Molecular interactions of the mammalian intermediate filament protein synemin with cytoskeletal proteins present in adhesion sites

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Molecular interactions of the mammalian intermediate filament protein synemin with cytoskeletal proteins present in adhesion sites

by

Ning Sun

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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The type VI intermediate filament (IF) protein synemin is a very large, unique member of the IF protein superfamily. Synemin associates with the major type III IF protein desmin and/or vimentin forming heteropolymeric intermediate filaments (IFs) within mammalian muscle cells. Previous studies in our lab showed that avian synemin interacts with vinculin and α-actinin, both of which are cytoskeletal proteins present in adhesion sites such as the focal adhesions within cells. Thus, synemin may link the heteropolymeric IFs to adhesion sites within mammalian muscle cells or within some non-muscle cells that express synemin. At least two isoforms of synemin, namely α- and β-synemin, are present in mammals. The larger α-synemin is identical with the smaller β-synemin, with the only exception being that the slightly smaller β-synemin lacks a 312 amino acid insert (SNTIII) near the end of the long C-terminal tail domain. Whether the two mammalian synemin isoforms have different functions is unclear. Studies in this dissertation provide evidence that the two large mammalian (human) synemin isoforms interact differentially with several cytoskeletal proteins and thereby may fulfill different cellular functions. Mapping the binding site(s) of human synemins within vinculin and talin by in vitro protein-protein interaction assays revealed that SNTIII, which is absent in β-synemin, interacts specifically with both vinculin and talin. Transient expression of enhanced green fluorescent protein (EGFP)-tagged SNTIII co-localized with vinculin and talin at focal adhesion sites within mammalian cells. Confocal microscope analysis of intracellular localizations of endogenous synemin and vinculin/talin indicated that synemin co-localized with vinculin and talin at the sites of costameres, which are considered specialized focal adhesions located periodically along and immediately subjacent to the sarcolemma of mammalian striated muscle cells. These results indicated that mammalian α-synemin, but not β-synemin, interacts directly with the cytoskeletal proteins vinculin and talin within mammalian cells. To elucidate the possible functions of, and to identify novel interacting proteins for, the smaller β-synemin within cells, yeast two-hybrid screening of a human adult skeletal muscle cDNA library was performed using the entire tail domain of human β-synemin (SNβT) as the bait. The LIM domain protein zyxin was identified as an interaction partner of β-synemin. The interaction was further confirmed by
several *in vitro* protein-protein interaction assays. Furthermore, over-expression of the zyxin-binding region of synemin within mammalian cells blocked the localizations of endogenous zyxin to the focal adhesions without disrupting normal cellular architectures. Knockdown synemin expression within cells by siRNA resulted in significantly compromised cell adhesion and migration. These results *in toto* indicate that mammalian (human) synemin isoforms participate in the focal adhesion dynamics and are essential for cell adhesion and cell motility.
GENERAL INTRODUCTION

Dissertation Organization

The manuscript-based format is used in my dissertation with the inclusion of three papers. The main body consists of one paper published in Biochemical Journal, a second paper published in Experimental Cell Research, and a third manuscript that will be submitted for publication in the journal Molecular Biology of the Cell. I was responsible for the design, implementation, and interpretation of almost all of the experiments in these three papers. I did receive initial help and guidance from Rahul Bhosle, a previous graduate student in our lab, and from Dr. Susan Veneziano, a postdoctoral assistant in the lab. Dr. Denise Paulin and Dr. Zhenlin Li at the University of Paris provided the full-length human synemin cDNAs used in my research. The references cited in the literature review are in the format of author/year style for ease of reading. Those references cited in the manuscripts are formatted in the styles according to the requirements of each journal and are listed in separate Reference sections corresponding with each manuscript. A comprehensive list of references with article titles is included at the end of the dissertation following the GENERAL CONCLUSIONS section.

Introduction

The overall goal of the research described in this dissertation was to increase our understanding of the cellular functions of the two large isoforms of the mammalian type VI intermediate filament (IF) protein synemin. It is generally known that the ~310 amino acid conserved central rod domain of cytoplasmic IF proteins mediates IF protein assembly by forming “coiled-coil” structures, whereas the head and tail domains of IF proteins contain most of the binding sites for non-IF protein partners. Thus, the unusually large C-terminal tails (933 amino acids in β-synemin and 1245 amino acids in α-synemin) of the mammalian (human) synemins likely contain most of the binding sites for their non-IF protein partners. Indeed, previous studies in our lab demonstrated that the long C-terminal tail domain of avian synemin, which is expressed as only one form, was shown to interact with α-actinin and
vinculin.

Avian synemin shares only ~35% sequence identity and ~53% sequence homology with the human synemin isoforms. Whether either of the two human synemin isoforms interact with vinculin has not been known. Furthermore, whether either synemin isoform interacts with metavinculin, which is a muscle specific isoform of vinculin, has also been unknown. The first manuscript (published in Biochemical Journal) included in this dissertation describes molecular interaction studies of the human α- and β- synemin isoforms with vinculin and metavinculin. The results demonstrate that the 312 amino acid insert (SNTIII), which is present only within α-synemin, interacts specifically with both vinculin and metavinculin, whereas regions from human β-synemin do not. Uyama et al. (2006, Gut 55:1276-1289) recently reported that synemin co-precipitates and co-localizes with talin, which is another key cytoskeletal protein regulating focal adhesion dynamics. However, information demonstrating direct interaction of synemin with talin has been unclear. The second manuscript (published in Experimental Cell Research) included in this dissertation demonstrates that SNTIII present in α-synemin, but not in human β-synemin, interacts specifically with the talin rod domain. Furthermore, SNTIII was found to be composed of seven ~39 amino acid tandem repeats. These studies in toto demonstrate that it is the human α-synemin isoform that interacts with vinculin and talin, thereby exhibiting functions not present within the smaller β-synemin isoform.

To increase our understanding of the role(s) of the smaller β-synemin, yeast two-hybrid screening of a human adult skeletal muscle cDNA library, using the entire tail domain of human β-synemin as the bait, was performed. These studies are described in the third manuscript included in this dissertation. The protein zyxin, an adhesion plaque protein, was discovered to be a novel interaction partner for both human α- and β-synemins. Subsequent interaction studies mapped the binding site for synemin within zyxin and the sites for zyxin within the synemins. By using siRNA studies, potential functions for synemin in cell adhesion and migration are also described in the third manuscript.
**Review of Literature**

**Intermediate Filament Proteins**

There are three major filamentous cytoskeletal components present within cytoskeletons of mammalian cells, namely the microfilaments composed primarily of actin monomers, the microtubules composed primarily of $\alpha$- and $\beta$-tubulins, and the intermediate filaments (IFs) comprising cell-type specific intermediate filament (IF) proteins (Robson, 1989; Fuchs and Weber, 1994). These three cytoskeletal components constitute an intricate three-dimensional network within the cytoplasm and the nucleus, and contribute to many important biological events occurring within eukaryotic cells. As for microfilaments and microtubules, research on IFs has long been of a focus of interest to many investigators. The name of IFs came from their $\sim 10$ nm diameter, which is intermediate between that of microfilaments (6 nm) and microtubules (23 nm) (Ishikawa et al., 1968). In contrast to microfilaments and microtubules, which are polar filaments composed of highly conserved globular monomers, IFs are composed of elongated IF protein subunits having no any obvious polarity (Strelkov et al., 2003). To date, at least 67 IF genes encoding individual IF proteins have been identified in the human genome (Hesse et al., 2001). The nucleotide sequences of these IF genes are highly diverse, and the proteins they encode are also diversely expressed in various tissues with different abundance (Fuchs and Cleveland, 1998). Nonetheless, all the IF proteins share a characteristic tripartite structural feature, namely a centrally conserved $\sim 310$ amino acid rod domain flanked by hypervariable head and tail domains (Coulombe et al., 2001). The conserved rod domain is composed primarily of $\alpha$-helical segments and is essential in mediating coiled-coil dimer formation as well as IF protein assembly (Strelkov et al., 2003; Herrmann and Aebi, 2004; Kreplak et al., 2004). The hypervariable head and tail domains, having various lengths and primary sequences, contribute to the diversity among the individual IF proteins (Coulombe and Wong, 2004), and may harbor most of the binding sites for their non-IF protein partners (Green and Simpson, 2007; Kim and Coulombe, 2007).

Intermediate filament proteins are classified into six major types based upon the nucleotide sequence homology within their rod domains (Coulombe et al., 2001). The acidic and basic keratins constitute the type I and type II IF proteins, respectively, and they usually form obligate heteropolymers upon IF formation (Herrmann and Aebi, 2004; Omary et al.,
The type III IF proteins consist of desmin, vimentin, glial fibrillary acidic protein (GFAP) and peripherin. These are able to self-assemble into homopolymeric 10 nm diameter IF filaments, but usually form heteropolymers with other IF proteins (Ivaska et al., 2007). The neurofilament triplet proteins (NF-L, NF-M, NF-H) and α-internexin, which are expressed in neurons, constitute the type IV IF proteins (Coulombe et al., 2001). The type V IF proteins comprise the nuclear lamins that form the nuclear lamina located along the inner surface of the nuclear envelope (Wilson et al., 2001). Notably, the lamins cannot form heteropolymers with the cytoplasmic IFs, possibly because of an extra 41 amino acid insert present within the lamin rod domain (Herrmann et al., 2000). Synemin and nestin constitute the type VI IF proteins, both of which contain an extremely short N-terminal head domain and a very long C-terminal tail domain. In contrast to the type III IF proteins, synemin and nestin are unable to self-assemble into homopolymeric IFs, but they do form heteropolymeric IFs with desmin and/or vimentin (Robson et al., 2004).

One of the most commonly recognized functions of all the IFs is that they provide mechanical structural support in living cells (Fuchs and Weber, 1994). A canonical example is that mutations in keratins K5 and K14, which are expressed within stratified dermal epithelium, causes Epidermolysis Bullosa Simplex, a severe skin blistering disease (Fuchs et al., 1994). Keratin K6 knockout neonatal mice also exhibit damaged cellular integrity of dorsal tongue epithelium after suckling from their mother (Wong et al., 2000). Novel functions of IFs have been emerging in recent years. Many studies have shown that IFs play essential roles in regulating cell adhesion and migration, apoptosis, protein and lipid targeting, and in organizing subcellular organelles such as mitochondria (Kim and Coulombe, 2007). Possible functions of IFs in cell adhesion and cell motility are focused upon herein this review of literature.

**IFs in cell adhesion and migration**

It has long been known that cytoplasmic IFs are attached at sites of desmosomes and hemidesmosomes within stratified and complex epithelia (Kottke et al., 2006; Litjens et al., 2006). Desmosomes are important cell-cell junctions that maintain the tissue integrity of epithelia and cardiac muscle cells (Green and Simpson, 2007), whereas hemidesmosomes
contribute to epithelial cell-extracellular basement membrane adhesions (Jones et al., 1998).

The major protein components that assemble desmosomes are cadherin-family proteins, armadillo proteins, and plakins. The transmembrane cadherins, desmogleins and desmocollins, establish the interface of desmosomes and bind to the armadillo proteins plakoglobins and plakophilins (Green and Simpson, 2007). Armadillo proteins, in turn, interact with the plakin family members, desmoplakins, which provide a platform for linking the IFs to desmosomes (Hatsell and Cowin, 2001). The desmoplakins bind specifically to the rod domains of the IF proteins keratin, vimentin, and desmin via the C-terminal plakin repeat domains (Fontao et al., 2003). Knockout animal models of, or mutations in, the desmoplakins resulted in disrupted desmosomes and defects in embryonic development, as well as tissue integrities of skin and cardiac muscles (Kottke et al., 2006; Green and Simpson, 2007). In hemidesmosomes, integrin \( \alpha_6\beta_4 \), collagen family member BP180, CD151, and two plakin family members plectin and BP230 are the major constituents (Litjens et al., 2006). Integrin \( \alpha_6\beta_4 \) contributes to the stable attachment of epithelial cells to the basement membrane via interactions with laminin-332, a major extracellular matrix protein expressed in the basement membrane (Wilhelmsen et al., 2006). Intermediate filament proteins are linked to hemidesmosomes via interactions with the C-terminal plakin repeats of plectin and BP230, which interacts with integrin \( \beta_4 \) (Koster et al., 2004). Disruption of hemidesmosomes leads to a spectrum of skin blistering phenotypes, indicating they are essential for maintaining epidermal tissue integrity (Zillikens, 1999). Some studies have also indicated that, during cell migration and cancer cell invasion, hemidesmosomes are disassembled and redistributed (Wilhelmsen et al., 2006). Thus, hemidesmosomes are not only important for cell-stromal membrane attachment, but also are associated with cell motility.

Accumulating evidence has shown that IFs can also be anchored at the classical cell-cell and cell-extracellular matrix adherens-type junctions (Kim and Coulombe, 2007). Kowalczyk et al. reported that desmoplakins were recruited to cell-cell adherens-type junctions along with VE-cadherin, and thereby may be able to link IFs to these sites (Kowalczyk et al., 1998). The type III IF protein vimentin was also shown to co-precipitate and co-localize with p120 catenin, which is another component of the cell-cell adherens-type junctions (Kim et al., 2005). In addition, fluorescent protein-tagged vimentin was shown to partially overlap with
integrin β3 within endothelial cells at the focal adhesions sites, which represent cell-substratum adherens-type junctions of cultured cells (Tsuruta and Jones, 2003). Another study also reported that, using leucine zipper mediated recombinant integrin α2β1 heterodimers, vimentin was found to interact with the α2β1 dimer in screening assays for putative binding partners. And, vimentin transiently co-localized with integrin α2β1 at the focal adhesion sites within endothelial cells (Kreis et al., 2005). Knockdown vimentin expression using RNAi led to smaller focal adhesions and decreased adhesions of endothelial cells upon shear stress (Tsuruta and Jones, 2003). These studies suggest that vimentin IFs contribute significantly to cell-substratum adhesions and regulate focal adhesion sizes.

The large type VI IF proteins nestin and synemin have also been shown to play essential roles in cell adhesion and migration. A recent study (Kleeberger et al., 2007) showed that nestin was up-regulated in several prostate cancer cell lines capable of metastases. RNAi studies showed that nestin knockdown markedly impaired prostate cancer cell metastasis and invasion, suggesting that nestin plays a role in regulating cell motility (Kleeberger et al., 2007). Studies done in our lab have also demonstrated interactions of synemin with vinculin, α-actinin, and dystrophin/utrophin within vertebrate muscle cells, suggesting that synemin is able to attach to adhesion sites enriched in these cytoskeletal proteins (Bellin et al., 1999; Bellin et al., 2001; Bhosle et al., 2006).

**Synemin**

The type VI IF protein synemin is a very large, unique member of the IF protein superfamily (Robson et al., 2004). Synemin was first discovered within avian smooth and skeletal muscle cells where it associates with the major type III IF proteins desmin and vimentin (Granger and Lazarides, 1980). It was at that time classified as an intermediate filament-associated protein (IFAP). Cloning and sequencing studies in our lab subsequently demonstrated that synemin is a bona fide IF protein containing the conserved characteristic IF protein rod domain (Becker et al., 1995). Sequencing studies of avian synemin in our lab indicated that it exhibits a very short, 10 amino acid head domain and a very long, more than 1,000 amino acid tail domain (Bellin et al., 1999). Results from our lab also demonstrated synemin is expressed within mammalian muscle tissues. Western blot analysis of porcine
muscle tissues using anti-avian synemin antibodies resulted in two bands at molecular weights of ~225 KDa and ~195 KDa (Bilak et al., 1998). Titeux et al. subsequently cloned and sequenced the human synemin gene and showed that it encodes two large splice variants, namely α-synemin (~180 KDa) and β-synemin (~150 KDa). Both α- and β-synemins co-localize with desmin and vimentin within muscle cells (Titeux et al., 2001). The mouse synemin gene was also cloned and sequenced recently and shown to encode three isoforms, namely H, M, and L-synemins, resulting from alternative splicing (Xue et al., 2004).

Although synemin is primarily expressed in muscle tissues, it is also expressed in many non-muscle tissues and cells. For example, synemin is found present within avian erythrocytes (Granger et al., 1982) and lens cells (Granger and Lazarides, 1984), rat and human astrocytes (Sultana et al., 2000; Jing et al., 2005), human glial cells and neurons (Hirako et al., 2003; Izmiryan et al., 2006), human retina and lens cells (Tawk et al., 2003), and in human hepatic stellate cells (Uyama et al., 2006). Also, there are accumulating reports of synemin expression in malignant cancer cells. These include human adrenal cortex adenocarcinoma cells (SW13 cells) (Bellin et al., 1999), hepatoma cells (HepG2 and J-5 cells) (Chan et al., 2007), malignant astrocytes (Jing et al., 2005), cervix epithelial adenocarcinoma cells (Hela cells) (Olsen et al., 2006), and malignant biliary epithelial cells (Schmitt-Graeff et al., 2006). The wide expression of synemin in various tissues and cancer cells suggests synemin has important cellular functions.

