmin phosphorylation, and that the abnormal electron-dense bodies that reacted with desmin antibodies may represent a storage form of phosphorylated and disassembled desmin.

Even though it has been difficult to ascertain/identify IFs in normal, adult
striated muscle, all the studies, when taken in toto, suggest a close structural association between IFs and the myofibrils. Unfortunately, this association remains poorly understood, i.e., it is not clear if IFs actually bind directly to myofibrils, if IFs bind or interact specifically with any of the myofibrillar proteins, or if there is an indirect attachment of IFs to myofibrils, such as via linker molecules. Isobe et al. [1988] have suggested, based solely upon three-dimensional analysis of α-actinin immunogold labeling in cultured cardiac muscle and non-muscle cells, that α-actinin is involved in interlinking IFs and actin filaments. Tokuyasu et al. [1985b], on the other hand, have discussed the evidence for and against whether IFs are directly in contact with the edges of the Z-line or are separated from the edges by an appreciable distance. They were unable to reach a firm conclusion, but their observations [Tokuyasu et al., 1983a, 1985b] indicated that no IFs were in direct contact with Z-lines, and led them to the supposition that the association of IFs with the Z-lines is established through intervening proteins over an appreciable distance (actual distance not given). Bard and Franzini-Armstrong [1991] also recently have suggested that there has been no direct demonstration of an IF/myofibril interaction, as has been proposed by some other investigators [e.g., Granger and Lazarides, 1978]. If there are additional crosslinking components between IFs and myofibrils, these might include involvement of one or more intermediate filament associated proteins (IFAPs) that characteristically cocycle, copurify, or
colocalize with IFs [for recent reviews, see Steinert and Roop, 1988; Robson, 1989].

Synemin is one of the IFAPs that has been localized in adult avian smooth and skeletal muscle cells [Granger and Lazarides, 1980], in developing avian smooth, skeletal, and cardiac muscle cells [Lazarides et al., 1982; Price and Lazarides, 1983], in avian erythrocytes [Granger and Lazarides, 1982; Granger et al., 1982], and in avian lens cells [Granger and Lazarides, 1984]. During myogenesis of skeletal muscle cells, desmin, vimentin, and synemin are present throughout the cytoplasm, primarily in a longitudinal array, until they begin to move to the Z-line periphery of myofibrils, in a transverse array, as the cells mature [Lazarides et al., 1982]. In mature avian skeletal muscle cells, the antibody labeling pattern of synemin was shown by Granger and Lazarides [1980] to be limited to Z-lines and very similar to that of desmin as shown by double immunofluorescence microscopy. By immuno-EM, using synemin antibodies followed by negative staining and low-angle shadowing of chicken erythrocytes, Granger and Lazarides [1982] observed a periodicity of synemin binding along vimentin filaments. This binding of synemin to vimentin filaments was more noticeable in areas where two IFs came together, suggesting that synemin may crosslink adjacent IFs.

Although synemin seems like an excellent candidate for possibly mediating desmin IF/myofibril interaction in skeletal muscle cells, other possible candidates
include the IFAP, plectin [Wiche, 1989; Wiche et al., 1991], and any of the proteins identified as peripheral Z-line proteins earlier in this section such as ankyrin [Nelson and Lazarides, 1983], spectrin [Langley and Cohen, 1986], and the non-muscle form of actin [Craig and Pardo, 1983; Otey et al., 1988; Bard and Franzini-Armstrong, 1991]. In the case of adult cardiac muscle (working muscle myocardium), the IFAP, paranemin [Breckler and Lazarides, 1982; Price and Lazarides, 1983] should also be considered a viable candidate for cross-linking IFs and myofibrils. One concern relative to synemin is that it has been reported that it is absent in adult avian cardiac muscle [Price and Lazarides, 1983] and in all mammalian muscles [Price and Lazarides, 1983; Price, 1987]. As shown in our laboratory [Bilak et al., 1990; Bright et al., 1991; and studies shown in this dissertation], however, synemin is present in mammalian muscle. Bilak et al. [1990] recently has shown by using purified synemin and purified desmin from avian gizzard that synemin interacts with desmin in vitro and disrupts the ability of desmin to assemble into normal, full-length, 10-nm diameter IFs. Also, synemin may copolymerize with desmin into heteropolymeric IFs [Granger and Lazarides, 1982], but this has not yet been shown clearly, and synemin seems unable by itself to assemble into an IF structure.

The objectives of the first part of my study were to clearly identify IFs in normal adult porcine skeletal muscle by using high resolution transmission
electron microscopy (TEM), and to examine the three-dimensional structural relationship of IFS to myofibrils by using stereomicroscopy. The objectives of the second part of my study were to demonstrate that the IFS I identified in the first part of my study were composed of desmin, and to determine if synemin colocalizes with desmin in adult mammalian skeletal and cardiac muscles by using immuno-EM techniques.

Explanation of Dissertation Format

This dissertation follows the alternate format, and has been prepared in the form of two manuscripts that will be submitted for publication. Some additional figures are included in this dissertation to provide additional examples and information. Most of these duplicates, however, will be omitted before submission of the manuscripts. The work for Section I was done solely by myself, with the advice of Drs. Richard M. Robson and Marvin H. Stromer. The experiments for Section II were primarily performed by myself, with the exception of the post-embedding immunogold labeling experiments, which also involved the expert technical help of Dr. Marvin H. Stromer. The General Introduction, which includes an extensive literature review, precedes Section I. The Literature Cited which follows Section II contains the references cited in the General Introduction and in the Overall Summary.
SECTION I. IDENTIFICATION OF INTERMEDIATE FILAMENTS IN
MATURE MAMMALIAN SKELETAL MUSCLE
IDENTIFICATION OF INTERMEDIATE FILAMENTS IN
MATURE MAMMALIAN SKELETAL MUSCLE

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ABSTRACT

In comparison to intermediate filaments (IFs) in many vertebrate cell types, IFs are not an obvious cytoskeletal filamentous system in vertebrate striated muscle cells. In fact, IFs rarely have been identified in normal mature mammalian skeletal muscle cells. We have systematically examined by electron microscopy samples of semitendinosus muscle removed immediately after death from healthy adult porcine animals. Tilted specimens for stereo pairs also were examined in order to elucidate the three-dimensional relationship of IFs and myofibrils. In all cells examined, myofibrils in most regions were too tightly packed to permit clear identification of IFs. However, in almost half the cells, some small areas, often near the cell periphery, could be found with increased intermyofibrillar space. In these regions, filaments with an average diameter of ~10-11 nm very frequently are observed in the intermyofibrillar spaces where they connect adjacent myofibrils at their Z-line levels. These IFs are especially evident when part of a myofibril leaves the sectioning plane, leaving only the periphery of the myofibrillar Z-line region. These results, plus examination of stereo pairs, indicate that IFs surround, rather than penetrate, the integral myofibrillar Z-line domains. IFs also were identified that connect the myofibrillar Z-lines to membranous structures such as mitochondria, and, in the case of myofibrils located near the cell periphery, also to nuclei and the sarcolemma.
Although not prominent in number, IPs are evident in normal mature mammalian skeletal muscle cells. These IPs primarily run transverse to the long axis of the cell in the intermyofibrillar spaces between Z-lines of adjacent myofibrils. At the Z-lines, IPs are attached or looped in a lateral, rather than end-on, fashion, and also connect myofibrils to other cellular structures including mitochondria, nuclei, and the sarcolemma.

Key words: intermediate filaments, desmin, skeletal muscle structure, cytoskeleton, myofibrils
Intermediate filaments (IFs) are one of the three major cytoskeletal protein filament systems in most vertebrate cells [Lazarides, 1980; Goldman et al., 1986]. The number of members of the IF protein family continues to grow [Skalli and Goldman, 1991] and can be divided by origin into at least six well-described groups [Steinert and Roop, 1988; Robson, 1989; Stewart, 1990]. Five are cytoplasmic IFs and include the keratins (epithelium), desmin (muscle), vimentin (mesenchyme), glial fibrillary acidic protein (GFAP; glia and astrocytes), and the neurofilament triplet (neurons). The sixth group consists of the lamins (nuclear lamina). The IF proteins also can be divided by comparison of primary structures into types I to V. Desmin, vimentin, GFAP and peripherin (peripheral nerves) share considerable homology and sequence principles, and represent type III IF proteins [Stewart, 1990]. Although many roles for IFs have been proposed, remarkably little is known about their specific function [Franke, 1987; Klymkowsky et al., 1989; Skalli and Goldman, 1991]. This is certainly true for desmin, the major IF protein of developed skeletal, cardiac, visceral and many vascular smooth muscle cells [Stromer, 1990]. The IFs of muscle cells often are ascribed a role in mechanical integration of cellular space and components [Lazarides, 1980], but the evidence remains unconvincing.

IFs are readily identified by electron microscopy in adult smooth muscle
cells, in specialized conducting cells of the heart (e.g., Purkinje fibers) [Thornell and Ericksson, 1981], in myogenic cells grown in culture, or in embryonic or immature cells in various stages of development [Bennett et al., 1979; Lazarides et al., 1982; Price and Sanger, 1983; Tokuyasu et al., 1985; Stromer, 1990; Chou et al., 1991], and are occasionally identified in adult striated muscle cells in a definitive disease process [Ferrans and Roberts, 1973; Edstrom et al., 1980; Stoeckel et al., 1981; Watkins et al., 1987; Pellissier et al., 1989]. In contrast, IFs have not easily been identified in normal adult vertebrate striated muscle cells [see discussions in Bennett et al., 1979; Bard and Franzini-Armstrong, 1991], even though immunofluorescence studies with desmin antibodies suggested already several years ago the consistent presence of desmin, mostly at the Z-lines, in adult striated muscle cells [Lazarides and Hubbard, 1976; Lazarides, 1980]. This lack of identification of IFs led to the suggestion that desmin may be present, but in a nonfilamentous form [Bennett et al., 1979; Lazarides et al., 1982].

