INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Molecular interactions of the intermediate filament protein synemin

by

Robert Milton Bellin

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biochemistry

Major Professor: Richard M. Robson

Iowa State University

Ames, Iowa

2000

UMI Number: 9962798

UMI®

ii

Graduate College
Iowa State University

This is to certify that the Doctoral dissertation of

Robert M. Bellin

has met the dissertation requirements of Iowa State University

Major Professor

For the Major Program

For the Graduate College

TABLE OF CONTENTS

ABSTRACT

Synemin is a large (~182 kDa by sequence, ~230 kDa by SDS-PAGE) cytoskeletal

protein that was originally identified based upon its co-localization and co-purification with

the intermediate filament (IF) proteins desmin and vimentin in muscle tissue. Studies in this

dissertation have focused on understanding the molecular properties and role of synemin.

Cloning and sequencing studies revealed that synemin itself is a large member of the IF

protein superfamily because it contains the domain structure typical of IF proteins, i.e., an

~310 amino acid, coiled-coil rod domain flanked by an N-terminal head domain, and a C-

terminal tail domain that is very large in the case of synemin. Based upon analysis of the

sequence, we developed the hypothesis that synemin forms heteropolymeric IFs with other IF

proteins such as desmin and/or vimentin, and acts to link, via its large tail domain, those IFs

to other cytoskeletal structures. Transfection studies utilizing the SW13 IF-mosaic cell line

indicate that synemin does, in fact, require the presence of another IF protein to assemble

into IFs. These tissue culture findings are supported by the results of *in vitro* co-

sedimentation studies using both purified proteins and expressed protein domains, which

indicate that synemin forms IFs with desmin via interactions of their rod domains.

Additionally, blot-overlay assays have identified interactions between the tail domain of

synemin and both the myofibrillar Z-line protein α-actinin and the costameric protein

vinculin. Furthermore, these interactions have been confirmed, and the protein sub-domains

involved in the interactions more specifically defined, by the use of the *Gal4* yeast two-

hybrid system. *In toto*, these results support a role for synemin as a component of obligate

heteropolymeric IFs, which may function to crosslink the IFs to other cytoskeletal structures.

In striated muscle cells, synemin may play an important role in directly linking the

heteropolymeric IFs to α-actinin within the myofibrillar Z-lines and to vinculin within the costameres. By these interactions, synemin-containing IFs may firmly link all adjacent myofibrils within the cell, and attach the peripheral layer of myofibrils to the muscle cell membrane, thereby promoting overall cytoskeletal integrity and contractile function.

GENERAL INTRODUCTION

Dissertation Organization

The alternate manuscript-based format is used in my dissertation with the inclusion of two papers. The main body consists of one extensive manuscript, which has been published in The Journal of Biological Chemistry, and a second manuscript, which will be submitted for publication in The Journal of Biological Chemistry. I was responsible for the design and implementation of almost all of the experiments described in these manuscripts. I received help with purification of proteins from muscle tissue, and with some of the *in vitro* binding studies from Suzanne W. Sernett, who was a Research Associate in our research group at the time of those experiments. The references cited in the Review of Literature, which are formatted in author/year style for ease of reading, and in each of the two manuscripts, which are formatted based on specific journal requirements, are listed in corresponding separate *References* sections with each of those three parts of the dissertation. A comprehensive bibliography, which includes titles of the articles, is included at the end of the dissertation.

Introduction

The overall focus of the research described in this dissertation was to advance the understanding of the intermediate filament (IF) protein synemin. It is our overall hypothesis that synemin functions within cells, as a component of heteropolymeric intermediate filaments (IFs), to enable these IFs to directly link or bind to other cytoskeletal components. In the case of striated muscle cells, synemin may specifically function to attach muscle cell IFs to the myofibrils and to the muscle cell membrane. This direct linkage would permit muscle cell IFs to link adjacent myofibrils together, and attach the peripheral layer of

myofibrils to the muscle cell membrane skeleton. The specific goals of this dissertation were to test this overall hypothesis by cloning and sequencing the synemin cDNA, and then conducting several molecular interaction studies to examine potential protein interactions involving synemin.

Included herein are one manuscript that was published in 1999 and one manuscript to be submitted. Together, they describe research that fulfills the goals of my dissertation research. The first manuscript describes the first published complete sequence of synemin, analysis of the sequence, cell transfection studies using the cDNA showing that synemin can not form IFs without another IF protein present, and molecular interaction studies demonstrating interaction of synemin with the IF protein desmin and the myofibrillar Z-line protein α-actinin. The second manuscript delves more deeply into the molecular interactions involving synemin by describing extensive testing of specific molecular interactions of synemin utilizing the *Gal4* yeast two-hybrid system. These studies demonstrate that synemin interacts with desmin, vimentin, α-actinin, and vinculin. The interaction between synemin and the costameric protein vinculin is also shown by using purified proteins. Additionally, this manuscript includes a schematic representation of how synemin-containing heteropolymeric IFs may link adjacent myofibrils together, and connect the peripheral layer of myofibrils to the muscle cell membrane in striated muscle cells.

Review of Literature

The cell cytoskeleton is believed to play many diverse and essential cellular roles, including those of maintaining the overall structure of the cell (Fuchs, 1994; Fuchs and Cleveland, 1998), permitting transport of cellular materials (Hackney, 1996), providing a conduit for mechanical signaling (Maniotis et al., 1997), and stabilizing the cell during the

stress of mechanical strain (Goldman et al., 1996). The cytoskeleton of most animal cells consists of the cell membrane skeleton and three distinct classes of filaments: the microfilaments composed primarily of actin, the microtubules composed primarily of tubulin, and the intermediate filaments (IFs) composed of members of a superfamily of cell-type specific IF proteins (Robson, 1989; Fuchs and Weber, 1994; Fuchs and Yang, 1999). These filament types are as diverse in their structure and assembly as they are in their functions.

The microfilaments are approximately 7 nm in diameter and composed primarily of long strings of globular actin monomers linked together to form a filament reminiscent of two strands of pearls twisted around each other (dos Remedios and Moens, 1995). The formation of the microfilaments occurs in an energy-dependent manner, and preferentially at the barbed end of the filament, resulting in a "directional" filament (Schafer and Cooper, 1995). The twisted structure of the microfilament results in the presence of two grooves along the microfilament (ben-Avraham and Tirion, 1995), which often serve as sites for binding of other proteins that can form part of the microfilaments in certain cell types, such as tropomyosin (Allen et al., 1978), troponin-I (Fowler, 1997), and nebulin (Wang, 1996) in skeletal muscle cells. The repeating structure of microfilaments also makes it possible for a specific class of motor proteins, the myosins, to use them as "tracks" for movement (Baker and Titus, 1998). Myosin molecules move along the microfilament by an energy-dependent mechanism in which they "walk" from one actin monomer to one closer to the barbed end of the filament, thus moving along the actin filament (Rayment et al., 1993). By this mechanism, myosin molecules are able to transport cellular cargo on actin filaments in non-muscle cells (Hackney, 1996; Baker and Titus, 1998), and cause muscle contraction along the specialized actin filaments in muscle cells (Hackney, 1996).

The microtubules are approximately 23 nm in diameter, with the structure primarily composed of tubulin molecules attached end-to-end and in an open-centered-bundle arrangement (Downing and Nogales, 1998). The energy-dependent formation of the structure results in a filament or tubule with a hollow center, and polarity due to the direction of the end-to-end protein attachments (Tran et al., 1997; Margolis and Wilson, 1998). Microtubules can be composed of a number of variants of tubulin, including the well known α-tubulin and β-tubulin, as well as γ-tubulin, and the recently identified δ-tubulin and ε-tubulin (Chang and Stearns, 2000), along with many microtubule associated proteins (MAPs) (Llanos et al., 1999). In a sense analogous to the actin-containing microfilaments, the tubulin-containing microtubules are used as "tracks" for the classes of motor proteins known as the kinesins and dyneins (Hackney, 1996). The several members of each of these classes of motor proteins are very diverse, but all kinesins have in common that they travel toward the "plus" end of the microtubule (Kirchner et al., 1999), whereas dyneins travel toward the "minus" end (Inoue, 1997). As with the myosin-microfilament system, the microtubule motor proteins act to transport cargo along the microtubule tracks (Kirchner et al., 1999; Sheetz, 1999). Although the necessity of two discrete intracellular transport systems may not be immediately obvious, a recent review (Brown, 1999) compared the two filament systems to the mechanisms by which freight is moved around the country. The microtubules are believed to be similar to the rail lines on which trains transport large cargo significant distances, whereas the microfilaments are likened to the roads on which trucks move smaller cargos, but to more specific locations than is possible with trains. By analogy, kinesin and dynein molecules move large cellular cargos within the cell, but often the delivery of cargo to a specific site is left to the myosin molecules working on the microfilaments.

The intermediate filaments (IFs) are approximately 10 nm in diameter (Fuchs and

Weber, 1994; Klymkowsky, 1995; Houseweart and Cleveland, 1998; Fuchs and Yang, 1999)

and were originally named in reference to their diameter being between that of the 7 nm thin

filaments (microfilaments) and 15 nm thick filaments in developing muscle cells, in which

they were originally identified (Ishikawa et al., 1968). The name is now often used to refer

to the fact that their diameter is between that of the microfilaments and microtubules found in

all cells. The IFs are composed of cell-type specific protein monomers that are believed to

assemble by a fairly complex, non-energy requiring mechanism (Ip et al., 1985; Herrmann

and Aebi, 1998). The steps in assembly begin with the formation of a parallel coiled-coil

dimer of two IF protein polypeptides because of interactions of their respective ~310 amino

acid long, α-helical rod domains (Herrmann and Aebi, 1998; Herrmann et al., 1999). The

dimers then associate to form a staggered tetramer composed of four IF polypeptides (Meng

et al., 1994). These tetramers are often considered to be the "true" building-block of IFs, and

are thought to then associate both laterally and longitudinally, resulting in very long

filaments that contain approximately 32 protein monomers in cross-section (Fuchs and

Cleveland, 1998; Herrmann and Aebi, 1998). Unlike the microtubules and microfilaments

that are known to play roles in transport of cellular material, no motor proteins have been

found that operate on IFs. In fact, because IFs lack overall directionality in their structure,

locomotion of a motor protein on their surface may be impossible. Instead, IFs appear to

play specialized roles in cellular stabilization and in the mechanical integration of cellular

space (Lazarides, 1980; Goldman et al., 1996). Indeed, it has been found that cells from

specific disease states lacking normal IF networks are easily damaged, and exhibit abnormal

alignment of organelles (Fuchs, 1994; Fuchs, 1996; Fuchs and Cleveland, 1998).

Additionally, there is some evidence suggesting that IFs may play a role in the relay of mechanical signals from the cell membrane to the nucleus, where they are then translated into signals that induce cellular changes (Maniotis et al., 1997). The IFs are now regarded as highly dynamic structures, and have been shown to shorten/lengthen *in vivo* at rates similar to those observed with microfilaments and microtubules (Goldman et al., 1999).

The actual protein composition of an individual IF is very dependent upon the cell type in which it is found (Fuchs and Weber, 1994). Additionally, because the ~60 members of the IF protein superfamily are so variable in properties and presumably impart IFs with unique traits, it is more difficult to make generalized comments about IFs than can be made about microfilaments or microtubules. The majority of the superfamily of IF proteins is now most often classified into six major classes or types: the Type I acidic keratins; the Type II basic keratins; the Type III class composed of vimentin, desmin, glial fibrillar acidic protein (GFAP) and peripherin; the Type IV neurofilament proteins NF-L, NF-M, NF-H and α-internexin; the Type V nuclear lamins (A/C and B); and the Type VI group currently composed only of nestin (Fuchs and Yang, 1999; Steinert et al., 1999; Herrmann and Aebi, 2000). This classification is based on sequence homology and genomic sequence intron mapping (Fuchs and Weber, 1994), in contrast to an earlier system of classifying IF proteins that predated widespread protein sequencing, and was based on their tissue source. There are, however, general correlations between the current classification system and specific cell type classification. All types of epithelial cells have heteropolymeric IFs composed of a Type I and a Type II IF protein (Fuchs, 1996). Many developing cell types, such as fibroblasts and myoblasts, contain IFs composed mostly of vimentin (Evans, 1998), and virtually all cells grown in culture express some vimentin. Mature muscle and glial cells contain IFs

composed primarily of desmin (Huiatt et al., 1980) and GFAP (Chen and Liem, 1994), respectively. Neuronal cells contain heteropolymeric IFs composed of the so-called neurofilament triplet proteins, NF-L, NF-M and NF-H, named based upon their relative molecular masses (Low, Medium, and High) (Lee et al., 1993; Carpenter and Ip, 1996). The Type V lamins are present as major components of the nuclear lamina in most nucleated cells (Glass et al., 1993). Nestin is found in many developing cell types, often as a component of vimentin-containing IFs (Sejersen and Lendahl, 1993; Sjoberg et al., 1994).

Steinert and co-workers have recently proposed (Steinert et al., 1999) that the Type VI definition be expanded to include a small number of other proteins that do not necessarily share sequence homology with nestin, but seem to have "functional" homology. It was proposed that the proteins paranemin, tanabin and synemin, the latter protein being the focus of this dissertation, be classified together with nestin because they all have relatively high molecular masses (~180 kDa) and seem to often act as components of heteropolymeric IFs containing a Type III or IV IF protein (Steinert et al., 1999). Although the existence of heteropolymeric IFs containing each of these high molecular weight IF proteins has been shown to exist, an actual function for only one of these four proteins, synemin, has been demonstrated (Bellin et al., 1999a). As shown herein, synemin is a component of heteropolymeric IFs, and in muscle cells may enable the IFs to link directly to other cytoskeletal structures by specific protein-protein interactions between synemin and cytoskeletal proteins within those structures.

A novel view of the cytoskeleton recently has emerged that envisions the three filament classes not as separate components of the cell, but rather as three interacting components of a vital network. This "tensegrity" model of the cytoskeleton was proposed by

Maniotis and co-workers (Maniotis et al., 1997) and is based upon measurements of the strength of an interconnected network of elements. The elucidation and characterization of cytoskeletal crosslinking proteins, such as plectin that is able to link microfilaments, microtubules and IFs (Wiche, 1998), and synemin that can link IFs to α-actinin-containing structures (described herein) help support the tensegrity model.

The muscle cells of animals are highly differentiated cells that play an important role in many functions of the body, including locomotion, blood circulation and digestion. In general, there are three distinct types of muscle tissue, including skeletal and cardiac muscle, which are both striated, and non-striated smooth muscle (Small et al., 1992). The terms "striated" and "non-striated" were derived from the appearance of different types of muscle tissue when viewed with the light microscope (reviewed in Pepe, 1975). These visual differences in appearance of striated and non-striated muscle tissue reflect significant differences in their cellular structures, especially with regard to arrangement of contractile elements. Skeletal and cardiac muscle cells are essentially packed with ordered contractile elements, known as the myofibrils. The ordered structural arrangement within muscle fibers, and the cross-connections that link and hold adjacent fibers in register, are responsible for the striated appearance of skeletal and cardiac muscle tissue. In contrast, the contractile elements in smooth muscle cells do not generally appear to be in precise register and generally do not contain cross-connecting elements (Small, 1995), and therefore lack the cross-banded pattern seen for skeletal and cardiac muscle.

The myofibrils enable muscle cells and tissues to perform their many functions in animal systems. In a sense, the myofibrils represent a highly specialized form of the microfilament-myosin motor system described earlier (Hackney, 1996). Within the myofibril

9

the actin-containing microfilaments are referred to as the "thin filaments", and include

several additional proteins that create the specialized filament required for muscle

contraction. First of all, the thin filaments of skeletal muscle cells have regulated lengths,

normally about 1 μm in human muscle (Kruger et al., 1991). The regulation of thin filament

length is thought to involve several proteins including the ruler-like proteins nebulin (Wang,

1996) and titin (Wang, 1996; Labeit et al., 1997), barbed-end capping protein CapZ (Schafer

et al., 1995) and the pointed-end capping protein tropomodulin (Fowler, 1997). Additionally,

the well characterized proteins tropomyosin (Hitchcock-DeGregori and An, 1996) and

troponin (Farah and Reinach, 1995; Gregorio and Fowler, 1995), which regulate contraction

of striated muscle in response to the presence or absence of Ca^{++} ions by modulating the

accessibility of the actin filament to myosin, are major components of the thin filaments

(Gergely, 1998).

The myosin in striated muscle myofibrils is organized in the form of the thick

filaments (Chew and Squire, 1995). The thick filaments are composed primarily of myosin,

but also contain several other proteins that likely give an ordered structure to the thick

filament (Barral and Epstein, 1999). These proteins are concentrated at specific regions of

the thick filament, and with periodicities in these regions. Two of these proteins are C-

protein (MyBP-C) and H-protein (MyBC-H), which may wrap around and stabilize the thick

filament structure and/or link titin to the thick filaments (Gilbert et al., 1999).

The beautiful structure of striated muscle cells is due primarily to the amazing degree

of organization of the thin and thick filaments within the myofibrils. That high degree of

organization is the key to enabling the generation of force during muscle contraction.

Adjacent thick filaments are linked together at sites called M-lines (Obermann et al., 1996;

10

Obermann et al., 1997). The M-line is actually a fairly wide zone in the middle of each myofibrillar sarcomere, and is distinguished in electron microscope studies by the presence of up to five transverse lines that are perpendicular to the long axis of the sarcomere or myofibril, depending on the species and type of muscle observed (Obermann et al., 1996). These transverse lines appear to be composed of thick-filament binding proteins, including M-protein (Obermann et al., 1996), myomesin (Obermann et al., 1996) and the C-terminal domain of titin molecules (Vinkemeier et al., 1993; Obermann et al., 1997; Gregorio et al., 1999). Together these proteins, and possibly about 5% of the creatine kinase in the cell (Stolz and Wallimann, 1998; Qin et al., 1998), act as an organization center for adjacent thick filaments at the middle of each sarcomere (Obermann et al., 1997).

The Z-lines (Z-discs) define the end of each repeating contractile unit, termed the sarcomere, of the myofibril. The denotation of "Z" comes from the German word "Zwischenscheibe" (Small et al., 1992) meaning "in the middle" of the sarcomere, which was where the Z-lines were originally thought to reside in early studies of muscle. The Z-lines are now considered as representing the ends of the sarcomere because adjacent Z-lines are pulled/pushed towards each other during muscle contraction. The Z-line structure contains several important muscle proteins including the N-terminus of titin (Labeit et al., 1997; Turnacioglu et al., 1997; Gregorio et al., 1998; Young et al., 1998; Gregorio et al., 1999), the C-terminus of nebulin (Wang et al., 1996; Millevoi et al., 1998), the actin capping protein Cap Z (Schafer et al., 1995) and the actin binding protein α-actinin (Suzuki et al., 1976; Yamaguchi et al., 1985). Of these proteins, α-actinin plays a very important role in the structure and function of the Z-line. The ~100 kDa α-actinin polypeptide consists of an N-terminal actin binding domain, a central domain composed of four spectrin-like repeats, and

a C-terminal tail domain containing two EF hand homology regions (Blanchard et al., 1989) that are believed to be non-functional in muscle cells (Arimura et al., 1988). The functional α-actinin molecule in muscle cells is an anti-parallel dimer of two polypeptides, which results in an actin-binding site present at each end of the molecule (Djinovic-Carugo et al., 1999). These molecules act to help anchor the thin filaments in the Z-lines by binding to actin (Goll et al., 1991). Because of its location within the Z-line and its ability to crosslink either parallel or anti-parallel actin filaments (Yamaguchi et al., 1985; Meyer and Aebi, 1990), α-actinin appears able to help link thin filaments in such a way that the thin filaments extending from opposite sides of the Z-line have the opposite polarity essential for their interaction with the bipolar thick filaments during muscle contraction. However, the overall arrangement of thin filaments extending from Z-lines may primarily be due to the presence of part of the giant protein titin within the Z-line. The N-terminal (Z-line) domains of titin molecules from adjacent sarcomeres overlap within the Z-line and then extend toward the M-lines in the adjacent sarcomeres bordering the Z-line (Gregorio et al., 1999). The N-terminal domain of each titin molecule is able to bind to two sites on the α-actinin molecule (Young et al., 1998). Therefore, it may ultimately be titin and α-actinin working together that are responsible for the anti-parallel orientation of thin filaments emanating from opposite sides of the Z-line. An additional structure at the Z-lines consists of the IFs, which contain the IF proteins desmin (Richardson et al., 1981), synemin (Bilak et al., 1998; Bellin et al., 1999a, b), and paranemin (Hemken et al., 1997). As reported in this dissertation, synemin is able to bind to α-actinin, thereby creating a potential direct link between the IFs and the myofibrillar Z-lines.

As its name reflects, smooth muscle is organized quite differently than striated muscle. Smooth muscle cells contain thin and thick filaments that slide past each during contraction, but the general organization of these filaments is not like that in a striated muscle myofibril (Small, 1995; Stromer, 1998). Rather than the Z-lines, smooth muscle cells contain what are often called cytoplasmic dense bodies (CDBs) (Chou et al., 1994), which act as attachment sites for thin filaments within the cytoplasmic space. The lack of "true Z-lines" and of precisely ordered, parallel filaments that extend from CDBs result in muscle that appears "smooth". In addition, smooth muscle cells lack the proteins nebulin and titin, which play significant roles in organization of the sarcomeric thick and thin filaments in striated muscle (Hill and Weber, 1986; Locker and Wild, 1986; Gregorio et al., 1999). The organization and structure of smooth muscle cells also include additional actin filament binding sites or structures immediately proximal to the cell membrane, which are called membrane-associated dense bodies (MADBs) (Small, 1985; Chou et al., 1992). The MADBs are somewhat similar to the cytoplasmic-located CDBs, but appear in a rib-like pattern around the interior surface of the smooth muscle cell membrane (Small, 1985). These ribs have been found to contain many of the proteins normally associated with cell matrix-type adhesion junctions such as vinculin and talin (Small, 1985; Stromer 1998), indicating that they have at least a potential relationship to those types of junctions. The MADBs are sites of actin filament attachment, and may help attach contractile components to the muscle cell membrane.

Structures in striated muscle cells called costameres (Pardo et al., 1983a,b), which are analogous to the MADBs of smooth muscle cells, are present along the cytoplasmic face of the muscle cell membrane (sarcolemma), and positioned at periodic sites positioned near the

Z-lines of the peripheral layer of myofibrils. These specialized costameric structures are, like the MADBs, considered analogous to the cell matrix-type adhesion junctions that exist in many cell types (Goncharova et al., 1992), and are thought to be important in the myofibril-sarcolemma linkage (Danowski et al., 1992; Porter et al., 1992). The costameres were originally described only as vinculin-staining, rib-like bands noted near the sarcolemma of striated muscle cells (Pardo et al., 1983a,b), and the precise structure, protein composition, and arrangement of proteins within the costameres still remain rather poorly understood.

Studies based upon antibody labeling have demonstrated the presence of many proteins in the costamere (Goncharova et al., 1992; Vohra et al., 1998), including the intracellular domains of transmembrane proteins such as the integrins (Pardo et al., 1983a,b; Anastasi et al., 1998). Among other costameric proteins is the actin-binding protein talin (Belkin et al., 1986; Hemmings et al., 1996), which binds to the cytoplasmic domains of the transmembrane integrins (McDonald et al., 1995). This linkage may act to help link the nearby actin cytoskeleton and peripheral layer of myofibrils to the sarcolemma in striated muscle cells (Schmidt et al., 1999). Talin also binds strongly to the costameric protein vinculin, which can also bind actin and α-actinin (Burridge and Chrzanowska-Wodnicka, 1996). Vinculin is a very interesting protein and the focus of many studies because it evidently is involved in the mechanisms linking signal transduction cascades to the dynamics of the actin cytoskeleton. Vinculin contains what are called cryptic (hidden) protein binding sites within its structure (Johnson and Craig, 1995; Steimle et al. 1999). The sites for binding to F-actin (Johnson and Craig, 1995), talin (Johnson and Craig, 1994), VASP (Huttelmaier et al., 1998), and α-actinin (Kroemker et al., 1994) are blocked when the small, extended tail domain of vinculin (Bakolitsa et al., 1999) wraps over the large globular head domain of

vinculin (Gilmore and Burridge, 1996). It has been shown recently that the binding of phosphoinositides to vinculin reverses the intramolecular head-tail association, thus making the talin-binding site available. Whether binding of the phosphoinositides to vinculin also makes the actin-binding site in the vinculin tail domain available (Gilmore and Burridge, 1996; Weekes et al., 1996) or blocks the actin-binding site (Steimle et al., 1999) remains unclear. In addition to binding the several proteins just discussed, vinculin contains binding sites for other proteins such as paxillin (Turner et al., 1990). As reported in this dissertation, the IF protein synemin also binds to the vinculin molecule.

Muscle cells are subjected to stresses and strains not encountered by other cell types due to their contractile nature. Because of these strains, and the need to have muscle cells operate efficiently, the extra-myofibrillar cytoskeleton of muscle cells is specially structured (Stromer, 1998). The muscle cell IFs, in particular, are thought to play a special role in the stabilization of the muscle cell during contraction (Stromer, 1998). Studies on striated muscle cells have demonstrated that IFs wrap around the periphery of each myofibrillar Z-line, and appear to link all of the myofibrils together at their Z-lines (Richardson, 1981; Tokuyasu et al., 1983). This linkage may be especially important for maintaining the alignment of Z-lines of adjacent myofibrils during contraction, which is important for efficient muscle contraction. Additionally, the IFs extend from the Z-lines of the peripheral layer of myofibrils to the costameric regions at the sarcolemma (Yagyu et al., 1990). This potential linkage to the sarcolemma could be an important conduit of strain from the myofibril to the sarcolemma (Price, 1984). Additionally, it has been reported that some IFs run adjacent and parallel to the myofibrils, and may play an additional stabilization role (Price and Sanger, 1984).

The IFs of muscle cells change in composition during muscle cell maturation (Bennett et al., 1979; Granger and Lazarides, 1979; Ngai et al., 1985). In immature myoblasts, the primary IF protein is vimentin (Furst et al., 1989). After fusion of myoblasts, most muscle cell types replace the vimentin with desmin (Bennett et al., 1979; Granger and Lazarides, 1979; Kim, 1996). Because it is essentially the first muscle specific protein to appear upon myoblast fusion, some studies have investigated the possibility that desmin acts as a type of signal for muscle cell differentiation (Hill et al., 1986; Li et al., 1993). Studies with a tissue culture model system showed that desmin was essential for myofibrillogenesis because cells, where desmin expression was blocked, failed to fuse and form mature myofibrils (Li et al., 1994). The advent of desmin knock-out mice has, however, proven this role for desmin is not correct, because desmin-null mice develop fairly normal muscle tissue (Agbulut et al., 1996; Capetanaki et al., 1997; Li et al., 1997).

The production of the desmin-null mouse, independently by two different labs, has provided a new model for the study of the role of IFs in muscle cells. Although desmin-null mice do not show gross morphological changes in their muscle structure, detailed observations of the effects on muscle tissue have provided significant findings to support and further our understanding of the role of IFs in muscle cells. As might have been expected because of the localization of IFs at the periphery of the myofibrillar Z-lines, desmin-null mice demonstrate within their muscle cells an overall loss of Z-line alignment (Capetanaki and Milner, 1998), which indicates the IFs play some role in maintenance of Z-lines and Z-line linkages. Additionally, it was noted that desmin-null mice are more prone to muscle cell damage, and especially so in highly worked muscle tissues, such as the diaphragm and heart (Li et al., 1996; Milner et al., 1996; Milner et al., 1999). Li et al. (1997) also reported that

muscle repair mechanisms seem to be absent, or greatly reduced in efficiency, in desmin-null mice. Overall, that muscle tissue develops, but appears to be easily damaged in the absence of desmin, indicates that desmin may not be essential for the development of muscle cells, but is necessary for stabilizing muscle cells when subjected to strain (Li et al., 1997; Sjuve et al., 1998).

Studies in the early 1980's on the IF proteins of muscle cells in Elias Lazarides' lab demonstrated the existence of two relatively high molecular weight proteins, synemin (Granger and Lazarides, 1980) and paranemin (Breckler and Lazarides, 1982), which were found in association with the IFs of developing and mature avian muscle cells. These two proteins were initially found to co-purify with preparations of desmin and vimentin, and antibodies produced against these two proteins were found to label muscle cell IFs (Price and Lazarides, 1983). This localization pattern of the two proteins led to the names synemin (Granger and Lazarides, 1980) and paranemin (Breckler and Lazarides, 1982), taking their names from Greek words meaning "with filaments". Because so little was known about the biochemical and molecular properties of these proteins, they were generally referred to as IF associated proteins (IFAPs) (Foisner and Wiche, 1991).