Avian and mammalian synemins are not able to assemble into homopolymeric IFs in vivo, but associate with other IF proteins such as vimentin, desmin, and neurofilament proteins forming heteropolymeric IFs (Bellin et al., 1999; Titeux et al., 2001; Xue et al., 2004; Izmiryan et al., 2006; Jing et al., 2007). Results from our lab indicated that the association is mediated by interactions between the conserved rod domain of synemin and those of desmin and/or vimentin (Bellin et al., 1999). We have also shown that the C-terminal tail domain of avian synemin interacts with the cytoskeletal proteins α-actinin and vinculin by blot overlay assays (Bellin et al., 1999; Bellin et al., 2001). Subsequent interaction studies in our lab using recombinant proteins of avian and human synemin regions and of dystrophin/utrophin regions showed that the tail domain of synemin binds to the cysteine rich region and specific spectrin-like repeat region within both dystrophin and utrophin (Bhosle et
Studies by Mizuno et al. (2001) demonstrated that the human synemin rod domain binds to α-dystrobrevin, a component of the dystrophin-associated protein complex that interacts directly with dystrophin (Mizuno et al., 2001). In mdx mice having no dystrophin, synemin mRNA expression was also found to be decreased (Mizuno et al., 2007). Interestingly, α-actinin, vinculin, dystrophin, and α-dystrobrevin are all present in cell adhesion sites such as the costameres within muscle cells. Costameres are multi-protein assemblies considered to be specialized cell-substratum adherens-type junction sites in register with the peripheral layer of cellular myofibrillar Z-lines, and are present periodically along and immediately subjacent to the sarcolemma (Craig and Pardo, 1983; Pardo et al., 1983). It is believed that forces generated by contracting myofibrils within a muscle cell are laterally transmitted to the extracellular matrix via the costameres (Ervasti, 2003). Therefore, synemin appears able to link the heteropolymeric IFs to the adhesion sites such as costameres within vertebrate striated muscle cells. Indeed, synemin has been shown to be enriched at the costameres and neuromuscular and myotendinous junctions within human skeletal muscle cells (Mizuno et al., 2004). Thus, it is evident that the synemin and desmin/vimentin heteropolymeric IFs encircle each myofibril at its Z-lines, thereby linking all adjacent myofibrils within the striated muscle cell, and linking the lateral outermost layer of myofibrils to the adhesion sites subjacent to sarcolemma. These linkages thereby contribute to maintaining the structural and functional integrity of the contracting myofibers.

Several studies from other labs working on synemin have provided information about other putative cellular functions of synemin. Jing et al. reported in a recent study that α-synemin was highly expressed in astrocytic tumors, and that both α- and β-synemins were newly synthesized by reactive and neoplastic astrocytes within human brain tissue (Jing et al., 2005). These authors also demonstrated that synemin co-localized with α-actinin within the membrane ruffles of glioblastoma cells, suggesting synemin may play a role in cell motility (Jing et al., 2005). Russell et al. reported that synemin interacts with protein kinase A (PKA) within human cardiac muscle cells, and suggested that synemin is an A-kinase anchoring protein (AKAP) providing temporal and spatial targeting of PKA (Russell et al., 2006). Schmitt-Graeff et al. showed that synemin expression was widespread in liver cell fibrosis and was up-regulated in malignant liver cells, indicating that synemin may be involved in
these disease processes (Schmitt-Graeff et al., 2006). However, a synemin knockout model, either in cells or animals, has not yet been established, and all of the exact overall cellular functions of synemin remain to be clarified.

**Vinculin and metavinculin**

Vinculin is a ubiquitously expressed important cytoskeletal protein present at the cytoplasmic face of cell-cell and cell-extracellular matrix adherens-type junctions (Demali, 2004; Ziegler et al., 2006). The 116 KDa vinculin molecule is composed of an ~90 KDa head domain, an ~27 KDa tail domain, and a proline rich linker region containing binding sites for SH3 domains (Bakolitsa et al., 1999). A recent report showed that vinculin actually exhibits an overall three dimensional “bundle of bundles” structure comprising five domains (D) of α-helical bundles (Bakolitsa et al., 2004). The N-terminal ~90 KDa head domain comprises four of the five domains (D1-D4) and the C-terminal ~27 KDa tail domain is the fifth domain itself (D5), with the loop-like proline rich linker region connecting the head and tail domains (Bakolitsa et al., 2004). Vinculin contains binding sites for many protein partners that are also present within adherens junctions, and thus it is believed to serve as an adapter for multi-protein assembly within adherens junctions (Jockusch and Rudiger, 1996; Critchley, 2000). For examples, the vinculin head domain contains binding sites for talin, α-catenin, and α-actinin, the tail domain binds to paxillin and F-actin, and the proline rich linker region between the head and tail domains harbors binding sites for vinexin, vasodilator stimulated phosphoprotein (VASP), and the ARP2/3 complex (Critchley, 2000; Ziegler et al., 2006). However, vinculin is usually held in an autoinhibited state by the intramolecular association between the head and the tail domains in the cytoplasm, which abolishes most of the interactions of the protein partners with vinculin (Johnson and Craig, 1995). When recruited to adhesion sites, vinculin is activated, which means that the head and the tail association is disrupted (Chen et al., 2005). Activation of vinculin is thought to be induced in a combinatory pathway by simultaneous binding of two or more partners (Bakolitsa et al., 2004), which exposes all of the binding sites for protein partners and recruits multiple proteins thereby forming complicated adhesion complexes. The role of vinculin in regulating cell adhesion and migration is illustrated by studies using vinculin-null cells. The latter cells
exhibited smaller focal adhesion sites, and closed wound more rapidly in comparison to wild type vinculin positive cells (Saunders et al., 2006). Especially when the vinculin head domains bind to talin, another important cytoskeletal protein regulating focal adhesions, they promote the recruitment and activation of integrins at focal adhesion sites (Humphries et al., 2007). Vinculin has also been shown to transiently recruit Arp2/3 to the far end of lamellipodia, thereby coupling cell adhesion with cell movement (DeMali and Burridge, 2003). As shown in (Xu et al., 1998), vinculin knockout mice died at E.10 and exhibited heart and brain defects during development, suggesting vinculin is also essential for embryonic development.

Vinculin has a splice variant, namely metavinculin, that is specifically expressed within muscle tissues (Belkin et al., 1988). The only difference between vinculin and metavinculin is that metavinculin contains an additional 68 amino acid insert within the C-terminal tail domain (Byrne et al., 1992; Koteliantsky et al., 1992). Both vinculin and metavinculin are present within costameres, intercalated discs, and dense bodies in muscle cells (Witt et al., 2004). Witt et al. also implicated the additional 68 amino acid insert interferes with the activation of the metavinculin molecule. The activities of metavinculin in bundling F-actin are also deferent from those of vinculin (Rudiger et al., 1998). We have shown that avian synemin interacts with the protein vinculin (Bellin et al., 2001). However, the interaction of both vinculin and metavinculin splice variants with mammalian synemin isoforms has remained unknown.

**Talin**

Talin is a very large (~230 KDa), elongated cytoskeletal protein present primarily in cell-substratum adherens junctions. Previous studies showed that talin is present within the costameres, neuromuscular junctions, and myotendinous junctions within mammalian muscle cells (Belkin et al., 1986; Sealock et al., 1986; Tidball et al., 1986). It is widely believed that talin plays a key role in regulating cell-extracellular matrix adhesions by coupling integrin adhesion molecules to the actin cytoskeleton at the cytoplasmic face (Critchley, 2005). Upon calpain digestion, talin can be cleaved into a ~47 KDa globular head domain and a ~190 KDa elongated rod domain (Hayashi et al., 1999). The talin head domain contains multiple basic
amino acids and hence serves as a very good surface for binding of the phospholipids, such as phosphatidylinositol-4, 5-bisphosphate (PIP2) (Rees et al., 1990). The talin head domain also contains a 4.1-ezrin-radixin-moesin homology (FERM) domain that interacts with F-actin (Lee et al., 2004), the β-integrin cytoplasmic domain (Garcia-Alvarez et al., 2003), focal adhesion kinase (FAK) (Borowsky and Hynes, 1998), and PIP kinase type-1γ (Di Paolo et al., 2002; Ling et al., 2002). The elongated tail domain contains another conserved F-actin binding domain (Tremuth et al., 2004) and multiple vinculin binding sites (Gingras et al., 2006). Thus, talin has been proposed to serve as an adaptor protein mediating multiple cytoskeletal protein interactions in focal adhesion assembly (Critchley, 2004; Nayal et al., 2004). The role of talin in focal adhesion dynamics has been demonstrated by talin knockout studies in cultured cells, which showed that down regulation of talin by antisense RNAs within cells led to inhibited focal adhesion assembly and cell spreading (Albiges-Rizo et al., 1995). Talin has also been shown to be indispensable for embryonic development because talin-null mice died at around E.9 and exhibited arrested gastrulation at around E.7 (Monkley et al., 2000). A recent study reported that human synemin co-localized with talin within cultured human hepatic stellate cells, and co-precipitated with talin from the cell extracts (Uyama et al., 2006). The latter suggests that synemin associates with talin within the same immunocomplex within cells. However, direct interaction of mammalian synemin isoforms with talin remains to be explored.

Zyxin

Zyxin is another protein present at cell-substratum attachments, such as the focal adhesions in cultured cells (Crawford and Beckerle, 1991). Western blotting analysis studies indicated that zyxin is highly expressed in heart, lung, and smooth muscle tissues, such as the small intestines (Macalma et al., 1996). The zyxin molecule is composed of an N-terminal proline rich region and a C-terminal region containing three tandem repeats of the LIM domains (Sadler et al., 1992). The LIM domain is a tandem zinc-finger structure present in many cytoskeletal proteins within the cytoplasm and in transcription factors within the nucleus (Kadrmas and Beckerle, 2004). The term of LIM was derived from the first letter of three proteins Lin-11, Isl1, and Mec-3, all of which are homeodomain proteins containing a
similar cysteine-rich sequence motif (the LIM domain) (Michelsen et al., 1993). It is now recognized that the LIM domains mediate important protein-protein interactions, thereby recruiting protein partners to specific subcellular locations and modulating their activities (Kadrmas and Beckerle, 2004). The LIM region of zyxin is necessary and sufficient to direct zyxin to focal adhesion sites (Nix et al., 2001), and it contains binding sites for several proteins such as cysteine-rich protein-1 (Schmeichel and Beckerle, 1994) and p130cas (Yi et al., 2002). Within the N-terminus of zyxin, it has been shown that the proline rich region resembles the proline rich repeats present in the Listeria monocytogenes ActA protein (Golsteyn et al., 1997), which facilitate the actin assembly required for Listeria movement in the hosts. The ActA repeat within zyxin interacts with the Ena/VASP protein family members and is indispensable for appropriate localization of Ena/VASP within focal adhesions (Drees et al., 2000). The N-terminus of zyxin also contains a binding site for the cytoskeletal protein α-actinin (Crawford et al., 1992; Drees et al., 1999; Li and Trueb, 2001). This latter interaction also contributes to directing zyxin to the focal adhesions and actin cytoskeletons within cells (Reinhard et al., 1999).

Interestingly, zyxin knockout mice exhibit normal development and activities after birth, without any defects within several examined tissues (Hoffman et al., 2003). In contrast, fibroblasts isolated from zyxin-null mice showed enhanced integrin-dependent adhesion, elevated cell migration, and mislocalization of the mena/VASP proteins (Hoffman et al., 2006). Applying unidirectional cyclic stretch or shear stress to fibroblast cells resulted in mobilization of zyxin from the focal adhesions to the actin stress fibers (Yoshigi et al., 2005). These latter studies indicate that zyxin is involved in actin remodeling and in regulation of cell motility. An additional interesting property of zyxin is that it is able to shuttle between the nucleus and the focal adhesions (Nix and Beckerle, 1997; Nix et al., 2001), suggesting that zyxin may help in relaying signals between these two sites within cells. Indeed, a recent study demonstrated that zyxin functions within the nucleus and interacts with the homeodomain transcription factor Xanf1/Hesx1, and modulates its activity in the anterior neural plate of the Xenopus laevis embryo (Martynova et al., 2008).

Recent studies have been focused on the possible function of zyxin in regulating the actin cytoskeleton. There have been no reports on the role of zyxin in modulating IFs within...
mammalian cells. Furthermore, reports of direct interaction of IF proteins with protein components of conventional adhesions sites are limited, and thus, whether IFs join in the dynamics of adhesion sites, especially focal adhesions, remains unclear. Studies included in my dissertation provide evidence of direct interactions of synemin with vinculin, talin and zyxin, all of which are proteins present within conventional cell-cell and cell-substratum adherens junctions. Therefore, synemin appears to be a unique IF protein that specifically participates in the dynamics of conventional adhesion sites, and may functionally link the heteropolymeric IFs to these sites. With regard to muscle cells, synemin isoforms likely link the heteropolymeric IFs to adhesion sites such as the costameres, dense bodies/dense plaques, neuromuscular junctions, and myotendinous junctions, thereby helping to provide overall integration of the muscle cell cytoskeleton. Studies in this dissertation also provide evidence that the mammalian synemin isoforms interact differentially with vinculin and talin, indicating the mammalian synemin isoforms may have differential functions within mammalian cells. In addition, identification of interactions of mammalian synemin isoforms with zyxin not only broadens our knowledge of the cellular function of mammalian synemins, but also may open a new field of research on the role of zyxin in modulating IF cytoskeletons.
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HUMAN α-SYNEMIN INTERACTS DIRECTLY WITH VINCULIN AND METAVINCULIN


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SYNOPSIS
Synemin is a very large, unique member of the intermediate filament (IF) protein superfamily. Association of synemin with the major IF proteins, desmin and/or vimentin, within muscle cells forms heteropolymeric IFs. We have previously identified interactions of avian synemin with α-actinin and vinculin. Avian synemin, however, is expressed as only one form, whereas human synemin is expressed as two major splice variants, namely α- and β-synemins. The larger α-synemin contains an additional 312 amino acid insert (SNTIII) located near the end of the long C-terminal tail domain. Whether α- and β-synemins have different cellular functions has been unclear. In this study we show, by in vitro protein-protein interaction assays, that SNTIII interacts directly with both vinculin and metavinculin. Furthermore, SNTIII interacts with vinculin in vivo, and this association is promoted by phosphatidylinositol-4,5-bisphosphate. SNTIII also specifically colocalizes with vinculin within focal adhesions when transiently expressed in mammalian cells. In contrast, other regions of synemin show distinct localization patterns in comparison to those of SNTIII, without labeling focal adhesions. Our results indicate α-synemin, but not β-synemin, interacts with both vinculin and metavinculin, thereby linking the heteropolymeric IFs to adhesion-type junctions, such as the costameres located within human striated muscle cells.

Key words: intermediate filaments, synemin, vinculin, metavinculin, costameres, focal adhesions
INTRODUCTION
Intermediate filaments (IFs) are very long, ~10-nm diameter, filamentous components that function in general as mechanical integrators of cellular space [1]. They help maintain both the morphology and integrity of animal cells [2]. Different types of IFs are composed of cell-type specific intermediate filament (IF) proteins that, in turn, give rise to the distinct properties of the filaments they form [3, 4]. There are more than 65 IF genes encoding distinct IF proteins within the human genome [5]. All of the IF proteins share a common tripartite structural feature, with a central ~310 amino acid conserved rod domain, flanked by hypervariable N-terminal head and C-terminal tail domains [4, 6-8].

Synemin is a very large, unique type VI member of the IF protein superfamily [9]. It is expressed in all three types (skeletal, cardiac, and smooth) of muscle cells [9-11], as well as in some specific non-muscle tissues and cells [10, 12-18]. The human synemin gene encodes two major isoforms, named α-synemin (180 kDa by SDS-PAGE; 172.7 kDa from sequence) and β-synemin (150 kDa by SDS-PAGE; 140.1 kDa from sequence), which are generated by alternative splicing [19]. The larger α-synemin contains an additional, unique 312 amino acid insert located near the end of the long C-terminal tail domain. This insert is absent in and not homologous to any of the sequence within the smaller β-synemin isoform [19]. In contrast to the α- and β- human synemin isoforms, avian synemin is expressed in only one form, which is considered the ortholog of human α-synemin. Interestingly, avian synemin and human α- and β-synemins all fail to assemble into homopolymeric filaments in cells, requiring the presence of the type III IF protein desmin and/or vimentin to form heteropolymeric IFs [19, 20].

Several proteins have been identified that interact with synemin. It was previously shown that the rod domain of both avian [20] and human [21] synemin interacts directly with desmin. The human synemin rod domain also interacts with α-dystrobrevin [21], and dystrophin and utrophin [22]. The avian synemin tail domain was originally shown to interact with vinculin and α-actinin [23]. Also, a fragment within the human α-synemin tail domain, sharing part of the β-synemin tail sequence, was reported to bind to dystrophin and utrophin [22]. Interestingly, the proteins found to interact with the synemin tail domain are all actin-binding proteins known to be present within myofibrils and/or within the costameric
structures located in striated muscle cells. The costameres represent specialized focal adhesion-type structures located periodically along and immediately subjacent to the sarcolemma [24-26]. Those findings, taken in toto, suggest that one of the functions of synemin is to link the heteropolymeric IFs to the sarcolemma.

Because avian synemin shares very low sequence identity (~35%) with the human synemins, and considering that the human synemin gene generates two major isoforms (α and β), resulting in differing lengths of their tail domains, the protein interactions of human and avian synemins may differ. Thus, it is important to characterize interactions of the human synemin isoforms with those proteins previously shown to bind to the avian synemin tail domain. Most importantly, these studies will help ascertain whether the two human synemin isoforms have different functions.