Of the few reports describing IFs in normal adult striated (working myocardium and skeletal) muscle cells, most have been in cardiac muscle cells [Ferrans and Roberts, 1973; Forbes and Sperelakis, 1980; Richardson et al., 1981; Tokuyasu, 1983; Watkins et al., 1987]. Identification of IFs in normal adult skeletal muscle by conventional electron microscopy has been very rare. In the reports of IFs (or likely IFs) in normal adult skeletal muscle, these have
included avian [Page, 1969; Richardson et al., 1981; Tokuyasu et al., 1983] and amphibian [Nunzi and Franzini-Armstrong, 1980] samples. Pierobon-Bormioli [1981] examined intact mammalian muscle (rat diaphragm) by conventional electron microscopy and described transverse filamentous structures, some of which connected adjacent myofibrils at their Z-lines, but the filaments were described as irregular in thickness and unmeasurable. The possibility also has been pointed out for differences in IF number, location, and arrangement between cardiac and skeletal muscle and between avian and mammalian muscle cells [Tokuyasu, 1983; Watkins et al., 1987]. Besides infrequent identification, questions regarding the three-dimensional relationship of IFs and myofibrils remain unresolved, e.g., do IFs penetrate or come in direct contact with myofibrillar Z-lines [Ferrans and Roberts, 1973; Behrendt, 1977; Granger and Lazarides, 1978], or is there a distinct gap between IFs and the edges of the Z-lines [Tokuyasu et al., 1983; Bard and Franzini-Armstrong, 1991]. To address these questions and to provide more complete structural evidence for the presence and possible functions of desmin IFs in mature mammalian skeletal muscle cells, we have systematically examined samples of semitendinosus muscle from healthy adult porcine animals. Preliminary reports including parts of this study have been presented at meetings [Yagyu et al., 1990a,b].
MATERIALS AND METHODS

Conventional Tissue Preparation

Samples of semitendinosus muscle from seven mature (95 to 109 kg) swine, which were examined and their health approved by a USDA veterinarian, were removed immediately after death at the Iowa State University Meat Laboratory. Thin slices from the middle, dark portion of the semitendinosus muscle were restrained isometrically in a clamp before removal from the muscle. Clamped samples were either fixed promptly at 25°C (results in Fig. 1 only), 2°C (results in Fig. 2 only), or stored at 2°C for 3 hr before fixation at 25°C (results in Figs. 3-6, 8-11) in Karnovsky’s fixative [Karnovsky, 1965].

After fixation for 1.5 hr, samples were cut into ~1 mm cubes in phosphate-buffered saline (PBS), and fixed for another 1.5 hr. The samples were then post-fixed in 1% osmium tetroxide for 1.5 hr, dehydrated in a graded acetone series, infiltrated, and embedded in Epon-Araldite resin. Thin sections (~600 to 900 Å unless otherwise noted) were cut with an LKB ultramicrotome from at least six randomly selected blocks per sample. Sections were stained with 2% aqueous uranyl acetate and with Reynolds’ lead citrate, and examined in a JEOL JEM-100CXII transmission electron microscope operated at 80 kV. All sections shown herein have longitudinal or slightly oblique orientation unless otherwise noted. Stereo pairs were obtained by tilting the grids with tilt angles of ±10 to 25°. Images were recorded on Kodak SO-163 electron image film.
Unlabeled Antibody Decoration Procedure

Glycerinated muscle strips also were prepared from small samples of porcine semitendinosus muscle and processed for antibody decoration as described by Tokuyasu et al. [1983], except that the glycerination was done in the presence of additional protease inhibitors (2.5 $\mu$M leupeptin, 10 mg/l trypsin inhibitor from chicken egg white, and 2 mM phenylmethyl sulfonyl fluoride; Sigma Chemical Co., St. Louis, MO). Just prior to use, the strips were teased into thin fiber bundles in PBS, and rinsed well with PBS for 1 hr at 4°C. The fibers were then incubated with unlabeled antibodies to porcine skeletal muscle desmin [O’Shea et al., 1981; characterized as shown by Bilak et al., 1991] or preimmune serum, with constant agitation at 4°C overnight. After thorough washes with PBS, the samples were fixed in 3% glutaraldehyde in Millonig’s phosphate buffer (5.4% glucose, 2.26% NaH$_2$PO$_4$, and 2.52% NaOH, pH 7.2) for 1 hr, stained en bloc with 2% aqueous uranyl acetate, processed conventionally, and embedded in Epon-Araldite resin.
RESULTS

Porcine semitendinosus muscle samples removed immediately after death and fixed at either 2°C or 25°C generally showed a typical appearance of skeletal muscle, i.e., cells were packed with myofibrils with little intermyofibrillar space present (e.g., Fig. 1, top cell). This tight packing of myofibrils and nearby organelles did not permit clear visualization of other structures. However, in about half the cells examined, small regions could be found with increased intermyofibrillar spaces (Fig. 1, bottom cell). Another characteristic, typical of the porcine skeletal muscle examined, is the presence of significant numbers of glycogen granules, especially when the samples were removed immediately after death and promptly fixed at 2°C (Fig. 2). The glycogen granules were present in the intermyofibrillar spaces, and some of them were associated with transversely-oriented filaments connecting Z-lines of adjacent myofibrils. Their presence often hindered clear identification of filaments and measurement of their diameter. Immediate postmortem fixation at 2°C, however, had no effect on incidence of expanded intermyofibrillar spaces and presence of filamentous structures in these areas (Fig. 2).

Storage of muscle removed at death for 3 hr at 2°C, followed by fixation at 25°C, lessened the problem with glycogen granules without otherwise altering morphology of the muscle cell; thus, this method was routinely introduced
Figure 1. Electron micrograph of porcine semitendinosus muscle showing two adjacent muscle cells with very different degrees of intermyofibrillar space.

This sample was removed immediately postmortem and fixed at 25°C. The sarcolemma of the top cell (black arrows pointing down) and of the bottom cell (black arrows pointing up) are marked. The bottom cell is running at an oblique angle. The top cell is typical in appearance, with myofibrils and nearby organelles packed closely together (white arrowheads). The bottom, adjacent cell exhibits cellular regions with larger intermyofibrillar spaces (black arrowheads). The triads (short white arrows) are positioned at the A-I junctions. Z = Z-lines, M = M-lines, MT = mitochondria. Bar = 1.0 μm.
Figure 2. Electron micrograph of porcine semitendinosus muscle removed immediately after death and fixed at 2°C.

Note the abundance of glycogen granules (G and curved arrows). Many of them demonstrate an affinity for transversely-oriented filaments (arrows) connecting Z-lines of adjacent myofibrils. Z = Z-lines. Bar = 0.5 \mu m.
(Figs. 3-6, 8-11). An overview of an area with expanded intermyofibrillar spaces is shown in Figure 3. Filamentous structures connecting adjacent myofibrils at their Z-lines, or located at the periphery of myofibrils that have left the plane of section, are clearly demonstrated, in part because glycogen granules do not obscure their appearance. These filamentous connections are more visible in sections grazing the periphery of myofibrils. At higher magnification, transversely-oriented filaments are clearly seen that traverse the intermyofibrillar space between two myofibrils at their Z-lines, as well as at the periphery of the Z-line area where part of one myofibril leaves the sectioning plane (Fig. 4a,b). These filaments have an average diameter of 10-11 nm, characteristic of IFs.

In cross sections of myofibrils, IFs were observed that wrap around the circumference or periphery of myofibrils at the Z-line level, especially in regions of cells with expanded intermyofibrillar space (Fig. 5). In some places, there is some space between the surrounding IFs and the edge of the Z-line, but in other places the IFs are either very close to, or directly in contact with, the edge of the Z-line. Filamentous structures (black arrowheads) also appeared to be connecting myofibrils to the sarcolemma and to adjacent myofibrils (Fig. 5). Although the diameters of these latter filaments were ~11-12 nm, they were not easily measured.

Examination of stereo pairs obtained from areas including IFs and adjacent
Figure 3. Electron micrograph of an overview of porcine semitendinosus muscle fixed at 25°C following 3 hr of storage at 2°C. Note that the sectioning plane was very close to the surface of some myofibrils, and that parts of the myofibrils are not included in the sectioning plane (✧). Filamentous structures (large arrows) in the intermyofibrillar spaces connecting myofibrils at the Z-line level, or located at the periphery of myofibrils that have left the plane of section, easily can be identified. Although most of these structures were filamentous in morphology, occasionally it was difficult to discern whether some structures were filamentous or membranous (black arrowheads) in nature. Z = Z-lines. Bar = 1.0 μm.
Figure 4. Electron micrographs of porcine semitendinosus muscle at higher magnification showing transversely-oriented IFs.  
a) Filamentous structures (large arrows) are clearly seen that traverse the intermyofibrillar space between the Z-lines of two myofibrils, and at the periphery of the Z-line region of the myofibril on the right that has partly left the sectioning plane ( ◊ ). Filaments (small arrow) also are seen connecting the myofibrillar Z-line to a mitochondrion. The filamentous structures have an average diameter of 10 to 11 nm and will be referred to hereinafter as IFs. \( Z = Z \)-lines, \( MT = \) mitochondria. \( \text{Bar} = 0.5 \ \mu m. \)
b) This is another example showing IFs (arrows) connecting two myofibrils at their Z-line levels, and also where part of one myofibril (on the left) partly leaves the sectioning plane (◊). Z = Z-lines.

Bar = 0.5 μm.
Figure 5. Electron micrograph of a semi-thin (~1200 Å) cross section of porcine semitendinosus muscle.

This example shows a cross section including a fiber sectioned through the Z-line plane. Filaments (arrows) having a diameter of IFs are visible around the periphery of the myofibril at its Z-line level. At many places, two rings of IFs can be seen (black arrows), and sometimes possibly three rings (white arrows). Some filamentous structures (arrowheads) also appear to be traversing the spaces between the myofibril sectioned at the Z-line and other myofibrils or the sarcolemma. Z = Z-lines, C2 = an adjacent cell. Bar = 0.25 μm.
myofibrils, especially when the sectioning plane was near the periphery of the
myofibrils (Fig. 6a,b), showed that the transversely-oriented IFs are not in the
same plane as that of the thin (actin) filaments. This suggests that the IFs
primarily surround the myofibrillar Z-lines in a lateral, rather than end-on,
fashion. Longitudinally-oriented IFs also were observed, but very infrequently
(Fig. 6b).