Most of the early studies on paranemin were primarily aimed at surveying its presence in avian tissue types and developmental stages. Paranemin was found primarily in immature muscle tissues (skeletal, cardiac and smooth), and remained to some degree only at the Z-lines and intercalated disks in adult cardiac muscle and in vascular muscle of the elastic vessels (Price and Lazarides, 1983). Recent studies in our lab have greatly expanded our understanding of paranemin. We demonstrated by cloning and sequencing the paranemin cDNA from 12 day embryonic chick skeletal muscle that the paranemin molecule contains

the ~310 amino acid rod domain characteristic of IF proteins, and thus paranemin is in fact an IF protein (Hemken et al., 1997). We also demonstrated by cell transfection experiments that paranemin is unable, by itself, to form IFs, but will form heteropolymeric IFs along with another major IF protein such as vimentin (Hemken et al., 1997). Additionally, antibodies produced in our lab from purified paranemin label a protein at the Z-lines of both adult avian and porcine skeletal and cardiac myofibrils, demonstrating the presence of a mammalian homologue of paranemin (Hemken et al., 1997). An interesting finding from our sequencing studies is that paranemin is identical to a protein called transitin present in developing rat brain cells (Hemken et al., 1997; Ma et al., 1998). These studies provide evidence that paranemin is present in another tissue type, but it again appears to be developmentally regulated (Ma et al., 1998).

As was the case for paranemin, many of the early studies on synemin focused on determining its tissue distribution (reviewed in Bellin et al., 1999b). Early antibody labeling studies demonstrated the presence of synemin in all types (skeletal, cardiac and smooth) of developing avian muscle (Price and Lazarides, 1983), in avian erythrocytes (Granger and Lazarides, 1982; Granger et al., 1982; Moon and Lazarides, 1983) and in avian lens cells (Granger and Lazarides, 1984). Additionally, early studies found that synemin remained in adult avian skeletal and smooth muscles, but not in adult cardiac muscle (Price and Lazarides, 1983). Studies from the same lab also demonstrated that synemin could be highly phosphorylated *in vitro* (Sandoval et al., 1983), and in the same report they showed by gel chromatography that purified avian smooth muscle synemin seemed to exist as a tetramer in physiological-like buffer conditions. More recent studies in our lab have clarified many of the molecular features of synemin. Our cloning and sequencing studies have demonstrated

synemin itself is an IF protein, because its sequence contains the ~310 amino acid rod domain of IF proteins (Becker et al., 1995; Bellin et al., 1999a, b). As is demonstrated in this dissertation, much has been learned about synemin from its cDNA sequence. The rod domain of synemin is flanked by a very short N-terminal head domain, and an extremely long C-terminal tail domain (Bellin et al., 1999a). In a search of GenBank with the synemin sequence, I found two unidentified human partial sequences with high homology to synemin, indicating there exists a human homologue of synemin (Bellin et al., 1999a).

Further studies into the tissue distribution of synemin have identified its presence in a number of additional species and tissue types. Studies in our lab demonstrated the presence of synemin at the Z-lines of adult porcine skeletal and cardiac muscle myofibrils (Bilak et al., 1998). As demonstrated in this dissertation, synemin was found in a human adrenal cortex adenocarcinoma cell line, providing further evidence of "human" synemin (Bellin et al., 1999a). Synemin has recently been identified in developing rat glial cells (Sultana et al., 2000), and because of its temporal pattern of expression, it likely plays a role in a specific stage of differentiation of Type I glial cells.

Some quite interesting observations were made on what happens to synemin in desmin knock-out mice. These findings were of special interest to those working on the desmin-null mice, because some of the early desmin knock-out papers attributed the mild phenotype to potential compensation by up-regulation of synemin expression (Milner et al., 1996). It has been found by antibody labeling that synemin loses its Z-line location in striated muscle tissue of desmin knock-out mice, and is located instead primarily at the sarcolemma (Carlsson et al., 1997), especially concentrated around the costameric regions (R. Bloch, University of Maryland, personal communication).

Studies described in this dissertation add support to the findings on the desmin knock-out mice because synemin appears unable to form IFs by itself, but will form heteropolymeric IFs in the presence of a major IF protein. In cosedimentation studies, synemin, and specifically the synemin rod domain, will pellet with the IF protein desmin much more readily than by itself. The latter findings indicate a synemin-desmin interaction. Synemin also interacts with IF proteins in blot overlay assays, as described herein. Results of blot overlay assays were also used herein to demonstrate interactions of synemin with the Z-line protein, α-actinin, and the costameric protein, vinculin. All of those molecular interactions involving synemin are additionally demonstrated herein within the confines of a living cell by the *Gal4* yeast two-hybrid system, which provide additional support for the validity of the *in vitro* findings.

As is evident from the literature reviewed, muscle cells are highly specialized and require an intricate cytoskeleton to maintain their overall structure. The studies presented in this dissertation support the overall hypothesis that the IF protein synemin is a key element in the muscle cell cytoskeleton. In striated muscle cells, synemin-containing heteropolymeric IFs may be firmly attached to (1) each myofibrillar Z-line via interactions between synemin and α-actinin, and (2) the costameric regions along the sarcolemma via interactions between synemin and vinculin. These linkages may enable the heteropolymeric IFs to connect all of the myofibrils together at their Z-lines, and additionally to help anchor the peripheral layer of myofibrils to the sarcolemma. Thus, synemin may contribute significantly to providing the overall structural support essential to muscle cell structure and function.

References

Agbulut, O., Li, Z., Mouly, V., and Butler-Browne, G. S. (1996). Analysis of skeletal and cardiac muscle from desmin knock-out and normal mice by high resolution separation of myosin heavy-chain isoforms. Biol Cell 88, 131-135.

Allen, R. E., Stromer, M. H., Goll, D. E., and Robson, R. M. (1978). Synthesis of tropomyosin in cultures of differentiating muscle cells. J Cell Biol 76, 98-104.

Anastasi, G., Cutroneo, G., Santoro, G., and Trimarchi, F. (1998). The non-junctional sarcolemmal cytoskeleton: the costameres. Ital J Anat Embryol 103, 1-11.

Arimura, C., Suzuki, T., Yanagisawa, M., Imamura, M., Hamada, Y., and Masaki, T. (1988). Primary structure of chicken skeletal muscle and fibroblast alpha- actinins deduced from cDNA sequences. Eur J Biochem 177, 649-655.

Baker, J. P., and Titus, M. A. (1998). Myosins: matching functions with motors. Curr Opin Cell Biol 10, 80-86.

Barral, J. M., and Epstein, H. F. (1999). Protein machines and self assembly in muscle organization. BioEssays 21, 813-823.

Becker, B., Bellin, R. M., Sernett, S. W., Huiatt, T. W., and Robson, R. M. (1995). Synemin contains the rod domain of intermediate filaments. Biochem Biophys Res Commun 213, 796-802.

Bellin, R. M., Sernett, S. W., Becker, B., Ip, W., Huiatt, T. W., and Robson, R. M. (1999a). Molecular characteristics and interactions of the intermediate filament protein synemin. Interactions with alpha-actinin may anchor synemin- containing heterofilaments. J Biol Chem 274, 29493-29499.

Bellin, R. M., Sernett, S. W., and Robson, R. M. (1999b). Synemin. In Guidebook to the cytoskeletal and motor proteins, second edition, T. Kreis and R. Vale, eds. (UK: Oxford Univ. Press), pp. 322-324.

ben-Avraham, D., and Tirion, M. M. (1995). Dynamic and elastic properties of F-actin: a normal-modes analysis. Biophys J 68, 1231-1245.

Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979). Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. J Cell Biol 82, 577-584.

Bilak, S. R., Sernett, S. W., Bilak, M. M., Bellin, R. M., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1998). Properties of the novel intermediate filament protein synemin and its identification in mammalian muscle. Arch Biochem Biophys 355, 63-76.

Blanchard, A., Ohanian, V., and Critchley, D. (1989). The structure and function of alpha-actinin. J Muscle Res Cell Motil *10*, 280-289.

Breckler, J., and Lazarides, E. (1982). Isolation of a new high molecular weight protein associated with desmin and vimentin filaments from avian embryonic skeletal muscle. J Cell Biol *92*, 795-806.

Brown, S. S. (1999). Cooperation between microtubule- and actin-based motor proteins. Annu Rev Cell Dev Biol *15*, 63-80.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol *12*, 463-518.

Capetanaki, Y., and Milner, D. J. (1998). Desmin cytoskeleton in muscle integrity and function. Subcell Biochem *31*, 463-495.

Capetanaki, Y., Milner, D. J., and Weitzer, G. (1997). Desmin in muscle formation and maintenance: knockouts and consequences. Cell Struct Funct *22*, 103-116.

Carlsson, L., Price, M. G., Robson, R. M., Breckler, J., Wiche, G., Li, Z., Paulin, D., and Thornell, L. E. (1997). Expression of IF-related proteins in desmin knock-out mice. Mol. Biol. Cell *8*, 391a.

Carpenter, D. A., and Ip, W. (1996). Neurofilament triplet protein interactions: evidence for the preferred formation of NF-L-containing dimers and a putative function for the end domains. J Cell Sci *109*, 2493-2498.

Chang, P., and Stearns, T. (2000). delta-Tubulin and varepsilon-tubulin: two new human centrosomal tubulins reveal new aspects of centrosome structure and function. Nat Cell Biol *2*, 30-35.

Chen, W. J., and Liem, R. K. (1994). The endless story of the glial fibrillary acidic protein. J Cell Sci *107*, 2299-2311.

Chew, M. W., and Squire, J. M. (1995). Packing of alpha-helical coiled-coil myosin rods in vertebrate muscle thick filaments. J Struct Biol *115*, 233-249.

Chou, R. G., Stromer, M. H., Robson, R. M., and Huiatt, T. W. (1992). Assembly of contractile and cytoskeletal elements in developing smooth muscle cells. Dev Biol *149*, 339-348.

Chou, R. G., Stromer, M. H., Robson, R. M., and Huiatt, T. W. (1994). Substructure of cytoplasmic dense bodies and changes in distribution of desmin and alpha-actinin in developing smooth muscle cells. Cell Motil Cytoskeleton *29*, 204-214.

Danowski, B. A., Imanaka-Yoshida, K., Sanger, J. M., and Sanger, J. W. (1992). Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. J Cell Biol *118*, 1411-1420.

Djinovic-Carugo, K., Young, P., Gautel, M., and Saraste, M. (1999). Structure of the alpha-actinin rod: molecular basis for cross-linking of actin filaments. Cell *98*, 537-546.

dos Remedios, C. G., and Moens, P. D. (1995). Actin and the actomyosin interface: a review. Biochim Biophys Acta *1228*, 99-124.

Downing, K. H., and Nogales, E. (1998). Tubulin and microtubule structure. Curr Opin Cell Biol *10*, 16-22.

Evans, R. M. (1998). Vimentin: the conundrum of the intermediate filament gene family. Bioessays *20*, 79-86.

Farah, C. S., and Reinach, F. C. (1995). The troponin complex and regulation of muscle contraction. FASEB J *9*, 755-767.

Foisner, R., and Wiche, G. (1991). Intermediate filament-associated proteins. Curr Opin Cell Biol *3*, 75-81.

Fowler, V. M. (1997). Capping actin filament growth: tropomodulin in muscle and nonmuscle cells. Soc Gen Physiol Ser *52*, 79-89.

Fuchs, E. (1996). The cytoskeleton and disease: genetic disorders of intermediate filaments. Annu Rev Genet *30*, 197-231.

Fuchs, E. (1994). Intermediate filaments and disease: mutations that cripple cell strength. J Cell Biol *125*, 511-516.

Fuchs, E., and Cleveland, D. W. (1998). A structural scaffolding of intermediate filaments in health and disease. Science *279*, 514-519.

Fuchs, E., and Weber, K. (1994). Intermediate filaments: structure, dynamics, function, and disease. Annu Rev Biochem *63*, 345-382.

Fuchs, E., and Yang, Y. (1999). Crossroads on cytoskeletal highways. Cell *98*, 547-550.

Furst, D. O., Osborn, M., and Weber, K. (1989). Myogenesis in the mouse embryo: differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. J Cell Biol *109*, 517-527.

Gergely, J. (1998). Molecular switches in troponin. Adv Exp Med Biol *453*, 169-176.

Gilbert, R., Cohen, J. A., Pardo, S., Basu, A., and Fischman, D. A. (1999). Identification of the A-band localization domain of myosin binding proteins C and H (MyBP-C, MyBP-H) in skeletal muscle. J Cell Sci *112*, 69-79.

Gilmore, A. P., and Burridge, K. (1996). Regulation of vinculin binding to talin and actin by phosphatidyl- inositol-4-5-bisphosphate. Nature *381*, 531-535.

Glass, C. A., Glass, J. R., Taniura, H., Hasel, K. W., Blevitt, J. M., and Gerace, L. (1993). The alpha-helical rod domain of human lamins A and C contains a chromatin binding site. EMBO J *12*, 4413-4424.

Goldman, R. D., Chou, Y. H., Prahlad, V., and Yoon, M. (1999). Intermediate filaments: dynamic processes regulating their assembly, motility, and interactions with other cytoskeletal systems. FASEB J *13 Suppl 2*, S261-265.

Goldman, R. D., Khuon, S., Chou, Y. H., Opal, P., and Steinert, P. M. (1996). The function of intermediate filaments in cell shape and cytoskeletal integrity. J Cell Biol *134*, 971-983.

Goll, D. E., Dayton, W. R., Singh, I., and Robson, R. M. (1991). Studies of the alpha-actinin/actin interaction in the Z-disk by using calpain. J Biol Chem *266*, 8501-8510.

Goncharova, E. J., Kam, Z., and Geiger, B. (1992). The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. Development *114*, 173-183.

Granger, B. L., and Lazarides, E. (1984). Expression of the intermediate-filament-associated protein synemin in chicken lens cells. Mol Cell Biol *4*, 1943-1950.

Granger, B. L., and Lazarides, E. (1982). Structural associations of synemin and vimentin filaments in avian erythrocytes revealed by immunoelectron microscopy. Cell *30*, 263-275.

Granger, B. L., and Lazarides, E. (1980). Synemin: a new high molecular weight protein associated with desmin and vimentin filaments in muscle. Cell *22*, 727-738.

Granger, B. L., and Lazarides, E. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z disc. Cell *18*, 1053-1063.

Granger, B. L., Repasky, E. A., and Lazarides, E. (1982). Synemin and vimentin are components of intermediate filaments in avian erythrocytes. J Cell Biol *92*, 299-312.

Gregorio, C. C., and Fowler, V. M. (1995). Mechanisms of thin filament assembly in embryonic chick cardiac myocytes: tropomodulin requires tropomyosin for assembly. J Cell Biol *129*, 683-695.

Gregorio, C. C., Granzier, H., Sorimachi, H., and Labeit, S. (1999). Muscle assembly: a titanic achievement? Curr Opin Cell Biol *11*, 18-25.

Gregorio, C. C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Suzuki, K., Obermayr, F., Herrmann, B., Granzier, H., Sorimachi, H., and Labeit, S. (1998). The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. J Cell Biol *143*, 1013-1027.

Hackney, D. D. (1996). The kinetic cycles of myosin, kinesin, and dynein. Annu Rev Physiol *58*, 731-750.

Hemken, P. M., Bellin, R. M., Sernett, S. W., Becker, B., Huiatt, T. W., and Robson, R. M. (1997). Molecular characteristics of the novel intermediate filament protein paranemin. Sequence reveals EAP-300 and IFAPa-400 are highly homologous to paranemin. J Biol Chem *272*, 32489-32499.

Herrmann, H., and Aebi, U. (1998). Intermediate filament assembly: fibrillogenesis is driven by decisive dimer-dimer interactions. Curr Opin Struct Biol *8*, 177-185.

Herrmann, H., and Aebi, U. (1998). Structure, assembly, and dynamics of intermediate filaments. Subcell Biochem *31*, 319-362.

Herrmann, H., Haner, M., Brettel, M., Ku, N. O., and Aebi, U. (1999). Characterization of distinct early assembly units of different intermediate filament proteins. J Mol Biol *286*, 1403-1420.

Hill, C., and Weber, K. (1986). Monoclonal antibodies distinguish titins from heart and skeletal muscle. J Cell Biol *102*, 1099-1108.

Hill, C. S., Duran, S., Lin, Z. X., Weber, K., and Holtzer, H. (1986). Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. J Cell Biol *103*, 2185-2196.

Hitchcock-DeGregori, S. E., and An, Y. (1996). Integral repeats and a continuous coiled coil are required for binding of striated muscle tropomyosin to the regulated actin filament. J Biol Chem *271*, 3600-3603.

Houseweart, M. K., and Cleveland, D. W. (1998). Intermediate filaments and their associated proteins: multiple dynamic personalities. Curr Opin Cell Biol *10*, 93-101.

Huiatt, T. W., Robson, R. M., Arakawa, N., and Stromer, M. H. (1980). Desmin from avian smooth muscle. Purification and partial characterization. J Biol Chem 255, 6981-6989.

Huttelmaier, S., Mayboroda, O., Harbeck, B., Jarchau, T., Jockusch, B. M., and Rudiger, M. (1998). The interaction of the cell-contact proteins VASP and vinculin is regulated by phosphatidylinositol-4,5-bisphosphate. Curr Biol 8, 479-488.

Inoue, S. (1997). The role of microtubule assembly dynamics in mitotic force generation and functional organization of living cells. J Struct Biol 118, 87-93.

Ip, W., Hartzer, M. K., Pang, Y. Y., and Robson, R. M. (1985). Assembly of vimentin in vitro and its implications concerning the structure of intermediate filaments. J Mol Biol 183, 365-375.

Ishikawa, H., Bischoff, R., and Holtzer, H. (1968). Mitosis and intermediate-sized filaments in developing skeletal muscle. J Cell Biol 38, 538-555.

Johnson, R. P., and Craig, S. W. (1994). An intramolecular association between the head and tail domains of vinculin modulates talin binding. J Biol Chem 269, 12611-12619.

Johnson, R. P., and Craig, S. W. (1995). F-actin binding site masked by the intramolecular association of vinculin head and tail domains. Nature 373, 261-264.

Kim, H. D. (1996). Expression of intermediate filament desmin and vimentin in the human fetal heart. Anat Rec 246, 271-278.

Kirchner, J., Woehlke, G., and Schliwa, M. (1999). Universal and unique features of kinesin motors: insights from a comparison of fungal and animal conventional kinesins. Biol Chem 380, 915-921.

Klymkowsky, M. W. (1995). Intermediate filaments: new proteins, some answers, more questions. Curr Opin Cell Biol 7, 46-54.

Kroemker, M., Rudiger, A. H., Jockusch, B. M., and Rudiger, M. (1994). Intramolecular interactions in vinculin control alpha-actinin binding to the vinculin head. FEBS Lett 355, 259-262.

Kruger, M., Wright, J., and Wang, K. (1991). Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles: correlation of thin filament length, nebulin size, and epitope profile. J Cell Biol 115, 97-107.

Labeit, S., Kolmerer, B., and Linke, W. A. (1997). The giant protein titin. Emerging roles in physiology and pathophysiology. Circ Res 80, 290-294.

Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. Nature *283*, 249-256.

Lee, M. K., Xu, Z., Wong, P. C., and Cleveland, D. W. (1993). Neurofilaments are obligate heteropolymers in vivo. J Cell Biol *122*, 1337-1350.

Li, H., Choudhary, S. K., Milner, D. J., Munir, M. I., Kuisk, I. R., and Capetanaki, Y. (1994). Inhibition of desmin expression blocks myoblast fusion and interferes with the myogenic regulators MyoD and myogenin. J Cell Biol *124*, 827-841.

Li, Z., Colucci, E., Babinet, C., and Paulin, D. (1993). The human desmin gene: a specific regulatory programme in skeletal muscle both in vitro and in transgenic mice. Neuromuscul Disord *3*, 423-427.

Li, Z., Colucci-Guyon, E., Pincon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D., and Babinet, C. (1996). Cardiovascular lesions and skeletal myopathy in mice lacking desmin. Dev Biol *175*, 362-366.

Li, Z., Mericskay, M., Agbulut, O., Butler-Browne, G., Carlsson, L., Thornell, L. E., Babinet, C., and Paulin, D. (1997). Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. J Cell Biol *139*, 129-144.

Llanos, R., Chevrier, V., Ronjat, M., Meurer-Grob, P., Martinez, P., Frank, R., Bornens, M., Wade, R. H., Wehland, J., and Job, D. (1999). Tubulin binding sites on gamma-tubulin: identification and molecular characterization. Biochemistry *38*, 15712-15720.

Locker, R. H., and Wild, D. J. (1986). A comparative study of high molecular weight proteins in various types of muscle across the animal kingdom. J Biochem *99*, 1473-1484.

Ma, X., Charron, F., Cole, G. J., Savard, P. E., and Vincent, M. (1998). The developmentally regulated avian protein IFAPa-400 is transitin. Brain Res Dev Brain Res *109*, 59-66.

Maniotis, A. J., Chen, C. S., and Ingber, D. E. (1997). Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci U S A *94*, 849-854.

Margolis, R. L., and Wilson, L. (1998). Microtubule treadmilling: what goes around comes around. Bioessays *20*, 830-836.

McDonald, K. A., Lakonishok, M., and Horwitz, A. F. (1995). Alpha v and alpha 3 integrin subunits are associated with myofibrils during myofibrillogenesis. J Cell Sci *108*, 975-983.

McGregor, A., Blanchard, A. D., Rowe, A. J., and Critchley, D. R. (1994). Identification of the vinculin-binding site in the cytoskeletal protein alpha-actinin. Biochem J *301*, 225-233.

Meng, J. J., Khan, S., and Ip, W. (1994). Charge interactions in the rod domain drive formation of tetramers during intermediate filament assembly. J Biol Chem *269*, 18679-18685.

Meyer, R. K., and Aebi, U. (1990). Bundling of actin filaments by alpha-actinin depends on its molecular length. J Cell Biol *110*, 2013-2024.

Milner, D. J., Taffet, G. E., Wang, X., Pham, T., Tamura, T., Hartley, C., Gerdes, A. M., and Capetanaki, Y. (1999). The absence of desmin leads to cardiomyocyte hypertrophy and cardiac dilation with compromised systolic function. J Mol Cell Cardiol *31*, 2063-2076.

Milner, D. J., Weitzer, G., Tran, D., Bradley, A., and Capetanaki, Y. (1996). Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. J Cell Biol *134*, 1255-1270.

Moon, R. T., and Lazarides, E. (1983). Synthesis and post-translational assembly of intermediate filaments in avian erythroid cells: vimentin assembly limits the rate of synemin assembly. Proc Natl Acad Sci U S A *80*, 5495-5499.

Ngai, J., Capetanaki, Y. G., and Lazarides, E. (1985). Expression of the genes coding for the intermediate filament proteins vimentin and desmin. Ann N Y Acad Sci *455*, 144-157.

Obermann, W. M., Gautel, M., Steiner, F., van der Ven, P. F., Weber, K., and Furst, D. O. (1996). The structure of the sarcomeric M band: localization of defined domains of myomesin, M-protein, and the 250-kD carboxy-terminal region of titin by immunoelectron microscopy. J Cell Biol *134*, 1441-1453.

Obermann, W. M., Gautel, M., Weber, K., and Furst, D. O. (1997). Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. EMBO J *16*, 211-220.

Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983a). Vinculin is a component of an extensive network of myofibril-sarcolemma attachment regions in cardiac muscle fibers. J Cell Biol *97*, 1081-1088.

Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983b). A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc Natl Acad Sci U S A *80*, 1008-1012.

Pepe, F. A. (1975). Structure of muscle filaments from immunohistochemical and ultrastructural studies. J Histochem Cytochem 23, 543-62.

Porter, G. A., Dmytrenko, G. M., Winkelmann, J. C., and Bloch, R. J. (1992). Dystrophin colocalizes with beta-spectrin in distinct subsarcolemmal domains in mammalian skeletal muscle. J Cell Biol 117, 997-1005.

Price, M. G. (1984). Molecular analysis of intermediate filament cytoskeleton--a putative load-bearing structure. Am J Physiol 246, H566-572.

Price, M. G., and Lazarides, E. (1983). Expression of intermediate filament-associated proteins paranemin and synemin in chicken development. J Cell Biol 97, 1860-1874.

Price, M. G., and Sanger, J. W. (1984). Reduction of density and anisotropic distribution of intermediate filaments occur during avian skeletal myogenesis. Am J Anat 171, 427-440.

Qin, W., Khuchua, Z., Cheng, J., Boero, J., Payne, R. M., and Strauss, A. W. (1998). Molecular characterization of the creatine kinases and some historical perspectives. Mol Cell Biochem 184, 153-167.

Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993). Structure of the actin-myosin complex and its implications for muscle contraction. Science 261, 58-65.

Richardson, F. L., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1981). Immunoelectron and immunofluorescence localization of desmin in mature avian muscles. Eur J Cell Biol 26, 91-101.

Robson, R. M. (1989). Intermediate filaments. Curr Opin Cell Biol 1, 36-43.

Sandoval, I. V., Colaco, C. A., and Lazarides, E. (1983). Purification of the intermediate filament-associated protein, synemin, from chicken smooth muscle. Studies on its physicochemical properties, interaction with desmin, and phosphorylation. J Biol Chem 258, 2568-2576.

Schafer, D. A., and Cooper, J. A. (1995). Control of actin assembly at filament ends. Annu Rev Cell Dev Biol 11, 497-518.

Schafer, D. A., Hug, C., and Cooper, J. A. (1995). Inhibition of CapZ during myofibrillogenesis alters assembly of actin filaments. J Cell Biol 128, 61-70.

Schmidt, J. M., Zhang, J., Lee, H. S., Stromer, M. H., and Robson, R. M. (1999). Interaction of talin with actin: sensitive modulation of filament crosslinking activity. Arch Biochem Biophys 366, 139-150.

Sejersen, T., and Lendahl, U. (1993). Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci *106*, 1291-1300.

Sheetz, M. P. (1999). Motor and cargo interactions. Eur J Biochem *262*, 19-25.

Sjoberg, G., Jiang, W. Q., Ringertz, N. R., Lendahl, U., and Sejersen, T. (1994). Colocalization of nestin and vimentin/desmin in skeletal muscle cells demonstrated by three-dimensional fluorescence digital imaging microscopy. Exp Cell Res *214*, 447-458.

Sjuve, R., Amer, A., Li, Z., Mies, B., Paulin, D., Schmittner, M., and Small, J. V. (1998). Mechanical alterations in smooth muscle from mice lacking desmin. J Muscle Res Cell Motil *19*, 415-429.

Small, J. V. (1985). Geometry of actin-membrane attachments in the smooth muscle cell: the localisations of vinculin and alpha-actinin. EMBO J *4*, 45-49.

Small, J. V. (1995). Structure-function relationships in smooth muscle: the missing links. Bioessays *17*, 785-792.

Small, J. V., Furst, D. O., and Thornell, L. E. (1992). The cytoskeletal lattice of muscle cells. Eur J Biochem *208*, 559-572.

Steinert, P. M., Chou, Y. H., Prahlad, V., Parry, D. A., Marekov, L. N., Wu, K. C., Jang, S. I., and Goldman, R. D. (1999). A high molecular weight intermediate filament-associated protein in BHK- 21 cells is nestin, a type VI intermediate filament protein. Limited co-assembly in vitro to form heteropolymers with type III vimentin and type IV alpha-internexin. J Biol Chem *274*, 9881-9890.

Steimle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999). Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. J Biol Chem *274*, 18414-18420.

Stolz, M., and Wallimann, T. (1998). Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK. J Cell Sci *111*, 1207-1216.

Stromer, M. H. (1998). The cytoskeleton in skeletal, cardiac and smooth muscle cells. Histol Histopathol *13*, 283-291.

Sultana, S., Sernett, S. W., Bellin, R. M., Robson, R. M., and Skalli, O. (2000). The intermediate filament protein synemin is transiently expressed in a subset of astrocytes during development. Glia *30*, 143-153.

Suzuki, A., Goll, D. E., Singh, I., Allen, R. E., Robson, R. M., and Stromer, M. H. (1976). Some properties of purified skeletal muscle alpha-actinin. J Biol Chem *251*, 6860-6870.

Tran, P. T., Joshi, P., and Salmon, E. D. (1997). How tubulin subunits are lost from the shortening ends of microtubules. J Struct Biol *118*, 107-118.

Turnacioglu, K. K., Mittal, B., Dabiri, G. A., Sanger, J. M., and Sanger, J. W. (1997). An N-terminal fragment of titin coupled to green fluorescent protein localizes to the Z-bands in living muscle cells: overexpression leads to myofibril disassembly. Mol Biol Cell *8*, 705-717.

Turner, C. E., Glenney, J. R., Jr., and Burridge, K. (1990). Paxillin: a new vinculin-binding protein present in focal adhesions. J Cell Biol *111*, 1059-1068.

Vinkemeier, U., Obermann, W., Weber, K., and Furst, D. O. (1993). The globular head domain of titin extends into the center of the sarcomeric M band. cDNA cloning, epitope mapping and immunoelectron microscopy of two titin-associated proteins. J Cell Sci *106*, 319-330.

Vohra, M. S., Komiyama, M., Hayakawa, K., Obinata, T., and Shimada, Y. (1998). Subcellular localization of dystrophin and vinculin in cardiac muscle fibers and fibers of the conduction system of the chicken ventricle. Cell Tissue Res *294*, 137-143.

Wang, K. (1996). Titin/connectin and nebulin: giant protein rulers of muscle structure and function. Adv Biophys *33*, 123-134.

Weekes, J., Barry, S. T., and Critchley, D. R. (1996). Acidic phospholipids inhibit the intramolecular association between the N- and C-terminal regions of vinculin, exposing actin-binding and protein kinase C phosphorylation sites. Biochem J *314*, 827-832.