The major purpose of this study was to identify and clarify the interactions of human α- and β-synemins with both vinculin and its muscle-specific isoform, metavinculin. The cytoskeletal protein vinculin is a major component of cell-cell and cell–extracellular matrix adhesion junctions [27-29]. It is composed of a 90 kDa N-terminal head domain (VH), a proline rich linker, and a 27 kDa C-terminal tail domain (VT) [30]. Metavinculin contains an additional 68 amino acid insert within the tail domain [31, 32]. It was originally shown that the VT interacts with avian synemin [23]. Whether there are interactions between the metavinculin tail domain (MVT) and human synemin isoforms has been unknown. Preliminary studies in our lab showed no interactions of human β-synemin with vinculin and metavinculin. We hypothesize that the unique 312 amino acid insert within α-synemin contains the binding site for vinculin and metavinculin, and therefore confers extra functions to α-synemin. We show herein that the specific 312 amino acid sequence present within the tail domain of human α-synemin, which is absent in β-synemin, interacts directly with the tail domains of both human vinculin and metavinculin. Taken in toto, our results indicate that α- and β-synemins have different functions within muscle cells. Based upon its direct interaction with vinculin and metavinculin, human α-synemin appears able to link the heteropolymeric IFs to adhesion-type junctions such as the costameres within striated muscle cells, thereby fulfilling an overall very important cytoskeletal role within the muscle cell cytoskeleton.
MATERIALS and METHODS

Generation of cDNA constructs

The full-length human α- and β-synemin cDNAs, which served as the templates for amplifying all of the human synemin regions by polymerase chain reaction (PCR), were described previously [22]. All primers used for PCR were synthesized at the DNA Sequencing and Synthesis Facility, Iowa State University (Ames, IA). cDNAs encoding human synemin tail (T) fragments (SNTIα, residues 321-579; SNTIβ, residues 580-920; SNTIII, residues 1151-1462; and SNβTII, residues 921-1251) were amplified by PCR using appropriate primer pairs, and subsequently cloned into pFLAG-ATS expression vectors (Sigma, St. Louis, MO) at 5’ Hind III and 3’ Bgl II sites as FLAG-tagged constructs. In order to increase solubility of the SNTless region (residues 1-329), it was cloned into pMAL-C2X maltose binding protein (MBP) expression vector (New England Biolabs, Ipswich, MA) at 5’ EcoR I and 3’ Sal I sites as a MBP-tagged fusion protein (MBP-SNTless).

The SNTIII region was subsequently divided into three consecutive sub-fragments named SNTIIIa (residues 1151-1243), SNTIIIb (residues 1244-1358), and SNTIIIc (residues 1359-1462). The three sub-fragments and the full-length SNTIII were PCR amplified using appropriate primer pairs containing 5’ BamH I and 3’ Hind III sites, and subsequently cloned into pMAL-C2X vectors as MBP-tagged proteins.

SNTIII was also PCR amplified and subcloned into the pEGFPc3, enhanced green fluorescent protein (EGFP) expression vector (BD Biosciences, San Jose, CA), at 5’ Hind III and 3’ BamH I sites. SNTless cDNA was digested out of the MBP-SNTless construct at the sites of 5’ EcoR I and 3’ Sal I and then inserted into the pEGFPc2 vector (BD Biosciences). The full-length human β-synemin tail domain (SNβT, residues 321-1251) was PCR amplified and subsequently cloned into the pEGFPc3 vector at 5’ Hind III and 3’ BamH I sites.

The cDNAs of the human vinculin tail (VT, residues 877-1066) and metavinculin tail (MVT, residues 877-1134) were amplified by reverse transcriptase-PCR from the Human Skeletal Muscle Total RNA Library (BD Biosciences). The amplified VT and MVT cDNAs were then cloned into the pGEX-4T2 glutathione S-transferase (GST) expression vector (GE
Healthcare, Pittsburgh, PA) at 5’ BamH I and 3’ Xho I sites.

Accuracy of all constructs was confirmed by automated sequencing in the DNA Sequencing and Synthesis Facility at Iowa State University.

**Expression and purification of recombinant proteins**

Each expression construct was transformed into *E. coli* BL21-Codon Plus (DE3) bacterial cells (Stratagene, La Jolla, CA). Protein expression was induced by Isopropyl-beta-D-thiogalactopyranoside (IPTG). FLAG-tagged synemin fusion proteins were purified by affinity chromatography using Anti-FLAG M2-Agarose Affinity Gel (Sigma). GST-VT and GST-MVT were batch purified using Glutathione Agarose (Sigma). MBP-tagged synemin fusion proteins were batch purified or purified by affinity chromatography using Amylose Resin (New England Biolabs).

**Antibodies**

Anti-synemin 2856 pAb has been described previously [20]. Anti-FLAG polyclonal antibody (pAb), anti-vinculin monoclonal antibody (mAb) hVIN-1, anti-desmin mAb DEU-10, anti-α-actinin mAb BM7.5, and anti-vimentin mAb V9 were obtained from Sigma. Anti-GST mAb, anti-GFP pAb, and horseradish peroxidase (HRP) conjugated anti-GFP pAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MBP pAb and HRP conjugated anti-MBP mAb were obtained from New England Biolabs. Alexafluor secondary antibodies and Alexafluor-594 phalloidin were obtained from Invitrogen (Carlsbad, CA).

**Western blots and blot overlay assays**

Western blots and blot overlay assays were conducted as previously described [20]. For blot overlay assays, equal picomoles of purified GST-VT, GST-MVT, bovine serum albumin (BSA), and GST were subjected to SDS-PAGE, and then transferred to nitrocellulose membranes, with BSA and GST serving as controls. The resulting blot containing the respective transferred proteins was overlaid with 10 μg/ml of purified synemin fusion protein in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), containing 0.1% (v/v) Tween-20 and 1% (w/v) non-fat milk powder. As a negative control, a
duplicate blot was overlaid with buffer containing no protein. Protein interactions were detected with anti-FLAG pAb or anti-MBP serum by chemiluminescence. Densitometric analysis from three independent experiments of the blot overlay assays was done using Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY). For Western blotting using human tissue protein lysates, human adult skeletal muscle and human uterine smooth muscle total protein lysates were purchased from Biochain (Hayward, CA).

**GST pull-down assays**

Reactions containing 50 µM of each GST fusion protein was incubated with 20 µl bed volume of glutathione agarose beads in binding buffer (50 mM Tris, 150 mM NaCl, 0.5% Triton-X 100, pH 7.4) for 1 hour at 4°C. Purified synemin fusion protein was then added to reach a final concentration of 50 µM and incubated for 12 hours at 4°C. After extensive washing with PBS containing 0.2% Tween-20 (PBST), the beads were eluted with 2x SDS sample buffer. Eluates were subjected to SDS-PAGE and analyzed by Western blotting with anti-FLAG pAb or anti-MBP serum.

**Solid phase binding assays by ELISA**

Solid phase binding assays were performed as previously described [33] with minor modifications. Assays were all performed in duplicate. Briefly, 96-well microtiter plates (Nunc, Rochester, NY) were coated overnight at 4°C with 100 nM purified GST-VT or GST-MVT diluted in PBS. Wells were then blocked with PBS containing 1% (w/v) non-fat milk powder for 4 hours at 37°C. Series of dilutions of MBP-tagged synemin fusion protein or of MBP alone ranging from 800 nM to 0 nM, i.e. buffer only, were then added and incubated for 1 hour at 25°C. After extensive washing with PBS containing 0.1% (v/v) Tween-20, bound MBP fusion proteins were detected with the HRP conjugated anti-MBP mAb and the HRP substrate 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) (Southern Biotechnology, Birmingham, Alabama), by measuring absorbance at 405 nm (A405). Data were then analyzed with Microsoft Excel.

**Cell culture**
A-10 cells, a rat aorta vascular smooth muscle cell line, were purchased from American Type Culture Collection (ATCC, Manassas, VA). NIH/3T3 cells were kind gifts of Dr. Janice E. Buss (Iowa State University). The A-10 cells were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, 4 mM L-glutamine, 100 U/ml penicillin G, and 100 U/ml streptomycin at 37°C (95% air, 5% CO₂). The NIH/3T3 cells were cultured under the same conditions as the A-10 cells, except 10% calf serum was used in place of 10% FBS in the growth media.

**Immunoprecipitation assays**

NIH/3T3 cells were grown to 50% confluence and then transfected with either EGFP-SNTIII plasmids, or the EGFP vectors alone, using JetPEI Transfection Reagent (Polyplus Transfection, San Marcos, CA). Cells were then lysed using the M-PER Mammalian Cell Extraction Buffer (Pierce Biotechnology, Rockford, IL) 24 hours post transfection. The resulting cell lysates were then incubated in the presence or absence of 100 μM phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) micelles at 4°C for 20 min. PtdIns(4,5)P₂ was obtained from Avanti Polar Lipids (Alabaster, AL). The PtdIns(4,5)P₂ micelles were prepared as previously described [34]. The samples were then incubated for 4 hours at 4°C with anti-GFP pAb coupled to Protein A Agarose (Santa Cruz). After extensive washing with PBST, the beads were eluted with 2x SDS sample buffer. The resulting eluates were subjected to SDS-PAGE and analyzed by Western blotting with anti-vinculin hVIN-1 mAb, or HRP conjugated anti-GFP pAb.

**Immunofluorescence, confocal microscopy and transfection studies**

Adult human skeletal muscle tissue sections were purchased from Biochain. The tissue sections were rinsed with PBS, permeabilized by 0.2% Triton-X 100 in PBS, and blocked with 10% BSA in PBS at 25°C. The sections were then incubated with anti-synemin 2856 pAb plus anti-vinculin mAb hVIN-1, anti-synemin 2856 pAb plus anti-α-actinin mAb BM7.5, or with anti-synemin 2856 plus anti-desmin mAb DEU-10, followed by incubation with appropriate Alexafluor secondary antibodies. After extensive washing with PBS, the sections were mounted with coverslips using Vectashield Mounting Medium (Vector Laboratories,
Burlingame, CA). For transfection studies, cells were seeded on collagen-coated coverslips to reach approximately 50% confluence. Cells were then transfected with EGFP-tagged synemin constructs, or with the EGFP vectors alone, using JetPEI Transfection Reagent. Twenty four to thirty six hours post transfection, the cells were fixed, permeabilized, and immunostained with anti-GFP pAb plus anti-vinculin hVIN-1, anti-GFP plus anti-vimentin mAb V9, or with anti-GFP pAb plus Alexafluor-594 phalloidin following procedures described immediately above. Epifluorescence microscopy was conducted using a LEICA DEMIRE2 inverted microscope equipped with a CCD camera in the Hybridoma Facility at Iowa State University. Confocal microscopy was conducted using a LEICA TCS NT Confocal Microscope in the Confocal Microscopy and Image Analysis Facility at Iowa State University.

RESULTS
Mapping the binding sites of vinculin and metavinculin in human synemin

How the large IF protein synemin contributes to the organization as well as functionality of the molecular contractile apparatus within mammalian muscle cells has long been a central interest of our studies. In order to examine the putative interactions of human synemin with vinculin and metavinculin, and to map the binding site(s) of vinculin and metavinculin within human synemin isoforms, polypeptides corresponding to specific regions spanning the entire amino acid sequences of human α- and β-synemins were generated as diagrammed in Fig. 1A. SNTless, SNTIa, SNTIb, and SNβTII are regions shared by both human α-synemin and β-synemin, whereas SNTIII is the additional 312 amino acid insert present only in α-synemin. Regions covering the C-terminal tail (SNTIa, SNTIb, SNTIII, and synβTII) were all cloned as FLAG-tagged fusion proteins. SNTless resulted from deleting the entire long C-terminal tail domain, and was expressed as a MBP-tagged fusion protein to increase its solubility during purification. These synemin fusion proteins were expressed in bacteria, affinity-purified, and analyzed by SDS-PAGE as shown in Fig. 2A. The identities of these bands were confirmed by Western blots with anti-FLAG and anti-MBP polyclonal antibody (pAb) (data not shown). Human VT and MVT were chosen in this study because we had previously determined that only the VT interacts with avian synemin whereas the VH does
not [23], and vinculin is highly conserved among different species, i.e. over 95% identity [35]. Human VT and MVT were expressed as GST-tagged fusion proteins (Fig. 1B).

Interactions of specific protein domains of human synemin with human VT and MVT were analyzed by blot overlay assays. As shown in Fig. 2B, blots containing purified GST-VT, GST-MVT, BSA, and GST were probed with each purified synemin fusion protein. Specific interactions of the synemin fusion proteins with GST-VT and/or GST-MVT were detected with anti-MBP and anti-FLAG pAb. Only SNTIII, which is the 312 amino acid insert present in α-synemin but absent in β-synemin, showed interactions with both VT and MVT. None of the other synemin fusion proteins showed any detectable interaction with VT or MVT (Fig. 2B). These results indicate that α-synemin harbors the binding site(s) for both vinculin and metavinculin.

GST pull-down assays were also conducted to examine possible interactions of synemin fusion proteins with VT or MVT, and to confirm the results of the blot overlay assays. The GST pull-down assays (Fig. 2, C and D) demonstrated that only SNTIII was specifically precipitated by both GST-VT (Fig. 2C) and GST-MVT (Fig. 2D). None of the other synemin fusion proteins were precipitated by either VT or MVT. GST alone did not precipitate any of the FLAG-tagged synemin fusion proteins. Interactions of MBP-SNTless with GST-VT and GST-MVT were also tested by GST pull-down assays. MBP-SNTless was not precipitated by either GST-VT or GST-MVT (data not shown). These results further indicated that SNTIII is the region that interacts directly with vinculin or metavinculin.

**Binding of SNTIII to VT is regulated by the intramolecular head-tail association of vinculin**

Vinculin cycles between “open” and “closed” conformations, which is regulated by the intramolecular association between the head and tail domains [36, 37]. The vinculin head-tail association is known to block most of the ligand binding sites, such as those for talin [38] and α-actinin [39] in the head domain, and for F-actin [40] in the tail domain. To confirm the in vivo interaction of SNTIII with the VT, NIH/3T3 cells were transfected with EGFP-tagged SNTIII plasmids and subjected to immunoprecipitation with anti-GFP pAb. Because PtdIns(4,5)P2 has been reported to induce a conformational change in vinculin that results in
increased binding to its ligands [34, 41, 42], immunoprecipitation assays also were conducted in the presence or absence of exogenous PtdIns(4,5)P2. As shown in Fig. 3, in the presence of PtdIns(4,5)P2, vinculin was co-precipitated with EGFP-SNTIII by the anti-GFP pAb, demonstrating an interaction between vinculin and SNTIII. Without exogenous PtdIns(4,5)P2, vinculin was not detected in the samples of EGFP-SNTIII precipitation, indicating that the association between vinculin and SNTIII is promoted by PtdIns(4,5)P2. These data are consistent with a model in which ligand binding can be regulated by the intramolecular head-tail association of the vinculin molecules.

The C-terminal 104 amino acid sequence of SNTIII contains the primary binding site for vinculin and metavinculin

To further define the region within human α-synemin that mediates interaction with VT and MVT, SNTIII was divided into three consecutive sub-fragments named SNTIIIa, SNTIIIb, and SNTIIIc (Fig. 1A). This division of SNTIII was done according to the regions within its cDNA sequence where optimal primer design was possible. The three sub-fragments were all cloned as MBP-tagged fusion proteins. Full-length SNTIII was also generated as an MBP-tagged fusion protein and was used in parallel as a positive control in subsequent experiments (Fig. 4A). Specific interaction of each sub-fragment with VT and MVT was analyzed by blot overlay assays. As shown in Fig. 4B, MBP-tagged full-length SNTIII demonstrated specific interactions with both VT and MVT as had the FLAG-tagged SNTIII. This result indicates the interactions are highly specific regardless of the nature of the attached recombinant tag. SNTIIIc, which is the C-terminal 104 amino acid sequence of SNTIII, showed similar binding activities as the full-length SNTIII with VT and MVT (Fig. 4, B and C). Relative weak binding of SNTIIIa, the N-terminal 93 amino acid sequence of SNTIII, to VT/MVT was observed (Fig. 4, B and C). No interaction of SNTIIIb, the central 115 amino acid sequence within SNTIII, with VT or MVT was observed in the blot overlay assays. These results indicate that the primary binding site for vinculin and metavinculin in human α-synemin is located in the C-terminal 104 amino acid sequence within SNTIII.

Interactions of VT and MVT with SNTIII/SNTIIIc are saturable and act in a
To determine whether the interactions of SNTIII and SNTIIIc with VT or MVT are saturable and to provide quantitative assessments of the interactions, solid phase binding assays by ELISA were conducted as previously described [33]. Increasing concentrations of MBP-SNTIII or MBP-SNTIIIc were added to 96-well plates coated with equal picomoles of GST-VT or GST-MVT per well. Duplicate plates incubated with increasing concentrations of purified MBP alone served as controls. As shown in Fig. 5, interactions of SNTIII with VT (Fig. 5A, circles) and MVT (Fig. 5B, circles) are both dose dependent and saturable. Determination of the concentrations of MBP-SNTIII at the half-maximal A405 yielded Kd values of 25 ± 4 nM and 53 ± 7 nM for SNTIII binding to VT and MVT, respectively. Similarly, interactions of SNTIIIc with VT (Fig. 5A, triangles) and MVT (Fig. 5B, triangles) were also dose dependent and saturable, yielding Kd values of 55 ± 9 nM and 70 ± 11 nM. No specific interactions were observed between MBP alone and the VT or MVT (Fig. 5, A and B, squares). Experiments using 96-well plates coated with GST alone also did not show any specific interactions of SNTIII or of SNTIIIc with GST itself (data not shown). These results provide further evidence that interactions of SNTIII or SNTIIIc with VT and MVT are highly specific.

We also determined whether VT and MVT compete with each other in binding to SNTIIIc. Purified MBP-SNTIIIc at a previously determined saturation concentration (300 nM, Fig. 5B) along with increasing concentrations of GST-VT were added to 96-well plates coated with GST-MVT. With increase in concentration of VT, the amount of SNTIIIc bound with MVT decreased as reflected by the A405 (Fig. 5C). This result indicates that VT competes with MVT for binding to SNTIIIc.