To obtain additional information about arrangement and protein composition
of IFs in adult mammalian skeletal muscle cells, very thin strips of glycerol-
extracted porcine semitendinosus muscle were teased into fiber bundles,
incubated with unlabeled anti-desmin antibodies, embedded and examined (Fig.
7). Bundles of transversely-oriented filaments were readily recognizable at
inter-Z-line regions, and at the myofibrillar Z-line periphery when the sectioning
plane was close to the surface of the myofibrils (Fig. 7). On the same grid,
cross-profiles of IFs also were observed in the intermyofibrillar space between
Z-lines (Fig. 7, inset). The results shown in Figure 7 were obtained from the
outer part of a muscle fiber bundle where antibodies had penetrated. Samples
obtained from the interior of fiber bundles did not routinely show bundles of IFs
that were as easily identified (not shown). The contours of the IFs visualized
by this protocol [Tokuyasu et al., 1983] appeared rather fuzzy, presumably due
to the attachment of antibodies to the filaments, as has also been shown in
studies on avian muscle [Tokuyasu et al., 1983].
Figure 6. Stereo pairs of porcine semitendinosus muscle showing IFs.

a) Transversely-oriented IFs (arrow) are located at the Z-line level. Parts of some of the myofibrils have left the plane of section, i.e., the section is near the outer surfaces of the myofibrils. IFs can be distinguished in the x-z direction with regard to the plane of the thin filaments, i.e., the IFs appear to go above the plane of the thin filaments. This suggests that the IFs surround or attach to the myofibrillar Z-line in a lateral fashion. Z=Z-lines. Tilt angle = ±25°. Bar = 1.0 μm.
b) This is another example showing IFs at the edge of the Z-lines. The transversely-oriented IFs are located under the plane of the thin filaments at the site (arrow) where part of one myofibril leaves the plane of section. In addition, a longitudinally-oriented IF (arrowhead) is present. Z = Z-lines, MT = mitochondria. Tilt angle = ±25°. Bar = 0.5 μm.
Figure 7. Electron micrograph of glycerinated porcine semitendinosus muscle fiber bundles incubated with anti-desmin antibodies before fixation and embedment.

Bundles of IFs (arrows) are enhanced and clearly visible at the levels of Z-lines in the intermyofibrillar spaces, and at the periphery of myofibrils leaving the sectioning plane. Many of the IFs appear somewhat fuzzy, presumably due to binding of antibodies. Inset: This micrograph is from the same grid and shows cross-profiles (arrow) of about six IFs in the intermyofibrillar space between Z-lines. Z = Z-lines. Bars = 0.5 μm.
IFs also were identified that connected myofibrils and membranous structures in the cell (Figs. 8-10). IFs often were recognized near mitochondria (Fig. 8). The filaments appear to be attached to the Z-line at one end and to mitochondria at the other. When IFs connect mitochondria to myofibrils, the mitochondria often were seemingly pulled into odd shapes, indicating strong connections (results not shown).

In sections that included areas near the sarcolemma, IFs were discerned that connect Z-lines of the outermost layer of myofibrils to the sarcolemma (Fig. 9a-c). Some filamentous connections between the M-lines and the sarcolemma also were observed (Fig. 10a,b), but with less frequency than the ~10 nm diameter IFs identified at the Z-lines. The filaments occasionally present at the M-line level generally had a smaller diameter (4-8 nm). Areas of cells between nuclei and the outermost layer of myofibrils also were examined. This perinuclear region often is filled almost entirely by membranous systems/organelles. Only when we found increased cellular space near nuclei, were IFs discerned connecting the nuclear envelope and the nearby myofibrils at their Z-line levels (Fig. 10).
Figure 8. Electron micrograph showing myofibrils and nearby mitochondria in the intermyofibrillar spaces of porcine semitendinosus muscle. IFs (arrows) emanate from the Z-line region of a myofibril and appear to be connected to mitochondria. Z=Z-lines, MT=Mitochondria. Bar = 0.5 µm.
Figure 9. Electron micrographs of porcine semitendinosus muscle showing myofibrils near the sarcolemma.

a) IFs (large arrows) extend from the Z-lines of the peripheral myofibril to the sarcolemma (arrowheads). Occasionally, very thin (~4-8 nm diameter) filamentous structures (small arrows) are observed that run from the myofibrillar M-line region to the sarcolemma. Z = Z-lines, M = M-lines, C = collagen. Bar = 0.5 μm.
Figure 9 (continued).

b) This is another example in which IFs (long black arrows) connect the Z-line regions of a peripherally located myofibril to the cell membrane (black arrowheads). This section contains the surface of a myofibril, and parts of it are not included in the section. Filamentous structures also are observed that connect the M-line regions (short black arrows) to the sarcolemma. Z = Z-lines, M = M-lines, Nu = nucleus, white arrows = sarcolemma of the top muscle cell. Bar = 1.0 μm.

c) This shows another example in which IFs (arrows) of a peripherally located myofibril, sectioned near its surface, connect the Z-line region of the myofibril to the sarcolemma (arrowheads). Z = Z-lines. Bar = 1.0 μm.
Figure 10. Electron micrograph of porcine semitendinosus muscle showing myofibrils near mitochondria and a nucleus.
IFs (arrows) can be followed from the Z-line region, along a mitochondrion, and then to sites on the nuclear envelope. Z = Z-lines, Nu = nucleus, MT = mitochondria. Bar = 0.5 μm.
DISCUSSION

Presence and Location of IFs in Normal, Mature Mammalian Skeletal Muscle Cells

We have utilized conventional electron microscopy, examination of stereo pairs, and examination of unlabeled desmin antibody-decorated samples in this study of IFs. Our three major findings are: (1) IFs readily can be identified in normal adult mammalian skeletal muscle cells if systematically examined, (2) there is a close structural relationship between IFs and myofibrillar Z-lines, with IFs connecting both the Z-lines of adjacent myofibrils and Z-lines with nearby membranous structures; the location and diameter of filamentous structures identified as IFs herein were: near Z-lines = 10.8 nm ± 0.33 nm, near mitochondria = 11.1 nm ± 0.39 nm, near sarcolemma = 10.7 nm ± 0.36 nm, and near nuclei = 11.6 ± 0.39 nm (numbers refer to means ± standard errors of the means); and, (3) IFs surround the myofibrillar Z-lines in a lateral, rather than end-on, fashion, i.e., the IFs evidently do not penetrate the integral Z-line domain.

Based primarily upon immunofluorescence localization studies with desmin antibodies [summarized in Lazarides, 1980; and see discussion in Richardson et al., 1981], it often has been assumed, albeit with little direct evidence, that desmin IFs are present in adult skeletal muscle cells. With the exception of the results shown herein, IFs have not readily been observed in healthy mammalian
skeletal muscle cells. One reason for difficulty in identifying IFs in these cells, in comparison to other types of adult muscle cells, is the amount of desmin present. Desmin comprises only ~0.16% of skeletal muscle protein [O’Shea et al., 1981], as compared to ~1-2% of cardiac muscle protein [Hartzer, 1984], and ~5% of visceral smooth muscle protein [Huiatt et al., 1980]. Secondly, tight packing of myofibrils and membranous structures/organelles in mature skeletal muscle cells leaves little space in which to identify other structures (e.g., IFs) present in small number. Samples were systematically and painstakingly examined in order to identify areas with increased intermyofibrillar spaces in this study. A third complicating factor was the presence of significant numbers of glycogen granules. Many of them demonstrated affinity for the IFs, in agreement with observations on amphibian skeletal muscle [Nunzi and Franzini-Armstrong, 1980] and canine cardiac conduction fibers [Rybicka, 1981]. To alleviate this problem with glycogen granules, which often partly obscured filament contours, extensive preliminary studies were conducted to compare several postmortem handling and fixation protocols. We found that simply storing muscle for 3 hr at 2°C, followed by fixation at 25°C, reduced the severity of the problem without altering either the incidence of areas with enlarged intermyofibrillar spaces or other important morphological aspects of the cell.

Difficulty in identifying IFs in normal adult skeletal muscle cells led to the
suggestion that desmin may be present, but in a non-filamentous form in these cells [Bennett et al., 1979; Lazarides et al., 1982]. Although we can not rule out completely the possibility of some nonfilamentous desmin in our samples, we believe the evidence [shown herein and in Bilak et al., 1991] indicates that desmin in normal mammalian skeletal muscle cells exists as IFs, in accord with our earlier report [Richardson et al., 1981] and that of Tokuyasu et al. [1983] on avian skeletal muscle. Whenever longitudinal sections of areas with enlarged intermyofibrillar spaces were examined (e.g., Figs. 2-4), IFs could be identified.

Two electron microscope studies on normal adult mammalian skeletal muscle and putative IFs, relevant to our studies, have been reported. Pierobon-Bormioli [1981] identified filamentous structures in rat diaphragm muscle that connected adjacent myofibrils at their Z-lines, and peripheral myofibrils to the sarcolemma at both Z- and M-lines, but the filaments were reported as being irregular in thickness, and filament diameters could not be measured. In a study of rabbit skeletal muscle, Wang and Ramirez-Mitchell [1983] examined residues remaining after extracting most of the thick and thin filaments with a KI solution. They observed filaments of about 10 nm diameter that ran in a direction transverse to the long myofibrillar axis, which seemingly connected Z-line remnants to Z-line remnants of adjacent myofibril residues or M-line remnants to M-line remnants of adjacent myofibril residues. It seems likely that
the transversely-oriented filaments observed at the Z-line by these workers were similar to the ones observed in our study. Although we also observed transversely-oriented filaments connecting M-lines of adjacent myofibrils, and M-lines of peripheral myofibrils to the sarcolemma, these filaments were not often found, and their diameters were less (~4-6 nm) than is typical of IFs. Wang and Ramirez-Mitchell [1983] also described longitudinally-oriented filaments of about 10 nm diameter that connected successive Z-line remnants within each myofibril residue. Whether those consisted of desmin, or possibly of remnants of the highly insoluble titin [Wang et al., 1979; Furst et al., 1988] and/or nebulin [Wang and Ramirez-Mitchell, 1988] filamentous systems, is not clear. We observed longitudinally-oriented IFs in this study, but infrequently.