Wiche, G. (1998). Role of plectin in cytoskeleton organization and dynamics. J Cell Sci *111*, 2477-2486.

Wilkins, J. A., Chen, K. Y., and Lin, S. (1983). Detection of high molecular weight vinculin binding proteins in muscle and nonmuscle tissues with an electroblot-overlay technique. Biochem Biophys Res Commun *116*, 1026-1032.

Yagyu, M., Robson, R. M., and Stromer, M. H. (1990). Identification of intermediate filaments in mature mammalian skeletal muscle by transmission electron microscopy. Proc 12th Intern Congr Electron Miscoscopy *3*, 454-455.

Yamaguchi, M., Izumimoto, M., Robson, R. M., and Stromer, M. H. (1985). Fine structure of wide and narrow vertebrate muscle Z-lines. A proposed model and computer simulation of Z-line architecture. J Mol Biol *184*, 621-643.

Young, P., Ferguson, C., Banuelos, S., and Gautel, M. (1998). Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin. EMBO J *17*, 1614-1624.

MOLECULAR CHARACTERISTICS AND INTERACTIONS OF THE INTERMEDIATE FILAMENT PROTEIN SYNEMIN
INTERACTIONS WITH α-ACTININ MAY ANCHOR SYNEMIN-CONTAINING HETEROFILAMENTS

Robert M. Bellin, Suzanne W. Sernett, Bruno Becker, Wallace Ip, Ted W. Huiatt
and Richard M. Robson

Summary

Synemin is a cytoskeletal protein originally identified as an intermediate filament (IF)-associated protein because of its colocalization and copurification with the IF proteins desmin and vimentin in muscle cells. Our sequencing studies have shown that synemin is an unusually large member (1,604 residues, 182,187 Da) of the IF protein superfamily, with the majority of the molecule consisting of a long C-terminal tail domain. Molecular interaction studies demonstrate that purified synemin interacts with desmin, the major IF protein in mature muscle cells, and with α-actinin, an integral myofibrillar Z-line protein. Furthermore, expressed synemin rod and tail domains interact, respectively, with desmin and α-actinin. Analysis of endogenous protein expression in SW13 clonal lines reveals that synemin is coexpressed and colocalized with vimentin IFs in SW13.C1 vim+ cells, but is absent in SW13.C2 vim- cells. Transfection studies indicate that synemin requires the presence of another IF protein, such as vimentin, in order to assemble into IFs. Taken *in toto*, our results suggest synemin functions as a component of heteropolymeric IFs and plays an important cytoskeletal crosslinking role by linking these IFs to other components of the cytoskeleton. Synemin in striated muscle cells may enable these heterofilaments to help link Z-lines of adjacent myofibrils and, thereby, play an important role in cytoskeletal integrity.

Introduction

Intermediate filaments (IFs)[1], along with actin-containing microfilaments and tubulin-containing microtubules, are one of the three major classes of cytoskeletal filaments in multicellular animals (1-4). The IFs, which are considered to play an important role in structure and mechanical integration of cellular space (5, 6), are composed of cell-type specific proteins that have been divided into classes based upon sequence comparisons (1, 3, 7). The members of this protein superfamily have within their sequence a conserved rod domain, which promotes coiled-coil interactions between two individual IF proteins and formation of an IF protein dimer, the first step in assembly of the ~10 nm diameter IFs (1, 3, 8-11). Flanking the rod domain are N-terminal head and C-terminal tail domains that vary considerably in size and sequence among the IF protein classes (1, 3, 4). Most IF proteins are grouped into five major classes or types (I-V) based upon sequence analysis (1-4). Some classes of IF proteins, such as the type I and II keratins (3) and the type IV neurofilament proteins (12, 13), are known to form obligate heteropolymers *in vivo*, resulting in IFs that consist of at least two different IF proteins. In contrast, IFs containing type III proteins, such as desmin or vimentin, often are considered homopolymeric IFs (3, 14) because each of these individually purified proteins readily assemble into synthetic IFs *in vitro* (8, 15-17).

The type III IF proteins vimentin and desmin are the major IF proteins of developing and mature striated muscle cells, respectively (18, 19). Synemin and paranemin, a pair of relatively high molecular weight proteins identified in the early 1980s, were initially described as IF-associated proteins because they co-purified in the initial purification steps with desmin and vimentin, and colocalized with them in muscle cells (2, 20-24). Recent cloning and sequencing studies in our lab, however, demonstrate that both synemin and

paranemin contain the ~310 amino acid rod domain characteristic of IF proteins and, therefore, are members of the IF protein superfamily (25, 26). Those results, along with their colocalization (20, 22, 26, 27), suggest synemin and paranemin may form heteropolymeric IFs with the type III proteins desmin and/or vimentin *in vivo* (26, 27).

Our hypothesis is that synemin acts as a component of heteropolymeric IFs with vimentin and/or desmin and helps attach these IFs to other cytoskeletal structures. Based upon localization of IFs at the periphery of, and between, Z-lines of adjacent myofibrils (19, 28, 29), synemin-containing heteropolymeric IFs may help link adjacent myofibrils in striated muscle cells. In this paper, we describe the complete sequence of synemin, which establishes it as a unique IF protein with a long C-terminal extension, which is not readily grouped with any of the well-established IF protein types. Transfection of full-length synemin into SW13 clonal lines demonstrates that synemin requires another IF protein for assembly into IFs. We demonstrate specific molecular interactions between synemin and desmin, the major IF protein present in most mature muscle cells (18, 19), and between synemin's large tail domain and α-actinin, an integral protein of myofibrillar Z-lines (30) and costameres (31) of striated muscle cells, and of adhesion plaques of many other cell types (32). *In toto*, the studies herein help establish synemin as an important member of the IF protein superfamily, and one that likely functions as a component of heteropolymeric IFs that can interact with α-actinin and, thereby, enable IFs to link other components of the cytoskeleton.

Experimental Procedures

Cloning and Sequencing of Avian Synemin cDNA— Initial cloning studies on synemin from our laboratory (25) described only sequence of the rod domain portion of

synemin. Additional clones encoding parts of the full-length synemin cDNA were retrieved from the same λgt11 library, prepared from adult chicken gizzard, by hybridization screening. Sequencing of the entire length of both strands of clones 47, 108, 135 and 244, and multiple internal sites of all other clones shown in Fig. 1, was done on ABI 373 and 377 sequencers at the Iowa State University Sequencing and Synthesis Facility. Confirmation of the 5' end of the sequence was done by 5' RACE (33) with a kit from Life Technologies by using a primer (nucleotides 144-162) from the 5' end of clone 108. The longest RACE clones produced start at the same nucleotide as the 244 clone. Computer analysis of the synemin cDNA sequence was carried out by using Version 10 of the Wisconsin Package, Genetics Computer Group (GCG), Madison, Wisconsin, and the NCBI BLAST server (34).

Antibodies— Synemin polyclonal antibodies (pAb) 2856 were produced in rabbits injected with native purified protein essentially as described (35). The pAbs were characterized by Western blotting, and they labeled only the 230 kDa synemin band present in fresh, avian whole muscle homogenates. Additionally, these antibodies labeled purified samples of both the expressed rod and C-terminal tail domains of synemin. Aliquots of these antibodies also were affinity purified by utilizing a column of purified, intact synemin coupled to CNBr-activated Sepharose 4B (Sigma). Vimentin monoclonal antibody (mAb) AMF-17b (developed by Dr. A. B. Fulton) was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under contract NO1-HD-2-3144 from the NICHD).

35

Immunocytochemistry and Western Blotting—Immunocytochemistry studies with SW13.C1 vim+ and SW13.C2 vim- cells were done similarly to those described in Hemken et al. (26), but utilized synemin pAb 2856. For Western blotting, cell lysates of SW13.C1 vim+ and SW13.C2 vim- cells were separated into supernatant and pellet fractions to concentrate the cytoskeletal proteins in the pellets by the method of Athlan et al. (36). The resulting samples were analyzed by standard procedures (37), using enhanced chemiluminescence (ECL) and blot stripping according to the manufacturer's procedure described in the ECL Western blotting protocols guidebook (Amersham Pharmacia Biotech).

Northern Blotting—Total RNA was prepared from SW13.C1 vim+ and SW13.C2 vim- cells by the standard guanidine isothiocyanate method (38), and blots were probed with avian cDNA probes corresponding to either the synemin rod or tail domain by using the GeneImages kit (Amersham Pharmacia Biotech). The kit instructions were followed, except that the gel transfer was carried out by using 50 mM NaOH as the transfer fluid, and the hybridization and final stringency washes were done at 47° C.

Cell Transfection Assays—Studies were carried out essentially as described (26), with the following changes. Full-length synemin cDNA was assembled from overlapping clones and inserted into the pcDNA3 eukaryotic expression vector (Invitrogen). The cDNA construct was transfected into SW13.C2 vim- cells with FuGENE 6 reagent (Boehringer Mannheim), utilizing an empirically determined ratio of 3 µl transfection reagent to 1 µg DNA for a 60 mm diameter dish. Proteins were visualized approximately 40 h after transfection by immunocytochemistry.

Protein Purification—All proteins purified from tissue were prepared from adult turkey gizzards quick frozen immediately postmortem to minimize proteolysis. Synemin, in particular, is highly susceptible to proteolytic degradation (27). Intact synemin (27), desmin (15) and α-actinin (39) were purified by standard methods. The rod (nucleotides 138-1047) and tail (nucleotides 1048-4917) domains of synemin were produced by bacterial expression using pProEX HT vectors (GibcoBRL). The rod domain was expressed in *E. coli* XL-I Blue (Stratagene) and purified by using Ni-NTA resin (Qiagen) in non-denaturing conditions. The tail domain was expressed in the protease-deficient *E. coli* strain BL21 (DE3) (Stratagene), and purified from inclusion bodies by dissolving the pellet fraction resulting from centrifugation (40,000 x g for 30 min) in 6 M urea, 10 mM Tris-HCl, pH 8.5, and dialyzing into 1 mM EGTA, 10 mM Tris-HCl, pH 8.5, before Ni-NTA (Qiagen) chromatography.

Cosedimentation Assays—The interaction of purified intact synemin with purified desmin was tested by using three different sets of conditions: (1) *Soluble desmin*: desmin and synemin were mixed in 10 mM Tris-HCl, pH 8.5; (2) *Filament forming conditions*: desmin and synemin were first mixed in 10 mM Tris-HCl, pH 8.5, and then the mixture was adjusted to IF-forming conditions by titrating the pH to 7.0 with addition of 2 M imidazole-HCl, pH 6.0, and by addition of $MgCl_2$ and NaCl to 1 mM and 100 mM, respectively; (3) *Pre-formed filaments*: desmin by itself was first assembled into filaments in 100 mM NaCl, 1 mM $MgCl_2$, 10 mM imidizole-HCl, pH 7.0, and then the desmin filaments and synemin were mixed. For each of the three sets of conditions, an equal amount by weight (25 µg) of purified intact synemin and purified intact desmin were used in each sample. Bovine serum albumin (BSA) (Sigma) was added (10 µg) to each sample as an internal control. High-speed

centrifugation conditions (100,000 x *g* for 20 min), chosen to sediment desmin filaments but not synemin alone, were used to sediment desmin IFs and any associated protein(s). The resulting supernatants and pellets were analyzed by SDS-PAGE. Individual samples of desmin and of synemin, in each of the three sets of conditions, also were subjected to the same high-speed centrifugation in order to test their sedimentation behavior in the absence of the other protein. The interaction of the synemin rod domain with desmin, under *filament-forming conditions*, also was tested as described for intact synemin.

Blot Overlay Assays— Highly purified samples of desmin, α-actinin, and synemin prepared from turkey gizzard, together with a sample of whole gizzard homogenate, were subjected to SDS-PAGE. The proteins were transferred electrophoretically to a nitrocellulose membrane (37, 40), which was then blocked by incubation in IF buffer (100 mM NaCl, 1 mM $MgCl_2$, 10 mM imidazole-HCl, pH 7.0) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder. Blots were incubated with purified synemin, or with bacterially-expressed synemin domains, at 10 μg/ml in IF buffer containing 0.1% (v/v) Tween 20 and 1% (w/v) non-fat milk powder, and then washed thoroughly with several fresh changes of the latter buffer. A control blot was treated identically, but incubated with buffer containing no synemin. Protein interactions were detected with affinity-purified synemin pAb 2856, diluted 1:10,000 in PBS containing 0.1% Tween 20 and 5% non-fat milk powder, and visualized by ECL.

Results

Sequence Analysis—Several overlapping cDNA clones that encompass the complete cDNA sequence for avian muscle synemin were obtained (Fig. 1). The full-length sequence is 8,615 bp (GenBank Accession Number U28143), which corresponds well with the 8.4 kb

transcript size we have shown previously by Northern blot analysis of avian smooth muscle RNA with a synemin cDNA probe (25). Analysis of the sequence reveals a single open reading frame followed by a long (3,330 bp) 3' untranslated region (UTR) (Fig. 1). The open reading frame (4,812 bp) of the complete sequence codes for a protein with a predicted molecular mass of 182,187 Da. The sequence mass is smaller than that estimated (230 kDa) for synemin by SDS-PAGE (20, 27). This size difference may be explained, as it was for the IF protein paranemin (26), by the acidic nature (pI = 4.85) of synemin, which has been shown for other proteins to repel SDS and result in a slower relative migration by SDS-PAGE (41). As we reported in a preliminary study (25), and as shown in Fig. 2, the synemin sequence contains the conserved, ~310 amino acid rod domain characteristic of IF proteins. Of the sequences in GenBank, this region has the highest sequence identity (~31 to 33%) with the type III IF proteins desmin, vimentin, peripherin, and GFAP, which is much lower than the >70% identity generally observed for IF proteins within the same "type" (3). Thus, synemin can not be classified as a specific "type" IF protein solely by cDNA sequence comparisons. The rod domain (304 amino acids) of synemin is flanked by a short N-terminal head domain of 10 amino acid residues, and a C-terminal tail domain of 1,290 amino acid residues (Fig. 2). This tail domain is extremely long for an IF protein, and lacks significant homology with all other proteins.

Other than the homology with the rod domains of other IF proteins, synemin's sequence shows no significant homology/identity to other known proteins in GenBank. There are, however, notable regions of identity with a human EST sequence (gb_est24:AB077476) and with an unidentified human cDNA clone (AB002351).

Synemin Transfection Studies—In order to determine the ability of synemin to

assemble into IFs *in vivo*, the SW13 cell line was utilized. This cell line has been separated

into specific clonal lines, including the SW13.C1 vim+, which has an endogenous vimentin

IF network, and the SW13.C2 vim-, which lacks any cytoplasmic IFs (42) These clonal lines

have been used in several studies (e.g., 26, 43) to characterize assembly of transfected IF

proteins into IFs in the presence and absence of the cytoplasmic IF protein vimentin. We first

characterized the cell line before we used it for the transfection studies. Western blot analysis

of lysates of these clonal lines (Fig. 3) showed, as expected, that synemin was absent in the

SW13.C2 vim- cells (Fig. 3, *panel A, lanes 4* and *5*), but surprisingly that synemin was

already present in the cytoskeletal protein-containing pellet fraction of SW13.C1 vim+ cells

(Fig. 3, *panel B, lane 3*). Northern blot analysis showed that the mRNA for synemin (~9 kb)

was present in SW13.C1 vim+ cells, but absent in the SW13.C2 vim- cells. Thus, synemin

has the same pattern of transcription as previously shown for the mRNA of vimentin in these

clonal lines (42). Double-label immunofluorescence of SW13.C1 vim+ cells, utilizing the

same antibodies as for the Western blotting experiments, also show that they express

synemin as a component of their vimentin-containing IF network (Fig. 4, *panels A* and *B*).

And, consistent with the Western and Northern blot analyses, immunofluorescence labeling

of the SW13.C2 vim- cells, known to lack an endogenous vimentin IF network, with synemin

pAbs showed no labeling of synemin (Fig. 4, *panels C* and *D*).

Transfection of SW13.C2 vim- cells with full-length synemin cDNA resulted in cells

that contain punctate aggregates when observed by immunofluorescence (Fig. 4, *panels E*

and *F*). The punctate aggregates were similar to those seen for paranemin expressed in the

same vimentin-negative cell line (26), and for assembly-deficient mutants of desmin in other

cells lacking IFs (44). These results suggest that synemin can not form an IF network without another IF protein, such as vimentin, present.

Protein Interaction Studies— Experiments were conducted to characterize interactions between synemin and desmin. Analysis of the interactions by cosedimentation of purified desmin and purified synemin is shown in Fig. 5. Under non-IF forming conditions (Fig. 5*A*), neither desmin alone (*panel 1*) nor synemin alone (*panel 2*) sedimented. However, even under these conditions that are unfavorable for IF formation, synemin and desmin interact as shown by the presence of a significant portion of both proteins in the pellet fraction (*panel 3*). Under conditions favorable for IF formation (Fig. 5*B*), desmin by itself (*panel 1*), but very little synemin by itself (*panel 2*), sedimented. When desmin and synemin were premixed under conditions unfavorable for IF formation, and then converted to IF forming conditions, nearly all of the synemin sedimented with the desmin (*panel 3*). In contrast to the results shown in Fig. 5*B* (*panel 3*), much less synemin interacted with "preformed" desmin filaments (Fig. 5*C, panel 3*). Overall, these results show that synemin and desmin interact (Fig. 5*A, B* and *C, panel 3*), and suggest that synemin has greater access and/or affinity for binding free desmin molecules available before and during filament formation (Fig. 5*B, panel 3*), in comparison to binding to desmin already incorporated into IFs (Fig. 5*C, panel 3*).

Interaction studies of the bacterially-expressed rod domain of synemin with purified desmin (pre-mixed at low ionic strength, and then the buffer adjusted to favor IF-forming conditions) are shown in Fig. 6. Whereas desmin by itself is sedimented (*panel 1*), only about half of the synemin rod domain by itself is sedimented (*panel 2*). However, essentially all of the synemin rod domain is sedimented in the presence of desmin (*panel 3*), confirming the

rod domain of synemin as a major site of interaction with desmin. The lack of BSA in the pellets (Figs. 5 and 6, all panels) in the cosedimentation assays indicates that the increased amount of synemin (Fig. 5) or of synemin rod (Fig. 6) in the pellets is due to a specific interaction with desmin.

Blot overlay analyses utilizing purified desmin, α-actinin, and synemin, as well as bacterially-expressed rod and tail domains of the synemin molecule, were done to identify specific protein interactions. α-Actinin was selected as a potential interaction partner both because it is a major integral Z-line protein (30), and because the synemin-containing IFs very closely encircle the Z-lines in muscle.[2] As shown in the control lacking any probe protein in the overlay (Fig. 7, *panel B*) the synemin pAb 2856 labeled only the purified synemin (*lane 4*) and the synemin in the gizzard homogenate (*lane 1*), but did not label either desmin (*lanes 1* or *2*) or α-actinin (*lanes 1* or *3*). As shown in Fig. 7 (*panel C*), probing blots of purified desmin and α-actinin with purified intact synemin reveals significant interactions of synemin with both desmin (*lanes 1* and *2*) and α-actinin (*lane 3*). If overloaded samples of gizzard homogenate were run in lane 1, an interaction between synemin and the α-actinin in the gizzard homogenate also was detected.[3] As shown in Fig. 7 (*panel D*), probing the blots with the bacterially-expressed synemin rod domain reveals that it interacts with desmin (*lanes 1* and *2*), but not with α-actinin (*lane 3*). As shown in Fig. 7 (*panel E*), probing blots with the bacterially-expressed synemin tail domain demonstrates it interacts with both desmin (*lanes 1* and *2*) and α-actinin (*lane 3*). Specificity of the blot overlays is indicated in that neither the synemin nor synemin domains bound to any of the several other proteins

42

(e.g., filamin, myosin heavy chains, and actin) in the gizzard homogenate (Fig. 7, *panels C-D, lane 1*).

Discussion

Analysis of the sequence of synemin suggests it is a unique member of the IF protein superfamily. The majority of synemin's huge size consists of an unusually long C-terminal tail domain. There is a small, but slowly increasing, number of "large" IF proteins, all of similar size with long C-terminal tail domains, including nestin (45), tanabin (46), paranemin (26), and now synemin. Previous to their discoveries, NF-H, the largest (~115 kDa) of the neurofilament triplet, was considered a large IF protein. Steinert et al. (47), in a recent report of studies involving nestin, proposed that these large IF proteins be included within an enlarged type VI IF class, which heretofore has included only nestin, because these four proteins may have similar function(s). Of these four novel IF proteins, nestin (48), paranemin (26), and synemin (studies herein) do appear to exist within particular cell types as components of heteropolymeric IFs, requiring the presence of a major IF protein, most often a type III, for assembly into IFs. All four proteins also have similar domain organizations within their sequences (i.e., very short N-terminal head, rod domain, and very long C-terminal tail). However, by sequence comparisons, these four proteins are rather diverse, and this is certainly so for synemin in comparison to the other three large IF proteins. Whereas the sequence homologies among nestin, tanabin, and paranemin are fairly high (e.g., within the rod domains paranemin has 63% and 48% amino acid identity to tanabin and nestin, respectively; (26)), synemin exhibits much less homology (i.e., synemin's rod domain shares only ~30% amino acid identity with the rod domains of nestin, tanabin, and paranemin). Synemin's tail domain shares only ~20% identity with the tail domains of these three

proteins. Overall, the tail domains of all four of the novel IF proteins vary greatly from each other. Paranemin, for instance, is the only IF protein discovered that contains a long, consecutive psuedo-heptad repeat region within its tail (26). Thus, assuming the tails are functionally important, these four novel IF proteins may play diverse roles. There also are notable differences among the four proteins within each of their short N-terminal head domains, which generally are considered to play a major role in IF assembly (3). The covalent modification of some residues within the head domain (e.g., phosphorylation of serine/theronine (49) and ADP-ribosylation of arginine (50)), in particular, are important in modulating assembly/disassembly of IFs. The short head domains of the four "large" IF proteins vary widely in the content of those particular residues. For instance, nestin's seven-residue head totally lacks all of those residues, whereas paranemin's 15-residue head contains one serine and one arginine, but no threonine, and synemin's ten-residue head has no serine or threonine residues, but contains two adjacent arginines. We have recently found that synemin is a good substrate *in vitro* for the skeletal muscle, arginine-specific mono-ADP-ribosyltransferase.[4] Thus, although nestin, tanabin, paranemin, and perhaps synemin, may behave functionally similar, this remains far from certain, and especially so for synemin.

The cDNA sequence identities of the avian synemin cDNA with the human EST (aligns with the last two-thirds of the synemin rod domain sequence with 58% sequence identity) and with human brain cDNA clone AB002351 (51) (aligns with the synemin sequence starting from within subdomain 2B near the end of the rod domain to the middle of the 3' UTR with 48% sequence identity), which in turn are 99% identical to each other at the nucleotide level, suggest that human synemin has been partially sequenced. If this does represent human synemin sequence, it exhibits considerable divergence from the avian

synemin sequence for much of the length of the sequence currently available. When the amino acid sequences predicted from these cDNA sequences are compared, avian synemin and the human AB002351 clone exhibit only 41% homology (33% amino acid identity). However, it is striking that the extreme C-terminal end (50 residues) of synemin is almost identical to the protein predicted from the AB002351 clone, including the same stop codon position. At a minimum, if this cDNA from human brain does not code for synemin, there exists a protein in human brain cells that exhibits significant homology to avian synemin. Additionally, our identification of endogenous synemin in SW13.C1 vim+ cells, a cell line derived from a human adrenal cortex adenocarcinoma, indicates the existence of human synemin. Western blotting of this endogenous human synemin indicated a molecular mass of approximately 225 kDa, slightly lower than observed for avian muscle synemin, but essentially identical to that we identified (225 kDa) in porcine muscle (27). Additionally, although the specific, full-length human synemin cDNA has not yet been sequenced, the approximate size of the human mRNA (~9.0 kb) we described herein is very close to the 8.4 kb identified for synemin in avian tissue (25).

With increasing interest in so-called 3' UTR "zip-codes", which have been shown to play a role in the localization of the mRNAs of other cytoskeletal proteins including specific actin isoforms and even the IF protein vimentin (52, 53), it is possible that such a sequence may exist within the notably large synemin 3' UTR. Unfortunately, in most cases no true sequence homology domains exist for the "zip-codes" making the identification of such a sequence difficult (53).

It has been speculated since its discovery (20), that synemin functions within cells in close association with the IF proteins desmin and/or vimentin (20, 22, 25, 27, 54). That the

sequence of synemin demonstrates it also is an IF protein strongly suggests that interactions between synemin and the type III IF proteins desmin and/or vimentin will include those of their rod domains. Our transfection studies in SW13 cells explored whether synemin, by itself, could assemble into IFs. Interactions that have been identified between two members of the IF protein superfamily (e.g., type I and II keratins, desmin and vimentin, NF-L with NF-M and/or NF-H) are known to involve their rod domains (3, 47). The colocalization of synemin and vimentin in the IF network in the SW13.C1 vim+ cells, and the lack of IF formation in the SW13.C2 vim- cells lacking vimentin, but expressing synemin from transfection, suggest that synemin forms heteropolymeric IFs along with another IF protein, but does not form IFs by itself. These *in vivo* findings are in concert with the *in vitro* cosedimentation results, which showed that synemin, by itself in IF forming conditions, remained in the supernatant (Fig. *5B, panel 2*), but was sedimented maximally in the presence of desmin when the two proteins were first mixed under conditions unfavorable for IF formation, and then converted to conditions favoring filament formation prior to centrifugation (Fig. *5B, panel 3*). That the assembly of synemin into IFs depends on the presence of another IF protein also provides additional insight into an early report (55), which showed that the rate of vimentin filament assembly limited the rate at which synemin became part of the insoluble fraction isolated from cell lysates (i.e., synemin required the presence of another IF protein for incorporation into IFs).

The results of our cosedimentation studies demonstrated a specific interaction between intact synemin and desmin (Fig. 5), and between the synemin rod domain and desmin (Fig. 6), especially under filament-forming conditions. Those interactions were verified by using a different technique, namely the blot overlay studies (Fig. 7). These

interactions are consistent with studies demonstrating colocalization and initial copurification of synemin with desmin from adult avian (27, 56) and mammalian (27) muscle tissue. The blot overlay studies also revealed an interaction between the synemin tail domain and desmin (Fig. 7), which may reflect the staggered-array of IF proteins present in the assembly intermediates involved in IF assembly (3, 8, 11, 17). Furthermore, specific sites within the C-terminal tails of IF proteins, such as vimentin, have been shown to interact with sites within the IF rod domain (57, 58), and to be important in IF formation, thus providing one explanation for synemin tail domain/desmin interaction.

Our results demonstrate an interaction between the tail domain of synemin and α-actinin. α-Actinin is a well characterized F-actin crosslinking and bundling protein (59, 60), and an integral myofibrillar Z-line component (30). This interaction suggests a functional crosslinking role for synemin's large C-terminal tail domain. The N-terminal head and C-terminal tail domains of IF proteins are generally believed to impart IFs with cell-type specific roles (3). The tail domains of IF proteins extend from the surface of the IFs (61). The presence of synemin, with its huge C-terminal tail domain, in heteropolymeric IFs may serve to directly link these particular IFs to other cytoskeletal structures, containing α-actinin, within the cell cytoskeleton. The discovery that synemin's tail domain binds to α-actinin reveals an entirely new mechanism for linkage/anchorage of IFs to other cytoskeletal structures containing α-actinin, an important component in actin attachment sites in eukaryotic cells. In striated muscle cells, synemin-containing IFs may be directly bound to myofibrillar Z-lines and costameres via synemin, which supports our overall hypothesis (24, 25, 27), as well as the recent suggestion (47) that these very large IF proteins may have

specific cross-linking roles in cells. The multi-purpose, cytoskeletal crosslinking protein plectin (62), which is located at the periphery of myofibrillar Z-lines in skeletal muscle cells (63), also may help provide an indirect linkage of IFs to the Z-lines (64, 65). Plectin has been shown to bind to the rod domains of IF proteins such as vimentin (62), and it will be interesting in future studies to define the interactions between plectin and synemin.

Overall, the interactions identified herein between synemin and the IF proteins vimentin and desmin, and between the synemin tail domain and the actin-binding protein α-actinin, support our working hypothesis for the role of synemin. As a component of heteropolymeric IFs, synemin would be able to directly link the IFs to α-actinin-containing structures. In the case of striated muscle cells, synemin may play an important role in directly linking the heteropolymeric IFs to α-actinin within the myofibrillar Z-lines and costameres. Thus, synemin-containing IFs could firmly link all adjacent myofibrils within the cell, and the peripheral layer of myofibrils to the muscle cell membrane. This linkage may help maintain overall cytoskeletal integrity and contractile function.