**Synemin colocalizes with vinculin/metavinculin within costameres of human adult skeletal muscle cells**

To examine the relationship of synemin and vinculin/metavinculin within a cellular context such as mammalian muscle cells, subcellular locations of synemin and vinculin/metavinculin within human adult skeletal muscle cells were analyzed by immunohistochemistry using confocal microscopy. Expression of both α- and β-synemins in human skeletal muscles was
first confirmed by reverse-transcriptase PCR amplifying synemin cDNA fragments from a human skeletal muscle total RNA library. The primer pair of 5'-GAAAAAGAAATTAAATACCCACGAA-3' and 5'-AAACCAATGCCCATCTTC-3' was used in the PCR, which will amplify a ~1 kb cDNA fragment from β-synemin mRNA and a ~2 kb fragment from α-synemin mRNA. The result indicated that both α-synemin and β-synemin mRNAs are present within human skeletal muscle cells (Fig. 6A, left panel). The two amplified fragments were also confirmed as synemin cDNAs by automated sequencing. Anti-synemin 2856 pAb was then used for immunolabeling of synemin within the human skeletal muscle cells. This antibody was previously shown to specifically recognize both human α- and β-synemins expressed within mammalian cells [43] as well as porcine α- and β-synemins [11]. We also analyzed the specificity of this antibody for human synemin by Western blotting. The results showed that the anti-synemin 2856 recognizes two specific bands at about 180 kDa and 150 kDa within both human skeletal muscle and human uterine smooth muscle total protein lysates (Fig. 6A, right panel). These molecular weights correspond in size to human α-synemin (180 kDa) and β-synemin (150 kDa). The relative expression levels of these two synemin isoforms are in agreement with previous studies reporting that the smaller β-synemin is expressed in a relatively higher amount than that of the larger α-synemin isoform in human skeletal muscle tissues, and is expressed in an approximately equal amount with that of α-synemin in human smooth muscle tissues [19, 21, 44].

Double immunofluorescence staining of synemin, and either desmin, α-actinin, or vinculin, within human adult skeletal muscle cells was then performed using anti-synemin 2856, anti-desmin DEU-10, anti α-actinin BM7.5, and anti-vinculin hVIN-1. Anti-vinculin hVIN-1 recognizes a specific epitope within the head domain of vinculin. Thus, this antibody also labels any metavinculin expressed within human skeletal muscles. In longitudinal sections, synemin colocalized with desmin in striated patterns at the myofibrillar Z-lines (Fig. 6, B-D). This result is in agreement with previous studies showing colocalization of synemin and desmin at the Z-lines within mammalian skeletal muscles [11, 19, 21], indicating the formation and presence of synemin/desmin heteropolymeric IFs. In both longitudinal and cross sections the labeling of vinculin was primarily restricted to
along the sarcolemma (Fig. 6, G and K). In cross sections, the labeling of synemin was not only within each muscle fiber, but also significantly colocalized with vinculin along the sarcolemma (Fig. 6, E-H, arrows). In longitudinal sections, striations of synemin labeling ended on the sarcolemma that was labeled with vinculin (Fig. 6, I-L), and colocalized with vinculin in dot-like structures consistent with the costameres present periodically along the sarcolemma (Fig. 6L, small window, arrows). These confocal microscopy results indicate association of synemin with vinculin and metavinculin within mammalian (human) skeletal muscle cells.

We previously reported the interaction of avian synemin with \( \alpha \)-actinin [20, 23]. Thus, localizations of human synemin and \( \alpha \)-actinin within human skeletal muscle tissues were also examined. Synemin co-localized with \( \alpha \)-actinin at the myofibrillar Z-lines as well as along the sarcolemma (Fig. 6, M-P, arrows), indicating co-localization of synemin with \( \alpha \)-actinin within mammalian (human) skeletal muscle cells.

**SNTIII and SNTIIIc colocalize with vinculin at focal adhesion (FA) sites within A-10 cells**

To further clarify whether SNTIII and SNTIIIc interact with vinculin and metavinculin within living cells, the subcellular localization of expressed SNTIII and SNTIIIc was examined by confocal microscopy. A-10 cells, a rat smooth muscle cell line, were transiently transfected with EGFP-SNTIII or EGFP-SNTIIIc plasmids and immunolabeled with anti-vinculin hVIN-1. Cells with moderate expression level of green fluorescent proteins were then examined. Transfection of the EGFP vector alone into A-10 cells served as a reference and showed classical non-specific localization throughout the cytoplasm (Fig. 7B). Localization of vinculin was restricted primarily to both the large focal adhesions (FAs) and the smaller focal complexes (Fig. 7, C, G and K). Both EGFP-SNTIII and EGFP-SNTIIIc clearly colocalized with vinculin within FAs (Fig. 7, H and L), further supporting our findings obtained in the *in vitro* protein-protein binding assays. In addition, the distribution of SNTIII within the FAs was clearly independent of vimentin IFs, as shown by confocal images of EGFP-SNTIII transfected cells immunolabeled with anti-vimentin V9 (Fig. 7, M-P). Previous results indicated that full-length human synemin associates with vimentin IFs
within transfected mammalian cells [19]. Thus, our results herein suggest that interaction of SNTIII with vinculin helps link the synemin/vimentin heteropolymeric IFs directly to the FAs.

**SNβT and SNTless did not localize to FAs**

To ascertain whether only SNTIII and SNTIIIc co-localize with vinculin in FAs, subcellular localizations of expressed protein domains of β-synemin that do not contain the SNTIII region were examined in A-10 cells using confocal microscopy. Human β-synemin was divided into two regions named SNβT and SNTless. SNβT is the full-length C-terminal tail domain of β-synemin, which does not contain the N-terminal head and rod domains. SNTless is the N-terminal head and rod domains of β-synemin. Both of the two regions were cloned into EGFP vectors and subsequently transfected into A-10 cells. Transient expression of EGFP-SNβT within the A-10 cells resulted in a diffuse cytoplasmic distribution without any specific FA-like structures (Fig. 8, A-D). Transfection of EGFP-SNTless into the A-10 cells resulted in numerous perinuclear aggregates within the cytoplasm that did not colocalize with vinculin (Fig. 8, E-G). The latter result agrees with the studies of “tail-less” vimentin [45], desmin [46], and glial fibrillary acidic protein (GFAP) [47], in which all three showed formation of cytoplasmic aggregates within the transfected cells. Clearly, neither SNβT nor SNTless colocalized with vinculin within FA sites (Fig. 8, D and G). The latter set of results demonstrated that only regions from α-synemin (SNTIII and SNTIIIc) specifically co-localized with vinculin in FAs.

**Expression of SNTIII within A-10 cells did not disrupt normal cellular architectures**

To examine whether exogenous expression of SNTIII may cause mislocalization of endogenous proteins, the overall appearances of FAs, actin stress fibers, and IFs within EGFP-SNTIII transfected A-10 cells were analyzed using epifluorescence microscopy. As shown in Fig. 9, the organizations of the FAs (Fig. 9B), actin stress fibers (Fig. 9D), and vimentin IFs (Fig. 9F) within EGFP-SNTIII expressing cells (Fig. 9, arrows) did not display significant changes in comparison with surrounding cells without EGFP-SNTIII expression. These results indicate that expression of SNTIII did not disrupt the normal cellular
architectures within the transfected cells. Therefore, co-localization of SNTIII with vinculin at FA sites as shown in Fig.7 is not a result of non-specific distribution of SNTIII within the cells.

DISCUSSION

In the present study we have defined the interactions of human α- and β-synemins with vinculin and metavinculin. Recombinant protein regions that include the entire amino acid sequence of human synemin were generated to map the vinculin/metavinculin binding site(s). Blot overlay assays showed that SNTIII, the 312 amino acid insert present only in α-synemin, interacts with both VT and MVT (Fig. 2B). These interactions are highly specific as indicated by the absence of interactions of SNTIII with both BSA and GST, which served as negative controls (Fig. 2B and Fig. 4B). Furthermore, both FLAG-tagged and MBP-tagged SNTIII showed specific interactions with VT and MVT (Fig. 2B and Fig. 4B), demonstrating that the interactions are not dependent on the nature of the attached fusion tag. In vitro GST pull-down assays provided additional information regarding the interactions of different regions of human synemin with VT and MVT, also demonstrating specifically that only SNTIII present in α-synemin interacts with both VT and MVT (Fig. 2, C and D).

The primary binding site for VT/MVT within SNTIII was further mapped to its C-terminal 104 amino acid sequence (SNTIIIc) (Fig. 4B). As shown in the solid phase binding assays (Fig. 5, A and B), SNTIIIc binds to VT and MVT with higher dissociation constants than those obtained with SNTIII. This indicates that the full-length SNTIII has higher affinities for VT and MVT than the truncated form (SNTIIIc). Also, SNTIIIa, albeit much weaker than that of SNTIIIc, has affinity to VT and MVT. Thus, the full-length SNTIII may exhibit optimal interactions with VT and MVT in vitro and/or in vivo.

Interactions of VT with SNTIII and with SNTIIIc also showed lower dissociation constants than those of MVT, indicating higher affinity of VT for α-synemin. The latter difference in the binding affinities could result from the presence of the additional 68 amino acid insert within the MVT, which may interfere with its binding to SNTIIIc. VT and MVT also compete with each other for binding to SNTIIIc (Fig. 5C), indicating that they bind to the same site on α-synemin.
We have also examined for the first time the subcellular localization of different regions of human synemin within mammalian muscle cells by confocal microscopy. The subcellular localization pattern of proteins, or protein domains, often reflects their actual cellular functions. We demonstrated by transfection studies that SNTIII and SNTIIIc colocalized with vinculin at FA sites within mammalian smooth muscle cells (Fig. 7, E-L). This provides additional evidence for the specific in vivo interactions of SNTIII and SNTIIIc with vinculin and metavinculin. SNβT and SNTless, which together cover the entire amino acid sequence of β-synemin, showed subcellular localizations distinct from those of SNTIII and SNTIIIc, by not labeling vinculin-containing FA sites (Fig. 8). These results further demonstrate that it is SNTIII, which is absent in β-synemin, that specifically interacts with vinculin and metavinculin, and thereby is targeted to FAs within mammalian muscle cells. The localizations of SNTIII and SNTIIIc within FAs were also independent of the vimentin IFs (Fig. 7, M-P). It has been reported that both full-length human α-synemin or β-synemin colocalized with vimentin when transiently expressed within mammalian cells [19]. These different localization patterns between SNTIII and full-length human synemin indicate that SNTIII is the region within human α-synemin that helps link synemin/vimentin heteropolymeric IFs to the FA sites. Confocal microscopy studies of subcellular localization of synemin and vinculin/metavinculin within human adult skeletal muscle cells also reveal that synemin colocalizes with vinculin within the costameres (Fig. 6L), which are considered specialized focal adhesions located along and subjacent to the sarcolemma [26]. Thus, α-synemin may directly link the synemin/desmin heteropolymeric IFs to the vinculin rich costameric structures within the mammalian (human) skeletal muscle cells.

We have provided evidence that interaction of α-synemin with VT may also be regulated by the molecular conformation of vinculin within a cellular context. The immunoprecipitation studies showed that vinculin was precipitated by SNTIII in the presence of exogenous PtdIns(4,5)P₂ (Fig. 3). This may reflect that the soluble pool of vinculin in the cell lysates was in the “closed” form, which would block the binding site of SNTIII within the vinculin tail domain. It is known that PtdIns(4,5)P₂ interferes with the “closed” form of vinculin [41, 42], which would thereby allow binding of SNTIII to the VT. It was recently shown that vinculin is in its activated (“open”) form when recruited in FAs [48]. Thus,
localization of SNTIII within FAs may result from the direct interaction with the VT of activated vinculin. Recent studies have reported close associations of FAs and IFs within mammalian cells [49-51]. It has also recently been shown that FAs are critical sites for the nascent assembly of vimentin [49] and keratin [50] IFs. However, direct interactions of IFs with specific components of FAs were not identified in those studies. Our results provide the first in vitro/in vivo evidence of direct interaction of IF proteins with components of FAs. Whether interaction of α-synemin with vinculin is able to direct the nascent formation of vimentin IFs at FAs remains to be explored.

Overall, the results of this study show that α-synemin, but not β-synemin, interacts directly with both vinculin and metavinculin. These findings indicate that the human α- and β-synemin isoforms have different cellular functions. The sequence of α-synemin is identical to β-synemin with the exception of the additional 312 amino acid insert in its tail domain. Our results indicate that this additional insert confers extra functions for α-synemin. Interaction of α-synemin with vinculin and metavinculin may directly anchor the heteropolymeric IFs to adhesion-type junctions, such as the costameric regions within striated muscle cells. The latter interactions within costameres would strengthen the linkage of the synemin/desmin heteropolymeric IFs to the cell membrane, and thereby help maintain the structural integrity and functional stability of the muscle cell cytoskeleton during muscle contraction.

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Section 1734 solely to indicate this fact.

FOOTNOTES

Abbreviations used: IF(s), intermediate filament(s); FA(s), focal adhesion(s); VH, vinculin head; VT, vinculin tail; MVT, metavinculin tail; MBP, maltose binding protein; GST, glutathione S-transferase; BSA, bovine serum albumin; PtdIns(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; pAb, polyclonal antibody; mAb, monoclonal antibody; SNT, synemin tail; SNβT, β-synemin tail.

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Figure 1. Schematic diagram of each protein and construct used in the in vitro binding studies. 

A, Schematic diagram of human synemin and the synemin constructs. Human synemin is composed of a short, ten-amino acid head domain (H), a conserved central 310 amino acid rod domain (rod), and a long C-terminal tail domain (Tail). Human α- and β-synemin sequences are identical except that α-synemin contains an additional 312 amino acid insert (SNTIII, ~33kDa) located near the end of the C-terminal tail. Human α- and β-synemins were divided as illustrated. The predicted molecular weight (without tags), and the relative position of each fragment in the amino acid sequence, are shown. Fragments spanning the human β-synemin tail domain (SNTIa, SNTIb, and SNβTII) were cloned into pFLAG-ATS vector as FLAG-tagged fusion proteins. SNTless and the three sub-fragments of SNTIII (SNTIIia, SNTIIib, and SNTIIIc) were cloned into pMALC2X vector as MBP fusion proteins. SNTIII was constructed as both a FLAG-tagged fusion protein and an MBP-tagged fusion protein. 

B, Schematic diagram of vinculin/metavinculin and the tail constructs. Vinculin and metavinculin are composed of a globular head domain, a proline rich linker, and an elongated tail domain. The metavinculin contains additional 68 amino acids (shaded oval) within the tail domain. Vinculin and metavinculin tail domains were cloned as GST fusion proteins.
Figure 2. SNTIII interacts with both vinculin and metavinculin tail domains in vitro. 