Nunzi and Franzini-Armstrong [1980] used a hypotonic solution to increase intermyofibrillar space in their studies of IFs in amphibian muscle. It is likely that one of the two sets of filaments they described (long transversely-oriented filaments that encircled the myofibrillar Z-lines and seemingly connected Z-lines of adjacent myofibrils) are homologous to the filaments described in our study. IFs also occasionally have been identified in diseased adult mammalian skeletal muscle cells [Edstrom et al., 1980; Stoeckel et al., 1981; Pellisier et al., 1989]. Thus, we obtained muscle samples only from animals examined and approved as healthy by a USDA veterinarian.

We often identified IFs that connect myofibrils to membranous structures
(Figs. 8-10), including mitochondria, nuclei, and the sarcolemma. And, often these IFs could be followed from myofibrillar Z-lines to the membranous site(s). These results are in general accord with those of others, who also have shown association of IFs with membranes. Goldman et al. [1986], for example, have shown in several cell types that IFs form networks between the nucleus and cell membrane. Specific binding sites for vimentin IFs have been identified at the cell membrane [Georgatos et al., 1985] and nuclear envelope [Georgatos and Blobel, 1987] of erythrocytes. Some variability has been described in the case of muscle cells. Ferrans and Roberts [1973] observed IFs in mammalian (canine) cardiac muscle cells that appeared to interconnect myofibrils, nuclei and the intercalated disk. Watkins et al. [1987], however, in a study on rat cardiac muscle cells, observed few IF connections between myofibrils and mitochondria, nuclei or the sarcolemma. In avian cardiac muscle, Tokuyasu [1983] observed IF connections between myofibrils and nearby mitochondria and nuclei, in accord with the study by Ferrans and Roberts [1973]. And, in avian smooth muscle cells, Stromer and Bendayan [1990] recently identified desmin IFs that form linkages with the nuclear envelope and with mitochondria. They suggested that these IFs did not simply pass near the organelles, but were directly linked to the surface of the membranes. Although the results in our study are in general accord with most of these studies [Ferrans and Roberts, 1973; Tokuyasu, 1983; Stromer and Bendayan, 1988], we can not state
unequivocally the exact nature (e.g., is attachment lateral or end-on?) of the IF-membrane attachment.

Structural Relationship of IFs to the Myofibrillar Z-line

Our studies on IFs in mammalian skeletal muscle cells show a definite and interesting relationship between myofibrillar Z-lines and IFs. It has been proposed that there is a direct attachment between IFs and Z-lines [Granger and Lazarides, 1978], and some reports on mammalian cardiac muscle cells [Ferrans and Roberts, 1973; Behrendt, 1977] have even indicated that IFs penetrate into myofibrils. Others, however, have found no evidence that IFs penetrate myofibrils [Tokuyasu, 1983; Tokuyasu et al., 1983], and some reports indicate no direct attachment of IFs to the edge of Z-lines [Tokuyasu et al., 1983; and see discussion in Bard and Franzini-Armstrong, 1991]. Most of the IFs identified in our study are transversely-oriented with respect to the long axis of the myofibrils and cell. They most easily can be identified in the intermyofibrillar space where they connect Z-lines of adjacent myofibrils. Provided the section includes the very outer periphery of myofibrils, the IFs also easily can be identified in regions where the myofibrillar Z-lines leave, or nearly leave, the plane of section. Thus, the IFs appear to run along the outer edge of the myofibrillar Z-lines, i.e., they form a collar-like arrangement around the Z-line. Examination of stereo pairs verified this arrangement, with the IFs
positioned above or below myofibrillar components in the Z-line region.

Examination of cross sections of myofibrils sectioned at their Z-lines further supported the location of IFs around the circumference of the Z-lines. Although discontinuities made it impossible to trace a given IF, at least two rows of IFs could be identified as forming "circles" around and near the edge of the Z-line (Fig. 5 and results not shown). Taking into account sectioning angle, and possible superimposition of structures within a section, it is difficult to unequivocally judge how close the IFs come to myofibrillar components. Certainly some distance, ~20 to 30 nm, separates some (the outermost ring) circumferentially arranged IFs from the Z-line edge, but in several places around the circumference of the Z-line, no detectable space between IFs and the edge of the Z-line could be ascertained, i.e., the IFs certainly come very close to, or actually impinge upon, the edge of the Z-line. Cross-profiles of IFs observed in longitudinal sections also were close (within ~8 nm) to the edge of the Z-line (e.g., Fig. 7). Our results on the relationship of IFs to the Z-line, taken in toto, (1) strongly suggest that the IFs are associated with Z-lines in a lateral, rather than end-on, fashion, much like the IFs associated with the desmosomes of intercalated disks [Kartenbeck et al., 1983; Tokuyasu, 1983], and (2) suggest that any distance separating some IFs and Z-lines is small enough that the end domains of desmin, known to be present at the surface of IFs [Kaufmann et al., 1985], or possibly IF-associated proteins [e.g., synemin, Granger and Lazarides,
1980; Sandoval et al., 1983], should be able to bridge gaps between the backbone core of the IFs and myofibrillar components. As will be shown in an accompanying paper [Bilak et al., 1991], immunoelectron microscope localization studies position synemin in the appropriate area necessary for such a possible crosslinking role. Evidence, however, for either direct desmin interaction with a Z-line protein, or of a desmin-synemin-Z-line protein interaction, is necessary to make this a more plausible scenario. Such studies are currently in progress.
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REFERENCES


SECTION II. IMMUNOELECTRON MICROSCOPE LOCALIZATION OF DESMIN AND SYNEMIN IN MATURE STRIATED MUSCLE
IMMUNOELECTRON MICROSCOPE LOCALIZATION OF DESMIN AND SYNEMIN IN MATURE STRIATED MUSCLE

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Running Title: Immunolocalization of Desmin and Synemin
Intermediate filaments (IFs) of mature striated (especially mammalian skeletal) muscle cells have not been readily identified. We have used antibodies to desmin and synemin (an IF-associated protein) and protein A-gold pre-embedding labeling of porcine skeletal and cardiac muscle myofibrils and post-embedding labeling of intact porcine skeletal muscle, to demonstrate localization of these proteins. Although the intensity of labeling with anti-synemin was generally lower than with anti-desmin, the pre-embedding labeling patterns of myofibrils obtained with the two antibodies were very similar, i.e., the gold labels were primarily transversely-oriented in the intermyofibrillar region between Z-lines of adjacent myofibrils, and at the periphery of the Z-line region of myofibrils that had been sectioned near their surface. Labeling also was observed at the intercalated disk region when present with cardiac muscle myofibrils. Occasionally, some longitudinally-oriented filaments in the intermyofibrillar spaces and close to the surface of the myofibrils, also were labeled, especially in the cardiac muscle samples. Intense labeling was observed with anti-desmin between myofibrils and associated sarcolemmal or nuclear remnants. Examination of stereo pairs suggests that IFs are arranged in a complex network around myofibrils and nearby nuclear remnants. Localization of desmin and synemin in post-embedded labeled intact muscle
samples was in accord with the results obtained in the pre-embedding labeling studies, indicating that the labeling patterns observed in the myofibril samples were representative of the proteins’ in situ locations. Results of this study, together with those of our companion electron microscope examination study of adult porcine skeletal muscle [Bilak et al., 1991a], support the cytoskeletal concept that desmin IFs link myofibrils together at their Z-lines, and the myofibrils to nearby membranous structures, in mature mammalian striated muscle cells. Synemin is either part of, or attached to, the desmin-IFs.

Key words: immunogold, desmin, synemin, intermediate filaments, striated muscle structure, cytoskeleton
Intermediate filaments (IFs) are prominent cytoskeletal components in differentiating vertebrate smooth, cardiac, and skeletal muscle cells, and in adult vertebrate smooth muscle and specialized conducting cells of the myocardium [for reviews, see Price and Sanger, 1983; Stromer, 1990]. The IFs have not been as readily identified in adult vertebrate cardiac (working myocardium) and skeletal muscle cells, where desmin, a type III IF-protein [Steinert and Roop, 1988; Robson, 1989; Stewart, 1990], comprises only about 1-2% [Hartzer, 1984] and 0.16% [O'Shea et al., 1981], respectively, of total muscle protein.

Immunofluorescence localization studies indicated over ten years ago [see Lazarides, 1980] that desmin was present in both types of adult striated muscle cells, and that it was located primarily at or near the periphery of the myofibrillar Z-lines of skeletal muscle and the Z-lines and intercalated disks of cardiac muscle cells. These light microscope studies left many unanswered questions, such as the filamentous vs. nonfilamentous state of desmin, due in large part to the rare identification of IFs in these mature cells, especially skeletal muscle cells [Bennett et al., 1979; but see accompanying paper, Bilak et al., 1991a]. Immunoelectron microscope (immuno-EM) studies, of avian skeletal [Richardson et al., 1981; Tokuyasu et al., 1983a, 1985], avian cardiac
Richardson et al., 1981; Tokuyasu, 1983; Tokuyasu et al., 1983b, 1985), and mammalian cardiac [Kartenbeck et al., 1983; Watkins et al., 1987; Sætersdal et al., 1989] muscle have shown that, at least in these specific cases, most, if not all, of the desmin is present in the form of IFs. In general, these studies have shown that the desmin IFs were present primarily in a transversely-oriented arrangement between the Z-lines of adjacent myofibrils, although some longitudinally-oriented IFs were described in avian cardiac [Tokuyasu, 1983] and in some [Sætersdal et al., 1989], but not all [Watkins et al., 1987], reports on mammalian cardiac muscle cells. Possible differences between avian and mammalian muscle, and between cardiac and skeletal muscle cells, in IF location and arrangement have been noted [Tokuyasu, 1983; Watkins et al., 1987].