Acknowledgments

References

1. Steinert, P. M., and Roop, D. R. (1988) *Annu. Rev. Biochem.* 57, 593-625

2. Robson, R. M. (1989) *Curr. Opin. Cell Biol.* 1, 36-43

3. Fuchs, E., and Weber, K. (1994) *Annu. Rev. Biochem.* 63, 345-382

4. Fuchs, E., and Cleveland, D. W. (1998) *Science* 279, 514-519

5. Lazarides, E. (1980) *Nature* **283**, 249-256

6. Goldman, R. D., Khuon, S., Chou, Y. H., Opal, P., and Steinert, P. M. (1996) *J. Cell Biol.* **134**, 971-983

7. Klymkowsky, M. W. (1995) *Curr. Opin. Cell Biol.* **7**, 46-54

8. Ip, W., Heuser, J. E., Pang, Y. Y., Hartzer, M. K., and Robson, R. M. (1985) *Ann. N.Y. Acad. Sci.* **455**, 185-199

9. Meng, J., Khan, S., and Ip, W. (1996) *J. Biol. Chem.* **271**, 1599-1604

10. Herrmann, H., and Aebi, U., (1998) *Curr. Opin. Struct. Biol.* **8**, 177-185

11. Herrmann, H., Haner, M., Brettel, M., Ku, N. O., and Aebi, U. (1999) *J. Mol. Biol.* **286**, 1403-1420

12. Lee, M. K., Xu, Z., Wong, P. C., and Cleveland, D. W. (1993) *J. Cell Biol.* **122**, 1337-1350

13. Ching, G. Y., and Liem, R. K. (1993) *J. Cell Biol.* **122**, 1323-1335

14. Abumuhor, I. A., Spencer, P. H., and Cohlberg, J. A. (1998) *J. Struct. Biol.* **123**, 187-198

15. Huiatt, T. W., Robson, R. M., Arakawa, N., and Stromer, M. H. (1980) *J. Biol. Chem.* **255**, 6981-6989

16. Geisler. N., and Weber, K. (1980) *Eur. J. Biochem.* **111**, 425-433

17. Ip, W., Hartzer, M. K., Pang, Y. Y., and Robson, R. M. (1985) *J. Mol. Biol.* **183**, 365-375

18. Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979) *J. Cell Biol.* **82**, 577-584

19. Granger, B. L., and Lazarides, E. (1979) *Cell* **18**, 1053-1063

20. Granger, B. L., and Lazarides, E. (1980) *Cell* **22**, 727-738

21. Breckler, J., and Lazarides, E. (1982) *J. Cell Biol.* **92**, 795-806

22. Price, M. G., and Lazarides, E. (1983) *J. Cell Biol.* **97**, 1860-1874

23. Foisner, R., and Wiche, G. (1991) *Curr. Opin. Cell Biol.* **3**, 75-81

24. Bellin, R. M., Sernett, S. W., and Robson, R. M. (1999) *Synemin* in *Guidebook to the Cytoskeleton and Motor Proteins, Second Edition* (T. Kreis and R. Vale, eds.), pp. 322-324, Oxford University Press, United Kingdom

25. Becker, B., Bellin, R. M., Sernett, S. W., Huiatt, T. W., and Robson, R. M. (1995) *Biochem. Biophys. Res. Commun.* **213**, 796-802

26. Hemken, P. M., Bellin, R. M., Sernett, S. W., Becker, B., Huiatt, T. W., and Robson, R. M. (1997) *J. Biol. Chem.* **272**, 32489-32499

27. Bilak, S. R., Sernett, S. W., Bilak, M. M., Bellin, R. M., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1998) *Arch. Biochem. Biophys.* **355**, 63-76

28. Richardson, F. L., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1981) *Eur. J. Cell Biol.* **26**, 91-101

29. Tokuyasu, K. T., Dutton, A. H., and Singer, S. J. (1983) *J. Cell Biol.* **96**, 1727-1735

30. Yamaguchi, M., Izumimoto, M., Robson, R. M., and Stromer, M. H. (1985) *J. Mol. Biol.* **184**, 621-644

31. Imanaka-Yoshida, K., Danowski, B. A., Sanger, J. M., and Sanger, J. W. (1996) *Cell Motil. Cytoskeleton* **33**, 263-275

32. Yamada, K., and Geiger, B. (1997) *Curr. Opin. Cell Biol.* **9**, 76-85

33. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8998-9002

34. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403-410

35. Knudson, K. A. (1985) *Anal. Biochem.* **147**, 285-288

36. Athlan, E. S., Sacher, M. G., and Mushynski, W. E. (1997) *J. Neurosci. Res.* **47**, 300-310

37. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350-4354

38. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1997) *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., New York

39. Craig, S. W., Lancashire, C. L., and Cooper J. A. (1982) *Methods Enzymol.* **85**, 316-321

40. Burnette, W. N. (1981) *Anal. Biochem.* 112, 195-203

41. Bryan, J. (1989) *J. Muscle Res. Cell Motil.* 10, 95-96

42. Sarria, A. J., Lieber, J. G., Nordeen, S. K., and Evans, R. M. (1994) *J. Cell Sci.* 107, 1593-1607

43. Chen, W.J., and Liem, R. K. (1994) *J. Cell Sci.* 107, 2299-2311

44. Raats, J. M., Pieper, F. R., Vree Egberts, W. T., Verrijp, K. N., Ramaekers, F. C., and Bloemendal, H. (1990) *J. Cell Biol.* 111, 1971-1985

45. Lendahl, U., Zimmerman, L. B., and McKay, R. D. (1990) *Cell* 60, 585-595

46. Hemmati-Brivanlou, A., Mann R. W., and Harland, R. M. (1992) *Neuron* 9, 417-428

47. Steinert, P. M., Chou, Y. H., Prahlad, V., Parry, D. A., Marekov, L. N., Wu, K. C., Jang, S. I., and Goldman, R. D. (1999) *J. Biol. Chem.* 274, 9881-9890

48. Marvin, M. J., Dahlstrand, J., Lendahl, U., and McKay, R. D. (1998) *J. Cell Sci.* 111, 1951-1961

49. Inagaki, M., Matsuoka, Y., Tsujimura, K., Ando, S., Tokui, T., Takahashi, T., and Inagaki, N. (1996) *Bioessays* 18, 481-487

50. Yuan, J., Huiatt, T. W., Liao, C. X., Robson, R. M., and Graves, D. J. (1999) *Arch. Biochem. Biophys.* 363, 314-322

51. Seki, N., Ohira, M., Nagase, T., Ishikawa, K., Miyajima, N., Nakajima, D., Nomura, D., and Ohara, O. (1997) *DNA Res.* 4, 345-349

52. Wilhelm, J., and Vale, R. (1993) *J. Cell Biol.* 123, 269-274

53. Oleynikov, Y., and Singer, R. H. (1998) *Trends Cell Biol.* 8, 381-383

54. Granger, B. L., and Lazarides, E. (1982) *Cell* 30, 263-275

55. Moon, R.T., and Lazarides, E. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 5495-5499

56. Sandoval, I.V., Colaco, C.A., and Lazarides, E. (1983) *J. Biol. Chem.* 258, 2568-2576

57. Kouklis, P. D., Hatzfeld, M., Brunkener, M., Weber, K., and Georgatos, S. D. (1993) *J. Cell Sci.* 106, 919-928

58. Makarova, I, Carpenter, D., Kahn, S., and Ip, W. (1994) *Cell Motil. Cytoskeleton* **28**, 265-277

59. Suzuki, A., Goll, D. E., Singh, I., Allen, R. E., Robson, R. M., and Stromer, M. H. (1976) *J. Biol. Chem.* **251**, 6860-6870

60. Hemmings, L., Barry, S. T., and Critchley, D. R. (1995) *Biochem. Society Trans.* **23**, 619-625

61. Lee, M. K., and Cleveland, D. W. (1996) *Annu. Rev. Neurosci.* **19**, 187-217

62. Steinbock, F. A., and Wiche, G. (1999) *Biol. Chem.* **380**, 151-158

63. Wiche, G., Krepler, R., Artlieb, U., Pytela, R., and Denk, H. (1983) *J. Cell Biol.* **97**, 887-901

64. Hijikata, T., Murakami, T., Imamura, M., Fujimaki, N., and Ishikawa, H. (1999) *J. Cell. Sci.* **112**, 867-876.

65. Schroder, R., Warlo, I., Herrmann, H., van der Ven, P. F., Klasen, C., Blumcke, I., Mundegar, R. R., Furst, D. O., Goebel, H. H., and Magin, T. M. (1999) *Eur. J. Cell Biol.* **78**, 288-295

Footnotes

[1]The abbreviations used are: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate; IF, intermediate filament; kb, kilobase(s); mAb, monoclonal antibody; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RACE, rapid amplification of cDNA ends; TRITC, tetramethylrhodamine isothiocyanate; UTR, untranslated region.

[2]M. M. Bilak and R. M. Robson, unpublished observations.

[3]S. W. Sernett and R. M. Robson, unpublished observations.

[4]T. W. Huiatt, D. J. Graves and R. M. Robson, unpublished observations.

Fig. 1. **Alignment of synemin cDNA clones.** Diagram showing the relative location of the library clones comprising the full-length synemin cDNA. Cross-hatched regions depict non-coding regions. The extent of the rod domain and tail domain bacterial expression constructs is labeled at the left as *Rod* and *Tail*, respectively.
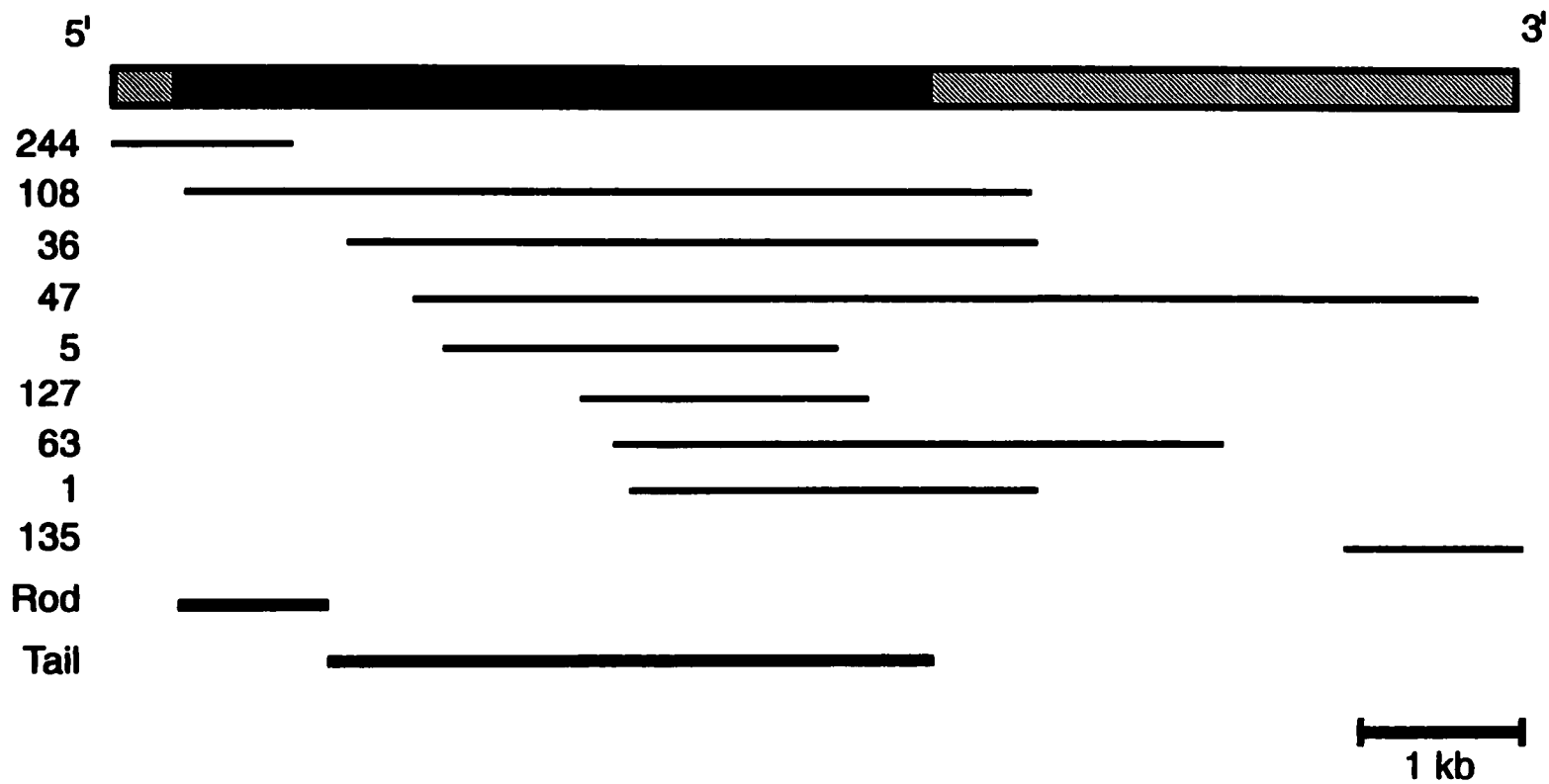
5'   3'

244
108
36
47
5
127
63
1
135
Rod
Tail

53

1 kb

Fig. 2. **Amino acid sequence of avian muscle synemin deduced from its cDNA sequence.**
Amino acids are designated by the one-letter code. *Bold type* denotes the extent of the
conserved rod domain typical of IF proteins. The extent of each of the subdomains within the
rod domain is as follows: 1A = residues 11 - 46, L1 = residues 47 - 57, 1B = residues 58 –
152, L12 = residues 153 – 166, 2A = residues 167 – 206, L2 = residues 207 – 217, and 2B =
residues 218 – 314. The "intermediate filament signature", identified with the GCG program
Motifs, is *underlined*. The nucleic acid sequence of synemin is available from GenBank
under Accession Number U28143.

```
   1  MWRRWEAGWDEKRELQELNSRLRVFVSRVRELEEENRFLARELAELREQELIGLRVPEQE    60

  61  LAWLRMQLEELSQAKLEAELERDGLRRELEQLQLLGAEVLATRRRLEPELAGQRQLLQRL   120

 121  QGECVALEELLLQLQDEHGHLAERQRREVVEIRELRMDLAALPPPLSALSLEELEETYEM   180

 181  LLSQSCQETLLRYQEQIQRLQEQEAQRSRENLELLREESRQCRQHLEDLHRQGQELCGLR   240

 241  ERLEQELLAMQDRHGAEVEEYQRIIDALEEEKQFLTMSITDYLRDYQELLQVKAGLILEI   300

 301  ETYRALLEGKSNQWVITWRDQLIGKLPQDIINTSYNYTDMYSTYQERNQNKTSPAIRITD   360

 361  TRHRIPATNTSSSARYSAQSTQAGSQTAVSGTAFGKGVLGSIYRPSTTIRKDERIVTDHK   420

 421  ELRTYTPGYSSWKHTETQQKTIPERKKTEVTSSSTVSFSKQSTHAERSDKDNKADTKPKI   480

 481  SESLRTKSNFSKFPTYELNTSSKPSVYEETITETRTTIKEKRGGSKPTEEGTSLPKEKDK   540

 541  LEKQTKEEKRKVDEKTFVEERSVNFGRQTDAKMGIEQKKYVREEVISQKVAESDISNART   600

 601  LKKESSKKDTDVSFESKSKEVIEIPITLERPAPDKVSQKKNEDISSQDFKTSRGRETGDH   660

 661  VTKPAEIHKIRVSESDLRDEEKTSDRSHKMKTLATESIAENIAADILKSFTPSSSSEVST   720

 721  DTKVTSSSKQEQPDDGKIRTEITVQSRVQENIDVSAEADRGSLSNQDVRKVLESAEGTPS   780

 781  KQEIENIVRHGLKGSGVAKNMSVNVEIVEETVDYTTDERTDFSTPFEVEEVEDTFPEREK   840

 841  RYGDDEQDITLTYEDLKKKKQRHESFTHVEEVTEEDDSPIEQKYFVSVPDDHPIINEKDD   900

 901  DSVYGQIHIEEESTIKYSWQDDFVQGTQSKRDEGVSSPEETYRVVGEEAAAHILKEEHPK   960

 961  AGTSHVESVVIEKEIKIPQEFQASIKGLLSKETKDPKQQLKEALEQLGGSLPESVKEELS  1020

1021  ALTKESQADSSNLEFDIKKVHQTEEGGLLTIVAEVNLSQTINADEFDIAQLGEIIAGEKE  1080

1081  KTTSHSLKRDKLERVAGGKISTETDVSARSDIYTPVANQEVYKSSTVRKTGSTGYHTTEK  1140

1141  VIYDSSILETANLGEDSHSPESTDESKSVRHIRVGPTEVQRFEQIVYEGPGSEMLGLSAT  1200

1201  EDVVQTEGTSEMSHSVKHFKLGPKEIQTTEEVIYRGPITTTVEEIDSGNLSQQTFSSDIN  1260

1261  RSTRHVTVGSRQVIEEVSFEGLGSDSLELNSSGHLSQSEGSVDVSRSVRQFRLHPKEIHS  1320

1321  EQVIFEGPISGKVEVSDSRDFSQTESSVRHIQLGPKEFMTAEKVIYQGPITEHIEISEVG  1380

1381  DQTHSESSVKHFRLGQTGVRTTERIIYQGSLSETPELINKGHLSENEGSSDISTSFRHVR  1440

1441  ITPVETQTEQIIFTRPISETLEDHSQARESSESSSSMKHVKVGSSGTSFTFQMDVSNVGG  1500

1501  VSMAESEEQATVVISNKQDPSVNQSQFRVESDHSGDSENKRSYIHSSFSQDHVENVVEKS  1560

1561  DFDKTVQLQRMVDQRSVISDDKKVALLYLDHEEEDDEENDGQWF   1604
```

Fig. 3. **Western blot analysis of endogenous expression of synemin and vimentin in SW13 cells.** *Panels A* and *B* depict the identical blot of fractionated cell lysates, with *A* probed with synemin pAb 2856, and *B* stripped and re-probed with vimentin mAb AMF-17b. *Lane 1*, avian gizzard homogenate (a stored sample exhibiting synemin and its proteolytic degradation products in *panel A* (27), and the small amount of expected vimentin in *panel B*); *Lane 2*, SW13.C1vim+ supernatant; *Lane 3*, SW13.C1vim+ pellet; *Lane 4*, SW13.C2vim- supernatant; and *Lane 5*, SW13.C2vim- pellet. The approximate migration distances of filamin (*250* kDa), myosin heavy chain (*205* kDa), α-actinin (*100* kDa), desmin (*53* kDa), and actin (*42* kDa) from the gizzard homogenate are indicated.

57
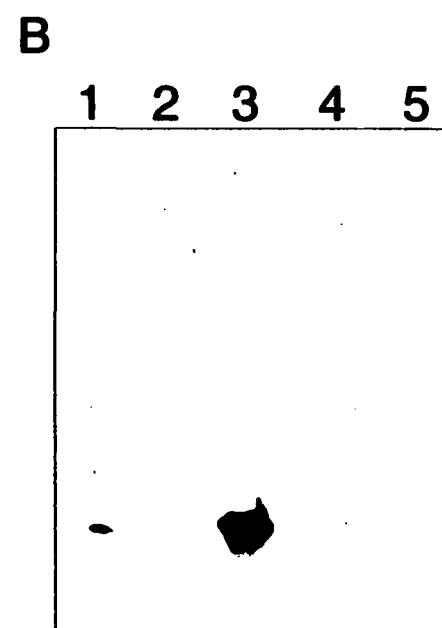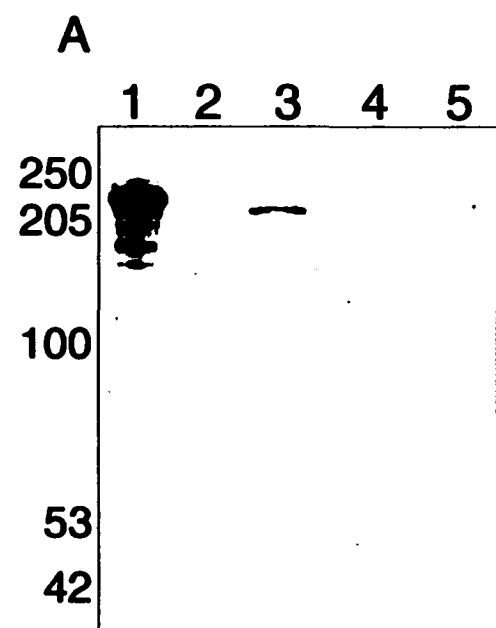
Fig. 4. **Immunofluorescence localization of synemin and vimentin in SW13 cells.** Panels on the left (*A*, *C*, and *E*) depict immunofluorescence labeling with synemin pAb 2856. Panels on the right (*B*, *D*, and *F*) depict fluorescence labeling with vimentin mAb AMF-17b. *Panels A* and *B* show endogenous synemin/vimentin expression in a filamentous pattern typical of IFs in the SW13.C1 vim+ cells. *Panels C* and *D* show the lack of endogenous synemin/vimentin expression in the SW13.C2 vim- cells. Note that synemin colocalizes with vimentin in SW13.C1 vim+ cells (*panels A* and *B*), but is absent from SW13.C2 vim- cells as is vimentin (*panels C* and *D*). *Panels E* and *F* show immunofluroescent labeling of SW13.C2 vim- cells after transfection with full-length synemin cDNA. Note that the synemin expressed in the SW13.C2 vim- cells appears in a non-filamentous, punctate pattern when vimentin is absent (*panel E*). *Bar* = 10 μm for *A-F*.
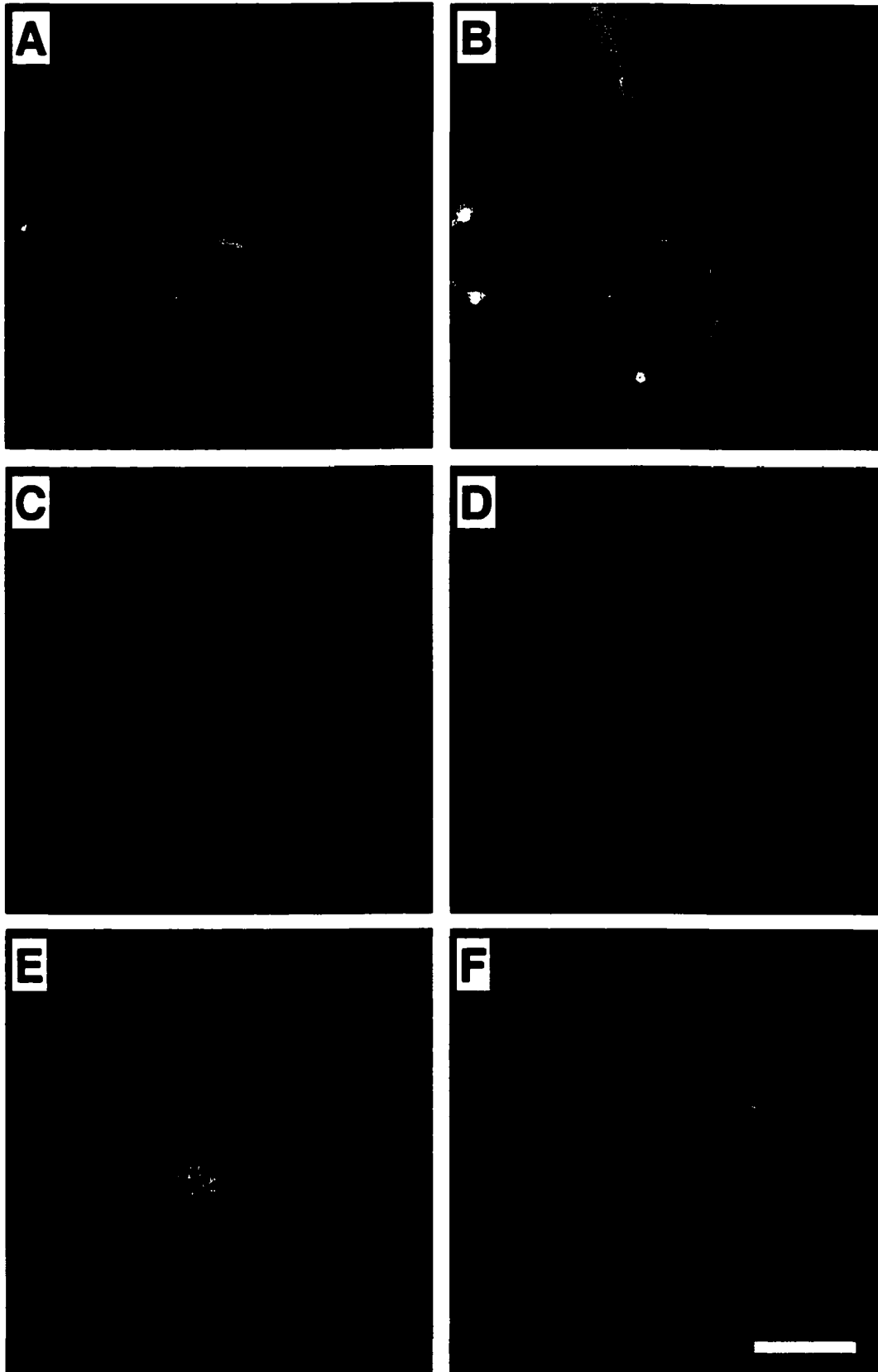
Fig. 5. **Cosedimentation of purified synemin with purified desmin.** *A,* SDS-PAGE analysis of results from mixing purified synemin with desmin in non-IF forming conditions (10 mM Tris-HCl, pH 8.5). *B,* SDS-PAGE analysis of results from mixing purified synemin with desmin in non-IF forming conditions, and then adjusting the buffer conditions to induce filament formation (100 mM NaCl, 1mM $MgCl_2$, pH 7.0) before centrifugation. *C,* SDS-PAGE analysis of results from mixing purified synemin with preformed desmin filaments. For *A, B,* and *C, panel 1* is desmin alone, without synemin, *panel 2* is synemin alone, without desmin, and *panel 3* is desmin and synemin mixed; *S,* supernatant; *P,* pellet. BSA was added to all samples to show that virtually no exogenous (unbound) protein (e.g., synemin) was simply trapped within the volume of the pellet(s).
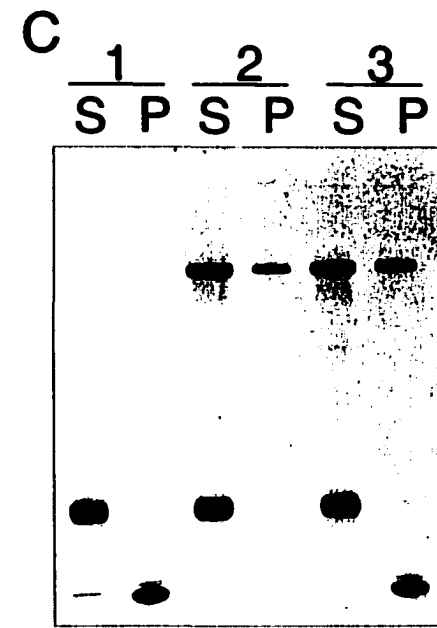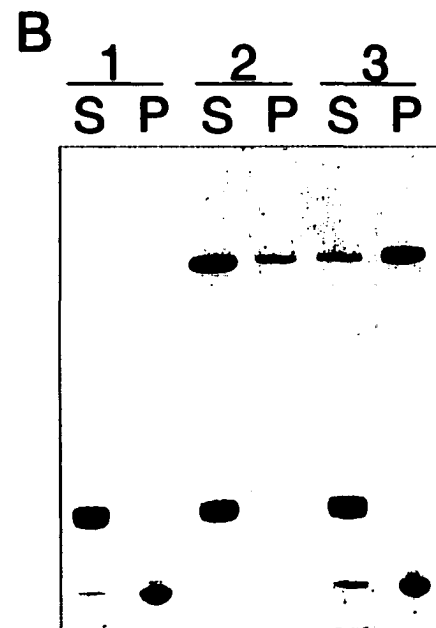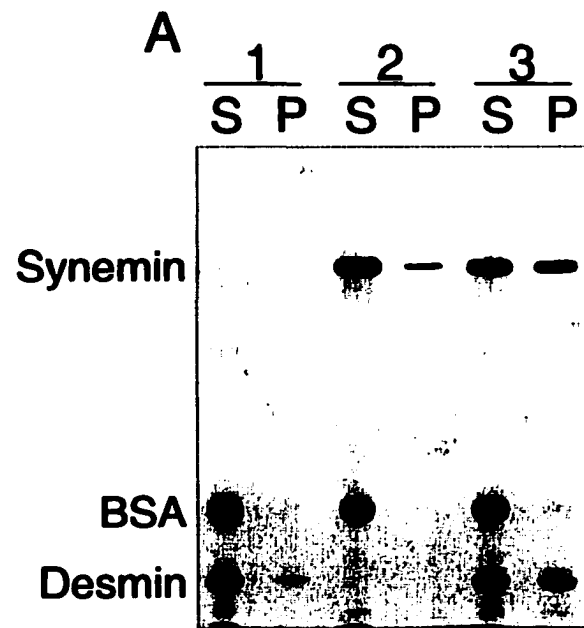
Fig. 6. Cosedimentation of bacterially-expressed synemin rod domain with purified desmin. SDS-PAGE analysis of results from mixing bacterially-expressed synemin rod domain with purified desmin under non-IF forming conditions, and then adjusting the buffer conditions to induce filament formation (100 mM NaCl, 1mM $MgCl_2$, pH 7.0) before centrifugation. *Panel 1* is desmin alone, without synemin rod; *panel 2* is synemin rod alone, without desmin; *panel 3* is desmin and synemin rod mixed; *S*, supernatant; *P*, pellet. In every case, BSA was added to show that virtually no exogenous (unbound) protein (e.g., synemin rod) was simply trapped within the volume of the pellet(s).
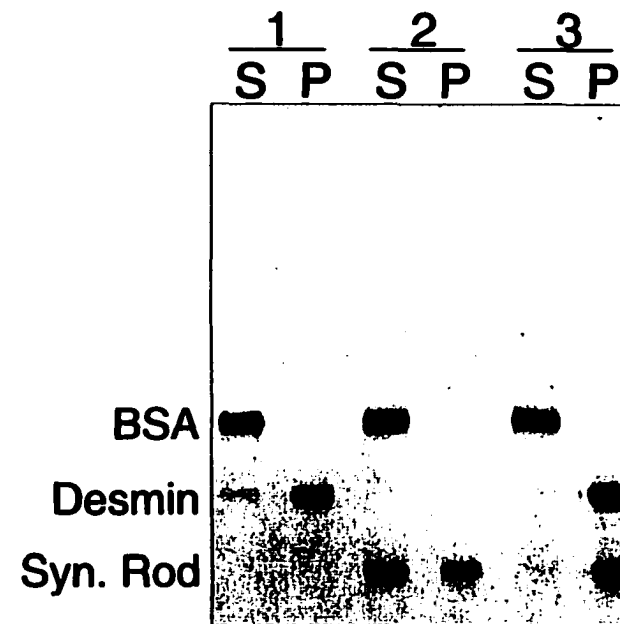
Fig. 7. **Blot overlay assay of purified synemin and of bacterially expressed synemin rod and tail domain interactions with desmin and α-actinin.** *Panel A*, SDS-PAGE gel with *lane 1*, whole gizzard homogenate; *lane 2*, purified desmin; *lane 3*, purified α-actinin; and *lane 4*, purified synemin. *Panels B-E* depict Western blots resulting from transfer of gels like that shown in *panel A*. In each case avian gizzard homogenate, purified desmin, purified α-actinin and purified synemin were subjected to SDS-PAGE and transferred to nitrocellulose. *B*, the blot was overlaid with buffer only before detection with synemin pAb 2856. *C-E*, the blots were treated identically as *B*, but were overlaid with purified synemin (*C*), bacterially-expressed synemin rod domain (*D*), and bacterially-expressed synemin tail domain (*E*) before washing and detection with synemin pAb 2856. The approximate migration distances of filamin (*250* kDa), myosin heavy chain (*205* kDa), α-actinin (*100* kDa), desmin (*53* kDa), and actin (*42* kDa) from the gizzard homogenate are indicated at the left. The approximate migration position of synemin corresponds to 230 kDa.