A, SDS-PAGE of purified synemin fusion proteins. B, Blot overlays. One hundred picomoles of purified GST-VT, GST-MVT, BSA, and GST alone were electrophoresed and transferred to nitrocellulose membranes. After blocking, membranes were overlaid with or without 10 μg/ml purified synemin fusion proteins in 1% milk-phosphate buffered saline, pH 7.4. Bound synemin fusion proteins were detected with anti-FLAG or anti-MBP pAb by chemiluminescence. Only SNTIII binds to both VT and MVT. C, GST-VT or GST alone was immobilized on glutathione agarose and incubated with each purified FLAG-tagged synemin tail fragment as described in the “Experimental Procedures” section. The resulting eluates were each divided into equal volumes and subjected to Western blot analysis with anti-FLAG pAb and anti-GST mAb, respectively. D. The same experimental design was followed as described in C, with the substitution of GST-MVT for GST-VT. SNTIII is specifically precipitated by GST-VT and GST-MVT.
Figure 3. Immunoprecipitation of vinculin with over-expressed SNTIII in NIH/3T3 cells. NIH/3T3 cells were transfected with EGFP-SNTIII plasmids or EGFP vector alone. Twenty four hours post transfection, the cells were lysed, and the resulting cell lysates were incubated with anti-GFP pAb immobilized on protein A agarose, in the presence or absence of 100 μM PtdIns(4,5)P$_2$. Each resulting immunoprecipitate was divided into equal volumes and subjected to Western blot analysis with anti-vinculin mAb hVIN-1 or HRP-conjugated anti-GFP pAb, respectively. Mock, which served as a negative control, denotes the incubation of mock transfected cell lysates with anti-GFP immobilized on protein A agarose. Lys denotes the load of non-transfected NIH/3T3 cell lysates. Vinculin was immunoprecipitated by anti-GFP pAb along with the EGFP-SNTIII in the presence of 100 μM PtdIns(4,5)P$_2$. 
Figure 4. The C-terminal 104 amino acid sequence of SNTIII is the primary binding site for both vinculin and metavinculin.  A, SDS-PAGE of purified MBP-SNTIII and the 3 MBP-tagged sub-fragments. B, Blot overlays. As in Fig. 2B, one hundred picomoles of purified GST-VT, GST-MVT, BSA, and GST alone were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then overlaid with or without 10 μg/ml purified MBP-tagged SNTIII sub-fragments. Bound proteins were detected with anti-MBP pAb. Overlay of MBP-tagged full-length SNTIII served as a positive control. C, Densitometry analysis of three independent replicates of blot overlay assays. Signal intensity of background on each blot was set as zero. SNTIIIc showed similar affinity with VT and MVT as the full-length SNTIII did.
Figure 5. Interactions of VT and MVT with SNTIII or with SNTIIIc are both saturable and act in a competitive manner. A and B, 96-well plates were coated with 100 nM purified GST-VT (A) or GST-MVT (B) and incubated with increasing concentrations of MBP-SNTIII (circles), MBP-SNTIIIc (triangles), or MBP alone (squares) for 1 hour at 25°C. After extensive washing, bound MBP fusion proteins were detected with HRP-conjugated anti-MBP mAb and the HRP substrate ABTS by measurement of A$_{405}$. Background A$_{405}$ values of wells incubated with buffer only were subtracted. The resulting A$_{405}$ values were plotted versus the concentration of incubated MBP fusion proteins. The values represent the mean ± s.d. from three independent experiments (the data were fitted to a moving average trendline with a period of 2). Interactions of VT and MVT with SNTIII, or with SNTIIIc, were both saturable. C, increasing concentrations of GST-VT, along with 300 nM purified MBP-SNTIIIc, were added to a 96-well plate coated with GST-MVT for 1 hour at 25°C. Bound SNTIIIc was then determined as described in A and B. Values represent the mean ± s.d. from three independent experiments. The values were plotted versus the concentration of GST-VT, and fitted to a linear regression trendline. The VT competes with the MVT for the interaction with SNTIIIc.
Figure 6. Confocal microscopy studies indicate synemin colocalizes with vinculin within human adult skeletal muscle cells. A. Left panel, reverse-transcriptase PCR amplifying a ~1 kb cDNA fragment from β-synemin mRNA and a ~2 kb cDNA fragment from α-synemin mRNA that present in a human skeletal muscle total RNA library. Right panel, Western blotting of human adult skeletal muscle (SK) and uterine smooth muscle (UT) total protein lysates with anti-synemin 2856 pAb. Fifty μg of each total protein lysate was separated by SDS-PAGE and subjected to Western blotting using anti-synemin 2856. B-D, Confocal microscopy analysis of the localization of synemin and desmin within longitudinal sections of human skeletal muscle. Synemin was labeled green with anti-synemin 2856 pAb and secondary Alexa Fluoro 488 goat anti-rabbit IgG. Desmin was labeled red with anti-desmin DEU-10 mAb, followed by staining with secondary Alexa Fluoro 594 goat anti-mouse IgG. Synemin colocalized with desmin at the myofibrillar Z-lines (D). E-L, Confocal microscopy analysis of synemin and vinculin within human skeletal muscle. Synemin was labeled green with anti-synemin 2856. Vinculin was labeled red with anti-vinculin hVIN-1 mAb. F, G, and H represent the boxed area in E. J, K, and L represent the boxed area in I. In cross sections (E-H), synemin colocalizes with vinculin along the sarcolemma (H, arrows). In longitudinal sections (I-L), synemin colocalized with vinculin within the costameres (L, small window, arrows). M-P, Confocal microscopy analysis of synemin and α-actinin within human skeletal muscle. Synemin was labeled green with anti-synemin 2856. α-Actinin was labeled red with anti-α-actinin mAb BM7.5. N, O, and P represent the boxed area in M. Synemin colocalized with α-actinin at the myofibrillar Z-lines as well as along the sarcolemma (P, arrows). (Bars, 25 μm)
Figure 7. SNTIII and SNTIIIc specifically localize within FAs in A-10 cells. A-L, A-10 cells were transfected with EGFP, EGFP-SNTIII, or EGFP-SNTIIIc plasmids. Vinculin was then labeled red 24 hours post transfection with anti-vinculin hVIN-1 plus secondary Alexa fluoro 594 goat anti-mouse IgG. The last three pictures in each row represent enlarged views of the boxed area in the first picture. SNTIII and SNTIIIc both co-localized with vinculin within FAs (H and L). M-P, A-10 cells were transfected with EGFP-SNTIII plasmids. Cells were then immunostained 24 hours post transfection with anti-vimentin V9 and secondary Alexa fluoro 594 goat anti-mouse IgG. N-P represents the boxed area in M. SNTIII localized within FA like structures at the cell periphery where vimentin was absent. (Bars, 25 μm)
Figure 8. SNβT and SNTless, which together cover the full-length amino acid sequence of β-synemin, do not colocalize with vinculin. A-D, Confocal microscopy of subcellular localization of SNβT within A-10 cells. A-10 cells were transfected with EGFP-SNβT plasmids and then immunostained with anti-vinculin hVIN-1. B-D represents enlarged views of the boxed area in A. E-G, Subcellular localization of EGFP-SNTless within A-10 cells. A-10 cells were transfected with EGFP-SNTless plasmids. Vinculin was then labeled red with anti-vinculin hVIN-1. Neither SNβT nor SNTless colocalized with vinculin within FAs. (Bars, 25 μm)
Figure 9. Expression of SNTIII did not result in significant changes in the cellular organization of FAs, actin stress fibers, and IFs. A-10 cells were transfected with EGFP-SNTIII plasmids followed by immunostaining of vinculin, F-actin, and vimentin to label the FAs (B), actin stress fibers (D), and IFs (F), respectively. B, D, and F represent the same microscope observation field as A, C, and E, respectively. Arrows indicate the identical cell(s) in the same microscope observation field. In comparison to the surrounding cells without EGFP-SNTIII expression, cells that express EGFP-SNTIII did not display any significant difference in the appearances of the FAs, actin stress fibers, and IFs. (Bars, 15 μm)
IDENTIFICATION OF A REPEATED DOMAIN WITHIN MAMMALIAN α-SYNEMIN THAT INTERACTS DIRECTLY WITH TALIN


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Footnotes
Abbreviations used: IF(s), intermediate filament(s); FA(s), focal adhesion(s); VT, vinculin tail; MBP, maltose binding protein; GST, glutathione S-transferase; BSA, bovine serum albumin; pAb, polyclonal antibody; mAb, monoclonal antibody; SNT, synemin tail; SNβT, β-synemin tail.

Abstract
The type VI intermediate filament (IF) protein synemin is a unique member of the IF protein superfamily. Synemin associates with the major type III IF protein desmin forming heteropolymeric intermediate filaments (IFs) within developed mammalian striated muscle cells. These IFs encircle and link all adjacent myofibrils together at their Z-lines, as well as linking the Z-lines of the peripheral layer of cellular myofibrils to the costameres located periodically along and subjacent to the sarcolemma. Costameres are multi-protein assemblies enriched in the cytoskeletal proteins vinculin, α-actinin, and talin. We report herein a direct interaction of human α-synemin with the cytoskeletal protein talin by protein-protein interaction assays. The 312 amino acid insert (SNTIII) present only within α-synemin binds to the rod domain of talin in vitro and co-localizes with talin at focal adhesion sites within mammalian muscle cells. Confocal microscopy studies showed that synemin co-localizes with talin within the costameres of human skeletal muscle cells. Analysis of the primary sequences of human α- and β-synemins revealed that SNTIII is composed of seven tandem repeats, each containing a specific Ser/Thr-X-Arg-His/Gln (S/T-X-R-H/Q) motif. Our results suggest human α-synemin plays an essential role in linking the heteropolymeric IFs to adherens-type junctions, such as the costameres within mammalian striated muscle cells, via
its interaction with talin, thereby helping provide mechanical integration for the muscle cell cytoskeleton.

**KEYWORDS**, intermediate filament, synemin, talin, cytoskeleton, costameres, muscle cell

**Introduction**
The cytoskeleton of mammalian cells is comprised primarily of three types of filamentous networks, namely microtubules, microfilaments, and intermediate filaments (IFs) [1, 2]. The long ~10 nm diameter IFs are believed to play essential roles in maintaining the morphological integrity and functional stability of living cells [3-5]. The IFs are heterogeneous in nature, and composed of cell-type specific IF proteins. Approximately 65 IF proteins have been identified in humans [6]. These have been classified into ~six major types based upon the degree of nucleotide sequence homology and the genomic structures of the IF genes [7].

Synemin is a very large, unique IF protein that is present primarily in the three types (skeletal, cardiac, and smooth) of muscle tissues [8]. The single human synemin gene encodes two large splice variants, α-synemin and β-synemin, with the larger α-synemin containing an additional 312 amino acid insert near the end of the long C-terminal tail domain [9]. Assembly of either α-synemin or β-synemin into IFs requires the presence of the major type III IF proteins desmin and/or vimentin, which then form heteropolymeric IFs within mammalian cells [8-10]. Synemin has previously been shown to interact with non-IF proteins including α-actinin, vinculin, α-dystrobrevin, and dystrophin, which are cytoskeletal proteins present in adherens-type junctions including costameres, neuromuscular junctions, and myotendinous junctions within striated muscle cells [11-14]. Thus, synemin appears able to link the heteropolymeric IFs to the adherens-type junctions within striated muscle cells.

Like synemin, the cytoskeletal protein talin is also localized in costameres, neuromuscular junctions, and myotendinous junctions within mammalian muscle cells [15-17]. Talin harbors multiple binding sites for different cytoskeletal proteins and signaling molecules, and appears to act as an adaptor protein within cell-extracellular matrix contacts [18, 19]. Talin also plays essential roles in relaying “outside-in” and “inside-out” signals across the plasma membrane,
and is critical for integrin activation [20, 21].

In the present study we provide evidence that a specific region located within the long rod domain of talin interacts with the 312 amino acid insert (SNTIII) present only within human α-synemin. Furthermore, analysis of the primary sequence of SNTIII reveals that it is composed of seven ~39 amino acid long tandem repeats containing specific Ser/Thr-X-Arg-His/Gln (S/T-X-R-H/Q) motifs, where X represents any of the five amino acid residues V, G, I, F, L. Results of our studies support a model in which α-synemin interacts directly with talin, and thereby helps to link the synemin/desmin heteropolymeric IFs to adherens-type junctions within developed mammalian (human) muscle cells.

Materials and methods

Generation of cDNA constructs

The human β-synemin cDNA fragment encoding amino acid residues 322-1251 (SNβT) and the human α-synemin cDNA fragment encoding amino acid residues 1153-1464 (SNTIII) were PCR amplified and subcloned into pFLAG-ATS expression vectors (Sigma, St. Louis, MO) at 5’ Hind III and 3’ Bgl II sites. Synemin cDNA fragment encoding amino acid residues 1-329 (SNTless) was PCR amplified and subcloned into pMAL-C2X maltose binding protein (MBP) expression vector (New England Biolabs, Ipswich, MA) at 5’ EcoR I and 3’ Sal I sites. SNTIII was also cloned into pMAL-C2X vector and into enhanced green fluorescent protein (EGFP) expression vector (Clontech, Mountain View, CA) at 5’ Hind III and 3’ BamH I sites. Human talin1 cDNAs encoding the head domain (amino acid residues 1-433, Head), and regions of the rod domain (amino acid residues 423-1326, R1; 1327-1948, R2; and 1942-2541, R3) were amplified by reverse transcriptase-PCR from the Human Skeletal Muscle Total RNA Library (BD Biosciences, San Jose, CA) and subcloned into pGEX-4T2 glutathione S-transferase (GST) expression vector (GE Healthcare, Pittsburgh, PA) at 5’ Sal I and 3’ Not I sites. The cDNA of the R2 region (amino acid residues 1327-1948) of human talin1 was also cloned into DsRed red fluorescent protein expression vector (Clontech) at 5’ Sal I and 3’ BamH I sites. Accuracy of each construct was confirmed by automated sequencing in the DNA Sequencing and Synthesis Facility at Iowa State University (Ames, IA).
**Expression and purification of recombinant proteins**

Recombinant fusion proteins were expressed in *E. coli* BL21-Codon Plus (DE3) bacterial cells (Stratagene, La Jolla, CA). FLAG-tagged synemin fusion proteins were affinity purified using Anti-FLAG M2-Agarose Affinity Gel (Sigma). GST-tagged talin1 fusion proteins were batch purified using Glutathione Agarose (Sigma). MBP-tagged synemin fusion proteins were batch purified using Amylose Resin (New England Biolabs). The concentration of each purified fusion protein was determined using Bio-Rad protein assays.

**Cell culture**

A-10 cells, a rat vascular smooth muscle cell line, were purchased from the American Type Culture Collection (Manassas, VA). Human uterine smooth muscle cells (UTSMC) were obtained from Lonza (Allendale, NJ). A-10 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37° C (95% air, 5% CO₂). Human UTSMC cells were maintained with Smooth Muscle Medium-2 Bulletkit (Lonza) at 37° C (95% air, 5% CO₂).

**Co-immunoprecipitation**

Co-immunoprecipitations were performed using the ProFound Mammalian Co-Immunoprecipitation Kit (Pierce, Rockford, IL) following the manufacturer’s instructions. Briefly, UTSMC cells were lysed with MPER Mammalian Protein Extraction Reagent (Pierce) and incubated with anti-talin 8d4 monoclonal antibody (mAb) covalently coupled to the Aminolink Plus Coupling Resin (Pierce). The Control Resin (Pierce) incubated with the same volume of UTSMC lysates was used in parallel as a negative control. The resins were then washed extensively with PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.1% Tween-20, pH 7.4) and then eluted with the ImmunoPure IgG Elution Buffer (Pierce). The eluates were then subjected to SDS-PAGE and analyzed by Western blotting using anti-synemin 2856 polyclonal antibody (pAb) [22] and anti-talin 8d4 mAb (Sigma).

**Blot overlay and dot blot overlay assays**

Blot overlay assays were conducted as previously described [22]. For the dot blot overlay assays, an equal molar amount of GST-talin R2, GST-VT, and GST alone were spotted onto a nitrocellulose membrane, with GST-VT and GST alone serving as a positive and a negative
control, respectively. The resulting membrane was blocked with PBST containing 5% (w/v) non-fat milk powder, and then overlaid with 10 μg/ml purified MBP-SNTIII or MBP alone in PBST containing 1% (w/v) milk. Bound proteins were detected with anti-MBP serum (New England Biolabs) by chemiluminescence.

**GST pull-down assays**

Two μg of GST-talin1-R2, or GST alone, were immobilized on glutathione agarose and then incubated with the same amount of purified FLAG-SNTIII in PBS for 12 h at 4°C. The beads were then precipitated, washed extensively with PBS containing 0.5% Tween-20, and eluted with 2X SDS sample buffer. The resulting eluates were subjected to SDS-PAGE and analyzed by Western blotting with anti-FLAG pAb (Sigma) and anti-GST mAb (Santa Cruz Biotechnology, Santa Cruz, CA).

**Solid phase binding assays by ELISA**

Duplicate 96-well microtiter plates (Nunc, Rochester, NY) were coated with either 100 nM purified GST-talin R2 or GST-VT for 16 h at 4°C. The wells were then blocked with PBS containing 1% (w/v) milk for 3 h at 37°C, and incubated with increasing concentrations of purified FLAG-tagged SNTIII fusion protein for 1 h at 25°C. Wells were extensively washed with PBST and then incubated with HRP-conjugated anti-FLAG M2 mAb (Sigma) for an additional 1 h at 25°C. After extensive washing with PBST, protein interactions were detected with the HRP substrate 2,2’-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) (Southern Biotechnology, Birmingham, Alabama) by measuring absorbance at 405 nm (A₄₀₅). Data were then plotted using Microsoft Excel.

**Transfection studies, immunofluorescence, and confocal microscopy**

For transfection studies, A-10 cells were seeded on rat tail collagen I (BD Biosciences)-coated glass coverslips to reach 50% confluence. Cells were then transfected with EGFP-SNTIII or co-transfected with EFGP-SNTIII and DsRed-talin R2 plasmids using JetPEI Transfection Reagent (Polyplus Transfection, San Marcos, CA), followed by incubation at 37°C for 24 h. For immunofluorescence, the transfected cells were fixed with 2% formaldehyde in PBS, permeabilized with 0.2% Triton-X 100 in PBS, and blocked with 5% bovine serum albumin (BSA) in PBS. The cells were then incubated with appropriate primary antibodies and Alexafluor secondary antibodies (Invitrogen, Carlsbad, CA). After
extensive washing with PBS, the slides were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Immunofluorescence using human adult skeletal muscle tissue sections (Biochain, Hayward, CA) was conducted following the procedures described for the immunofluorescence of the transfected cells without the fixation step. Confocal microscopy was conducted using a LEICA TCS NT Confocal Microscopy System in the Confocal Microscopy Facility at Iowa State University.

**Secondary protein structure prediction and repeat sequence analysis**

For prediction of secondary protein structure and analysis of repeat sequences, the reference sequences of human $\alpha$-synemin (NCBI accession no. **NP_663780**) and $\beta$-synemin (NCBI accession no. **NP_056101**) from the protein database of the National Center for Biotechnology Information (NCBI) were used. To predict the secondary structure of SNTIII, the 312 amino acid sequence corresponding to the unique $\alpha$-synemin insert, was submitted to the PredictProtein Server (http://www.predictprotein.org) [23]. For detection of repeat sequences, the primary sequence of human $\alpha$-synemin was submitted to the Rapid Automatic Detection and Alignment of Repeats (RADAR) [24] at the European Bioinformatics Institute (EBI). Similarly, mouse H-synemin (NCBI accession no. **NP_964001**) and chicken synemin (NCBI accession no. **NP_990140**) sequences were also submitted to RADAR for repeat sequence analysis. Data of the RADAR alignments and the secondary structure predictions were submitted to ESPript 2.2 [25, 26] to generate postscript image files.