Another key question involves the nature of the three-dimensional relationship between IFs and myofibrils. Some reports have suggested that IFs may penetrate myofibrillar Z-lines [Ferrans and Roberts, 1973; Behrendt, 1977; Granger and Lazarides, 1978], while others have indicated no penetration [Tokuyasu, 1983; Watkins et al., 1987; Sætersdal et al., 1989], or that there is even a distinct gap between IFs and the edges of the Z-lines and that perhaps another protein(s) may crosslink these two cytoskeletal elements [Tokuyasu et al., 1983a, 1985; also see discussion in Bard and Franzini-Armstrong, 1991]. In our accompanying paper [Bilak et al., 1991a], we present evidence that IFs
certainly come very close to, if not in direct contact with, the Z-lines of adult mammalian skeletal muscle cells.

Granger and Lazarides [1980] demonstrated by using immunofluorescence localization that an IF-associated protein (IFAP), synemin, is present in both developing myogenic and mature skeletal muscle cells. Synemin is an incompletely characterized, 230 kD protein shown to be present in several avian cell types, including all developing muscle cells (regardless of their type), adult smooth and skeletal muscle cells, cardiac conducting fibers [Price and Lazarides, 1983], erythrocytes [Granger et al., 1982; Price and Lazarides, 1983], lens cells, and ependymal tanycytes [Granger and Lazarides, 1984]. Because synemin is found together with one or the other of two type III IF proteins, desmin or vimentin, several crosslinking roles (e.g., crosslinking IFs, crosslinking IFs to other cellular structures, etc.) [Granger and Lazarides, 1980, 1982, 1984] have been proposed for synemin. Unfortunately, as is the case for IF proteins in general [Bloemendal and Pieper, 1989; Klymkowsky et al., 1989; Skalli and Goldman, 1991], none of the roles proposed for synemin have been confirmed. Nevertheless, that synemin is colocalized with desmin in adult skeletal muscle cells, raises the intriguing possibility that synemin may crosslink IFs and myofibrils, perhaps by bridging any small gap between IFs and the edge of the myofibrillar Z-lines.

In an accompanying report [Bilak et al., 1991a], we have described for the
first time in healthy adult mammalian skeletal muscle cells, the clear identification of IFs by using conventional electron microscopy. In the present study, we report the first immuno-EM localization of desmin in mature mammalian skeletal muscle cells and the first immuno-EM localization of synemin in any type of vertebrate muscle cell. Two complementary immuno-EM localization approaches, namely pre-embedding labeling of glycerinated porcine skeletal and cardiac myofibrils with anti-desmin or anti-synemin, and post-embedding labeling of intact porcine skeletal muscle with the two antibodies, have been employed. A preliminary report, including part of this study, was presented at the 1990 American Society for Cell Biology meeting [Yagyu et al., 1990].
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MATERIALS AND METHODS

Immunological Reagents

Rabbit anti-desmin antibodies were prepared by the procedure of Richardson et al. [1981] to highly purified porcine skeletal muscle desmin [O'Shea et al., 1981]. Rabbit anti-synemin antibodies to highly purified turkey gizzard synemin [Bilak et al., 1991b] were prepared by the method of Knudsen [1985], and affinity-purified by electroblotting a thin strip of SDS-polyacrylamide gel, which contained only the 230 kD synemin band, to APT (aminophenylthioether) paper (Schleicher & Schuell, Keene, NH) using the procedure supplied by the manufacturer. Monospecificity of both antibodies was shown by Bilak et al. [1991b] and confirmed in this study by immunofluorescence labeling of porcine striated muscle myofibrils and by Western blot analysis [Towbin et al., 1979] of IF-enriched fractions from porcine striated muscle. After the electrophoretic transfer of the proteins onto nitrocellulose paper by the method of Towbin et al. [1979], the strips of nitrocellulose paper were blocked with 1% Blotto in Tris-buffered saline (TBS; 0.5 M NaCl, 0.02 M Tris-HCl, pH 7.0) for 30 min at room temperature, with constant agitation, and then incubated with either a 1:20 dilution of anti-desmin or a 1:10 dilution of affinity-purified anti-synemin overnight at 4°C, with constant agitation. After three washes in TBS, the strips were then incubated with horseradish-peroxidase-goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) for 1 hr at 37°C, with constant agitation. The
reactive bands were visualized by 4-chloro-1-naphthol and hydrogen peroxide. The IF-enriched fractions used for immunoblotting were prepared from porcine semitendinosus and cardiac muscle myofibrils by the method of O'Shea et al. [1981]. Briefly, myofibril samples were extracted twice with 1 M KI, 20 mM imidazole-HCl, pH 7.1, and once in H2O [corresponds to Step IX in O'Shea et al., 1981], and the resulting sediment was dissolved in 6M urea. SDS-polyacrylamide gels (8.5% acrylamide) were run by the procedure of Laemmli [1970]. Additional details will be given in the results.

Immunofluorescence Labeling

Samples of semitendinosus and heart (right ventricle and papillary) muscles from six mature (~100-110 kg) swine, which were examined and their health approved by a USDA veterinarian at the Iowa State University Meat Laboratory, were removed immediately after death. Muscle strips of ~2-5 mm diameter were cut from the tissues, slightly stretched, and tied to wooden sticks, at 4°C. The strips were soaked overnight at 4°C, with gentle stirring, in 10 vol (wt/vol; wt refers to wet weight in gm of muscle strip) of a solution containing 75 mM KCl, 1 mM MgCl2, 2-6 mM ethylene glycol-bis(ß-aminoethyl ether) N,N,N′,N′′-tetraacetic acid (EGTA) (2 mM for skeletal and 6 mM for cardiac; Sigma Chemical Co., St. Louis, MO), 10 mM potassium phosphate, 2.5 μM leupeptin (Sigma), 2 mM phenylmethyl sulfonyl fluoride (PMSF), 10 mg/l trypsin inhibitor
from chicken egg white (Sigma), 0.5% (v/v) Triton X-100, pH 7.0 (Buffer A).
In the case of semitendinosus muscle samples, after overnight extraction, the
strips were minced into small cubes and homogenized for 30 sec in 10 vol of
Buffer A without Triton in a small Waring blender at 4°C. The samples were
then hand homogenized by using 50 strokes in a glass Dounce hand
homogenizer. In the case of heart muscle samples, after the overnight
extraction, the strips were minced into small cubes and homogenized in 10 vol
of Buffer A without Triton at 4°C by using 50 strokes with a motor driven
Teflon-glass homogenizer (Talboys Engineering Corp., Emerson, N.J.), and then
50 strokes in a glass Dounce hand homogenizer. After these relatively mild
homogenizations, both skeletal and cardiac muscle samples were then
centrifuged at 1,400 xg, and the pelleted myofibrils were resuspended in 50%
(v/v) glycerol, 1 mM EGTA, 2.5 μM leupeptin, 2 mM PMSF, in phosphate-
buffered saline (PBS), and stored at -29°C until ready for use (1 to 2 weeks).

For immunofluorescence labeling, 1-2 ml of the glycerinated myofibrils (~8 mg/ml) were washed three times with PBS at 4°C by repeated centrifugation at
1,400 xg and resuspension of the pellets. A few drops of the washed
myofibrils were allowed to settle down on microscope slides, and were fixed at
2°C with methanol for 5 min. After three washes in PBS, the myofibrils were
incubated with primary antibodies (anti-desmin or anti-synemin) diluted 1:50
with PBS containing 1% goat serum (Sigma) for 30 min at 37°C in a moist
chamber. After three washes with PBS, they were incubated with rhodamine-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA) that had been diluted 1:100 with PBS containing 1% goat serum for 45 min at 20°C. After three washes in PBS, the samples were examined in a Zeiss Photomicroscope III equipped with epifluorescence optics. Photographs were recorded with Kodak Tri-X film.

Pre-embedding Immunoelectron Microscopy

Samples of washed (PBS) myofibrils (from the same stocks as used in the immunofluorescence studies) were lightly fixed at 4°C for 30 min in a solution containing 0.25% paraformaldehyde, 1% (v/v) Triton X-100, and then washed three times with PBS at 4°C. The fixed myofibrils were incubated with 1:5 dilutions of primary antibodies for 30 min at 37°C (anti-desmin) or for 5 hr at 20°C (anti-synemin). Myofibril samples also were incubated with rabbit preimmune sera as controls. After three washes with PBS at 4°C, the myofibrils were incubated for 45 min at 37°C with 5-nm colloidal gold-Protein A-complex (Amersham, Corp., Arlington Heights, IL), which had been diluted 1:20 with 1% goat serum in PBS just prior to use. After three washes with PBS at 4°C, the immunolabeled myofibrils were sedimented at 1,400 xg for 10 min. The pellets were fixed in 3% glutaraldehyde in PBS overnight at 4°C, post-fixed with 1% osmium tetroxide for 1.5 hr, conventionally processed, and
embedded in Epon-Araldite resin. Sections of desired thickness (~600-900 Å for thin sections, ~1000-1200 Å for semi-thin sections, ~1/4 μm for thick sections) were lightly stained with 2% aqueous uranyl acetate and with Reynold’s lead citrate to slightly add contrast, and examined in a JEOL JEM-100CXII transmission electron microscope operated at 80 or 100 kV.