A

250
205

100

53
42

1 2 3 4

B   1 2 3 4

C   1 2 3 4

D   1 2 3 4

E   1 2 3 4

65
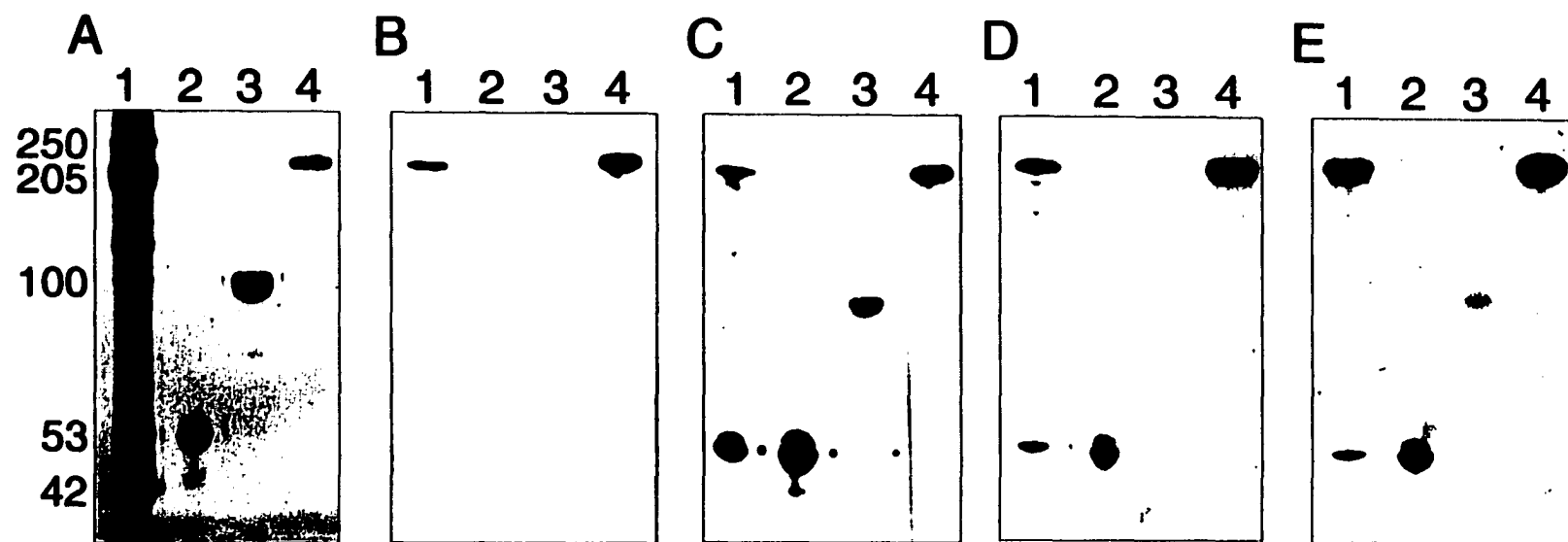
SYNEMIN MAY FUNCTION TO DIRECTLY LINK MUSCLE CELL INTERMEDIATE FILAMENTS TO BOTH MYOFIBRILLAR Z-LINES AND COSTAMERES

A paper to be submitted to The Journal of Biological Chemistry

Robert M. Bellin, Suzanne W. Sernett, and Richard M. Robson

Summary

Synemin is a large intermediate filament (IF) protein that has been identified in all types of muscle tissue in association with desmin- and/or vimentin-containing IFs. Our previous studies (Bellin et al., *J. Biol. Chem.*, 1999, 274:29493-29499) demonstrated that synemin forms heteropolymeric IFs with major IF proteins, and contains, within its large C-terminal tail domain, a binding site for the myofibrillar Z-line protein α-actinin. By utilizing blot-overlay assays, we show herein that synemin also interacts with the costameric protein vinculin. Furthermore, extensive assays utilizing the *Gal4* yeast two-hybrid system demonstrate interactions of synemin with desmin, vimentin, α-actinin, and vinculin within a cellular context. Results of the two-hybrid analyses also more precisely define the protein sub-domains involved in the synemin/α-actinin and synemin/vinculin interactions. The C-terminal ~300 amino acids of synemin have affinity for the N-terminal head and central spectrin-like rod domains of α-actinin. The C-terminal ~300 amino acids of the synemin tail and the synemin IF rod domain have affinity for the small ~150 amino acid C-terminal tail, but not the large head domain, of vinculin. Overall, these interactions indicate an important function for synemin in striated muscle cells, i.e., synemin may anchor heteropolymeric IFs to myofibrillar Z-lines via interactions with α-actinin, and to costameres at the sarcolemma

via interactions with vinculin. These linkages would enable the IFs, which closely encircle each myofibrillar Z-line, and also extend to costameric regions at the sarcolemma, to directly attach and link all cellular myofibrils, and to anchor the peripheral layer of myofibrils to the costameres. This IF-based linkage would maintain and promote the overall cytoskeletal integrity needed in highly contractile cells.

Introduction

Intermediate filaments (IFs)[1], along with microfilaments and microtubules, comprise the three cytoskeletal filament classes found in animal cells (1-5). The IFs are composed of cell-type specific proteins that are assembled *in vivo* into very long ~10 nm-diameter filaments (5-9). The superfamily of IF proteins currently contains ~60 members (10), and novel members continue to be identified (10-12). Because of the diversity among members of the protein superfamily, it is possible that IFs within different cell types may be formed that have diverse functions (5). Additionally, because the majority of IFs found in living cells are heteropolymeric in nature (i.e., composed of more that one IF protein member), even more diverse functionality of an individual IF is possible (5).

The IFs have long been considered to play an important role as mechanical integrators of cellular space (2, 13). The actual mechanism for how this cytoskeletal integration within cells occurs, however, remains poorly understood. For IFs to connect cell components, some form or type of attachment of IFs to other cytoskeletal components are necessary. These attachments could be indirect, via a crosslinking protein such as plectin that binds to several cytoskeletal proteins (14), or direct, by linking IF proteins themselves to protein components in other cellular structures.

Synemin was initially described as an IF-associated protein, because it colocalized and copurified with the IF proteins desmin and vimentin (15-17). We have, however, recently demonstrated that synemin is, in fact, an IF protein because the sequence contains the ~310 amino acid rod domain typical of IF proteins (12, 18). Furthermore, we demonstrated synemin is a novel member of the IF protein superfamily that contains an unusually large C-terminal tail domain. Synemin has been localized with desmin- and/or vimentin-containing IFs in all types of developing chick (19) and adult avian and porcine muscle cells (20); in chick lens cells (21, 22); in human SW13 adrenal cortex adenocarcinoma cells (12); and, most recently, in rat radial glial cells (23). Because synemin has so far always been found in the presence of at least one major IF protein, and because it fails to form IFs by itself *in vitro* (20) and when transfected into cells that lack other IF proteins (12), we believe it is very likely that synemin only functions within cells as a component of heteropolymeric IFs.

We have shown previously by blot-overlay assays that synemin interacts with both desmin and α-actinin (12). In this paper we now show, by blot-overlay assays, a direct interaction between synemin and the tail domain of vinculin. Furthermore, we demonstrate those interactions, and further define the specific binding sites for these interactions, by using the *Gal4* yeast two-hybrid system. We believe these specific interactions further support a role for synemin, as a component of heteropolymeric IFs, in directly attaching these IFs to other cytoskeletal structures via protein-protein interactions involving synemin.

A major interest in our lab is to define how the cytoskeletal elements are organized and attached to each other within muscle cells. We present a schematic showing how synemin in striated muscle cells may serve as a cytoskeletal crosslinking component by

enabling synemin-containing heteropolymeric IFs to directly bind to myofibrillar Z-lines and thereby link all cellular myofibrils via synemin/α-actinin interactions, and to anchor the peripheral layer of cellular myofibrils to the costameric sites at the sarcolemma via synemin/vinculin interactions.

Experimental Procedures

Protein Purification—Synemin (12, 20) and vinculin (24) were purified from avian smooth muscle as described. Proteolytic fragments of vinculin were prepared by treatment with V8 protease (25). The precise cleavage site(s) in vinculin was determined by protein microsequencing from PVDF membrane by the Iowa State University Protein Facility. The specific rod and tail domains of synemin were expressed in bacteria and purified (12).

Blot Overlay Assays—Blots of samples of highly purified synemin, desmin, vinculin, and proteolytically digested vinculin, plus a sample of avian smooth muscle homogenate, were probed, by using blot-overlay procedures (12) with synemin, expressed synemin rod, and expressed synemin tail domains. The polyclonal antibody (pAb) 2856 has been characterized previously (12), and recognizes intact synemin, and both the rod and tail domains of synemin.

Preparation of Yeast Two-Hybrid Constructs—Constructs used for desmin (26) and vimentin (27) have been described. Constructs encoding the entire synemin molecule, as well as specific domains of synemin, α-actinin and vinculin, were generated by PCR and cloned into both the pPC86 and pPC97 yeast two-hybrid vectors originally described by Chevray and Nathans (28). The pPC86 and pPC97 vector pair have been used in other two-hybrid studies on IF proteins (26, 27, 29).

Yeast Transformations—The PCY2 strain of yeast (28) was co-transformed with the Alkali-Cation Yeast Transformation kit (Bio101) using the manufacturer's protocol. An empirically-determined optimal amount of 400 ng of plasmid DNA was used for each construct.

Fluorescence-Based Quantitative Two-Hybrid Assays—Quantitative determinations of all two-hybrid interactions were carried-out by using the FluorAce β-galactosidase Reporter Assay Kit (BioRad) modified for use with yeast cells as follows. Yeast lysates were prepared from 2 ml yeast cultures (in double drop-out Leu⁻ Trp⁻ media, 180 RPM at 30° C for 72 h, followed by 3 h of protein induction in galactose containing Leu⁻ Trp⁻ media, 180 RPM at 30°C), by freezing the yeast pellet in liquid nitrogen for 5 min and resuspending in 100 µl of Lysate Buffer (1 mM $MgCl_2$, 0.1% NaN_3, 10 mM sodium phosphate, pH 7.0). An empirically-determined, constant amount of yeast lyaste (20 µl) was mixed with 50 µl of the kit's 1X Reaction Buffer, including 4-MUG and β-mercaptoethanol, directly in the wells of a black microtiter plate kept on ice. The plate was incubated for 3 h at 37° C, followed by addition of 150 µl of the kit's 1X Stop Buffer. The plate was then read with a Titertek Fluoroskan fluorescent microplate reader with excitation at 355 nm and emission at 460 nm. The specificity of interactions was confirmed by performing vector-swap experiments for each protein pair. Both the original vector pair and the vector-swap pair were independently tested twice, with the numbers for each determination resulting from reaction wells set-up in triplicate and averaged. The quantitative data reported in the figures were calculated by using an A_{600} measurement of each individual culture to normalize the respective fluorescence reading. The resulting data were then used to calculate relative interaction

affinities by comparing each reading with the value determined for the synemin rod/desmin interaction which was included with each set of assays.

Results

Blot Overlay Assays—Analyses utilizing purified synemin and vinculin, as well as bacterially-expressed rod and tail domains of synemin, and the proteolytic products of purified vinculin resulting from V8 protease treatment, were done to identify specific protein domain interactions. Vinculin was selected as a potential interaction partner for synemin both because it is a key cytoskeletal protein potentially involved in signal transduction pathways (30, 31) and because it is a component of the costameres (32), one of the attachment sites for striated muscle cell IFs (33). Results from protein microsequencing of the fragments resulting from V8 protease digestion of vinculin identified that the newly generated N-termini of two low molecular weight bands (~24 kDa and ~22 kDa in Fig. 1) have the sequences: LAPPKPPLPE and GEVPPPRPPP. These sequences agree precisely with residue numbers 858-867 and 868-877, respectively, in the avian vinculin sequence (GenBank accession number Y00312), therefore showing clearly that they represent tail fragments from vinculin. As is shown in the "buffer overlay control" blots lacking any probe protein in the overlay solution (Fig. 1, *panels A* and *D*), the synemin pAb 2856 labeled synemin present in the gizzard homogenate (*lane 1*), purified synemin (*panel A, lane 2*), and expressed synemin rod domain (*panel D, lane 2*), but did not label purified vinculin (*panels A* and *D, lane 3*), the small amount of intact purified vinculin remaining after V8 protease treatment (*lane 4*), nor any other proteins in the gizzard homogenate (*lane 1*). As is shown in Fig. 1 (*panel B*), probing blots of vinculin and V8-digested vinculin with purified intact synemin in the overlay reveals an interaction of synemin with purified intact vinculin (*lane*

3), the very small amount of intact vinculin remaining in the V8 protease digest of vinculin (*lane 4*) and with the small tail domain fragment(s) of vinculin produced by the V8 protease digestion (*lane 4*), but not with the large head domain fragment of vinculin that migrates with an approximate size corresponding to 90 kDa, (compare *panel B*, *lane 4* with the Coomasie-stained gel of digested vinculin sample showing the vinculin head (*H*) and tail fragments (*T*) in *panel F*). Additionally, an interaction between the probe synemin in the overlay solution and desmin in the gizzard homogenate lane (*panel B, lane 1*), can be seen as we reported earlier (12). As is shown in Fig. 1, probing blots with expressed synemin tail (*panel C*) and rod domains (*panel E*) results in similar binding patterns to that from using intact synemin, although only very weak binding to the vinculin tail domain is seen (*panels C and E, lane 4*). Specificity of the synemin binding to vinculin, specifically the vinculin tail domain, is evident in Fig.1 in that neither synemin (*panel B*) nor synemin domains (*panels C and E*) bound to any of the other proteins (e.g., filamin, myosin heavy chains, and actin) present in the gizzard homogenate (*lane 1*)

Two-hybrid Analysis—Identification and analysis of interactions by the *Gal4* two-hybrid system must include significant controls to demonstrate "seemingly positive" interactions are not due to "false positives" of the assay system. This is especially a concern when testing helical proteins, such as an IF protein rod domain, because a charged helix attached to the DNA-binding portion of *Gal4* (as is present in the pPC97 vector) has been shown, in some cases, to activate β-galactosidase reporter gene expression (34). The constructs used herein are shown in Fig. 2. As demonstrated in Fig. 3, when each of the constructs encoding a domain of synemin (see Fig. 2) is co-transfected with a negative control consisting of an empty two-hybrid vector (e.g., synemin rod insert in pPC86, but no

insert in pPC97), the values resulting from the fluoresence assays were very low, which indicate that none of the synemin constructs activates transcription by itself.

Results from two-hybrid assays testing synemin/desmin interactions are shown in Fig. 4. The rod domain of synemin interacts strongly with desmin (*column 1*), in comparison with the vector control (*column 9*). This is not unexpected because other studies have shown the rod domains of IFs are the primary interaction domains between IF protein pairs (reviewed in (5)). Interestingly, the synemin rod domain/desmin interaction (*column 1*) is somewhat weaker than the desmin/desmin dimerization (*column 10*). Results shown in Fig. 4 (*columns 2-8*), demonstrate that none of the tail domain constructs of synemin exhibit interactions with desmin in these assays.

The results of the two-hybrid assays of the interactions between synemin and vimentin (Fig. 5) are very similar to those seen between synemin and desmin (Fig. 4), as may be expected because of the high sequence homology between the Type III IF proteins vimentin and desmin (35). The rod domain of synemin reacts most strongly with vimentin (*column 1*), when compared with the vector control (*column 9*), and this interaction is slightly weaker than the dimerization of vimentin (*column 10*), but is approximately the same strength as synemin rod dimerization (*column 11*). The results shown in Fig. 5 (*columns 2-8*) demonstrate that none of the tail domain constructs of synemin interact with vimentin, akin to the lack of synemin tail interactions with desmin (Fig. 4).

Results from two-hybrid analysis of interactions between synemin and α-actinin are shown in Fig. 6. Analysis of these interactions needs to take into account that the full-length α-actinin pPC97 construct activated transcription by itself to a moderate degree (Fig. 6, *column 9*). As shown in Fig. 6, when compared to the vector controls (*column 9*), the

interaction of synemin with α-actinin is specific for the tail domain of synemin (*column 2*). Analysis of the constructs of the synemin tail domain split into four non-overlapping parts indicates that SN Tail IIb has the strongest interaction (*column 8*), thus mapping the interaction domain to the last quarter of the C-terminal tail of synemin. Additionally, testing this tail domain construct with domains of α-actinin demonstrates interaction of the SN Tail IIb with the N-terminal head domain of α-actinin (*column 10*), which contains the actin binding site, and with the rod domain of α-actinin (*column 12*), which contains four spectrin-like repeats (36). Each of these interactions are much stronger than the respective vector control results (c.f., compare *columns 10* and *11;* and *12* and *13*). Two-hybrid assays of the remaining synemin constructs with the three α-actinin constructs revealed no other strong interactions.[2]

The results of the two-hybrid assays of the interaction between synemin and vinculin are shown in Fig. 7. Because the head and tail domains within a single vinculin molecule are known to interact in a fashion that blocks some protein interaction sites in solution (25, 37), interactions with vinculin were tested on sub-domains of vinculin, rather than on the whole molecule. In agreement with the blot overlay assays shown earlier (Fig. 1), the synemin rod (*column 7*) and synemin tail domains (*columns 8, 10* and *12*) interact most strongly with the vinculin tail domain in comparison with the vector control (*column 13*). Within subdomains of the synemin tail domain, the strongest interaction with the vinculin tail was found with the SN Tail IIb construct (*column 12*). Thus, the end of the tail domain, as well as the rod domain (*column 7*), of synemin show affinity for the vinculin tail domain. Two-hybrid assays of the remaining tail domain constructs of synemin with the vinculin constructs revealed no other strong interactions.[2]

Discussion

We believe the newly identified interaction between synemin and vinculin is an important advancement in our understanding of the cytoskeleton. Although it has long been known that IFs attach or insert into, and are presumably somehow linked to, protein-rich cytoskeletal/membrane attachment sites, the only well established mechanism for attachment of IFs involves members of the plakin family (29, 38-40). Vinculin is a component of both cell-cell and cell-matrix type adhesion plaques (41). The cell-matrix adhesion plaques include the membrane-associated dense bodies in smooth muscle, the neuromuscular and myotendenious junctions in skeletal muscle, and the costameres of striated muscle (32, 42). Thus, the interaction between synemin and vinculin may serve as a mechanism of IF attachment to the costameres. Additionally, because vinculin, in turn, can be anchored to the cell membrane via an interaction with the integrin-binding protein talin (41, 43), the synemin-vinculin-talin interaction may also serve as a linkage of IFs to the cell membrane.

The results of the *Gal4* yeast two-hybrid assays, using specific interaction pairs, provide direct confirmation of the interactions of synemin within the context of a living cell, which originally were identified by methods using purified proteins ((12) and studies herein). The yeast two-hybrid system serves this purpose well because it is complementary in approach to the blot-overlay based *in vitro* methods initially used to identify these interactions. Although concerns can be raised over the validity of results from blot-overlay interaction assays because one protein is immobilized on the surface of a membrane, protein interactions identified by the two-hybrid system occur within a cellular environment. Conversely, the results obtained by the *in vitro* assays using tissue-purified proteins nicely complement results from the two-hybrid assays because the former utilized proteins that were

prepared from their normal tissue source (i.e., synemin, desmin, α-actinin and vinculin purified from muscle tissue). They, therefore, likely contain native protein post-translational modifications, which are not expected within the context of a yeast cell. Thus, the combination of approaches and results reported herein and previously (12) involving interactions of synemin provide complementary findings obtained by at least two different methods for the interactions of synemin with desmin, α-actinin and vinculin.

Although the usual and most basic application of the yeast two-hybrid system is to determine if two proteins have affinity for each other (44), use of the yeast two-hybrid system in conjunction with a quantitative detection method, as was used herein, additionally provides a relative measurement of the strength of the protein-protein interaction. The interactions between IF proteins were the strongest of those we tested. To provide a useful "ruler" of relative protein interaction strength, all values reported in this paper are relative to the value of the interaction measured between the synemin rod domain and desmin. That interaction was very similar in strength to that obtained for both the interaction between the synemin rod and vimentin and for the self-dimerization of the synemin rod. Although it might be expected that all IF protein interactions would be of similar strength because of the relatively high sequence homology and conservation in overall length of the cytoplasmic IF rod domains, the strength of the self-dimerization interactions of desmin and of vimentin were significantly higher than for all of those that included the synemin rod domain. A possible explanation for this finding is that, in contrast to the vimentin and desmin constructs, the synemin rod domain construct lacks any flanking sequences, which may play a role in further stabilization of the IF protein interaction. Regions of the N-terminal head and C-

terminal tail domains both have been found to be important in proper IF assembly in studies on both desmin and vimentin (45-47).

It is notable that, based on strength of interactions obtained herein, synemin is as likely to interact with desmin or vimentin as with itself. Based upon studies of the co-polymerization of the large IF protein nestin with vimentin (48), it has been suggested that the mode of integration of these large IF proteins into mature IFs initially involves formation of a heterodimer rather than a simple homodimer of the large IF protein (i.e., synemin/desmin, rather than synemin/synemin). It has been suggested that the large IF protein-containing heterodimers, together with homodimers of the small IF proteins (e.g., desmin/desmin), then assemble into filaments that contain more small IF protein subunits than large IF protein subunits (5). That the total amount of synemin is only ~1% of the total amount of desmin in an adult muscle cell (15) is consistant with that model for assembly of heteropolymeric IFs.

In comparison to the measured strength of the IF protein/IF protein interactions herein, the interactions between synemin and either α-actinin or vinculin are considerably weaker. Considering the nature of the protein interactions, however, this finding is not unexpected. The IF proteins have been shown to interact with each other by strong coiled-coil interactions between their ~310 amino acid long rod domains (6, 8, 9). The major force driving this protein interaction is thought to be due to interactions of a hydrophobic stripe of apolar residues along each of the α-helical rod domains of two polypeptides (7, 49), as well as charge-charge interactions that further stabilize the rod domain interactions (50, 51). By comparison, the interactions of synemin with α-actinin and with vinculin map to much smaller protein domains. Furthermore, they are unlikely to result from coiled-coil

interactions because the tail domain of synemin lacks any strongly helical domains needed for participation in a coiled-coil interaction (12).

The results of the two-hybrid studies herein further define the precise protein domains involved in the interactions we recently reported between synemin and α-actinin (12). The α-actinin molecule is composed of an N-terminal head domain containing an actin-binding site, a central rod domain composed of four spectrin-link repeats, and a C-terminal tail domain containing two EF hand homology regions (36, 52). Assays utilizing several constructs of domains of both the synemin tail and of α-actinin revealed the smallest interacting domains between the two proteins to reside within the C-terminal ~300 amino acids of the synemin tail, and within both the head and rod domains of α-actinin (Fig. 6). The binding site for α-actinin in synemin seems logical, because the end of the synemin tail may be the domain extended furthest from the surface of the 10 nm core of the IF, and therefore most likely to reach α-actinin molecules at the Z-line. It is not currently obvious why this domain of the synemin tail binds to two domains of α-actinin. Because these two domains are adjacent within α-actinin, and the strength of synemin binding to each domain is approximately half that of the entire α-actinin molecule (see Fig. 6), it is possible that the actual synemin binding domain of α-actinin spans both the α-actinin head and rod domains.

The blot overlay results demonstrate an interaction between synemin and vinculin. The two-hybrid assays provide additional important information about the specific protein domains involved in the synemin/vinculin interaction. The use of three separate constructs, two of which each code for approximately half of the large vinculin head domain, and one that codes for the small C-terminal vinculin tail domain, reveals that only the tail domain of

vinculin has affinity for synemin (Fig. 7). This small ~150 amino acid domain is a very interesting part of the vinculin molecule. The crystal structure of the tail of vinculin was recently solved, revealing it to most likely exist as a highly extended domain (53). The tail domain of vinculin contains two actin-binding sites, and their affinity for actin is regulated by binding of the tail domain to a site on the large head domain of vinculin, an interaction that, in turn, can be controlled by the binding of specific phospholipids (30, 31, 37, 54). Additionally, it was recently reported that the vinculin tail domain may act as a dimerization domain for vinculin, with actin filaments inducing the dimerization by stablizing interactions between the tail domains of two vinculin molecules (55). Whether some of these dynamic activities of the vinculin tail also affect their binding to synemin is unknown. The question of whether vinculin head-tail association also regulates synemin binding was not directly addressed in the blot-overlay assays in this paper (see Fig. 1) because full length vinculin exists in an "open" conformation when blotted to a membrane (56). In preliminary *in vitro* studies, however, we have found that purified full-length vinculin does not bind when it is overlaid onto synemin (i.e., the vinculin would be in the closed conformation), in contrast to a positive interaction when bacterially-expressed vinculin tail is overlaid onto synemin.[3] That the tail domain of vinculin has affinity for both the rod domain and the C-terminal ~300 amino acids of the very long tail domain of synemin is intriguing. Why vinculin has affinity for two distinct domains of the synemin molecule is unknown, but it is reasonable to suggest that these discrete vinculin binding sites in synemin may bind to separate vinculin molecules in the costamere, which would form a matrix/network between the IFs and vinculin, and result in strong anchoring of the IFs to these sites.

The binding sites for synemin on both α-actinin and vinculin exist within protein domains that also contain actin binding sites. Whereas these findings may indicate that α-actinin and vinculin act as "tight linkers" between synemin-containing heteropolymeric IFs and actin filaments, it is also possible that these IFs compete with actin filaments for binding to these proteins. The latter option raises the possibility that signal transduction pathways that effect actin filament dynamics could also alter binding to synemin-containing IFs. Conversely, it is possible that dynamic changes in the IF proteins, such as those induced by covalent modifications including phosphorylation (57) and ADP-ribosylation (58), may also impact the actin cytoskeleton. Synemin itself has been found to be an excellent substrate for both phosphorylation (16) and ADP-ribosylation (12) reactions.

Although the interactions identified for synemin with α-actinin and vinculin demonstrate a possible mechanism(s) of direct linkage between IFs and other cytoskeletal structures, some recent studies have implicated the protein plectin as an indirect mechanism for anchoring muscle cell IFs to other cytoskeletal structures (59, 60). Plectin is a member of the plakin family of proteins that also includes the protein desmoplakin, which is involved in anchoring of IFs to desmosomes (29, 38-40). Plectin contains binding sites for protein components of all three of the cytoskeletal filament classes (microtubules, microfilaments and IFs) (14, 61), so it often is called a universal cytoskeletal crosslinking protein (14, 62). A recent study showed that plectin interacts with desmin (63). Thus, it appears likely that plectin helps link IFs to other structures in muscle cells. Because plectin has been shown to be such a general crosslinking protein, synemin may play the essential role in establishing the direct linkages between heteropolymeric IFs and the Z-line and costameric regions. Once

direct linkages are established, plectin may provide additional structural support at these sites.