**Results**

**SNTIII interacts directly with the talin rod domain**

To examine the putative interaction of synemin with talin in cells, co-immunoprecipitation experiments using cultured human uterine smooth muscle cell (UTSMC) lysates were performed. The UTSMC cells were selected in the present study because it was previously shown that smooth muscle tissues express a relatively high level and approximately equal amounts of both $\alpha$- and $\beta$-synemins [9, 10]. Using the anti-talin mAb (clone 8d4) covalently coupled to the Aminolink resin, both human $\alpha$- and $\beta$-synemins immunoprecipitated along with talin from the UTSMC cell lysates (Fig. 2A), indicating that synemin and talin are closely associated within the same immunocomplex.
To examine direct interaction between human synemin and talin, as well as to map the respective binding site(s) within the two molecules, interactions between regions of human synemin isoforms and regions of human talin were investigated. Human $\alpha$- and $\beta$-synemins were divided into specific regions (shown in Fig. 1A), which include the full length primary sequences of the two molecules. SNTless and SN$\beta$T are regions shared by both $\alpha$- and $\beta$-synemins. SNTIII represents the additional 312 amino acid insert that is present only in the larger $\alpha$-synemin. Each region was expressed as a FLAG-tagged recombinant protein except SNTless that was expressed as an MBP-tagged fusion protein to increase its solubility during purification. Four overlapping regions, which span the full length amino acid sequence of talin, were generated and expressed as GST-tagged fusion proteins as diagrammed in Fig. 1B.

Blot overlay assays were conducted to examine the specific interactions between the synemin and talin regions. Blots containing GST-tagged talin fusion proteins, as well as GST-tagged vinculin tail (VT) and GST alone (Fig. 2B, top panel), were overlaid with each of the purified synemin recombinant regions, with GST-VT and GST alone serving as positive and negative controls, respectively. The bound synemin fusion proteins were then detected with anti-FLAG pAb or anti-MBP serum. As shown in Fig. 2B, SNTIII interacted specifically with the talin R2, as well as with the positive control VT [27], whereas SNTless and SN$\beta$T did not bind. These results indicate that the amino acid sequence 1327-1948 (R2) within the talin rod domain interacts with SNTIII.

Interaction of talin R2 with SNTIII was further confirmed by GST pull-down assays (Fig. 2C) and by dot blot overlay assays (Fig. 2D). In the GST pull-down assays, purified FLAG-tagged SNTIII was precipitated by GST-talin R2 immobilized on glutathione agarose beads. The negative controls of GST alone and of beads alone did not result in precipitation of any FLAG-SNTIII (Fig. 2B). In dot blot overlay assays, purified GST-talin R2, along with the positive control GST-VT and the negative control GST alone, were spotted on a nitrocellulose membrane. The resulting membrane was then overlaid with purified MBP-tagged SNTIII or with MBP alone. As shown in Fig. 2D, SNTIII bound to talin R2 and the positive control VT, but not to the negative control of GST alone. In toto, these protein-protein binding assays demonstrated that SNTIII, the 312 amino acid insert within $\alpha$-synemin, interacts specifically with talin R2. In addition, the specific interaction of SNTIII
with talin R2 is independent of the nature of the two attached fusion tags.

**Talin R2 competes with VT in binding to SNTIII**

To provide quantitative assessments of the interaction of SNTIII with talin R2, solid phase binding assays by ELISA were conducted. Equal molar concentrations of purified GST-talin R2, the positive control of GST-VT, and the negative control of GST alone were absorbed onto 96-well plates, and these were then incubated with increasing concentrations of purified FLAG-tagged SNTIII. Protein interactions were then measured as described in the “Material and Methods” section. The interaction of talin R2 with SNTIII was dose-dependent and saturable as was that of VT with SNTIII, which served as the positive control (Fig. 3A). The negative control of GST alone did not show any significant interaction with SNTIII. Determination of the concentration of FLAG-SNTIII at half-maximal A405 yielded a K_d value of 52 ± 11 nM for the talin R2 and SNTIII interaction. The K_d value for the VT and SNTIII interaction was 30 ± 8 nM, which is close to the value obtained in our previous study (25 ± 4 nM) [27]. Our present results indicate that talin R2 binds to, and has similar affinity for, SNTIII *in vitro* as does the VT.

To determine whether talin R2 and VT can bind simultaneously to SNTIII, competitive binding assays by ELISA were conducted. Increasing concentrations of GST-VT, along with 400 nM purified FLAG-SNTIII (a saturated concentration obtained from Fig. 3A), were added to 96-well plates coated with purified GST-talin R2. As shown in Fig. 3B, VT competes with talin-R2 in binding to SNTIII. This result indicates that interaction of talin R2 and VT with SNTIII are mutually exclusive.

**Synemin co-localizes with talin within the costameres in mammalian skeletal muscle cells**

To examine the relationship of synemin and talin within a cellular context, localization of synemin and of talin within human adult skeletal muscle tissues were analyzed by immunofluorescence and confocal microscopy. The anti-synemin 2856 pAb used in the study was previously verified as specifically recognizing both human and porcine α- and β-synemin [28, 29]. Double immunofluorescence labeling of synemin and desmin showed
that synemin colocalized with desmin at the myofibrillar Z-lines (Fig. 4, A-C). This result agrees with previous reports showing colocalization of synemin and desmin within mammalian skeletal muscle cells [9, 10, 13, 28, 29]. In cross sections, double immunofluorescence labeling of synemin and talin showed that synemin colocalized with talin along the sarcolemma (Fig. 4, D-F). In longitudinal sections, the striated synemin labeling ended at, and co-localized with, talin labeling within dot-like structures (Fig. 4, G-I, arrows). The latter structures are consistent with the location of the costameres present periodically along and immediately subjacent to the sarcolemma. These results of confocal microscopy indicate an intracellular association of synemin and talin within human striated muscle cells.

**SNTIII co-localizes with talin as well as talin R2 within transfected cells**

The ability of SNTIII and SNβT to localize to specific cellular structures was examined by transfecting A-10 rat smooth muscle cells with the appropriate EGFP-tagged expression constructs. Cells with moderate levels of GFP expression were examined. EGFP alone showed a classical non-specific distribution across the cytoplasm (data not shown). EGFP-tagged SNTIII displayed specific co-localization with talin within the focal adhesion (FA) sites (Fig. 5, A-D), while EGFP-tagged SNβT did not (Fig. 5, E-H). This result is consistent with our results obtained from the in vitro protein-protein interaction assays, which showed that it was SNTIII, but not SNβT, that interacted specifically with the talin rod domain.

To examine whether SNTIII and talin R2 associate within living cells, EGFP-SNTIII and DsRed-talin R2 plasmids were co-transfected into A-10 cells. DsRed-tagged talin R2 exhibited a typical subcellular localization within the FAs, which is in agreement with results from previous studies [30, 31]. EGFP-SNTIII co-localized with DsRed-talin R2 at FA sites within the transfected A-10 cells (Fig. 5, I-L). The results of these cellular transfection studies demonstrated a close intracellular association of SNTIII with talin R2 within living cells.

**Primary sequence analysis reveals that SNTIII is composed of seven tandem repeats.**
That SNTIII interacts specifically with both the vinculin tail domain and talin rod domain could be reflected by its unique primary amino acid sequence, as well as its specific secondary structure. We therefore analyzed the amino acid sequence and predicted the secondary structure of SNTIII by using selected bioinformatics tools. In the search against the NCBI protein databases using BLASTp, the primary sequence of SNTIII (amino acid residues 1153-1464) was not homologous to any of the individual protein sequences in the current protein database. Also, SNTIII does not contain any defined, conserved domains/motifs as reflected in the search against the current NCBI Conserved Domain Database (CDD). However, by using the Rapid Automatic Detection and Alignment of Repeats (RADAR) [24] at the European Bioinformatics Institute (EBI), a specific region (amino acid residues 1107-1464), which was composed of eight tandem repeated sequences, was identified within the human α-synemin tail domain, but absent within the β-synemin tail domain (Fig. 6A). Interestingly, the amino acid sequence from the start of the second repeat to the end of the last repeat is the primary sequence of SNTIII, i.e., the 312 amino acid insert present only in the human α-synemin isoform. Each repeat within SNTIII contains a unique sequence motif of Ser/Thr-X-Arg-His/Gln (S/T-X-R-H/Q, where X represents any of the five amino acid residues V, G, I, F, L) (Fig. 6A). Furthermore, similar tandem repeated sequences are also present in synemins from different species, such as mouse H-synemin and chicken synemin (both considered orthologs of human α-synemin) as shown in the RADAR output results in Fig. 6, B and C, respectively. Surprisingly, the tandem repeat sequences of the chicken synemin ortholog is more similar to the human tandem repeats than is that of the mouse H-synemin. However, in terms of the entire protein sequences, the overall homology of the mouse H-synemin compared to the human α-synemin (67% identity, 78% homology) is higher than that of the chicken synemin to human α-synemin (35% identity, 53% homology). Multiple sequence alignment of the primary sequences of human α-synemin, mouse H-synemin, and chicken synemin using CLUSTAL W [32] and MULTALIN [33] also revealed that most of the S/T-X-R-H/Q motifs are conserved among the different species (Fig. 6D). Secondary protein structure prediction for SNTIII was also performed using the PROFPHD at the PredictProtein Server [23, 34]. The results showed that the secondary structure of SNTIII contains 0% α-helix, 45% β-strands, and 55% random loops.
Interestingly, the S/T-X-R-H/Q motifs are all present within the predicted β-strands (Fig. 6A).

**Discussion**

We have identified and characterized the direct interaction of human synemin with the cytoskeletal protein talin. In the co-immunoprecipitation experiment (Fig. 2A), both human α- and β-synemin isoforms were precipitated along with the talin from the human uterine smooth muscle cell extracts, indicating close association of the human synemins with talin within living muscle cells. This result is in agreement with a recent study, which reported co-immunoprecipitation of talin with synemin from human hepatic stellate cell extracts [35]. Subsequent protein-protein interaction assays using recombinant protein regions of both talin and human synemin isoforms identified that a specific region within the talin rod domain (R2) interacts directly with SNTIII, the 312 amino acid insert present only within the human α-synemin isoform (Fig. 2, B-D). No interaction of human β-synemin regions with talin regions were observed, indicating that α-synemin is the only isoform that interacts with talin. Precipitation of β-synemin in the Co-IP experiment (Fig. 2A) may result from the direct association of β-synemin with α-synemin, or from the indirect association with other IF proteins (e.g., vimentin) that associate with α-synemin via their conserved rod domains. It is generally known that the rod domain of IF proteins mediate α-helical coiled-coil formation and IF assembly [36, 37].

We previously reported that SNTIII also interacts with vinculin [27], another important cytoskeletal protein, and is present in both cell-cell and cell-extracellular matrix adherens-type junctions [38-40]. Talin is found primarily within cell-extracellular matrix adherens-type junctions [20, 41]. In the present study the talin R2 region exhibited a similar affinity for SNTIII as did the vinculin tail (VT) in the solid phase ELISA binding assays (Fig. 3A). Also, talin R2 competed with VT in binding to SNTIII (Fig. 3B). These results suggest that human α-synemin does not bind simultaneously to both vinculin and talin. Interestingly, talin also contains multiple vinculin binding sites within its extended rod domain, several of which are located within the talin R2 region [42]. Thus, it is possible that multiple synemin
molecules bind with multiple talin and vinculin molecules, thereby forming protein complexes that provide structural support for living muscle cells (Fig. 7).

Results of confocal microscopy studies provided additional evidence regarding the interaction of synemin and talin within human skeletal muscle cells. Synemin and talin co-localized within the costameric structures located periodically along and subjacent to the sarcolemma (Fig. 4). In addition, EGFP-tagged SNTIII co-localized with DsRed-tagged talin R2 at the focal adhesion sites within transfected A-10 cells. These results in toto indicate an intracellular association between human synemin and talin.

We have identified for the first time that there is a region within the tail domain of human \( \alpha \)-synemin containing eight tandem repeated sequences. Seven of these tandem repeats comprise the entire region of SNTIII. Furthermore, each of these seven repeats contains a specific S/T-X-R-H/Q motif, in which the arginine residues are highly conserved. Additionally, similar regions containing tandem repeats and motifs similar to the S/T-X-R-H/Q motifs are also present in synemins of differing species, suggesting these repeats have been conserved in evolution. A recent study of molecular evolution of type VI IF proteins showed that nestin, tanabin, and transitin all contain a repeated domain within their C-terminal tail [43]. Thus, our findings also provide additional support for classification of synemin as a type VI IF protein.

Overall, the results of this study show that the 312 amino acid insert (SNTIII), which is present only in human \( \alpha \)-synemin, interacts specifically with a region (R2) within the long rod domain of the cytoskeletal protein talin. That SNTIII interacts with both vinculin and talin may be reflected by its unique primary sequence, which is composed of seven tandem repeats containing unique S/T-X-R-H/Q motifs. Our results indicate that the additional 312 amino acid insert confers extra functions for the human \( \alpha \)-synemin isoform. Both vinculin and talin are actin-binding proteins [19, 40] that are present within adherens-type junctions, such as the costameres in striated muscle cells. Thus, our results suggest that human \( \alpha \)-synemin, via its interaction with either vinculin or talin, is able to link the desmin/synemin heteropolymeric IFs to the muscle cell sarcolemma, and thereby helps provide overall integration of the contractile apparatus within mammalian muscle cells.
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References


Figure 1. Schematic diagram of human synemin and talin, and the constructs used in the in vitro binding studies. A, Human synemin molecule and the three synemin constructs (SNTless, SNβT, and SNTIII). Synemin is composed of a short, ten-amino acid head domain (H), a conserved central 310 amino acid rod domain (Rod), and a long C-terminal tail domain (Tail). Human α- and β-synemin sequences are identical with the exception that α-synemin contains an additional 312 amino acid insert (SNTIII, ~33 kDa) located near the end of the long C-terminal tail. The entire β-synemin tail (SNβT, amino acid residues 322-1251, ~102 kDa) was cloned into the pFLAG-ATS vector as a FLAG-tagged fusion protein. SNTless (amino acid residues 1-329, ~37 kDa) was cloned into the pMALC2X vector as an MBP fusion protein. SNT III was cloned into both the pFLAG-ATS vector and the PMALC2X vector as a FLAG-tagged fusion protein and an MBP-tagged fusion protein, respectively. B, Schematic diagram of human talin and the GST talin constructs.
Figure 2. The 312 amino acid insert (SNTIII) within human α-synemin interacts directly with the rod domain of talin. A, Co-immunoprecipitation of human synemin isoforms with talin by anti-talin 8d4 mAb from UTSMC extracts. Lys denotes the load of the UTSMC lysates. Both α- and β-synemins were precipitated along with talin. B, Blot overlay assays using GST-tagged talin fusion proteins and the positive control of GST-VT overlaid with each of the purified synemin recombinant proteins. GST alone served as a negative control. The migration position of each GST-tagged talin fusion protein on the blot is marked with an asterisk. SNTIII interacts with both the talin R2 and VT, whereas SNβT and SNTless did not. C, GST pull-down assays. GST-talin R2 was immobilized on glutathione agarose and incubated with purified FLAG-SNTIII. GST alone and beads alone served as negative controls. Immobilized GST-talin R2 precipitated FLAG-SNTIII, whereas GST alone or beads alone did not. D, Dot blot overlay assays using GST-talin R2 and the positive control of GST-VT overlaid with purified MBP-tagged SNTIII. A duplicate membrane overlaid with MBP alone served as a negative control. SNTIII interacts specifically with the talin R2 and the VT.
Figure 3. Talin R2 interacts with SNTIII and competes with the VT in binding to SNTIII. A, Ninety six-well plates coated with 100 nM purified GST-talin R2 (squares), GST-VT (circles), or GST alone (triangles) were incubated with increasing concentrations of purified FLAG-SNTIII for 1 h at 25°C. After extensive washing, the bound SNTIII was detected with HRP-conjugated anti-FLAG mAb and the HRP substrate 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) by measurement at A_{405}. Background A_{405} values of wells incubated with buffer only were subtracted. The resulting A_{405} values were plotted versus the concentrations of incubated purified FLAG-SNTIII. The values represent the mean ± s.d. from three independent experiments. The data were then fitted to a moving average trendline with a period of 2. Interactions of talin R2 and VT with SNTIII were both saturable, with estimated K_D values of 52 ± 11 nM and 30 ± 8 nM, respectively. B, Increasing concentrations of GST-VT, along with 400 nM purified FLAG-SNTIII, were added to a 96-well plate coated with GST-talin R2, and incubated for 1 h at 25°C. Bound SNTIII was then determined as described in A. Values represent the mean ± s.d. from three independent experiments. The data were then plotted versus the concentration of GST-VT, and fitted to a moving average trendline with a period of 2. VT competes with talin R2 for binding to SNTIII.
Figure 4. Confocal microscopy studies indicate synemin colocalizes with talin within human adult skeletal muscle cells. A-C, Confocal microscopy analysis of the localization of synemin and desmin within longitudinal sections of human skeletal muscle. Synemin was labeled green with anti-synemin 2856 pAb and secondary Alexa Fluoro 488 goat anti-rabbit IgG. Desmin was labeled red with anti-desmin DEU-10 mAb, followed by staining with secondary Alexa Fluoro 594 goat anti-mouse IgG. Synemin colocalized with desmin at the myofibrillar Z-lines (C). D-I, Confocal microscopy analysis of synemin and talin within human skeletal muscle. Synemin was labeled green with anti-synemin 2856. Talin was labeled red with anti-talin 8d4 mAb. In cross sections (D-F), synemin colocalized with talin along the sarcolemma (F). In longitudinal sections (G-I), synemin colocalized with talin within the costameres (I, small window, arrows). (Bars, 25 μm)
Figure 5. SNTIII co-localizes with talin as well as with talin R2 at FA sites within transfected A-10 cells. A-D, A-10 cells were transfected with EGFP-SNTIII plasmids. Talin was then labeled red 24 hours post transfection with anti-talin 8d4 mAb plus secondary Alexa fluoro 594 goat anti-mouse IgG. B-D represents the boxed area in A. SNTIII co-localized with talin within FAs (A and D). E-H, A-10 cells were transfected with EGFP-SNβT plasmids. F-H represent the boxed area in E. SNβT did not co-localize with talin at the FA sites labeled by anti-talin 8d4 mAb. I-L, A-10 cells were co-transfected with EGFP-SNTIII and DsRed-talin R2 plasmids. J-L represents the boxed area in I. SNTIII co-localized with talin R2 at FA sites. (Bars, 15 μm)
Figure 6. **SNTIII is composed of seven tandem repeats.** Analysis of the primary sequences of human α-synemin, mouse H-synemin, and chicken synemin using Rapid Automatic Detection and Alignment of Repeats (RADAR) at the European Bioinformatics Institute (EBI) identified a similar domain containing tandem repeated sequences within the C-terminal tail of each synemin ortholog. The relative position of each repeat within the amino acid sequence of each synemin ortholog is shown at the left side of the alignment. The well aligned residues are boxed. A, The repeated domain (amino acid residues 1107-1464) within the human α-synemin tail. SNTIII (amino acid residues 1153-1464) comprises the last seven repeats. A sequence motif of S/T-X-R-H/Q (where X represents any of the five amino acid residues of V, G, I, F, L) within each repeat of the SNTIII is well aligned. Secondary structure prediction using PROFPHD for each repeat is shown at the top of the alignment. The dashed lines with arrows denote β-strands (β1). All of the S/T-X-R-H/Q motifs are within the predicted β-strands. B and C, The repeated domain identified within the mouse H-synemin tail (B) and the chicken synemin tail (C), respectively. The well aligned arginine residues (3 lysines instead of arginines in chicken synemin) are marked with asterisks. D, Alignment of the amino acid sequences corresponding to human, mouse, and chicken SNTIII using MULTALIN. The conserved S/T-X-R-H/Q motifs are boxed in the resulting consensus sequence. At least five of the seven S/T-X-R-H/Q motifs are conserved.
Figure 7. Schematic diagram showing that desmin/synemin heteropolymeric IFs are linked to the costameric protein assemblies. The green ovals within the α-synemin tail domains represent the 312 amino acid insert. Different α-synemin molecules are shown interacting with talin and vinculin, which attach the desmin/synemin heteropolymeric IFs to the costameres. The rod domains and tail domains of both α- and β-synemins are able to interact with α-dystrobrevin and dystrophin, respectively, which represents an alternative way of attaching the desmin/synemin heteropolymeric IFs to the costameres.
SYNEMIN INTERACTS WITH THE LIM DOMAIN PROTEIN ZYXIN AND IS ESSENTIAL FOR CELL ADHESION AND MIGRATION