Post-embedding Immunoelectron Microscopy

Samples of porcine semitendinosus muscle were removed immediately after death from five of the same healthy mature animals obtained at the Iowa State University Meat Laboratory and used for the immunofluorescence studies. Thin slices of tissue from the dark, inner portion of the muscle were restrained isometrically in a surgical clamp before the ends of the slices were cut, and then fixed in 2% paraformaldehyde, 1.5% glutaraldehyde in Millonig’s phosphate buffer (5.4% glucose, 2.26% NaH₂PO₄, 2.52% NaOH, pH 7.2) for 2.5 hr at 2°C. Samples were cut into ~1 mm cubes and rinsed in 0.15 M glycine to quench free aldehyde groups. They were then dehydrated in a graded series of methanol, embedded in Lowicryl K4M resin, and polymerized in a UV chamber at -29°C for 7-10 days [Stromer and Bendayan, 1988]. Sections of ~600-1200 Å thickness were placed on Formvar/carbon-coated grids. Colloidal gold particles (~14-nm) were coupled with protein A (Sigma) by the procedure of Bendayan [1984]. The labeling also was essentially done
according to the method described by Bendayan [1984]. Controls, either substituting PBS or preimmune serum for the anti-serum, were included. Labeled sections were lightly stained with 2% aqueous uranyl acetate and with Reynold's lead citrate to increase contrast, and examined in a JEOL JEM-100CXII transmission electron microscope operated at 80 kV.
RESULTS

Immunofluorescence Labeling and Immunoblotting

The anti-desmin antibodies used in this study specifically label the Z-lines of adult porcine striated muscle myofibrils by indirect immunofluorescence labeling (Fig. 1a,b). Anti-desmin labeling shows the familiar punctate staining pattern [Richardson et al., 1981]. The staining pattern obtained with anti-synemin (Fig. 1c,d) is very similar to that obtained with anti-desmin. Specificity of the antibodies also was shown by Western blot analysis (Fig. 2A-C). As shown in Figure 2, the polyclonal anti-desmin we used recognizes avian smooth as well as mammalian skeletal and cardiac desmins (Fig. 2B). The antibodies show no cross-reactivity to other myofibrillar/cytoskeletal proteins, including synemin, in the porcine striated muscle fractions (Fig. 2B). The mammalian skeletal and cardiac muscle desmins have a slightly slower mobility than that of avian desmin in SDS-polyacrylamide gels run by the Laemmli [1970] procedure, a result which we previously have shown [O'Shea et al., 1979]. The anti-synemin we used recognizes avian synemin but not desmin (Fig. 2C). The antibodies to avian synemin show cross-reactivity only with synemin (a major synemin band at 225 kD and a minor synemin band at 195 kD) in the porcine skeletal and cardiac muscle fractions (Fig. 2C), as has been described in detail in Bilak et al. [1991b]. Mammalian synemin is either composed of two isoforms of synemin, or more likely, the lower band (195 kD) represents a proteolytic
Figure 1. Immunofluorescence localization of desmin and synemin in porcine semitendinosus muscle myofibrils.

(a,b) Paired phase (a) and immunofluorescence (b) images of myofibrils labeled with polyclonal anti-desmin show characteristic punctate anti-desmin staining at the Z-lines.  

(c,d) Paired phase (c) and immunofluorescence (d) images of myofibrils labeled with polyclonal anti-synemin show that the anti-synemin labels the Z-lines in a fashion similar to that of anti-desmin.  Arrows = Z-lines.  Bar = 10 μm.
Figure 2. Characterization of anti-desmin and anti-synemin antibodies by Western blot analysis.

(A) SDS-polyacrylamide gel stained with Coomassie Blue; (B) Western blot of a duplicate gel shown in (A) incubated with anti-desmin; (C) Western blot of a duplicate gel (except for lane 1 as described below) shown in (A) incubated with anti-synemin. Lane 1 = purified turkey gizzard synemin [Bilak et al., 1991b]; lane 2 = purified turkey gizzard desmin [Huiatt et al., 1980]; lane 3 = IF-enriched fraction from porcine semitendinosus muscle [O'Shea et al., 1981]; and, lane 4 = IF-enriched fraction from porcine cardiac muscle myofibrils prepared as described for the sample shown in lane 3. As shown in (B), anti-desmin antibodies recognize turkey gizzard desmin (lane 2) as well as the homologous porcine skeletal (lane 3) and cardiac (lane 4) desmins, but not avian (lane 1) or porcine (lanes 3, 4) synemins or other proteins present. As shown in (C), anti-synemin antibodies recognize avian synemin (lane 1), and the homologous mammalian synemin (two bands) in porcine skeletal (lane 3) and cardiac (lane 4) samples, but not avian (lane 2) or porcine (lanes 3, 4) desmins or other proteins present. Lane 1 in the gel used for the immunoblot in (C) was loaded with one tenth as much synemin as in lane 1 of (A) and (B) in order to show a strong reaction product with avian synemin, yet still be able to resolve the difference between avian 230 kD synemin and the major mammalian 225 kD synemin band.
fragment of 225 kD synemin [Bilak et al., 1991b].

Pre-embedding Immunogold Labeling of Porcine Skeletal Muscle Myofibrils

By transmission electron microscopy, control samples of both skeletal and cardiac muscle myofibrils incubated with preimmune rabbit serum and Protein A-gold complex are nearly devoid of reaction products (Fig. 3a,b), demonstrating a very low level of background labeling. Anti-desmin labeling of isolated porcine skeletal myofibrils showed that desmin is located in the Z-line region (Fig. 4a,b). In sections near the surface of skeletal muscle myofibrils (Fig. 4a), bundles of labeled filaments were readily discernible at the Z-line level, where this part of the myofibril has left the plane of section, and in the intermyofibrillar spaces connecting Z-lines of adjacent myofibrils. In the semi-thin section (~1200 Å) shown in Figure 4b, which includes primarily the interior of several myofibrils, the reaction products were confined to a transversely-oriented labeling pattern in the intermyofibrillar space between Z-lines of adjacent myofibrils. The anti-synemin labeling pattern (Fig. 5) appeared strikingly similar to the anti-desmin labeling pattern (Fig. 4a).

Examination of stereo pairs of a thick section (~1/4 μm) taken at an angle of ±10° infrequently revealed a desmin-labeled IF network emanating from the Z-line regions that appeared to surround the myofibrils (Fig. 6). This type of IF network was only seen in thick sections and when parts of the myofibrils left
Figure 3. Electron micrographs of porcine skeletal and cardiac muscle

myofibril controls that had been incubated with rabbit preimmune
serum and Protein A-gold complex before fixation and embedment.
Both skeletal (a) and cardiac (b) muscle myofibrils show very few
(if any) gold particles (arrows), demonstrating very low nonspecific
background labeling. The cardiac myofibril sample shown includes
an intercalated disk region. Z = Z-lines, ID = intercalated disk.
Bars = 1.0 μm.
Figure 4. Electron micrographs of porcine semitendinosus muscle myofibrils that had been incubated with anti-desmin and Protein A-gold complex before fixation and embedment.

a) This section includes the periphery of several myofibrils and shows anti-desmin labeling (arrows). Labeling is primarily at the Z-line region of the periphery of myofibrils that have left the sectioning plane, and in the intermyofibrillar spaces between Z-lines of adjacent myofibrils. Z = Z-lines. Bar = 0.25 μm.
b) This semi-thin section of several myofibrils shows anti-desmin labeling (arrows) in an intermyofibrillar pattern connecting adjacent myofibrils at their Z-lines. Z = Z-lines. Bar = 0.5 μm.
Figure 5. Electron micrograph of porcine semitendinosus muscle myofibrils that had been incubated with anti-synemin and Protein A-gold complex before fixation and embedment. This section includes the periphery of several myofibrils and shows anti-synemin labeling (arrows). Most of the labeling is at the Z-line region of the periphery of myofibrils that have left the sectioning plane, and in the intermyofibrillar spaces between Z-lines of adjacent myofibrils. Some labeling occasionally was observed at the intermyofibrillar region along the I-band. Z = Z-lines. Bar = 0.5 μm.
Figure 6. An electron micrograph and a stereo pair of porcine semitendinosus muscle myofibrils that had been incubated with anti-desmin and Protein A-gold complex.

a) The electron micrograph of this thick section (~1/4 μm) includes an extensive IF network that is labeled with gold particles (arrows). The IFs emanate from the Z-line regions.

b) A stereo pair of part of (a) shows that the IF network surrounds the myofibrils. Examples like this one were infrequently observed, and were evident only in thicker sections and when parts of the myofibrils dropped out of the section. Z = Z-lines. Tilt angle = ±10°. Bar = 0.5 μm.
the sectioning plane. Based upon results of Bilak et al. [1991a], it seems likely that these myofibrils originated from peripheral areas of the cell with expanded intermyofibrillar spaces.

Desmin labeling also was observed in regions near nuclei (Fig. 7a). Although the overnight soaking of muscle strips in Buffer A (contains Triton X-100) and subsequent storage of myofibrils in glycerol-containing solution have partly removed the nuclear envelope, and altered nuclear morphology, the nuclei (or their remnants) are still recognizable. Desmin-labeled filaments were present in the space between the nucleus and Z-lines of nearby myofibrils. The labeling pattern observed with anti-synemin (Fig. 7b) was similar to that obtained with anti-desmin (Fig. 7a), but labeling intensity was lower with anti-synemin.

Examination of stereo pairs from a thick section (~1/4 μm) that includes parts of nuclei (or their remnants) and nearby myofibrils shows an apparent connection of Z-lines and the nucleus via a desmin-labeled, complex IF network (Fig. 8).

Although only remnants of the sarcolemma, attached to some of the myofibrils, survived the soaking and storage steps, they were still evident. Fairly heavy labeling with anti-desmin was evident in areas near the remaining sarcolemma (Fig. 9a,b). Filaments in this region also are labeled with anti-synemin, but intensity is much lower (Fig. 10a,b). For both desmin (Fig. 9a,b) and synemin (Fig. 10a,b), the gold particles follow filamentous structures that
Figure 7. Electron micrographs of porcine semitendinosus muscle myofibrils and nearby nuclei (or their remnants) that had been incubated with anti-desmin (a) or anti-synemin (b), and Protein A-gold complex before fixation and embedment.

a) In this desmin-labeled sample, the gold particles (arrows) primarily were associated with, and followed, filamentous structures present in the space between the nucleus and Z-lines of the closest myofibril. Z = Z-line, Nu = nucleus. Bar = 0.5 \mu m.

b) In this synemin-labeled sample, the gold particles also were associated with filamentous structures between the nucleus and nearby myofibrils. Although this labeling pattern was similar to that obtained with anti-desmin (a), the intensity of the synemin labeling was lower. Z = Z-line, Nu = nucleus. Bar = 0.5 \mu m.
Figure 8. An electron micrograph and a stereo pair of porcine semitendinosus muscle myofibrils and nearby nucleus (or its remnant) that had been incubated with anti-desmin and Protein A-gold complex before fixation and embedment.

a) The electron micrograph of this thick section (~1/4 μm) shows part of a nucleus and the surrounding area. An extensive IF network (arrows), emanating from Z-lines of a nearby myofibril and extending to the nucleus, is labeled with gold particles. Z = Z-lines, Nu = nucleus. Bar = 0.5 μm.