Recent studies indicate that synemin is a component of heteropolymeric IFs in striated muscle cells (12, 20). The IFs in striated muscle cells are located at the periphery of myofibrillar Z-lines, link adjacent myofibrils at their Z-line levels, and extend from the Z-lines of the peripheral layer of cellular myofibrils to the costameres along the inside of the sarcolemma (33, 64-66). Based upon this localization and the interactions of synemin with (a) the IF proteins vimentin and desmin, (b) the myofibrillar Z-line protein α-actinin, and (c) the costameric protein vinculin, a likely role for synemin in striated muscle cells can be proposed (see Fig. 8 for a schematic representation). The synemin/desmin-containing heteropolymeric IFs encircle each myofibrillar Z-line, and may be firmly anchored to the Z-lines by interactions between the end of the synemin tail domain and domains within α-actinin. Additionally, the IFs extending from the Z-lines of the peripheral myofibrils to the costameric regions may be anchored there by interactions between synemin and the tail domain of vinculin molecules. Because of the strong linkage between vinculin-talin and the muscle cell membrane, the direct synemin/vinculin interaction would effectively connect the peripheral layer of myofibrils to the cell membrane. The importance of these linkages has been shown in the muscle cells of desmin knock-out mice, which still contain synemin (67), but lack IFs because synemin by itself is unable to form filaments (12). Structural observations of striated muscle from desmin knock-out mice reveal an extreme loss of Z-line alignment and strain-induced muscle cell damage (68-73). Based upon our proposed role for the importance of synemin-containing heteropolymeric IFs in muscle cells, those findings are what would be expected in muscle cells lacking either desmin "or" synemin. Overall, the

synemin-based linkages in striated muscle cells may enable the heteropolymeric IFs to maintain Z-line alignment, and to reduce stress on myofibrils during muscle contraction by acting as a conduit to transmit some of the mechanical strain from the myofibrils to the muscle cell membrane.

Acknowledgments

We thank Dr. Wallace Ip and his lab, University of Cincinnati Medical Center, for the desmin and vimentin two-hybrid constructs and for providing initial help with the two-hybrid system, and Dr. David R. Critchley, University of Leicester, for the α-actinin and vinculin cDNAs.

References

1. Robson, R. M. (1989) *Curr Opin Cell Biol* 1, 36-43

2. Fuchs, E., and Weber, K. (1994) *Annu Rev Biochem* 63, 345-382

3. Klymkowsky, M. W. (1995) *Curr Opin Cell Biol* 7, 46-54

4. Fuchs, E., and Yang, Y. (1999) *Cell* 98, 547-550

5. Herrmann, H., and Aebi, U. (2000) *Curr Opin Cell Biol* 12, 79-90

6. Herrmann, H., Haner, M., Brettel, M., Muller, S. A., Goldie, K. N., Fedtke, B., Lustig, A., Franke, W. W., and Aebi, U. (1996) *J Mol Biol* 264, 933-953

7. Herrmann, H., and Aebi, U. (1998) *Curr Opin Struct Biol* 8, 177-185

8. Herrmann, H., and Aebi, U. (1998) *Subcell Biochem* 31, 319-362

9. Herrmann, H., Haner, M., Brettel, M., Ku, N. O., and Aebi, U. (1999) *J Mol Biol* 286, 1403-1420

10. Fuchs, E., and Cleveland, D. W. (1998) *Science* 279, 514-519

11. Hemken, P. M., Bellin, R. M., Sernett, S. W., Becker, B., Huiatt, T. W., and Robson, R. M. (1997) *J Biol Chem* 272, 32489-32499

12. Bellin, R. M., Sernett, S. W., Becker, B., Ip, W., Huiatt, T. W., and Robson, R. M. (1999) *J Biol Chem* **274**, 29493-29499

13. Lazarides, E. (1980) *Nature* **283**, 249-256

14. Wiche, G. (1998) *J Cell Sci* **111**, 2477-2486

15. Granger, B. L., and Lazarides, E. (1980) *Cell* **22**, 727-738

16. Sandoval, I. V., Colaco, C. A., and Lazarides, E. (1983) *J Biol Chem* **258**, 2568-2576

17. Granger, B. L., and Lazarides, E. (1984) *Mol Cell Biol* **4**, 1943-1950

18. Becker, B., Bellin, R. M., Sernett, S. W., Huiatt, T. W., and Robson, R. M. (1995) *Biochem Biophys Res Commun* **213**, 796-802

19. Price, M. G., and Lazarides, E. (1983) *J Cell Biol* **97**, 1860-1874

20. Bilak, S. R., Sernett, S. W., Bilak, M. M., Bellin, R. M., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1998) *Arch Biochem Biophys* **355**, 63-76

21. Granger, B. L., and Lazarides, E. (1982) *Cell* **30**, 263-275

22. Granger, B. L., Repasky, E. A., and Lazarides, E. (1982) *J Cell Biol* **92**, 299-312

23. Sultana, S., Sernett, S. W., Bellin, R. M., Robson, R. M., and Skalli, O. (2000) *Glia* **30**, 143-153

24. Evans, R. R., Robson, R. M., and Stromer, M. H. (1984) *J Biol Chem* **259**, 3916-3924

25. Johnson, R. P., and Craig, S. W. (1995) *Biochem Biophys Res Commun* **210**, 159-164

26. Carpenter, D. A., and Ip, W. (1996) *J Cell Sci* **109**, 2493-2498

27. Meng, J. J., Khan, S., and Ip, W. (1996) *J Biol Chem* **271**, 1599-1604

28. Chevray, P. M., and Nathans, D. (1992) *Proc Natl Acad Sci U S A* **89**, 5789-5793

29. Meng, J. J., Bornslaeger, E. A., Green, K. J., Steinert, P. M., and Ip, W. (1997) *J Biol Chem* **272**, 21495-21503

30. Johnson, R. P., and Craig, S. W. (1994) *J Biol Chem* **269**, 12611-12619

31. Weekes, J., Barry, S. T., and Critchley, D. R. (1996) *Biochem J* **314**, 827-832

32. Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983) *Proc Natl Acad Sci U S A* **80**, 1008-1012

33. Craig, S. W., and Pardo, J. V. (1983) *Cell Motil* **3**, 449-462

34. Giniger, E., and Ptashne, M. (1987) *Nature* **330**, 670-672

35. Steinert, P. M., and Roop, D. R. (1988) *Annu Rev Biochem* **57**, 593-625

36. Arimura, C., Suzuki, T., Yanagisawa, M., Imamura, M., Hamada, Y., and Masaki, T. (1988) *Eur J Biochem* **177**, 649-655

37. Gilmore, A. P., and Burridge, K. (1996) *Nature* **381**, 531-535

38. Stappenbeck, T. S., Bornslaeger, E. A., Corcoran, C. M., Luu, H. H., Virata, M. L., and Green, K. J. (1993) *J Cell Biol* **123**, 691-705

39. North, A. J., Bardsley, W. G., Hyam, J., Bornslaeger, E. A., Cordingley, H. C., Trinnaman, B., Hatzfeld, M., Green, K. J., Magee, A. I., and Garrod, D. R. (1999) *J Cell Sci* **112**, 4325-4336

40. Kowalczyk, A. P., Bornslaeger, E. A., Norvell, S. M., Palka, H. L., and Green, K. J. (1999) *Int Rev Cytol* **185**, 237-302

41. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) *Annu Rev Cell Dev Biol* **12**, 463-518

42. Belkin, A. M., Ornatsky, O. I., Glukhova, M. A., and Koteliansky, V. E. (1988) *J Cell Biol* **107**, 545-553

43. Critchley, D. R., Holt, M. R., Barry, S. T., Priddle, H., Hemmings, L., and Norman, J. (1999) *Biochem Soc Symp* **65**, 79-99

44. Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991) *Proc Natl Acad Sci U S A* **88**, 9578-9582

45. Raats, J. M., Pieper, F. R., Vree Egberts, W. T., Verrijp, K. N., Ramaekers, F. C., and Bloemendal, H. (1990) *J Cell Biol* **111**, 1971-1985

46. Kouklis, P. D., Hatzfeld, M., Brunkener, M., Weber, K., and Georgatos, S. D. (1993) *J Cell Sci* **106**, 919-928

47. Makarova, I., Carpenter, D., Khan, S., and Ip, W. (1994) *Cell Motil Cytoskeleton* **28**, 265-277

48. Steinert, P. M., Chou, Y. H., Prahlad, V., Parry, D. A., Marekov, L. N., Wu, K. C., Jang, S. I., and Goldman, R. D. (1999) *J Biol Chem* **274**, 9881-9890

49. Shoeman, R. L., and Traub, P. (1993) *Bioessays* **15**, 605-611

50. Meng, J. J., Khan, S., and Ip, W. (1994) *J Biol Chem* **269**, 18679-18685

51. Letai, A., and Fuchs, E. (1995) *Proc Natl Acad Sci U S A* **92**, 92-96

52. Djinovic-Carugo, K., Young, P., Gautel, M., and Saraste, M. (1999) *Cell* **98**, 537-546

53. Bakolitsa, C., de Pereda, J. M., Bagshaw, C. R., Critchley, D. R., and Liddington, R. C. (1999) *Cell* **99**, 603-613

54. Steimle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999) *J Biol Chem* **274**, 18414-18420

55. Johnson, R. P., and Craig, S. W. (2000) *J Biol Chem* **275**, 95-105

56. Adey, N. B., and Kay, B. K. (1997) *Biochem J* **324**, 523-528

57. Foisner, R. (1997) *Bioessays* **19**, 297-305

58. Yuan, J., Huiatt, T. W., Liao, C. X., Robson, R. M., and Graves, D. J. (1999) *Arch Biochem Biophys* **363**, 314-322

59. Hijikata, T., Murakami, T., Imamura, M., Fujimaki, N., and Ishikawa, H. (1999) *J Cell Sci* **112**, 867-876

60. Schroder, R., Warlo, I., Herrmann, H., van der Ven, P. F., Klasen, C., Blumcke, I., Mundegar, R. R., Furst, D. O., Goebel, H. H., and Magin, T. M. (1999) *Eur J Cell Biol* **78**, 288-295

61. Steinbock, F. A., and Wiche, G. (1999) *Biol Chem* **380**, 151-158

62. Svitkina, T. M., Verkhovsky, A. B., and Borisy, G. G. (1996) *J Cell Biol* **135**, 991-1007

63. Reipert, S., Steinbock, F., Fischer, I., Bittner, R. E., Zeold, A., and Wiche, G. (1999) *Exp Cell Res* **252**, 479-491

64. Granger, B. L., and Lazarides, E. (1979) *Cell* **18**, 1053-1063

65. Richardson, F. L., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1981) *Eur J Cell Biol* **26**, 91-101

66. Tokuyasu, K. T., Dutton, A. H., and Singer, S. J. (1983) *J Cell Biol* **96**, 1736-1742

67. Carlsson, L., Price, M. G., Robson, R. M., Breckler, J., Wiche, G., Li, Z., Paulin, D., and Thornell, L. E. (1997) *Mol. Biol. Cell* **8**, 391a

68. Agbulut, O., Li, Z., Mouly, V., and Butler-Browne, G. S. (1996) *Biol Cell* **88**, 131-135

69. Li, Z., Colucci-Guyon, E., Pincon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D., and Babinet, C. (1996) *Dev Biol* **175**, 362-366

70. Li, Z., Mericskay, M., Agbulut, O., Butler-Browne, G., Carlsson, L., Thornell, L. E., Babinet, C., and Paulin, D. (1997) *J Cell Biol* **139**, 129-144

71. Milner, D. J., Weitzer, G., Tran, D., Bradley, A., and Capetanaki, Y. (1996) *J Cell Biol* **134**, 1255-1270

72. Thornell, L., Carlsson, L., Li, Z., Mericskay, M., and Paulin, D. (1997) *J Mol Cell Cardiol* **29**, 2107-2124

73. Capetanaki, Y., Milner, D. J., and Weitzer, G. (1997) *Cell Struct Funct* **22**, 103-116

Footnotes

[1]The abbreviations used are: IF, intermediate filament; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis

[2]R. M. Bellin and R. M. Robson, unpublished observations.

[3]S. W. Sernett, R. M. Bellin, and R. M. Robson, unpublished observations.

Fig 1. Blot overlay analysis of the interactions of synemin and of specific synemin domains with vinculin. *Panels A-C* depict

blots resulting from transfer of SDS-PAGE gels (*lane 1*, whole gizzard homogenate; *lane 2*, purified synemin; *lane 3*, purified

vinculin; and *lane 4*, V8 protease digested vinculin) and then overlaid with buffer only (*panel A*), with purified, intact synemin

(*panel B*), and bacterially-expressed synemin rod domain (*panel C*), before washing and detection with synemin pAb. *Panels D*

and *E* depict blots resulting from transfer of SDS-PAGE gels (*lane 1*, whole gizzard homogenate; *lane 2*, expressed synemin rod

domain; *lane 3*, purified vinculin; and *lane 4*, V8 protease digested vinculin), and then overlaid with buffer only (*Panel D*) and

synemin rod domain (*Panel E*) before washing and detection with synemin pAb. *Panel F* is a Coomasie blue-stained SDS-PAGE

gel of V8 protease digested vinculin, with *H* corresponding to the large head domain, and *T* corresponding to two sizes of tail

domain.

A B C D E F

1 2 3 4   1 2 3 4   1 2 3 4   1 2 3 4   1 2 3 4

205
116
97
66
42
29

⟷

H

T

88

Control buffer
overlay

Intact synemin
overlay

Synemin tail
overlay

Control buffer
overlay

Synemin rod
overlay
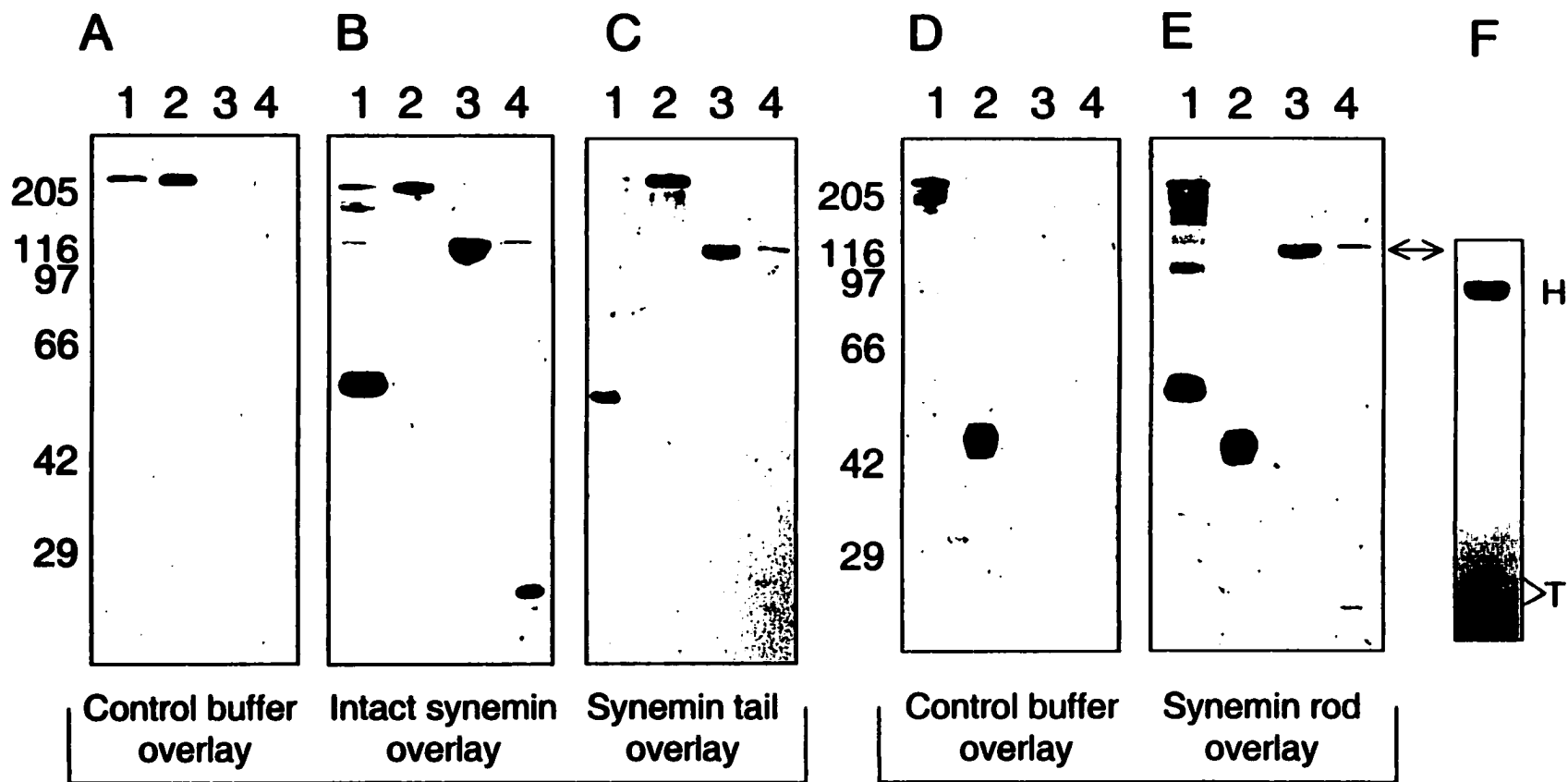
Fig. 2. **Constructs used in two-hybrid studies.** *SN* (avian synemin; GenBank accession number U28143), *Desmin* (murine desmin; GenBank accession number L22550), *Vimentin* (murine vimentin; GenBank accession number M24849), *α-Actinin/α-Act*, (avian α-actinin; GenBank accession number J03486), and *Vinc* (avian vinculin; GenBank accession number Y00312). *Numbers* at the right of each construct refer to amino acid residues in the construct, with the N-terminus of the protein corresponding to residue number 1.

SN Rod ━━━━━━ (10-314)

SN Tail ━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━ (315-1604)

SN Tail I ━━━━━━━━━━━━━━━ (315-971)

SN Tail II ━━━━━━━━━━━━━━ (972-1604)

SN Tail Ia ━━━━━━ (315-632)

SN Tail Ib ━━━━━━━ (633-971)

SN Tail IIa ━━━━━━ (972-1291)

SN Tail IIb ━━━━━ (1292-1604)

Desmin ━━━━━━ (1-468)

Vimentin ━━━━━━ (1-464)

α-Actinin ━━━━━━━━━━━━━━━ (1-888)

α-Act Head ━━━ (1-269)

α-Act Rod ━━━━━━━━ (270-740)

α-Act Tail ━━ (741-888)

Vinc Head I ━━━━━━ (1-398)

Vinc Head II ━━━━━━━ (399-880)

Vinc Tail ━━━ (881-1066)