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Running Head: Interaction of synemin with zyxin

Abbreviations: IF(s), intermediate filament(s); FA(s), focal adhesion(s); GST, glutathione S-transferase; BSA, bovine serum albumin; pAb, polyclonal antibody; mAb, monoclonal antibody; SNT, synemin tail; SNβT, β-synemin tail; SNαT, α-synemin tail; DB, DNA binding domain; AD, activation domain; CO-IP, co-immunoprecipitation.

Abstract
Synemin is a very unique cytoplasmic intermediate filament protein with limited understanding of its exact cellular functions. The single human synemin gene encodes at least two splice variants named α-synemin and β-synemin, with the larger α-synemin containing an additional 312 amino acid insert within the C-terminal tail domain. We report herein that, by using the entire tail domain of the smaller β-synemin as the bait in a yeast two-hybrid screen of a human skeletal muscle cDNA library, the LIM domain protein zyxin was identified as an interaction partner of human synemin. Co-immunoprecipitation of synemin and zyxin from lysates of cultured cells provided additional evidence for in vivo association of these two proteins. Synemin binding site in human zyxin was subsequently mapped to the C-terminal three tandem repeats of the LIM domains, whereas the binding site for zyxin within β-synemin was within the C-terminal 332 amino acid region (SNβTII) at the end of
the tail domain. Transient expression of SNβTII markedly reduced the zyxin protein level within mammalian cells and blocked localization of zyxin at the focal adhesion sites. Knockdown synemin expression with siRNAs within Hela cells resulted in significantly compromised cell adhesion and cell motility. Our results suggest that synemin participates in focal adhesion dynamics and is essential for cell adhesion and migration.

Introduction
Intermediate filaments (IFs) are ~10 nm in diameter, filamentous cytoskeletal polymers that provide crucial structural support within mammalian cells (Erber et al., 1998). In addition to the traditional mechanical scaffolding role, recent studies indicated that IFs may have novel functions in many important cellular processes such as cell adhesion/migration, signal transduction, targeting of proteins and lipids, and organization of subcellular organelles (Ivaska et al., 2007; Kim and Coulombe, 2007). The IFs, which are heterogeneous in nature, are composed of cell-type specific intermediate filament (IF) proteins (Fuchs and Weber, 1994; Coulombe and Wong, 2004; Omary et al., 2004). In humans, more than 67 IF genes encoding individual IF proteins have been identified, all of which compose the IF protein superfamily (Hesse et al., 2001).

Synemin is a very large, unique member within the IF protein superfamily. The molecular structure of the synemin molecule is characteristic of an IF protein, with a central 312 amino acid conserved α-helical rod domain flanked by a very short, 10 amino acid N-terminal head domain and a very long, ~1000 amino acid C-terminal tail domain (Becker et al., 1995; Bellin et al., 1999). Although first discovered within muscle cells (Granger and Lazarides, 1980), synemin is also found expressed in many non-muscle cells and some types of cancer cells. These include vertebrate erythrocytes (Granger and Lazarides, 1982; Granger et al., 1982), lens cells (Granger and Lazarides, 1984; Tawk et al., 2003), normal and malignant astrocytes (Sultana et al., 2000; Jing et al., 2005; Jing et al., 2007), glia and neurons (Izmeryan et al., 2006), hepatic stellate cells (Uyama et al., 2006), human hepatoma cells (Liu et al., 2004), human malignant biliary epithelial cells (Schmitt-Graeff et al., 2006), and even cultured Hela cells (Olsen et al., 2006), which is a human cervix epithelial adenocarcinoma cell line. The wide expression profile of synemin, and particularly, expression in cancer cells,
suggests that synemin has important, fundamental cellular function.

Synemin is not able to self-assemble into homopolymeric filaments in vivo (Bellin et al., 1999; Titeux et al., 2001; Xue et al., 2004; Jing et al., 2007). Association of synemin with the major type III IF proteins, such as desmin and/or vimentin, through the conserved α-helical rod domains forms heteropolymeric IFs within mammalian cells (Robson et al., 2004). Synemin also interacts with non-IF proteins α-actinin (Bellin et al., 1999; Bellin et al., 2001), vinculin (Bellin et al., 2001; Sun et al., 2008a), talin (Uyama et al., 2006; Sun et al., 2008b), α-dystrobrevin (Mizuno et al., 2001), dystrophin/utrophin (Bhosle et al., 2006), as well as protein kinase A (Russell et al., 2006). Thus, synemin seems to be capable of linking the heteropolymeric synemin/desmin or synemin/vimentin IFs to various subcellular structures via interactions with those non-IF protein binding partners.

The single human synemin gene encodes two splice variants named α-synemin (180 kDa by SDS-PAGE; 172.7 kDa from sequence) and β-synemin (150 kDa by SDS-PAGE; 140.1 kDa from sequence) (Titeux et al., 2001). The larger α-synemin, with the only difference of an additional 312 amino acid insert near the end of the long C-terminal tail domain, is identical to the smaller β-synemin (Titeux et al., 2001). In previous studies, we have shown that the 312 amino acid insert within α-synemin interacts with human vinculin/metavinculin and talin, thereby conferring extra functions for the larger α-synemin (Sun et al., 2008b; Sun et al., 2008a). However, the possible role(s) of the smaller β-synemin within living cells remained unclear.

Using the entire tail domain of human β-synemin (SNβT) as the bait, zyxin was identified as a binding partner of synemin in a yeast two-hybrid screen of a human skeletal muscle cDNA library. Zyxin is a LIM domain protein present primarily at sites of actin-membrane interactions (Beckerle, 1986; Crawford and Beckerle, 1991; Kadrmas and Beckerle, 2004). Several lines of evidences suggest that zyxin plays important roles in regulating cell adhesion and migration, actin filament assembly, and in nucleus-cytoplasm communications (Beckerle, 1986; Nix and Beckerle, 1997; Nix et al., 2001; Yoshigi et al., 2005; Hansen and Beckerle, 2006; Hoffman et al., 2006). Our results suggest that, via direct interaction with zyxin, synemin participates in actin cytoskeleton dynamics within living cells and is essential for cell adhesion and migration.
Materials and Methods

Generation of cDNA Constructs

The human α- and β-synemin cDNAs were described previously (Titeux et al., 2001). For yeast two-hybrid assays, human synemin cDNA fragments encoding amino acid residues 321-579 (SNTIa), 580-920 (SNTIb), 1153-1464 (SNTIII), 922-1253 (SNβTII), 922-1565 (SNαTII), and the entire tail domain of β-synemin (SNβT, amino acid residues 322-1253) (Fig. 2D) were amplified by PCR and were introduced into the yeast two-hybrid bait vector pDEST32 (Invitrogen) by Gateway Recombination Technology using LR Clonase (Invitrogen). The human zyxin cDNA fragment encoding amino acid residues 1-383 (Zyx 1-383) was PCR amplified from the Human 3-Frame Skeletal Muscle cDNA Library (Invitrogen) and was cloned into the yeast prey vector pDEST22 (Invitrogen). The resulting plasmids were all sequenced in the DNA Sequencing and Synthesis Facility at Iowa State University, and were all confirmed in frame with the DNA binding domain (DB, pDEST32 constructs) or the activation domain (AD, pDEST22 constructs) of the Gal4 protein.

For expression of recombinant proteins, the cDNA fragments encoding SNTIa, SNTIb, SNTIII, SNβTII, and SNβT were PCR amplified and subcloned into the pFLAG-ATS expression vector (Sigma) at 5’ Hind III and 3’ Bgl II sites as FLAG-tagged expression constructs. The cDNA fragments encoding full-length human zyxin (Zyx 1-572, amino acid residues 1-572), Zyx 1-383, and the three zyxin LIM domains (Zyx 375-572, amino acid residues 375-572) were PCR amplified from the Human 3-Frame Skeletal Muscle cDNA Library and were subcloned into the pGEX-4T2 GST expression vector (GE Healthcare) at 5’ BamH I and 3’ Xho I sites. All the expression constructs were confirmed accurate by automated sequencing in the DNA Sequencing and Synthesis Facility at Iowa State University.

Yeast Two-Hybrid Screening

Yeast Two-hybrid screening was performed following instructions of the ProQuest Two-Hybrid System with Gateway Technology manual (Invitrogen). Briefly, 10 μg of both the pDEST32-SNβT plasmids and the human 3-frame skeletal muscle cDNA library were
co-transformed into the yeast strain MaV203, plated onto the SC-Leu-Trp-His plates containing 25 mM 3-aminotriazole (3-AT), and incubated for 72 hr at 30°C. Plates containing growing yeast colonies were replica-cleaned and incubated for another 48 hr at 30°C. Each of the resulting yeast colonies growing on the selective plates was then streaked onto a fresh SC-Leu-Trp plate and incubated at 30°C for 48 hr. Plasmids were isolated from the positive yeast clones and were sequenced using the sequencing primer pair for the pDEST22 vector. To confirm the positive interactions, the identified pDEST22 plasmids containing zyxin cDNA fragments were co-transformed with the pDEST32-SNβT plasmids into the MaV203 yeast cells. Five master plates were then created by streaking the colonies onto SC-Leu-Trp plates along with the five yeast control strains (controls A-E) (Invitrogen) and the two self-activation control yeast strains (the “bait only” control containing DB-SNβT plus empty AD vectors and the “prey only” control containing AD-Zyx 1-572 plus empty DB vectors). The master plates were then patching onto Whatman filter paper # 541 for X-gal assays, and were replica-plated onto plates of SC-Leu-Trp-Ura, SC-Leu-Trp containing 0.2% 5-fluoroorotic acid (5-FA), and of SC-Leu-Trp-His containing 100 mM 3-AT for observation of growth phenotypes.

β-Galactosidase Liquid Culture Assays

β-Galactosidase liquid culture assays using chlorophenol red-β-D-galactopyranoside (CPRG) (Roche Diagnostics) as the substrate were performed following instructions of the Clontech Yeast Protocol Handbook (Clontech, 2000). The yeast control stains (controls A-E) used in the assays came from the ProQuest Two-Hybrid System with Gateway Technology Kit (Invitrogen). These five controls represented increasing strength of interactions in the β-Galactosidase liquid culture assays and served as the standards. The experiments were performed in triplicates and the calculated mean of β-galactosidase units ± S.D. for each assay was plotted using Microsoft Excel.

Expression and Purification of Recombinant Proteins

Recombinant proteins were all expressed in *E.coli* BL21-Codon Plus (DE3) bacterial cells (Stratagene). FLAG-tagged synemin fusion proteins were affinity purified using Anti-FLAG
M2-Agarose Affinity Gel (Sigma). GST-tagged zyxin fusion proteins were batch purified using Glutathione Agarose (Sigma).

**Antibodies**

The anti-synemin 2856 polyclonal antibody (pAb) has been described previously (Bellin et al., 1999). Anti-vimentin monoclonal antibody (mAb) V9, horseradish peroxidase (HRP) conjugated anti-FLAG M2 mAb, anti-α-tubulin mAb, and anti-vinculin mAb hVIN-1 were purchased from Sigma. Anti-GFP pAb and HRP conjugated anti-GST mAb were obtained from Santa Cruz Biotechnology. Anti-human zyxin mAb, Alexafluor secondary antibodies, and Alexafluor-594 phalloidin were obtained from Invitrogen. Anti-VASP mAb were obtained from BD Biosciences.

**Co-Immunoprecipitation (Co-IP), FLAG Immunoprecipitation, and GST Pull-Down Assays**

For Co-IP assays, 1x10⁷ Hela cells were lysed with the MPER Mammalian Protein Extraction Reagent (Pierce) and incubated with 5 µg anti-human zyxin mAb for 1 hr at 4°C. Fifty µl 25% Protein A/G Plus Beads (Santa Cruz Biotechnology) were then added to the reaction for incubation of another 1 hr at 4°C. The same amount of Hela cell lysates incubated with 5 µg anti-GAPDH mAb (Cell Signaling) plus 50 µl Protein A/G Plus Beads, or with 50 µl beads alone, were served as two negative controls. The reactions were washed twice with 1× PBS and then eluted with 2× SDS sample buffer. The resulting eluates were subjected to SDS-PAGE and analyzed by Western blotting with anti-synemin 2856 pAb, and then reprobed with anti-human zyxin mAb.

FLAG immunoprecipitation assays were performed following instructions of the Anti-FLAG M2 Affinity Gel manual (Sigma). Briefly, 4 µg of each GST-tagged zyxin fusion protein was incubated in 500 µl 1× PBS containing 4 µg purified FLAG-SNβT and 40 µl of the pre-cleaned Anti-FLAG M2 beads for 3 hr at 4°C. The beads were then centrifuged, washed twice with 1× PBS containing 0.1% Tween-20 (PBST), and eluted with 2× SDS sample buffer. The resulting eluates were subjected to SDS-PAGE and analyzed by Western blotting with HRP conjugated anti-GST mAb and HRP conjugated anti-FLAG M2 mAb.

GST-pull down assays were performed essentially as previously described (Sun et al.,
Surface Plasmon Resonance

Surface plasmon resonance was performed using Biacore T100 (GE Healthcare) in the Proteomics Facility at Iowa State University. Purified GST-Zyx 1-572 fusion protein was immobilized on the activated flowcell 2 of a CM5 sensor chip using the GST Capture Kit (GE Healthcare) at an Rmax value of ~100. GST alone immobilized on the activated flowcell 1 served as a reference in the experiments. Increasing concentrations (from 250 nM to 3000 nM) of purified FLAG-SNβT were then injected over the flowcells for an association phase of 180 sec at a flow rate of 10 μl/min and followed by a 100 sec disassociation phase. The protein-protein interactions were then detected in realtime by the Biacore T100 control software (GE Healthcare). The resulting sensorgrams, as well as affinity of the interaction, was analyzed by the Biacore T100 evaluation software (GE Healthcare) using the reference subtracted (flowcell 2-1) data sets.

Cell Culture

Hela cells were kindly provided by Dr. Marit Nilsen-Hamilton at Iowa State University. A-10 cells, a rat vascular smooth muscle cell line, were purchased from American Type Culture Collection (ATCC). Human uterine smooth muscle cells (UTSMC) were obtained from Lonza. Hela cells and A-10 cells were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C (95% air, 5% CO2). Human UTSMC cells were maintained with Smooth Muscle Medium-2 Bulletkit (Lonza) at 37°C with 95% air and 5% CO2.

Immunofluorescence, Transfection Studies, and Flowcytomery

Immunofluorescence staining of cultured cells were performed essentially as previously described (Sun et al., 2008a). For transfection studies, cells that seeded on collagen-coated coverslips with optimal confluence were transfected with mammalian expression constructs using JetPEI Transfection Reagent (Polyplus Transfection). Cells were then immunostained with appropriate primary and Alexafluor secondary antibodies. Immunofluorescence
microscopy was conducted using a LEICA DEMIRE2 inverted microscope equipped with a CCD camera in the Hybridoma Facility at Iowa State University. Cells that express EGFP-SNβTII were collected by flow cytometry using a Beckman Coulter EPICS Altra Flow Cytometer in the Hybridoma Facility at Iowa State University.