b) A stereo pair of part of (a) that shows that the complex IF network labeled with gold particles connects the Z-lines of the nearest myofibril to the surface of the nucleus. Z = Z-lines, Nu = nucleus. Tilt angle = ±10°C. Bar = 0.5 μm.
Figure 9. Electron micrographs of porcine semitendinosus muscle myofibrils and associated remnants of the sarcolemma that had been incubated with anti-desmin and Protein A-gold complex before fixation and embedment.

a) This section includes myofibrils with attached remnants of the original sarcolemma (arrowheads). Gold particles (arrows) are evident between the remnants of the sarcolemma and the Z-lines of the adjacent myofibril. Label also is fairly heavy on filaments emanating from the Z-line (lower left corner) of the lower myofibril. Z = Z-lines. Bar = 0.5 μm.

b) This is another example of a section that includes myofibrils and attached remnants of the original sarcolemma (arrowheads). Gold particles (arrows) are evident between the sarcolemmal remnants and the Z-line regions of the attached myofibril. Part of the myofibril in the Z-line region shown in the upper right corner of the micrograph is not included in the sectioning plane, and label also is associated with filamentous structures present at this site. Z = Z-lines. Bar = 0.5 μm.
Figure 10. Electron micrographs of porcine semitendinosus muscle myofibrils and associated remnants of the sarcolemma that had been incubated with anti-synemin and Protein A-gold complex before fixation and embedment.

a) This section includes myofibrils with remnants of the sarcolemma (arrowheads). The intensity of synemin labeling (arrows) is lower than that observed with anti-desmin (Fig. 9a,b). However, the gold particles primarily are observed between the sarcolemmal remnant and the Z-line regions of the nearest myofibril. Some labeling also is evident at the Z-line region where part of the myofibril has left the plane of section (upper right corner). The label is associated with filamentous structures. Z=Z-lines. Bar = 0.5 μm.

b) This example shows another section that includes myofibrils and attached sarcolemmal remnants similar to that shown in (a). Again, labeling (arrows) is rather light, the pattern of labeling is similar to that shown in (a), and the gold particles are associated with filamentous structures. Z=Z-lines. Bar = 0.5 μm.
primarily run from the Z-line region to the sarcolemmal remnants.

Pre-embedding Immunogold Labeling of Porcine Cardiac Muscle Myofibrils

In cardiac muscle myofibrils, desmin exhibited a strong transversely-oriented labeling pattern at the Z-lines (Fig. 11a,b) and occasionally some longitudinally-oriented labeling along the edge of the myofibril (Fig. 11b). In sections of cardiac myofibrils that include remnants of intercalated disks (Fig. 11c,d), the reaction product obtained with anti-desmin also was concentrated in these intercalated disk regions, especially near the desmosomes (Fig. 11d). The synemin labeling pattern (Fig. 12a,b) obtained with cardiac myofibrils was similar to the desmin labeling pattern obtained with cardiac myofibrils (Fig. 11a), and to the synemin labeling pattern obtained with skeletal muscle myofibrils (Figure 5). Anti-synemin also labeled the intercalated disk remnants when they were present with myofibrils (Fig. 12b).

Post-embedding Immunogold Labeling of Intact Porcine Skeletal Muscle

In order to confirm the locations of desmin and synemin in intact adult mammalian skeletal muscle cells, immunogold labeling also was conducted by the post-embedding labeling method using samples embedded in Lowicryl resin. Control samples incubated with rabbit preimmune serum and Protein A-gold complex showed a very low level of background labeling (Fig. 13). Samples
Figure 11. Electron micrographs of porcine cardiac muscle myofibrils that had been incubated with anti-desmin and Protein A-gold complex before fixation and embedment.

a) This section includes the periphery of several myofibrils and shows anti-desmin labeling (arrows). The labeling is associated with filamentous structures and is concentrated primarily in the intermyofibrillar space connecting Z-lines of adjacent myofibrils, and at the Z-line region where parts of the myofibrils are not included in the sectioning plane. This desmin labeling pattern in cardiac muscle was very similar to that observed in skeletal muscle (Fig. 4a). Z = Z-lines. Bar = 0.5 μm.
b) This example shows a section near the periphery of several myofibrils and shows anti-desmin labeling. Longitudinal (arrowheads) as well as transverse (arrows) patterns of intermediate filaments are evident in this example. The longitudinally-oriented labeling pattern is along the edge of a myofibril. \( Z = Z \text{-line} \). Bar = 0.5 \( \mu \text{m} \).
c) This section of cardiac myofibrils includes remnants of an intercalated disk. The myofibrils have been sectioned near their surface. Labeling with anti-desmin is observed at regions of the intercalated disk remnant (large arrows) as well as in the Z-line regions (short arrows) of myofibrils that have partly left the sectioning plane. The presence of longitudinally-oriented filaments (arrowheads) also are evident. Z = Z-lines, ID = intercalated disk. Bar = 0.5 μm.
d) This section also includes the remnants of an intercalated disk, including a desmosome, associated with cardiac myofibrils. The desmin labeling pattern shows gold particles associated with filamentous structures (arrows) emanating from the Z-line region. Considerable label also is associated with the edges of the desmosome. Z = Z-lines, D = desmosome. Bar = 0.5 μm.
Figure 12. Electron micrographs of porcine cardiac muscle myofibrils that had been incubated with anti-synemin and Protein A-gold complex before fixation and embedment.

a) This example includes a section near the very periphery of myofibrils and shows transversely-oriented anti-synemin labeling (arrows) in an intermyofibrillar pattern connecting adjacent myofibrils at Z-line regions where myofibrils have left the plane of section. Z = Z-lines. Bar = 0.5 \mu m.
b) This section of cardiac myofibrils includes remnants of an intercalated disk. Anti-synemin labeling (arrows) is associated with filaments located near the intercalated disk as well as at the Z-line level (short arrows) where parts of myofibrils have left the plane of section. The immunogold labeling pattern with anti-synemin was similar to that obtained with anti-desmin, but the intensity with anti-synemin was lower. Z = Z-lines, ID = intercalated disk. Bar = 0.5 μm.
Figure 13. Electron micrograph of Lowicryl-embedded porcine semitendinosus muscle post-incubated with rabbit preimmune serum and Protein A-gold complex.

Transversely-oriented, ~10 nm-diameter filaments (arrows) traverse the space where the Z-lines of myofibrils that are not included in the section would have been located. This typical control shows a very low level of nonspecific labeling (short arrow). Z=Z-lines, Tr=triads. Bar =0.5 μm.
labeled with anti-desmin showed that desmin is located primarily at the periphery of the myofibrillar Z-lines (Fig. 14, and insets). Some approximately longitudinally-oriented labeling also was present in the intermyofibrillar space in some samples (results not shown). Desmin labeling also was occasionally evident around the periphery of mitochondria near Z-lines (Fig. 14, bottom inset). Samples labeled with anti-synemin (Fig. 15) exhibited a labeling pattern similar to that shown with anti-desmin (Fig. 14), except the intensity of anti-synemin labeling was generally somewhat lower.

The labeling patterns observed in the post-embedding labeling experiments were qualitatively similar to, and supportive of, the results obtained with the pre-embedding labeling experiments. The amount of labeling, however, was lower in the post-embedding experiments.
Figure 14. Electron micrograph of Lowicryl-embedded porcine semitendinosus muscle post-incubated with anti-desmin and Protein A-gold complex. Gold particles were associated with desmin IFs (arrows) at the Z-line level. Some gold particles also were found in the nearby Z-line region when the plane of section was near the periphery of myofibrils. Top inset: This is another example showing desmin labeling at and near the end (edge) of a transverse Z-line. Bottom inset: In this section, desmin labeling was at the end (edge) of a transverse Z-line and around the periphery of a nearby mitochondrion. Insets are at the same magnification as the micrograph. Z = Z-lines. Bar = 0.5 μm.
Figure 15. Electron micrograph of Lowicryl-embedded porcine semitendinosus muscle post-incubated with anti-synemin and Protein A-gold complex.

Gold particles (arrows) were primarily found in the Z-line region, especially near the ends of the Z-lines. Gold particles also were found near the periphery of myofibrils when they were sectioned near their surface. Inset: This is another example showing synemin labeling (arrows) at the Z-line region when the myofibril has been sectioned very close to its surface. Z = Z-lines. Bars = 0.5 μm.
We have used antibodies to desmin and to synemin labeled with protein A-coupled to colloidal gold particles to examine the location of these two proteins in healthy, mature mammalian striated muscle cells. The major thrust of the study involved skeletal muscle, with some studies on cardiac muscle included for comparison. Two overall, complementary approaches were used. First, myofibrils (primarily consisting of myofibril bundles), obtained by mild homogenization of muscle strips that had been soaked overnight in Triton X-100-containing solution and subsequently stored in glycerol-containing solution, were prepared for pre-embedding labeling. Such samples permitted good penetration of immunoreagents, yet were shown by examination to have retained many of the desired structural features (e.g., myofibril to myofibril registry and intermyofibrillar connections, and some myofibrils with attached/associated remnants of other cellular structures such as nuclei and the cell membrane skeleton). In the second approach, post-embedding labeling studies were conducted on intact skeletal muscle in order to verify the in situ location of antigens.

The specificity of the primary antibodies was shown by immunoblotting. The relative location of primary antibodies also was shown by both immunofluorescence labeling of myofibrils shown herein and of cryosections
from porcine skeletal and cardiac muscle elsewhere [Bilak et al., 1991b].
Several controls were included in our immunolabeling studies with desmin and
synemin. These included substitution of PBS or preimmune sera for primary
antibodies, and pre-absorption of the primary antibodies with appropriate
antigen, which eliminated specific binding. The background labeling was
consistently low throughout.