Fig. 3. **Negative controls for two-hybrid assays of synemin constructs.** The *labels* at the X-axis refer to the construct names defined in Fig. 2. *Vector* refers to a two-hybrid vector without a cDNA insert, which is included as a negative control. All fluorescence values were calculated relative to the value (defined as 100) obtained in the interaction of synemin rod domain with desmin (*column 9*) as described in the Experimental Procedures. Results from vector-swap tests (i.e., pairs 1-9) of each interaction are shown as a pair of open and solid bars, with error bars representing the standard error of the mean. *Open bars* represent assays in which the synemin insert is in the pPC97 vector, and a control vector (*columns 1-8*), or desmin (*column 9*) is in the pPC86 vector. *Solid bars* represent assays in which the synemin insert is in the pPC86 vector and a control vector (*columns 1-8*), or desmin, (*column 9*) is in the pPC97 vector. Note that none of the synemin constructs tested with a control (empty) vector yielded significant amounts of transcription of the β-galactoside reporter.
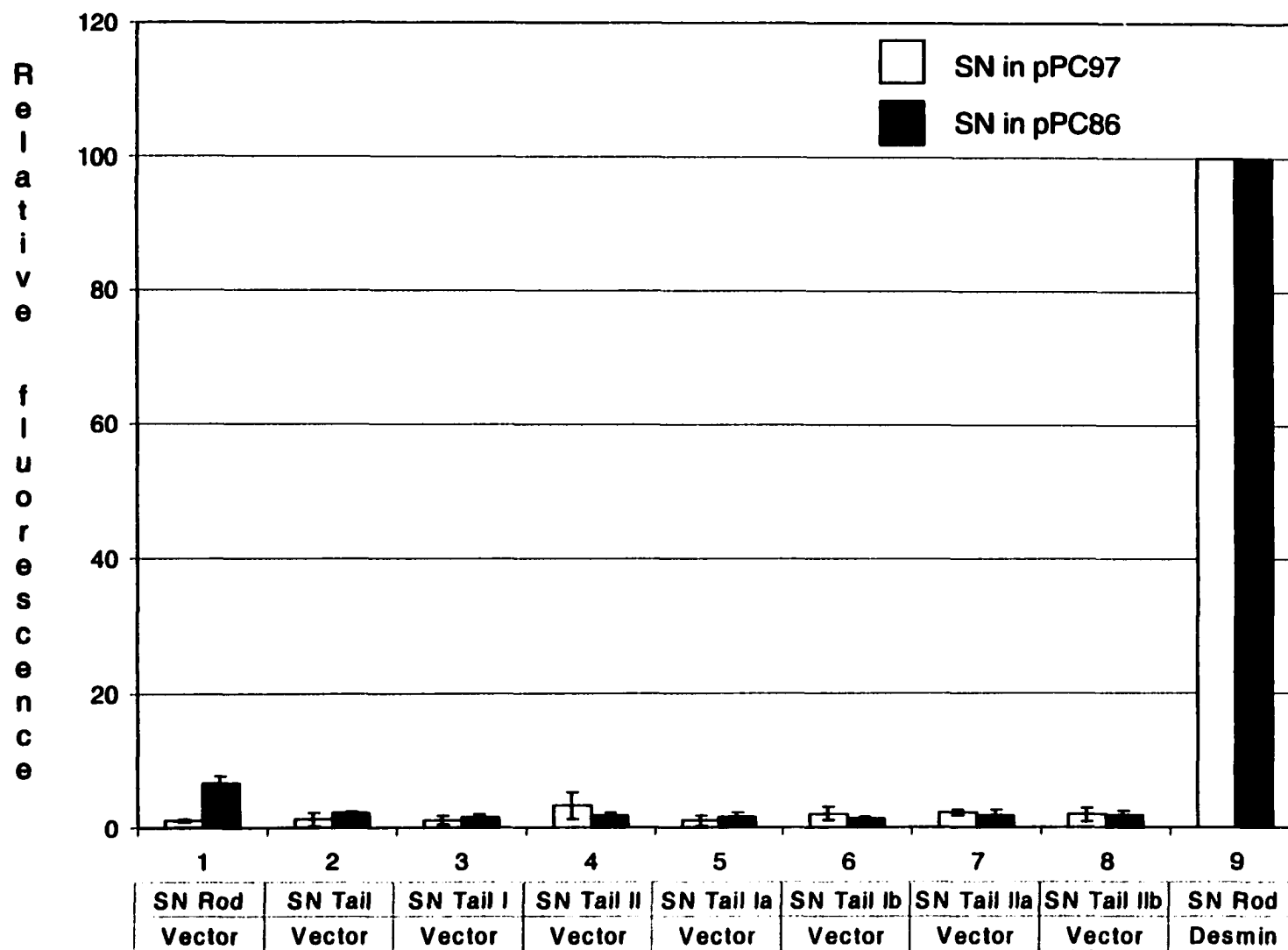
Fig. 4. **Two-hybrid assays of interactions involving synemin and desmin.** *Hatched bar* represents a test of desmin dimerization, so no vector-swap test is possible. All fluorescence values were calculated relative to the value (defined as 100) obtained in the interaction of synemin rod domain with desmin as described in the Experimental Procedures. Note that only the rod domain of synemin interacts significantly with desmin. For details on graph format and labeling see the Legend to Fig. 3.
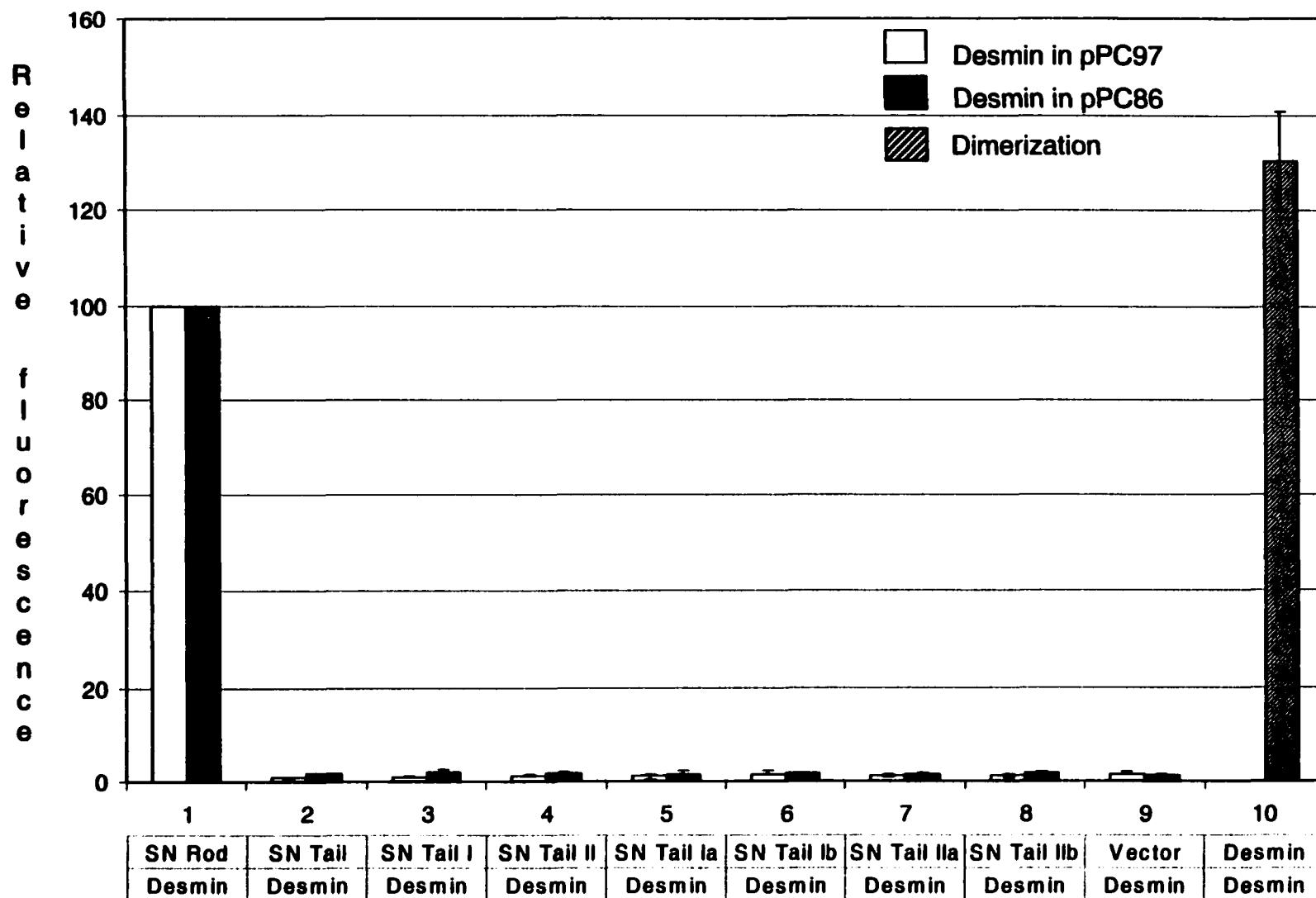
Fig. 5. **Two-hybrid assays of interactions involving synemin and vimentin.** *Hatched bars* represent tests of protein dimerization, so no vector-swap tests are possible. All fluorescence values were calculated relative to the value (defined as 100) obtained for the interaction of synemin rod domain with desmin as described in the Experimental Procedures. Note that only the rod domain of synemin interacts with vimentin. For details on graph format and labeling, see the Legend to Fig. 3.
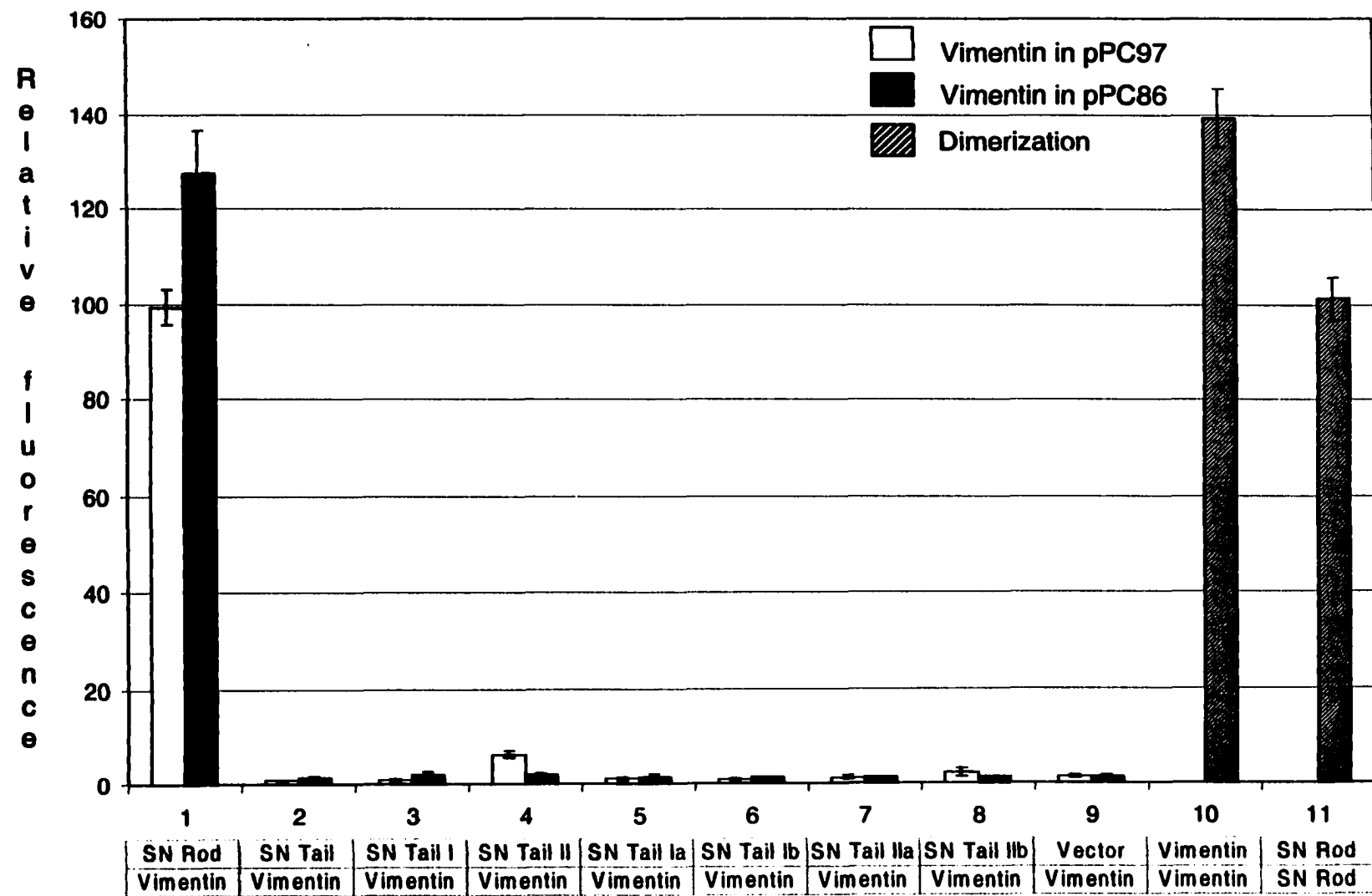
Fig. 6. Two-hybrid assays of interactions involving synemin and α-actinin. All fluorescence values were calculated relative to the value (defined as 100) obtained in the interaction of synemin rod domain with desmin as described in the Experimental Procedures. Analysis of interactions with the full-length α-actinin construct (*columns 1-8*) require careful comparison to the "negative" control in *column 9 (open bar)* because the pPC97 α-actinin construct activates transcription of the reporter to a moderate level (~20%) by itself. This did not occur with the pPC86 α-actinin construct (*solid bar, column 9*). Note the positive interactions between α-actinin and synemin tail (*column 2*), synemin tail II (*column 4*) and synemin tail IIb (*column 8*). Furthermore, synemin tail IIb interacts with both the α-actinin head (*column 10*) and rod (*column 12*) domains. For details on graph format and labeling, see the Legend to Fig. 3.
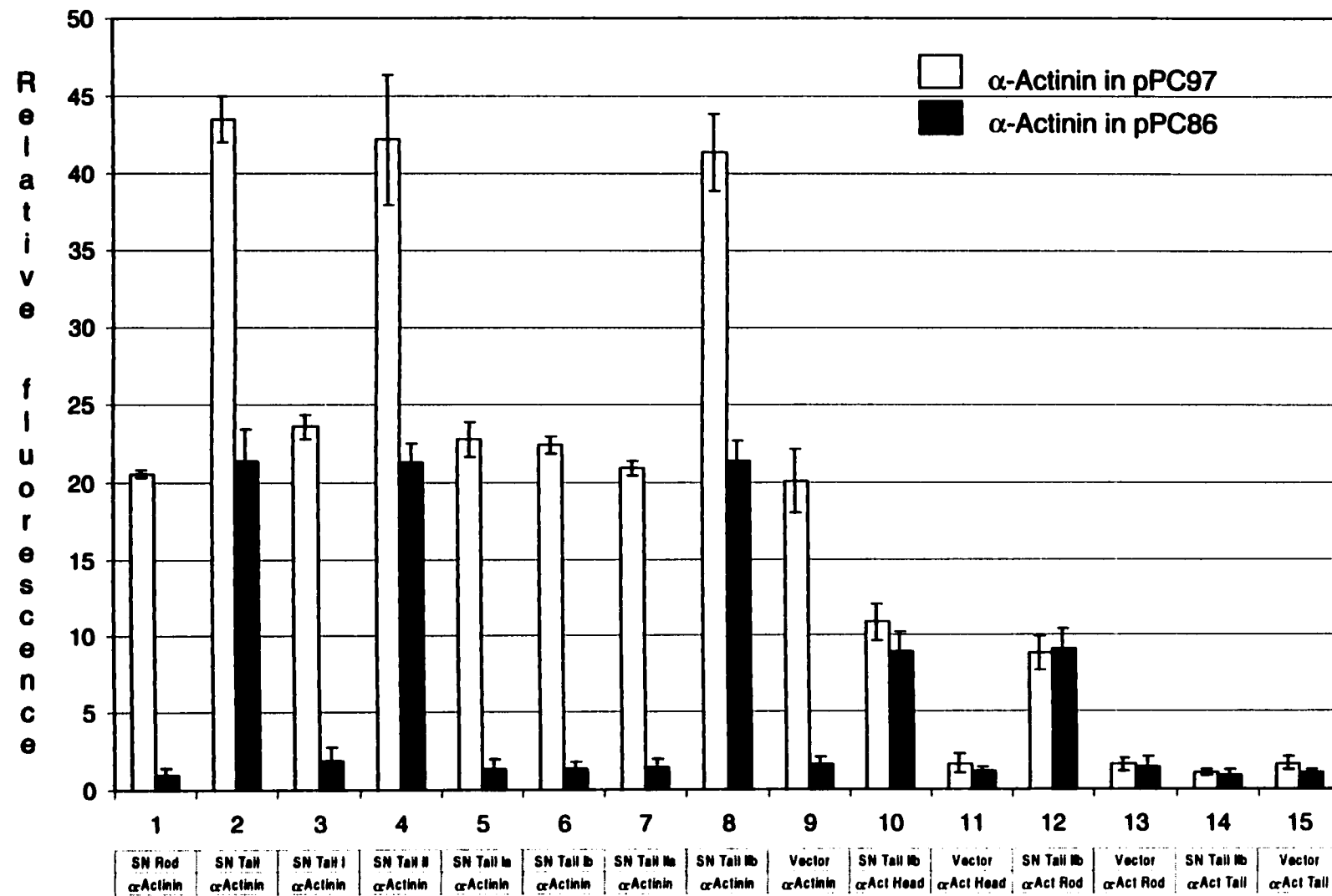
Fig. 7. **Two-hybrid assays of interactions involving synemin and vinculin.** All fluorescence values were calculated relative to the value (defined as 100) obtained in the interaction of synemin rod domain with desmin as described in the Experimental Procedures. Note that interactions are positive for both the rod (*column 7*) and tail domains (*columns 8, 10* and *12*) of synemin with the small vinculin tail domain. For details on graph format and labeling, see Fig. 3.
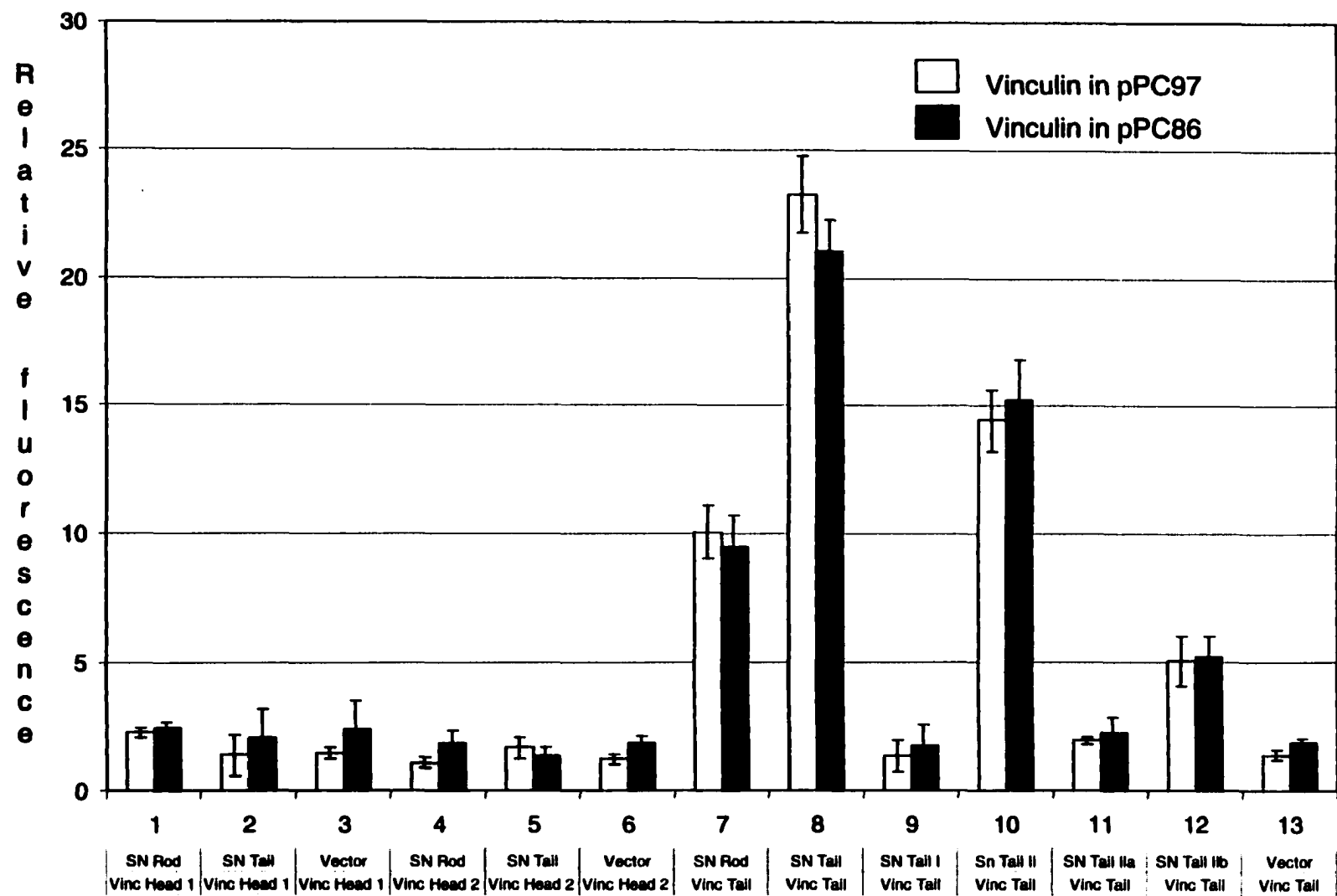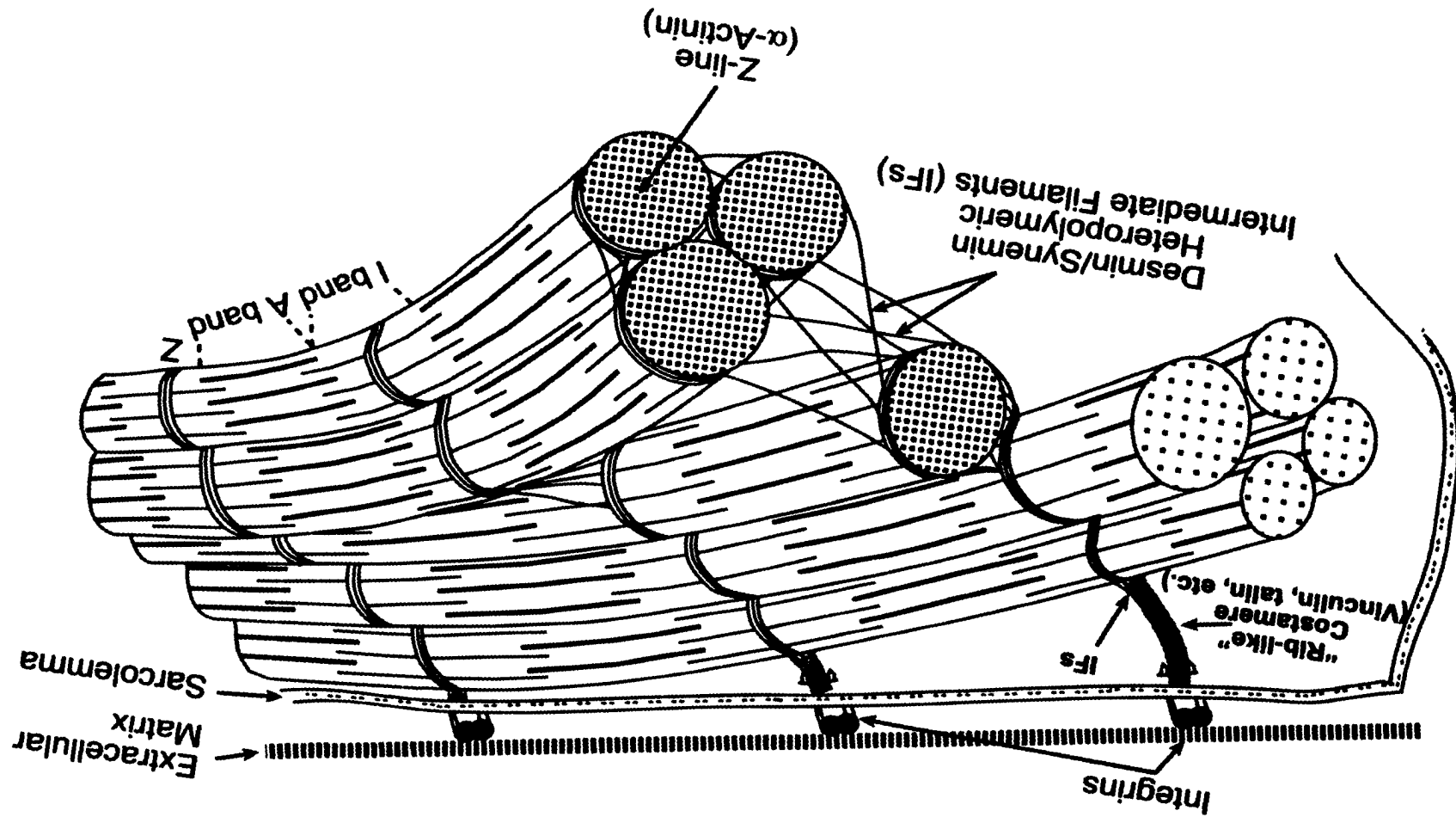
Fig. 8. Schematic showing a possible role for synemin in striated muscle cells. The diagram shows the known location of heteropolymeric IFs at the Z-lines of striated muscle cells. Also depicted are attachment points of IFs to structures containing proteins that we have found interact with synemin. Our hypothesis is that heteropolymeric IFs are firmly anchored to (a) the myofibrillar Z-lines by interactions between synemin and α-actinin, an integral Z-line protein (this interaction would enable heteropolymeric IFs to link all myofibrils together at their Z-lines), and (b) the costameres by interactions between synemin and vinculin (this interaction would enable heteropolymeric IFs to firmly anchor the peripheral layer of myofibrils to the costameres at the cell membrane). Adapted from (13).

102

Z-line
(α-Actinin)

Desmin/Synemin
Heteropolymeric
Intermediate Filaments (IFs)

I band A band Z

"Rib-like"
Costamere
(Vinculin, talin, etc.)

IFs

Sarcolemma

Extracellular
Matrix

Integrins

OVERALL SUMMARY

In the research presented in this dissertation, major advancements were made in our understanding of the protein synemin, and how it may function in cells as a component of heteropolymeric IFs. The cloning and sequencing of the avian synemin cDNA revealed that synemin contains the rod domain typical of IFs. This finding established synemin as a member of the superfamily of IF proteins. The sequencing studies also established that the primary structure of synemin consists of a small N-terminal head domain, the rod domain, and a very long C-terminal tail domain similar in size to that of other large IF proteins such as nestin and paranemin. An interesting fact is that although synemin is similar in domain structure to nestin and paranemin, it lacks significant overall sequence homology to those proteins. Comparison of the synemin sequence with GenBank records revealed homology with two human-derived sequences, strongly indicating the existence of at least a human synemin homologue.

The cloning of the synemin cDNA made possible further investigation of synemin as a component of IFs. Previous attempts to explore the assembly of synemin into IFs by using purified synemin in an *in vitro* assembly assay were essentially inconclusive. Use of the full-length synemin cDNA clone, in conjunction with clonal lines of the IF-mosaic SW13 cell line, made it possible to establish that synemin will assemble into heteropolymeric IFs with another major IF protein, but that it is unable to form IFs by itself within a cellular context.

The synemin cDNA clones also proved invaluable in the exploration of molecular interactions of synemin. Initial studies were conducted on synemin purified from avian muscle, and revealed that synemin interacts with the IF protein desmin, the Z-line protein α-

actinin, and the costameric protein vinculin. The synemin cDNA clones made possible the production of bacterially-expressed domains of the synemin molecule. By using these expressed synemin domains, it was possible to establish the synemin rod domain as the primary domain for interaction with other IF proteins. Additionally, the expressed tail domain of synemin was found to interact with α-actinin and vinculin. Potentially the greatest utility of the synemin cDNA has resulted from the use of the *Gal4* yeast two-hybrid system to test protein interactions within a cellular context. The two-hybrid studies have resulted in a completely independent test of the synemin interactions discovered by *in vitro* methods using purified proteins. The results of these latter studies have, in fact, confirmed all interactions demonstrated by the *in vitro* assays, and have significantly extended those studies by further mapping the specific domains involved in the interactions.

It is our overall hypothesis that synemin functions as a component of heteropolymeric IFs to enable them to anchor/bind to specific cytoskeletal structures via interactions between synemin and other cytoskeletal proteins. In striated muscle cells, synemin-containing heteropolymeric IFs may be firmly attached to (a) every myofibrillar Z-line via interactions between synemin and α-actinin, and (b) the costameric regions via interactions between synemin and vinculin. Together, these linkages would enable the heteropolymeric IFs to connect all of the myofibrils together at their Z-lines, and to anchor the Z-lines of the peripheral layer of myofibrils to the costameres at the sarcolemma. Taken *in toto*, results of the studies described in this dissertation substantiate our hypothesis and indicate an important role for synemin in the maintenance of overall cytoskeletal integrity and function in muscle cells.

COMPREHENSIVE BIBLIOGRAPHY

Abumuhor, I. A., Spencer, P. H., and Cohlberg, J. A. (1998). The pathway of assembly of intermediate filaments from recombinant alpha-internexin. J Struct Biol *123*, 187-198.

Adey, N. B., and Kay, B. K. (1997). Isolation of peptides from phage-displayed random peptide libraries that interact with the talin-binding domain of vinculin. Biochem J *324*, 523-528.

Agbulut, O., Li, Z., Mouly, V., and Butler-Browne, G. S. (1996). Analysis of skeletal and cardiac muscle from desmin knock-out and normal mice by high resolution separation of myosin heavy-chain isoforms. Biol Cell *88*, 131-135.

Allen, R. E., Stromer, M. H., Goll, D. E., and Robson, R. M. (1978). Synthesis of tropomyosin in cultures of differentiating muscle cells. J Cell Biol *76*, 98-104.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J Mol Biol *215*, 403-410.

Anastasi, G., Cutroneo, G., Santoro, G., and Trimarchi, F. (1998). The non-junctional sarcolemmal cytoskeleton: the costameres. Ital J Anat Embryol *103*, 1-11.

Arimura, C., Suzuki, T., Yanagisawa, M., Imamura, M., Hamada, Y., and Masaki, T. (1988). Primary structure of chicken skeletal muscle and fibroblast alpha- actinins deduced from cDNA sequences. Eur J Biochem *177*, 649-655.

Athlan, E. S., Sacher, M. G., and Mushynski, W. E. (1997). Associations between intermediate filament proteins expressed in cultured dorsal root ganglion neurons. J Neurosci Res *47*, 300-310.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1997). Current protocols in molecular biology (New York: John Wiley & Sons Inc.).

Baker, J. P., and Titus, M. A. (1998). Myosins: matching functions with motors. Curr Opin Cell Biol *10*, 80-86.

Bakolitsa, C., de Pereda, J. M., Bagshaw, C. R., Critchley, D. R., and Liddington, R. C. (1999). Crystal structure of the vinculin tail suggests a pathway for activation. Cell *99*, 603-613.

Barral, J. M., and Epstein, H. F. (1999). Protein machines and self assembly in muscle organization. BioEssays *21*, 813-823.

Becker, B., Bellin, R. M., Sernett, S. W., Huiatt, T. W., and Robson, R. M. (1995). Synemin contains the rod domain of intermediate filaments. Biochem Biophys Res Commun *213*, 796-802.

Belkin, A. M., Ornatsky, O. I., Glukhova, M. A., and Koteliansky, V. E. (1988). Immunolocalization of meta-vinculin in human smooth and cardiac muscles. J Cell Biol *107*, 545-553.

Belkin, A. M., Zhidkova, N. I., and Koteliansky, V. E. (1986). Localization of talin in skeletal and cardiac muscles. FEBS Lett *200*, 32-36.

Bellin, R. M., Sernett, S. W., Becker, B., Ip, W., Huiatt, T. W., and Robson, R. M. (1999). Molecular characteristics and interactions of the intermediate filament protein synemin. Interactions with alpha-actinin may anchor synemin- containing heterofilaments. J Biol Chem *274*, 29493-29499.

Bellin, R. M., Sernett, S. W., and Robson, R. M. (1999). Synemin. In Guidebook to the cytoskeletal and motor proteins, second edition, T. Kreis and R. Vale, eds. (UK: Oxford Univ. Press), pp. 322-324.

ben-Avraham, D., and Tirion, M. M. (1995). Dynamic and elastic properties of F-actin: a normal-modes analysis. Biophys J *68*, 1231-1245.

Bennett, G. S., Fellini, S. A., Toyama, Y., and Holtzer, H. (1979). Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. J Cell Biol *82*, 577-584.

Bilak, S. R., Sernett, S. W., Bilak, M. M., Bellin, R. M., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1998). Properties of the novel intermediate filament protein synemin and its identification in mammalian muscle. Arch Biochem Biophys *355*, 63-76.

Blanchard, A., Ohanian, V., and Critchley, D. (1989). The structure and function of alpha-actinin. J Muscle Res Cell Motil *10*, 280-289.

Breckler, J., and Lazarides, E. (1982). Isolation of a new high molecular weight protein associated with desmin and vimentin filaments from avian embryonic skeletal muscle. J Cell Biol *92*, 795-806.

Brown, S. S. (1999). Cooperation between microtubule- and actin-based motor proteins. Annu Rev Cell Dev Biol *15*, 63-80.

Bryan, J. (1989). Caldesmon, acidic amino acids and molecular weight determinations. J Muscle Res Cell Motil *10*, 95-96.

Burnette, W. N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem *112*, 195-203.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol *12*, 463-518.

Capetanaki, Y., and Milner, D. J. (1998). Desmin cytoskeleton in muscle integrity and function. Subcell Biochem *31*, 463-495.

Capetanaki, Y., Milner, D. J., and Weitzer, G. (1997). Desmin in muscle formation and maintenance: knockouts and consequences. Cell Struct Funct *22*, 103-116.

Carlsson, L., Price, M. G., Robson, R. M., Breckler, J., Wiche, G., Li, Z., Paulin, D., and Thornell, L. E. (1997). Expression of IF-related proteins in desmin knock-out mice. Mol Biol Cell *8*, 391a.

Carpenter, D. A., and Ip, W. (1996). Neurofilament triplet protein interactions: evidence for the preferred formation of NF-L-containing dimers and a putative function for the end domains. J Cell Sci *109*, 2493-2498.

Chang, P., and Stearns, T. (2000). delta-Tubulin and varepsilon-tubulin: two new human centrosomal tubulins reveal new aspects of centrosome structure and function. Nat Cell Biol *2*, 30-35.

Chen, W. J., and Liem, R. K. (1994). The endless story of the glial fibrillary acidic protein. J Cell Sci *107*, 2299-2311.

Chevray, P. M., and Nathans, D. (1992). Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc Natl Acad Sci U S A *89*, 5789-5793.

Chew, M. W., and Squire, J. M. (1995). Packing of alpha-helical coiled-coil myosin rods in vertebrate muscle thick filaments. J Struct Biol *115*, 233-249.

Chien, C. T., Barlet, P. L. , Sternglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Sci U S A *88*, 9578-9582.

Ching, G. Y., and Liem, R. K. (1993). Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. J Cell Biol *122*, 1323-1335.

Chou, R. G., Stromer, M. H., Robson, R. M., and Huiatt, T. W. (1992). Assembly of contractile and cytoskeletal elements in developing smooth muscle cells. Dev Biol *149*, 339-348.

Chou, R. G., Stromer, M. H., Robson, R. M., and Huiatt, T. W. (1994). Substructure of cytoplasmic dense bodies and changes in distribution of desmin and alpha-actinin in developing smooth muscle cells. Cell Motil Cytoskeleton *29*, 204-214.

Craig, S. W., Lancashire, C. L., and Cooper, J. A. (1982). Preparation of smooth muscle alpha-actinin. Methods Enzymol *85*, 316-321.

Craig, S. W., and Pardo, J. V. (1983). Gamma actin, spectrin, and intermediate filament proteins colocalize with vinculin at costameres, myofibril-to-sarcolemma attachment sites. Cell Motil *3*, 449-462.

Critchley, D. R., Holt, M. R., Barry, S. T., Priddle, H., Hemmings, L., and Norman, J. (1999). Integrin-mediated cell adhesion: the cytoskeletal connection. Biochem Soc Symp *65*, 79-99.

Danowski, B. A., Imanaka-Yoshida, K., Sanger, J. M., and Sanger, J. W. (1992). Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. J Cell Biol *118*, 1411-1420.

Djinovic-Carugo, K., Young, P., Gautel, M., and Saraste, M. (1999). Structure of the alpha-actinin rod: molecular basis for cross-linking of actin filaments. Cell *98*, 537-546.

dos Remedios, C. G., and Moens, P. D. (1995). Actin and the actomyosin interface: a review. Biochim Biophys Acta *1228*, 99-124.

Downing, K. H., and Nogales, E. (1998). Tubulin and microtubule structure. Curr Opin Cell Biol *10*, 16-22.

Evans, R. M. (1998). Vimentin: the conundrum of the intermediate filament gene family. Bioessays *20*, 79-86.

Evans, R. R., Robson, R. M., and Stromer, M. H. (1984). Properties of smooth muscle vinculin. J Biol Chem *259*, 3916-3924.

Farah, C. S., and Reinach, F. C. (1995). The troponin complex and regulation of muscle contraction. Faseb J *9*, 755-767.

Foisner, R. (1997). Dynamic organisation of intermediate filaments and associated proteins during the cell cycle. Bioessays *19*, 297-305.

Foisner, R., and Wiche, G. (1991). Intermediate filament-associated proteins. Curr Opin Cell Biol *3*, 75-81.

Fowler, V. M. (1997). Capping actin filament growth: tropomodulin in muscle and nonmuscle cells. Soc Gen Physiol Ser *52*, 79-89.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci U S A *85*, 8998-9002.

Fuchs, E. (1996). The cytoskeleton and disease: genetic disorders of intermediate filaments. Annu Rev Genet *30*, 197-231.

Fuchs, E. (1994). Intermediate filaments and disease: mutations that cripple cell strength. J Cell Biol *125*, 511-516.

Fuchs, E., and Cleveland, D. W. (1998). A structural scaffolding of intermediate filaments in health and disease. Science *279*, 514-519.

Fuchs, E., and Weber, K. (1994). Intermediate filaments: structure, dynamics, function, and disease. Annu Rev Biochem *63*, 345-382.

Fuchs, E., and Yang, Y. (1999). Crossroads on cytoskeletal highways. Cell *98*, 547-550.

Furst, D. O., Osborn, M., and Weber, K. (1989). Myogenesis in the mouse embryo: differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. J Cell Biol *109*, 517-527.

Furst, D. O., Vinkemeier, U., and Weber, K. (1992). Mammalian skeletal muscle C-protein: purification from bovine muscle, binding to titin and the characterization of a full-length human cDNA. J Cell Sci *102*, 769-778.

Geisler, N., and Weber, K. (1980). Purification of smooth-muscle desmin and a protein-chemical comparison of desmins from chicken gizzard and hog stomach. Eur J Biochem *111*, 425-433.

Gergely, J. (1998). Molecular switches in troponin. Adv Exp Med Biol *453*, 169-176.

Gilbert, R., Cohen, J. A., Pardo, S., Basu, A., and Fischman, D. A. (1999). Identification of the A-band localization domain of myosin binding proteins C and H (MyBP-C, MyBP-H) in skeletal muscle. J Cell Sci *112*, 69-79.

Gilmore, A. P., and Burridge, K. (1996). Regulation of vinculin binding to talin and actin by phosphatidyl- inositol-4-5-bisphosphate. Nature *381*, 531-535.

Giniger, E., and Ptashne, M. (1987). Transcription in yeast activated by a putative amphipathic alpha helix linked to a DNA binding unit. Nature *330*, 670-672.

Glass, C. A., Glass, J. R., Taniura, H., Hasel, K. W., Blevitt, J. M., and Gerace, L. (1993). The alpha-helical rod domain of human lamins A and C contains a chromatin binding site. Embo J *12*, 4413-4424.

Goldman, R. D., Chou, Y. H., Prahlad, V., and Yoon, M. (1999). Intermediate filaments: dynamic processes regulating their assembly, motility, and interactions with other cytoskeletal systems. FASEB J *13 Suppl 2*, S261-265.

Goldman, R. D., Khuon, S., Chou, Y. H., Opal, P., and Steinert, P. M. (1996). The function of intermediate filaments in cell shape and cytoskeletal integrity. J Cell Biol *134*, 971-983.

Goll, D. E., Dayton, W. R., Singh, I., and Robson, R. M. (1991). Studies of the alpha-actinin/actin interaction in the Z-disk by using calpain. J Biol Chem *266*, 8501-8510.

Goncharova, E. J., Kam, Z., and Geiger, B. (1992). The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. Development *114*, 173-183.

Granger, B. L., and Lazarides, E. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z disc. Cell *18*, 1053-1063.

Granger, B. L., and Lazarides, E. (1984). Expression of the intermediate-filament-associated protein synemin in chicken lens cells. Mol Cell Biol *4*, 1943-1950.

Granger, B. L., and Lazarides, E. (1982). Structural associations of synemin and vimentin filaments in avian erythrocytes revealed by immunoelectron microscopy. Cell *30*, 263-275.

Granger, B. L., and Lazarides, E. (1980). Synemin: a new high molecular weight protein associated with desmin and vimentin filaments in muscle. Cell *22*, 727-738.

Granger, B. L., Repasky, E. A., and Lazarides, E. (1982). Synemin and vimentin are components of intermediate filaments in avian erythrocytes. J Cell Biol *92*, 299-312.

Gregorio, C. C., and Fowler, V. M. (1995). Mechanisms of thin filament assembly in embryonic chick cardiac myocytes: tropomodulin requires tropomyosin for assembly. J Cell Biol *129*, 683-695.

Gregorio, C. C., Granzier, H., Sorimachi, H., and Labeit, S. (1999). Muscle assembly: a titanic achievement? Curr Opin Cell Biol *11*, 18-25.

Gregorio, C. C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Suzuki, K., Obermayr, F., Herrmann, B., Granzier, H., Sorimachi, H., and Labeit, S. (1998). The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. J Cell Biol *143*, 1013-1027.

Hackney, D. D. (1996). The kinetic cycles of myosin, kinesin, and dynein. Annu Rev Physiol *58*, 731-750.

Hemken, P. M., Bellin, R. M., Sernett, S. W., Becker, B., Huiatt, T. W., and Robson, R. M. (1997). Molecular characteristics of the novel intermediate filament protein paranemin. Sequence reveals EAP-300 and IFAPa-400 are highly homologous to paranemin. J Biol Chem *272*, 32489-32499.

Hemmati-Brivanlou, A., Mann, R. W., and Harland, R. M. (1992). A protein expressed in the growth cones of embryonic vertebrate neurons defines a new class of intermediate filament protein. Neuron *9*, 417-428.

Hemmings, L., Barry, S. T., and Critchley, D. R. (1995). Cell-matrix adhesion: structure and regulation. Biochem Soc Trans *23*, 619-626.

Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O., and Critchley, D. R. (1996). Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J Cell Sci *109*, 2715-2726.

Herrmann, H., and Aebi, U. (1998). Intermediate filament assembly: fibrillogenesis is driven by decisive dimer-dimer interactions. Curr Opin Struct Biol *8*, 177-185.

Herrmann, H., and Aebi, U. (1999). Intermediate filament assembly: temperature sensitivity and polymorphism. Cell Mol Life Sci *55*, 1416-1431.

Herrmann, H., and Aebi, U. (2000). Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. Curr Opin Cell Biol *12*, 79-90.

Herrmann, H., and Aebi, U. (1998). Structure, assembly, and dynamics of intermediate filaments. Subcell Biochem *31*, 319-362.

Herrmann, H., Haner, M., Brettel, M., Ku, N. O., and Aebi, U. (1999). Characterization of distinct early assembly units of different intermediate filament proteins. J Mol Biol *286*, 1403-1420.

Herrmann, H., Haner, M., Brettel, M., Muller, S. A., Goldie, K. N., Fedtke, B., Lustig, A., Franke, W. W., and Aebi, U. (1996). Structure and assembly properties of the

intermediate filament protein vimentin: the role of its head, rod and tail domains. J Mol Biol *264*, 933-953.

Hijikata, T., Murakami, T., Imamura, M., Fujimaki, N., and Ishikawa, H. (1999). Plectin is a linker of intermediate filaments to Z-discs in skeletal muscle fibers. J Cell Sci *112*, 867-876.

Hill, C., and Weber, K. (1986). Monoclonal antibodies distinguish titins from heart and skeletal muscle. J Cell Biol *102*, 1099-1108.

Hill, C. S., Duran, S., Lin, Z. X., Weber, K., and Holtzer, H. (1986). Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. J Cell Biol *103*, 2185-2196.

Hitchcock-DeGregori, S. E., and An, Y. (1996). Integral repeats and a continuous coiled coil are required for binding of striated muscle tropomyosin to the regulated actin filament. J Biol Chem *271*, 3600-3603.

Houseweart, M. K., and Cleveland, D. W. (1998). Intermediate filaments and their associated proteins: multiple dynamic personalities. Curr Opin Cell Biol *10*, 93-101.

Huiatt, T. W., Robson, R. M., Arakawa, N., and Stromer, M. H. (1980). Desmin from avian smooth muscle. Purification and partial characterization. J Biol Chem *255*, 6981-6989.

Huttelmaier, S., Mayboroda, O., Harbeck, B., Jarchau, T., Jockusch, B. M., and Rudiger, M. (1998). The interaction of the cell-contact proteins VASP and vinculin is regulated by phosphatidylinositol-4,5-bisphosphate. Curr Biol *8*, 479-488.

Imanaka-Yoshida, K., Enomoto-Iwamoto, M., Yoshida, T., and Sakakura, T. (1999). Vinculin, Talin, Integrin alpha6beta1 and laminin can serve as components of attachment complex mediating contraction force transmission from cardiomyocytes to extracellular matrix. Cell Motil Cytoskeleton *42*, 1-11.

Inoue, S. (1997). The role of microtubule assembly dynamics in mitotic force generation and functional organization of living cells. J Struct Biol *118*, 87-93.

Ip, W., Hartzer, M. K., Pang, Y. Y., and Robson, R. M. (1985). Assembly of vimentin in vitro and its implications concerning the structure of intermediate filaments. J Mol Biol *183*, 365-375.

Ip, W., Heuser, J. E., Pang, Y. Y., Hartzer, M. K., and Robson, R. M. (1985). Subunit structure of desmin and vimentin protofilaments and how they assemble into intermediate filaments. Ann N Y Acad Sci *455*, 185-199.

Ishikawa, H., Bischoff, R., and Holtzer, H. (1968). Mitosis and intermediate-sized filaments in developing skeletal muscle. J Cell Biol *38*, 538-555.

Johnson, R. P., and Craig, S. W. (2000). Actin activates a cryptic dimerization potential of the vinculin tail domain. J Biol Chem *275*, 95-105.

Johnson, R. P., and Craig, S. W. (1995). The carboxy-terminal tail domain of vinculin contains a cryptic binding site for acidic phospholipids. Biochem Biophys Res Commun *210*, 159-164.

Johnson, R. P., and Craig, S. W. (1994). An intramolecular association between the head and tail domains of vinculin modulates talin binding. J Biol Chem *269*, 12611-12619.

Kim, H. D. (1996). Expression of intermediate filament desmin and vimentin in the human fetal heart. Anat Rec *246*, 271-278.

Kirchner, J., Woehlke, G., and Schliwa, M. (1999). Universal and unique features of kinesin motors: insights from a comparison of fungal and animal conventional kinesins. Biol Chem *380*, 915-921.

Klymkowsky, M. W. (1995). Intermediate filaments: new proteins, some answers, more questions. Curr Opin Cell Biol *7*, 46-54.

Knudsen, K. A. (1985). Proteins transferred to nitrocellulose for use as immunogens. Anal Biochem *147*, 285-288.

Kouklis, P. D., Hatzfeld, M., Brunkener, M., Weber, K., and Georgatos, S. D. (1993). In vitro assembly properties of vimentin mutagenized at the beta-site tail motif. J Cell Sci *106*, 919-928.

Kowalczyk, A. P., Bornslaeger, E. A., Norvell, S. M., Palka, H. L., and Green, K. J. (1999). Desmosomes: intercellular adhesive junctions specialized for attachment of intermediate filaments. Int Rev Cytol *185*, 237-302.

Kroemker, M., Rudiger, A. H., Jockusch, B. M., and Rudiger, M. (1994). Intramolecular interactions in vinculin control alpha-actinin binding to the vinculin head. FEBS Lett *355*, 259-262.

Kruger, M., Wright, J., and Wang, K. (1991). Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles: correlation of thin filament length, nebulin size, and epitope profile. J Cell Biol *115*, 97-107.

Labeit, S., Kolmerer, B., and Linke, W. A. (1997). The giant protein titin. Emerging roles in physiology and pathophysiology. Circ Res *80*, 290-294.

Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. Nature *283*, 249-256.

Lee, M. K., and Cleveland, D. W. (1996). Neuronal intermediate filaments. Annu Rev Neurosci *19*, 187-217.

Lee, M. K., Xu, Z., Wong, P. C., and Cleveland, D. W. (1993). Neurofilaments are obligate heteropolymers in vivo. J Cell Biol *122*, 1337-1350.

Lendahl, U., Zimmerman, L. B., and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585-595.

Letai, A., and Fuchs, E. (1995). The importance of intramolecular ion pairing in intermediate filaments. Proc Natl Acad Sci U S A *92*, 92-96.

Li, H., Choudhary, S. K., Milner, D. J., Munir, M. I., Kuisk, I. R., and Capetanaki, Y. (1994). Inhibition of desmin expression blocks myoblast fusion and interferes with the myogenic regulators MyoD and myogenin. J Cell Biol *124*, 827-841.

Li, Z., Colucci, E., Babinet, C., and Paulin, D. (1993). The human desmin gene: a specific regulatory programme in skeletal muscle both in vitro and in transgenic mice. Neuromuscul Disord *3*, 423-427.

Li, Z., Colucci-Guyon, E., Pincon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D., and Babinet, C. (1996). Cardiovascular lesions and skeletal myopathy in mice lacking desmin. Dev Biol *175*, 362-366.

Li, Z., Mericskay, M., Agbulut, O., Butler-Browne, G., Carlsson, L., Thornell, L. E., Babinet, C., and Paulin, D. (1997). Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. J Cell Biol *139*, 129-144.

Llanos, R., Chevrier, V., Ronjat, M., Meurer-Grob, P., Martinez, P., Frank, R., Bornens, M., Wade, R. H., Wehland, J., and Job, D. (1999). Tubulin binding sites on gamma-tubulin: identification and molecular characterization. Biochemistry *38*, 15712-15720.

Locker, R. H., and Wild, D. J. (1986). A comparative study of high molecular weight proteins in various types of muscle across the animal kingdom. J Biochem *99*, 1473-1484.

Ma, X., Charron, F., Cole, G. J., Savard, P. E., and Vincent, M. (1998). The developmentally regulated avian protein IFAPa-400 is transitin. Brain Res Dev Brain Res *109*, 59-66.

Makarova, I., Carpenter, D., Khan, S., and Ip, W. (1994). A conserved region in the tail domain of vimentin is involved in its assembly into intermediate filaments. Cell Motil Cytoskeleton 28, 265-277.

Maniotis, A. J., Chen, C. S., and Ingber, D. E. (1997). Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci U S A 94, 849-854.

Margolis, R. L., and Wilson, L. (1998). Microtubule treadmilling: what goes around comes around. Bioessays 20, 830-836.

Marvin, M. J., Dahlstrand, J., Lendahl, U., and McKay, R. D. (1998). A rod end deletion in the intermediate filament protein nestin alters its subcellular localization in neuroepithelial cells of transgenic mice. J Cell Sci 111, 1951-1961.

McDonald, K. A., Lakonishok, M., and Horwitz, A. F. (1995). Alpha v and alpha 3 integrin subunits are associated with myofibrils during myofibrillogenesis. J Cell Sci 108, 975-983.

McGregor, A., Blanchard, A. D., Rowe, A. J., and Critchley, D. R. (1994). Identification of the vinculin-binding site in the cytoskeletal protein alpha-actinin. Biochem J 301, 225-233.

Meng, J. J., Bornslaeger, E. A., Green, K. J., Steinert, P. M., and Ip, W. (1997). Two-hybrid analysis reveals fundamental differences in direct interactions between desmoplakin and cell type-specific intermediate filaments. J Biol Chem 272, 21495-21503.

Meng, J. J., Khan, S., and Ip, W. (1994). Charge interactions in the rod domain drive formation of tetramers during intermediate filament assembly. J Biol Chem 269, 18679-18685.

Meng, J. J., Khan, S., and Ip, W. (1996). Intermediate filament protein domain interactions as revealed by two-hybrid screens. J Biol Chem 271, 1599-1604.

Meyer, R. K., and Aebi, U. (1990). Bundling of actin filaments by alpha-actinin depends on its molecular length. J Cell Biol 110, 2013-2024.

Millevoi, S., Trombitas, K., Kolmerer, B., Kostin, S., Schaper, J., Pelin, K., Granzier, H., and Labeit, S. (1998). Characterization of nebulette and nebulin and emerging concepts of their roles for vertebrate Z-discs. J Mol Biol 282, 111-123.

Milner, D. J., Taffet, G. E., Wang, X., Pham, T., Tamura, T., Hartley, C., Gerdes, A. M., and Capetanaki, Y. (1999). The absence of desmin leads to cardiomyocyte hypertrophy and cardiac dilation with compromised systolic function. J Mol Cell Cardiol 31, 2063-2076.

Milner, D. J., Weitzer, G., Tran, D., Bradley, A., and Capetanaki, Y. (1996). Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. J Cell Biol *134*, 1255-1270.

Moon, R. T., and Lazarides, E. (1983). Synthesis and post-translational assembly of intermediate filaments in avian erythroid cells: vimentin assembly limits the rate of synemin assembly. Proc Natl Acad Sci U S A *80*, 5495-5499.

Ngai, J., Capetanaki, Y. G., and Lazarides, E. (1985). Expression of the genes coding for the intermediate filament proteins vimentin and desmin. Ann N Y Acad Sci *455*, 144-157.

North, A. J., Bardsley, W. G., Hyam, J., Bornslaeger, E. A., Cordingley, H. C., Trinnaman, B., Hatzfeld, M., Green, K. J., Magee, A. I., and Garrod, D. R. (1999). Molecular map of the desmosomal plaque. J Cell Sci *112*, 4325-4336.

Obermann, W. M., Gautel, M., Steiner, F., van der Ven, P. F., Weber, K., and Furst, D. O. (1996). The structure of the sarcomeric M band: localization of defined domains of myomesin, M-protein, and the 250-kD carboxy-terminal region of titin by immunoelectron microscopy. J Cell Biol *134*, 1441-1453.

Obermann, W. M., Gautel, M., Weber, K., and Furst, D. O. (1997). Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. EMBO J *16*, 211-220.

Ohtsuka, H., Yajima, H., Maruyama, K., and Kimura, S. (1997). The N-terminal Z repeat 5 of connectin/titin binds to the C-terminal region of alpha-actinin. Biochem Biophys Res Commun *235*, 1-3.

Oleynikov, Y., and Singer, R. H. (1998). RNA localization: different zipcodes, same postman? Trends Cell Biol *8*, 381-383.

Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983). Vinculin is a component of an extensive network of myofibril-sarcolemma attachment regions in cardiac muscle fibers. J Cell Biol *97*, 1081-1088.

Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983). A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc Natl Acad Sci U S A *80*, 1008-1012.

Pepe, F. A. (1975). Structure of muscle filaments from immunohistochemical and ultrastructural studies. J Histochem Cytochem *23*, 543-562.

Porter, G. A., Dmytrenko, G. M., Winkelmann, J. C., and Bloch, R. J. (1992). Dystrophin colocalizes with beta-spectrin in distinct subsarcolemmal domains in mammalian skeletal muscle. J Cell Biol *117*, 997-1005.

Price, M. G. (1984). Molecular analysis of intermediate filament cytoskeleton--a putative load-bearing structure. Am J Physiol *246*, H566-572.

Price, M. G., and Lazarides, E. (1983). Expression of intermediate filament-associated proteins paranemin and synemin in chicken development. J Cell Biol *97*, 1860-1874.

Price, M. G., and Sanger, J. W. (1984). Reduction of density and anisotropic distribution of intermediate filaments occur during avian skeletal myogenesis. Am J Anat *171*, 427-440.

Qin, W., Khuchua, Z., Cheng, J., Boero, J., Payne, R. M., and Strauss, A. W. (1998). Molecular characterization of the creatine kinases and some historical perspectives. Mol Cell Biochem *184*, 153-167.

Raats, J. M., Pieper, F. R., Vree Egberts, W. T., Verrijp, K. N., Ramaekers, F. C., and Bloemendal, H. (1990). Assembly of amino-terminally deleted desmin in vimentin-free cells. J Cell Biol *111*, 1971-1985.

Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993). Structure of the actin-myosin complex and its implications for muscle contraction. Science *261*, 58-65.

Reipert, S., Steinbock, F., Fischer, I., Bittner, R. E., Zeold, A., and Wiche, G. (1999). Association of mitochondria with plectin and desmin intermediate filaments in striated muscle. Exp Cell Res *252*, 479-491.

Richardson, F. L., Stromer, M. H., Huiatt, T. W., and Robson, R. M. (1981). Immunoelectron and immunofluorescence localization of desmin in mature avian muscles. Eur J Cell Biol *26*, 91-101.

Robson, R. M. (1989). Intermediate filaments. Curr Opin Cell Biol *1*, 36-43.

Sandoval, I. V., Colaco, C. A., and Lazarides, E. (1983). Purification of the intermediate filament-associated protein, synemin, from chicken smooth muscle. Studies on its physicochemical properties, interaction with desmin, and phosphorylation. J Biol Chem *258*, 2568-2576.

Sarria, A. J., Lieber, J. G., Nordeen, S. K., and Evans, R. M. (1994). The presence or absence of a vimentin-type intermediate filament network affects the shape of the nucleus in human SW-13 cells. J Cell Sci *107*, 1593-1607.

Schafer, D. A., and Cooper, J. A. (1995). Control of actin assembly at filament ends. Annu Rev Cell Dev Biol *11*, 497-518.

Schafer, D. A., Hug, C., and Cooper, J. A. (1995). Inhibition of CapZ during myofibrillogenesis alters assembly of actin filaments. J Cell Biol *128*, 61-70.

Schmidt, J. M., Zhang, J., Lee, H. S., Stromer, M. H., and Robson, R. M. (1999). Interaction of talin with actin: sensitive modulation of filament crosslinking activity. Arch Biochem Biophys *366*, 139-150.

Schroder, R., Warlo, I., Herrmann, H., van der Ven, P. F., Klasen, C., Blumcke, I., Mundegar, R. R., Furst, D. O., Goebel, H. H., and Magin, T. M. (1999). Immunogold EM reveals a close association of plectin and the desmin cytoskeleton in human skeletal muscle. Eur J Cell Biol *78*, 288-295.

Sejersen, T., and Lendahl, U. (1993). Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci *106*, 1291-1300.

Seki, N., Ohira, M., Nagase, T., Ishikawa, K., Miyajima, N., Nakajima, D., Nomura, N., and Ohara, O. (1997). Characterization of cDNA clones in size-fractionated cDNA libraries from human brain. DNA Res *4*, 345-349.

Sheetz, M. P. (1999). Motor and cargo interactions. Eur J Biochem *262*, 19-25.

Shoeman, R. L., and Traub, P. (1993). Assembly of intermediate filaments. BioEssays *15*, 605-611.

Sjoberg, G., Jiang, W. Q., Ringertz, N. R., Lendahl, U., and Sejersen, T. (1994). Colocalization of nestin and vimentin/desmin in skeletal muscle cells demonstrated by three-dimensional fluorescence digital imaging microscopy. Exp Cell Res *214*, 447-458.

Sjuve, R., Arner, A., Li, Z., Mies, B., Paulin, D., Schmittner, M., and Small, J. V. (1998). Mechanical alterations in smooth muscle from mice lacking desmin. J Muscle Res Cell Motil *19*, 415-429.

Small, J. V. (1985). Geometry of actin-membrane attachments in the smooth muscle cell: the localisations of vinculin and alpha-actinin. EMBO J *4*, 45-49.

Small, J. V. (1995). Structure-function relationships in smooth muscle: the missing links. BioEssays *17*, 785-792.

Small, J. V., Furst, D. O., and Thornell, L. E. (1992). The cytoskeletal lattice of muscle cells. Eur J Biochem *208*, 559-572.

Sorimachi, H., Freiburg, A., Kolmerer, B., Ishiura, S., Stier, G., Gregorio, C. C., Labeit, D., Linke, W. A., Suzuki, K., and Labeit, S. (1997). Tissue-specific expression and alpha-actinin binding properties of the Z-disc titin: implications for the nature of vertebrate Z-discs. J Mol Biol *270*, 688-695.

Stappenbeck, T. S., Bornslaeger, E. A., Corcoran, C. M., Luu, H. H., Virata, M. L., and Green, K. J. (1993). Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. J Cell Biol *123*, 691-705.

Steimle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999). Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. J Biol Chem *274*, 18414-18420.

Steinbock, F. A., and Wiche, G. (1999). Plectin: a cytolinker by design. Biol Chem *380*, 151-158.

Steinert, P. M., Chou, Y. H., Prahlad, V., Parry, D. A., Marekov, L. N., Wu, K. C., Jang, S. I., and Goldman, R. D. (1999). A high molecular weight intermediate filament-associated protein in BHK- 21 cells is nestin, a type VI intermediate filament protein. Limited co-assembly in vitro to form heteropolymers with type III vimentin and type IV alpha-internexin. J Biol Chem *274*, 9881-9890.

Steinert, P. M., Marekov, L. N., and Parry, D. A. (1999). Molecular parameters of type IV alpha-internexin and type IV-type III alpha-internexin-vimentin copolymer intermediate filaments. J Biol Chem *274*, 1657-1666.

Steinert, P. M., and Roop, D. R. (1988). Molecular and cellular biology of intermediate filaments. Annu Rev Biochem *57*, 593-625.

Stolz, M., and Wallimann, T. (1998). Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK. J Cell Sci *111*, 1207-1216.

Stromer, M. H. (1998). The cytoskeleton in skeletal, cardiac and smooth muscle cells. Histol Histopathol *13*, 283-291.

Sultana, S., Sernett, S. W., Bellin, R. M., Robson, R. M., and Skalli, O. (2000). The intermediate filament protein synemin is transiently expressed in a subset of astrocytes during development. Glia *30*, 143-153.

Suzuki, A., Goll, D. E., Singh, I., Allen, R. E., Robson, R. M., and Stromer, M. H. (1976). Some properties of purified skeletal muscle alpha-actinin. J Biol Chem *251*, 6860-6870.

Svitkina, T. M., Verkhovsky, A. B., and Borisy, G. G. (1996). Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. J Cell Biol *135*, 991-1007.

Thornell, L., Carlsson, L., Li, Z., Mericskay, M., and Paulin, D. (1997). Null mutation in the desmin gene gives rise to a cardiomyopathy. J Mol Cell Cardiol *29*, 2107-2124.

Tokuyasu, K. T., Dutton, A. H., and Singer, S. J. (1983). Immunoelectron microscopic studies of desmin (skeletin) localization and intermediate filament organization in chicken cardiac muscle. J Cell Biol *96*, 1736-1742.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A *76*, 4350-4354.

Tran, P. T., Joshi, P., and Salmon, E. D. (1997). How tubulin subunits are lost from the shortening ends of microtubules. J Struct Biol *118*, 107-118.

Turnacioglu, K. K., Mittal, B., Dabiri, G. A., Sanger, J. M., and Sanger, J. W. (1997). An N-terminal fragment of titin coupled to green fluorescent protein localizes to the Z-bands in living muscle cells: overexpression leads to myofibril disassembly. Mol Biol Cell *8*, 705-717.

Turner, C. E., Glenney, J. R., Jr., and Burridge, K. (1990). Paxillin: a new vinculin-binding protein present in focal adhesions. J Cell Biol *111*, 1059-1068.

Vaughan, K. T., Weber, F. E., Einheber, S., and Fischman, D. A. (1993). Molecular cloning of chicken myosin-binding protein (MyBP) H (86-kDa protein) reveals extensive homology with MyBP-C (C-protein) with conserved immunoglobulin C2 and fibronectin type III motifs. J Biol Chem *268*, 3670-3676.

Vinkemeier, U., Obermann, W., Weber, K., and Furst, D. O. (1993). The globular head domain of titin extends into the center of the sarcomeric M band. cDNA cloning, epitope mapping and immunoelectron microscopy of two titin-associated proteins. J Cell Sci *106*, 319-330.

Vohra, M. S., Komiyama, M., Hayakawa, K., Obinata, T., and Shimada, Y. (1998). Subcellular localization of dystrophin and vinculin in cardiac muscle fibers and fibers of the conduction system of the chicken ventricle. Cell Tissue Res *294*, 137-143.

Wang, K. (1996). Titin/connectin and nebulin: giant protein rulers of muscle structure and function. Adv Biophys *33*, 123-134.

Weekes, J., Barry, S. T., and Critchley, D. R. (1996). Acidic phospholipids inhibit the intramolecular association between the N- and C-terminal regions of vinculin, exposing actin-binding and protein kinase C phosphorylation sites. Biochem J *314*, 827-832.

Wiche, G. (1998). Domain structure and transcript diversity of plectin. Biol Bull *194*, 381-382; discussion 383.

Wiche, G. (1998). Role of plectin in cytoskeleton organization and dynamics. J Cell Sci *111*, 2477-2486.

Wiche, G., Krepler, R., Artlieb, U., Pytela, R., and Denk, H. (1983). Occurrence and immunolocalization of plectin in tissues. J Cell Biol *97*, 887-901.

Wilhelm, J. E., and Vale, R. D. (1993). RNA on the move: the mRNA localization pathway. J Cell Biol *123*, 269-274.

Wilkins, J. A., Chen, K. Y., and Lin, S. (1983). Detection of high molecular weight vinculin binding proteins in muscle and nonmuscle tissues with an electroblot-overlay technique. Biochem Biophys Res Commun *116*, 1026-1032.

Yagyu, M., Robson, R. M., and Stromer, M. H. (1990). Identification of intermediate filaments in mature mammalian skeletal muscle by transmission electron microscopy. Proc 12th Intern Congr Electron Miscoscopy *3*, 454-455.

Yamada, K. M., and Geiger, B. (1997). Molecular interactions in cell adhesion complexes. Curr Opin Cell Biol *9*, 76-85.

Yamaguchi, M., Izumimoto, M., Robson, R. M., and Stromer, M. H. (1985). Fine structure of wide and narrow vertebrate muscle Z-lines. A proposed model and computer simulation of Z-line architecture. J Mol Biol *184*, 621-643.

Young, P., Ferguson, C., Bauelos, S., and Gautel, M. (1998). Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin. EMBO J *17*, 1614-1624.

Yuan, J., Huiatt, T. W., Liao, C. X., Robson, R. M., and Graves, D. J. (1999). The effects of mono-ADP-ribosylation on desmin assembly-disassembly. Arch Biochem Biophys *363*, 314-322.

122

ACKNOWLEDGEMENTS

I thank my major professor Richard Robson for offering me so many ideas, providing me with a wealth of learning opportunities and supplying me with a great deal of support and encouragement during my graduate studies. I also thank the members of my graduate committee and several other Iowa State University faculty members that have provided me with ideas and equipment needed for my research.

I have had the opportunity to work with many wonderful people in the Muscle Biology Group, all of which enhanced my graduate studies in some way. There were several that had a particular influence on this dissertation. Dr. Bruno Becker, who was a postdoctoral assistant in the lab when I joined the group, taught me a great deal about molecular biology through our work together starting the cloning and sequencing of synemin. Suzy Sernett, who was a research associate in the lab until the summer of 1999, worked a great deal on the purification of muscle cytoskeletal proteins. Together we did a great number of the *in vitro* protein interaction studies and had many thoughtful discussions about protein interactions involving synemin. Stephanie Seiler and Ho-Sup Lee, the other two current graduate students in the Robson lab, have been excellent colleagues for discussion of research ideas and for helping solve the day-to-day experimental procedure problems that can stall research progress if they are not worked out.

I am also indebted to a number of individuals outside of the Muscle Biology Group. Dr. Wally Ip and his lab, at the University of Cincinnati, helped me learn to use the two-hybrid system on IF proteins during a two-week trip to their lab. Dr. David Critchley, at the University of Leicester, provided the cDNAs for α-actinin and vinculin that were used to