Localization of Desmin in Adult Mammalian Striated Muscle

With the exception of the results shown in the accompanying paper [Bilak et
al., 1991a], IFs have not been clearly identified by electron microscopy in
healthy mature mammalian skeletal muscle cells. In this study, we have
localized desmin in skeletal and cardiac muscle myofibrils by pre-embedding
labeling and in intact skeletal muscle by post-embedding labeling. To our
knowledge, this is the first immuno-EM report on desmin localization in mature
mammalian skeletal muscle. Our results demonstrate that in porcine skeletal, as
well as in cardiac, muscle cells, desmin is present in the form of IFs, in accord
with conclusions reached in earlier immuno-EM studies conducted on avian
skeletal [Richardson et al., 1981; Tokuyasu et al., 1983a], avian cardiac
[Richardson et al., 1981; Tokuyasu, 1983; Tokuyasu et al., 1983b], and
mammalian cardiac [Watkins et al., 1987; Sætersdal et al., 1989] muscle cells.
Anti-desmin labeling of isolated skeletal muscle myofibrils showed that
desmin is primarily transversely-oriented with respect to the long myofibrillar axis and is located in the intermyofibrillar spaces on filaments connecting the Z-lines of adjacent myofibrils, and on filaments at or near the periphery of the myofibrillar Z-lines if the sectioning plane is close to, or at, the surface of myofibrils at their Z-line regions. If, however, the plane of the section is through the interior of adjacent myofibrils, then only the intermyofibrillar labeling pattern was observed. These results were qualitatively similar to those shown earlier in our laboratory [Richardson et al., 1981] and in others [Tokuyasu et al., 1983a] in studies of avian skeletal muscle, and suggest that desmin IFs are wrapped around myofibrillar Z-lines, run between Z-lines of adjacent myofibrils, but do not penetrate the integral Z-line domains. When the myofibrils, which had been prepared by mild homogenization, had nuclear or cell membrane skeleton remnants still attached, desmin-labeled filaments were prominent between the myofibrillar Z-lines and these remnants. Observations from stereo pairs of thick sections indicated that the labeled filaments often comprised a complex IF network in these regions. These results are supportive of the electron microscope observations on intact porcine skeletal muscle of IFs connecting Z-lines and adjacent membranous structures [Bilak et al., 1991a]. Tokuyasu et al. [1983a] also obtained immuno-EM evidence for desmin IF linkages between myofibrillar Z-lines and nearby membranous structures, including mitochondria, nuclei, and the sarcolemma. The results of our post-
embedding labeling studies on intact porcine skeletal muscle were supportive of our pre-embedding labeling results.

In general, the pre-embedding anti-desmin labeling of porcine cardiac myofibrils yielded results similar (strong transversely-oriented pattern of labeling) to those obtained with the porcine skeletal muscle myofibrils. In addition, some longitudinally-oriented labeling between adjacent myofibrils, but close to the surface of the myofibrils, was evident, as was labeling of remnants of the intercalated disk, especially the desmosomes, when they were associated with the myofibrils being examined. These results are in general agreement with previous reports on avian cardiac muscle cells [Richardson et al., 1981; Tokuyasu, 1983; Tokuyasu et al., 1983b]. Three immuno-EM studies on mature mammalian heart muscle (all conducted on myocytes isolated from rat hearts), relevant to our study, have been reported. Watkins et al. [1987] localized desmin as part of their study on myocytes isolated from normal and hypertrophied rat heart muscle. In the normal heart myocytes, they observed transverse connections between adjacent Z-lines, but very few longitudinally-oriented IFs. Sætersdal et al. [1989] also observed the desmin staining pattern indicative of an overall transverse orientation at the Z-line levels, but observed longitudinally directed profiles as well. Both of these studies [Watkins et al., 1987; Sætersdal et al., 1989] observed no desmin labeling within the integral Z-line domain. Kartenbeck et al. [1983] and Watkins et al. [1987] both
observed desmin labeling in the desmosomal region of the intercalated disk, a result we also consistently observed herein.

Localization of Synemin in Adult Mammalian Striated Muscle

We are interested in the localization of synemin because this IFAP was shown previously [Granger and Lazarides, 1980; Price and Lazarides, 1983] to be present in mature avian skeletal muscle cells, and is the only protein present in these cells that we know clearly will interact with desmin [Bilak et al., 1990]. Insofar as we are aware, this is the first immuno-EM report on localization of synemin in muscle cells. Overall, we have demonstrated that the labeling pattern obtained with anti-synemin is strikingly similar to that obtained with anti-desmin in both porcine skeletal or cardiac muscle samples, i.e., the labeling is primarily in a transverse orientation to the long myofibrillar axis, and is at the Z-line region of the periphery of myofibrils that have left the plane of section, and in the intermyofibrillar spaces between Z-lines of adjacent myofibrils. Like the anti-desmin labeling results, anti-synemin also labeled intercalated disk remnants when associated with myofibrils in the pre-embedding labeling studies. The post-embedding anti-synemin labeling studies of intact porcine skeletal muscle were qualitatively similar to the results obtained by the pre-embedding labeling approach. In general, the labeling intensity resulting from anti-synemin antibodies was lower than that obtained with anti-desmin. This is
not surprising in view of the much lower content of synemin, in comparison to
desmin, in muscle cells [Granger and Lazarides, 1980; Bilak et al., 1991b].

The vertebrate myofibrillar Z-line is itself a complex structure, but one with
a high degree of structural order [Yamaguchi et al., 1985]. The complete
protein composition of the Z-line is unknown, and the nature of the protein
interactions of even those known Z-line components are complex [Goll et al.,
1991]. In general, the Z-line proteins can be divided into two categories,
peripheral and integral [see discussion in Goll et al., 1991]. Our studies herein
on synemin do not prove, but strongly suggest, that all of the desmin IFs found
at the periphery of myofibrillar Z-lines in these muscle cells also have synemin
present. We can not ascertain from these studies, however, whether synemin
is arranged along the desmin filaments with a distinct periodicity, or whether
synemin (or part of the synemin molecule) protrudes or projects from the
surface of the desmin IFs. Certainly, synemin is located together with the
desmin IFs around the circumference of the myofibrillar Z-lines. Thus, it is in a
position to possibly mediate desmin IF/myofibril interaction. Synemin binds to
desmin IFs. Perhaps future studies will show that synemin also can interact
with an integral Z-line protein.
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OVERALL SUMMARY

The objectives of the first section of my study were to: (1) clearly identify IFs in healthy mature mammalian skeletal muscle, and (2) study the three-dimensional structural relationship of IFs and the myofibrillar Z-lines. High resolution transmission electron microscopy, using conventional techniques combined with examination of stereo pairs, was used. In addition, samples of glycerinated muscle fibers were incubated with unlabeled anti-desmin in order to aid in visualization of IFs.

The major conclusions that can be drawn from the results obtained in this section are:

1. IFs readily can be identified in healthy adult mammalian skeletal muscle cells if systematically examined, especially in areas of cells where myofibrils are less tightly packed.

2. Visualization of IFs can be enhanced by decoration of glycerinated myofibrils with unlabeled anti-desmin.

3. A close structural relationship can be observed between IFs and myofibrillar Z-lines, with IFs transversely-oriented to the long myofibrillar axis, and connecting the Z-lines of adjacent myofibrils. Diameter of the filamentous structures near the Z-lines and identified as IFs herein was 10.8 nm ± 0.33 nm (mean ± standard error of the mean).
(4) IPs also connect the myofibrillar Z-lines with nearby membranous structures. The location and diameter of these filamentous structures identified as IPs herein were: near mitochondria = 11.1 nm ± 0.39 nm; near sarcolemma = 10.7 nm ± 0.36 nm; and, near nuclei = 11.6 ± 0.39 nm (numbers refer to means ± standard errors of the means).

(5) IPs evidently do not penetrate the integral myofibrillar Z-line domain, i.e., IPs surround the myofibrillar Z-lines in a lateral, rather than end-on, fashion.

The objectives of the second section of my study were to: (1) elucidate whether the IPs in mature mammalian striated muscle cells are composed of desmin and, if so, to localize desmin IPs in these muscle cells, and (2) determine if the IFAP, synemin, colocalizes with desmin in these muscle cells. Two immuno-EM approaches were used. Firstly, pre-embedding immunogold labeling with anti-desmin and anti-synemin antibodies was conducted by using mildly homogenized myofibrils from either porcine semitendinosus, or heart, muscle. Secondly, post-embedding immunogold labeling with the two antibodies was performed on intact porcine semitendinosus muscle that had been embedded in Lowicryl resin in order to confirm the in situ location of these proteins in intact muscle cells.

The major conclusions that can be drawn from the results obtained in this section are:
(1) Anti-desmin labeling of isolated porcine skeletal muscle myofibrils demonstrated that desmin primarily is transversely-oriented with respect to the long myofibril axis, and is located primarily in the intermyofibrillar space between the Z-lines of adjacent myofibrils. When the sectioning plane is near the surface of myofibrils, labeled filaments also could be readily observed at the periphery of the myofibrillar Z-lines.

(2) Anti-desmin antibodies labeled some longitudinally-oriented IFs, in addition to the transversely-oriented IFs, in isolated porcine cardiac muscle myofibrils. Longitudinally-oriented IFs also were observed in porcine skeletal muscle myofibrils, but much less frequently. Anti-desmin antibodies also label the desmosomal region of the intercalated disk remnants when associated with cardiac muscle myofibrils.

(3) The anti-synemin labeling pattern is very similar to the anti-desmin labeling pattern in both skeletal and cardiac muscle myofibrils. However, the intensity of the labeling was generally lower with anti-synemin than with anti-desmin.

(4) Labeling with both anti-desmin and anti-synemin also was concentrated near nuclei (or nuclear remnants) and sarcolemma (or sarcolemmal remnants).

(5) Examination of stereo pairs obtained from thick sections (~1/4 μm) occasionally revealed a complex three-dimensional network of IFs surrounding the myofibrils in the intermyofibrillar region, and especially in the region
between nuclei (or nuclear remnants) and adjacent myofibrils. This network appeared to connect myofibrils at their Z-lines to nuclei (or nuclear remnants).

(6) The post-embedding immunogold labeling patterns of intact porcine semitendinosus muscle obtained with both anti-desmin and anti-synemin antibodies were supportive of those patterns obtained in the pre-embedding labeling studies, indicating that the locations of these proteins determined in the pre-embedding labeling studies were representative of their in situ locations in intact muscle cells.


Nikkari, S.T., Koistinaho, J., and Jaakkola, O. (1990): Changes in the


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