**RNA Interference and Quantitative-PCR**

The Hs_Dmn_4_HP siRNA specific for both human α- and β-synemins and the Alexa Fluor 488 Labeled Negative Control siRNA were obtained from Qiagen. Hela cells cultured in 6-well plates (Corning COSTAR) were transfected with 5 nM of the each siRNA using the HiPerFect Transfection Reagent (Qiagen). Total RNA and cDNA of the siRNA treated Hela cells were then prepared 72 hr post transfection using the RNeasy Mini Kit (Qiagen) and the QuantiTect Reverse Transcription Kit (Qiagen), respectively, following the manufacturer’s instructions. Quantitative-PCR measuring synemin mRNA level was done with Hs_Dmn_1_SG QuantiTect Primer Assay (Qiagen) using a Stratagene Mx4000 Multiplex Quantitative PCR System (Stratagene) in the DNA Sequencing and Synthesis Facility at Iowa State University. Protein expression levels were analyzed by Western blotting using appropriate antibodies 72 hr post transfection of the siRNAs.

**Cell Adhesion and Migration Assays**

Cell adhesion assays were performed as previously described (Zhang et al., 2006) with modifications. Briefly, \(1 \times 10^5\) Hela cells that had been treated for 72 hr with 5 nM of the Alexa Fluor 488 Labeled Negative Control siRNA or with 5 nM of the Hs_Dmn_4_HP siRNA were seeded onto wells of a 12-well CellBIND surface tissue culture microplate (Corning Life Sciences) for 1 hr. Cells were then washed gently with 1xPBS for 5 times, detached from the plate with 0.25% Trypsin-EDTA, and counted with the Guava Viacount (Guava Technology) at the Hybridoma Facility at Iowa State University. The experiments were performed in triplicate. The adhesion rate was calculated as number of adherent cells/\(1 \times 10^5\). Data were plotted using Microsoft Excel. The 2-D wound healing assay and the 3-D migration modified Boyden chamber assay using non-coated transwell inserts (6.5 mm 24-well format with 8 μm pore size, Corning Life Sciences) were performed essentially as
previously described (Kleeberger et al., 2007). For the modified Boyden chamber assay,
$1 \times 10^5$ Hela cells that had been treated with siRNAs for 72 hr were seeded on the upper side
of the membrane of the transwell inserts within serum free DMEM media, while DMEM
media containing 10% FBS was added at the bottom side. After incubation for 48 hr at 37°C,
cells were fixed with 2% formaldehyde in 1× PBS and stained with 1.67 mmol/L CellTrace
Calcein AM (Invitrogen). Cells on the upper side of the membranes were then removed by
gently scraping with a wet cotton swab. The resulting membranes were then cut out of the
transwell inserts, mounted on glass slides with Vectashield Mounting Medium (Vector
Laboratories), and were observed under the microscope. Numbers of cells from 5 different
high-power fields (HPF) (20× objective) on each membrane from triplicate experiments were
counted. Data (mean ± S. D.) were plotted with Microsoft Excel.

Results

Zyxin Was Identified to Interact with Synemin by Yeast Two-Hybrid Screening Using SNβT as
the Bait

The hypervariable N-terminal head and C-terminal tail domains of IF proteins often are the
regions mediating interactions with non-IF protein binding partners (Green et al., 2005; Kim
and Coulombe, 2007). In the case of the large type VI IF protein synemin, it contains a very
short, 10 amino acid head domain and a very long, ~1000 amino acid tail domain. Thus,
chances are that the long tail domain of synemin harbors most of the binding sites for its
putative non-IF protein interaction partners. To identify novel proteins that interact with
synemin, the entire tail domain (amino acid residues 322-1253) of human β-synemin was
used as the bait (DB-SNβT) in a yeast two-hybrid screen of a human skeletal muscle cDNA
library. A total of approximately $2 \times 10^6$ transformants were screened and resulted in 15
positive colonies growing on SC-Leu-Trp-His plates containing 25 mM 3-aminotriazole.
Nine of them hosted prey plasmids containing cDNA fragments encoding various regions of
human zyxin in frame with the Gal4 activation domains. Interestingly, these zyxin regions
exactly constituted a series of N-terminal deletions of the full-length zyxin, with the smallest
one (Zyx 375-572) representing the three tandem repeats of the LIM domains at the
C-terminal end of human zyxin (Fig. 1A).
Yeast two-hybrid screening sometime resulted in false positives. In addition, although there is no evidence that the LIM domains of zyxin can bind directly to double strand DNA, a well list of transcription factors containing specific LIM domains have been shown to be able to do so (Matthews and Sunde, 2002), which may directly activate the reporter genes of the yeast two-hybrid system. To exclude the possibility that our results obtained from the yeast two-hybrid screening were false positives, the MaV203 yeast cells were co-transformed with each AD-Zyxin construct plus the DB-SNβT plasmid, and interactions were then tested using the three reporter genes (LacZ, His3, and Ura3) of the ProQuest yeast two-hybrid system. The five control yeast strains (Controls A-E, ProQuest yeast two-hybrid system), as well as the self-activation controls of the bait only (DB-SNβT plus AD vector) and the prey only (AD-Zyxin 1-572 plus DB vector) were also included. Similar to the positive controls (Controls B-D), the yeast strains containing various AD-Zyxin constructs plus DB-SNβT demonstrated strong β-galactosidase activities, grew vigorously on SC-Leu-Trp-Ura and SC-Leu-Trp-His + 100 mM 3-AT selective plates, and exhibited inhibited growth on SC-Leu-Trp-Ura plates containing 0.2% 5-Fluoroorotic acid. In contrast, the negative control (DB vector plus AD vector) and the two self-activation controls (DB-SNβT plus AD vector and AD-Zyxin 1-572 plus DB vector) showed phenotypes contrary to the positive controls (Fig. 1B). Therefore, these results confirmed that the interaction of SNβT with zyxin regions within the yeast two-hybrid system were not false positives. Furthermore, the primary binding site for synemin on zyxin could be within the three tandem repeats of the LIM domains.

Interaction of synemin with zyxin was further illustrated by CO-IP assays. Hela cells were used for the CO-IP assays because they express synemin (Olsen et al., 2006) and are relatively easy to handle with. As shown in Fig. 1C, synemin was specifically precipitated along with zyxin by immobilized anti-human zyxin mAb from the Hela cell lysates. Lack of β-synemin in the lane of CO-IP with anti-human zyxin mAb may result from a very small amount of β-synemin precipitated, and thereby could not be detected by Western blotting. In toto, this result indicated an in vivo association of human synemin with zyxin. 

Mapping the Synemin Binding Site in Human Zyxin
To confirm the binding site of synemin in zyxin and to provide quantitative estimates of the interactions, co-transformants of DB-SNβT plus each zyxin region in AD vector (AD-Zyx 1-572, AD-Zyx 82-572, AD-Zyx 197-572, AD-Zyx 375-572, and AD-Zyx 1-383) were analyzed for β-galactosidase activity with liquid culture assays using CPRG. These AD-Zyxin constructs represented overlapping regions which together include the full length amino acid sequence of zyxin (Fig. 2A). The five control yeast strains (controls A-E) with interaction strengths ranging from none (control A, DB vector plus AD vector) to very strong (control E, AD vector plus DB-full length Gal4) were also included in the assay as standards to indicate relative interaction strengths. The co-transformant of DB-SNβT plus AD vector served as a negative control. As shown in Fig. 2B, human zyxin regions Zyx 1-572, Zyx 82-572, Zyx 197-572, and Zyx 375-572 all showed positive interactions with SNβT as compared to the positive, weak interaction control B (DB-human RB + AD-human E2F1). In contrast, zyxin region 1-383 did not show any interaction with SNβT, confirming that the zyxin LIM domains are required for zyxin/synemin interaction. In addition, interaction of the full-length zyxin (Zyx 1-572) with SNβT displayed relatively moderate interaction strength as compared to the control C (DB-Drosophila DP + AD-Drosophila E2F) which represented a moderate-strong interaction. Interestingly, deleting the N-terminal 81 amino acids of zyxin (Zyx 82-572) resulted in an even higher affinity for SNβT than that of the full length zyxin (Zyx 1-572). This may reflect an intramolecular interference caused by the N-terminal 81 amino acids of zyxin in its interaction with human synemin. In comparison with other zyxin regions, the zyxin LIM domains (Zyx 375-572) displayed relatively weak interaction with SNβT, indicating that sequences in the zyxin N-terminal region are required for the optimal interaction with SNβT.

Interactions of different zyxin regions with SNβT were also examined in vitro by immunoprecipitation assays. GST-tagged full-length zyxin (GST-Zyx 1-572) and zyxin LIM domains (GST-Zyx 375-572), but not the N-terminal 383 amino acids of zyxin (GST-Zyx 1-383), were specifically precipitated by FLAG-SNβT immobilized on anti-FLAG M2 resins (Fig. 2C). These results were consistent with the results obtained from the yeast two-hybrid β-galactosidase liquid culture assays (Fig. 2B) and further confirmed that the zyxin LIM
domains contain the binding site for synemin.

**Mapping the Zyxin Binding Site in Human Synemin**

To narrow down the binding site for zyxin in human synemin, SNβT was further divided into three consecutive regions named SNTIa, SNTIb, and SNβTII as diagrammed in Fig. 2D. The 312 amino acid insert present only in α-synemin (SNαTII) and the region includes both SNβTII and SNTIII in the α-synemin tail (SNαTII) (Fig. 2D) were also included in the mapping study to examine whether they interact with zyxin. The cDNAs encoding SNTIa, SNTIb, SNβTII, SNTIII and SNαTII were introduced into the yeast two-hybrid DB vectors and were co-transformed with AD-Zyx 82-572 plasmids into the MaV203 yeast cells. Interactions were examined using β-galactosidase liquid culture assays. Co-transformants of DB-SNβT plus AD-Zyx 82-572 and of DB vector plus AD-Zyx 82-572 served as the positive control and the negative control, respectively. As shown in Fig. 2E, SNβTII displayed very strong interaction with zyxin as compared with the positive control. SNTIa, SNTIb and SNTIII did not interact with zyxin. These results demonstrated that the binding site for zyxin in synemin is within the C-terminal 332 amino acids at the end of the β-synemin tail domain. SNαTII showed similar interaction strength as SNβT does (Fig. 2E), indicating that presence of the 312 amino acid insert within α-synemin tail does not abolish the interaction. Thus, α-synemin also interacts with zyxin.

The interactions of synemin regions with zyxin were further tested in vitro by GST pull-down assays. SNβTII was specifically precipitated by immobilized GST-Zyx 1-572, while SNTIa, SNTIb, and SNTIII were not (Fig. 2F). These results confirmed the data obtained in the yeast two-hybrid β-galactosidase liquid culture assays.

**Analysis of the Interaction Affinity**

To provide a quantitative assessment of the interaction of SNβT with zyxin, surface plasmon resonance (SPR) were conducted as described in the “Materials and Methods” section. The purified GST tagged full length zyxin (GST-Zyx 1-572) was used as the ligand in the SPR analysis, whereas the purified recombinant FLAG-SNβT was used as the analyte. Zyxin
displayed positive interactions with increasing concentrations of SNβT as reflected by the sensorgrams (Fig. 3A). When the concentration of SNβT was high, the interaction reached saturation. The $K_d$ value of the interaction was calculated as $5.68 \times 10^{-8}$ M (Fig. 3B). This SPR analysis result not only provided quantitative information about the interaction but also further confirmed the *in vitro* interaction of human zyxin with synemin.

*Over Expression of SNβTII Significantly Blocked Localization of Endogenous Zyxin at Focal Adhesion (FA) Sites within the Transfected Cells*

To further examine the interaction of synemin with zyxin within a cellular context, Hela cells were transfected with EGFP-tagged SNβTII plasmids. Subcellular localizations of EGFP-SNβTII and zyxin were then examined with epifluorescence microscopy. Cells with moderate GFP expression level were selected for analysis. In comparison with surrounding cells, transient expression of EGFP-SNβTII within the transfected Hela cells resulted in significantly reduced zyxin localization at the FA sites (Fig. 4, D-F). Expression of EGFP alone within the transfected cells did not alter the localization pattern of endogenous zyxin (Fig. 4, A-C), indicating that zyxin localization to the focal adhesions (FAs) was blocked specifically by SNβTII. Note that loss of zyxin at the FAs was not due to disruption of the normal architecture of the FAs themselves, as illustrated by the intact FAs labeled with vinculin within the EGFP-SNβTII transfected Hela cells (Fig. 4, G-I), nor due to disruption of the normal architectures of other cytoskeletal components including vimentin IFs (Fig. 4, M-O) and actin cytoskeletons (Fig. 4, P-R). These results *in toto* suggested that interaction of exogenous SNβTII with zyxin blocked localization of endogenous zyxin at the FA sites within the transfected cells. Previous studies have shown that positioning of vasodilator-activated phosphoprotein (VASP) at FA sites is dependent on appropriate zyxin localization (Drees *et al.*, 1999; Drees *et al.*, 2000). We therefore examined whether loss of zyxin at FA sites by exogenous SNβTII would also block VASP localization to the FAs. Indeed, localization of VASP to the FAs was significantly reduced within EGFP-SNβTII-expressing cells (Fig. 4, J-L), indicating that it was a result of loss of zyxin at the FA sites.

To examine whether loss of zyxin at the FA sites by exogenous SNβTII would also occur
in other mammalian cellular contexts, A-10 cells, a rat vascular smooth muscle cell line, and NIH/3T3 cells were also transfected with EGFP-SNβTII plasmids. Similar results of significantly reduced zyxin localization at the FA sites were also observed in A-10 cells (Fig. 4, S-V) and NIH/3T3 cells (data not shown) transfected with EGFP-SNβTII, suggesting a general mechanism underpinning these phenotypes. By using fluorescent activated cell sorting (FACS), EGFP-SNβTII positive Hela cells were collected, lysed, and subjected to Western blot analysis. The results indicated that the zyxin, but not the vinculin, protein level within EGFP-SNβTII-expressing cells were significantly reduced (Fig. 4W), whereas the zyxin mRNA level was normal as indicated by RT-PCR when compared with the zyxin mRNA level within wild type Hela cells (Fig. 4X). Thus, loss of zyxin at the FA sites was resulted from a reduced zyxin protein level within the EGFP-SNβTII positive cells.

Knockdown Synemin Did not Alter Zyxin Expression and Localization within Hela Cells

To investigate whether loss of synemin will influence zyxin expression and localization within cells, synemin-knockout models were generated using siRNA specific to both human α- and β-synemins. Although it was previously shown that synemin is expressed in cultured Hela cells by mass spectrometry (Olsen et al., 2006), we also characterized and confirmed synemin expression in Hela cells by Western blotting (Fig. 5A), reverse transcriptase-PCR (Fig. 5B), and immunofluorescence microscopy (Fig. 5C). Western blotting showed that, compared to the relative equal and low amount of α- and β-synemins in both A-10 and UTSMC cells, α-synemin was highly expressed in Hela cells and β-synemin expression was very low (Fig. 5A). Reverse transcriptase-PCR using a specific primer pair that amplifies a ~1 kb fragment from β-synemin mRNA and a ~2 kb fragment from α-synemin mRNA also showed that α-synemin was highly expressed in Hela cells (Fig. 5B). Synemin primarily co-localized with vimentin IFs within Hela cells as indicated by double immunofluorescence labeling of synemin and vimentin (Fig. 6C). Thus, these results in toto confirmed synemin expression in cultured Hela cells.

Transfection of the Hela cells with the Hs_Dmn_4_HP siRNA for 72 hr (Dmn_4-Hela cells) resulted in a significant decrease in the synemin protein level, with no obvious change in protein levels of both zyxin and vinculin (Fig. 5E). Immunofluorescence staining of synemin
within Dmn_4-Hela cells indicated that synemin was absent in >98% of the cells (data not shown). Double immunofluorescence labeling of endogenous synemin and vimentin, or of synemin and zyxin, within Dmn_4-Hela cells showed that both vimentin filaments and zyxin localization remained intact. Other cytoskeletal architectures such as microfilaments and microtubules were also normal (data not shown). These results indicated that zyxin expression and localization is not affected when synemin is absent within cells.

Syne

Within Dmn_4-Hela cells indicated that synemin was absent in >98% of the cells (data not shown). Double immunofluorescence labeling of endogenous synemin and vimentin, or of synemin and zyxin, within Dmn_4-Hela cells showed that both vimentin filaments and zyxin localization remained intact. Other cytoskeletal architectures such as microfilaments and microtubules were also normal (data not shown). These results indicated that zyxin expression and localization is not affected when synemin is absent within cells.

Synemin Is Essential for Cell Adhesion and Migration

Intermediate filaments such as vimentin IFs (Ivaska et al., 2007) and keratin filaments (Green and Simpson, 2007; Magin et al., 2007) have been shown to play essential roles in cell adhesion and migration. Especially Kleeberger et al., recently reported that nestin, another mammalian type VI IF protein that shares similar molecular structure with synemin, is important for the migration and metastasis of prostate cancer cells (Kleeberger et al., 2007). This report raised our interest in examining whether synemin may play a role in cell adhesion and migration as well.

In the cell adhesion assays, the calculated adhesion rate for the Dmn_4-Hela cells was 29.9%, while for Hela cells transfected with the fluorescent negative control siRNA (Fl siRNA-Hela cells) was 54.8% (Fig. 6A). Statistical analysis indicated that Dmn_4-Hela cells exhibited significant compromised ability in adhering to the CellBIND surface tissue culture microplates (p < 0.05). The decreased adhesion rate of Dmn_4-Hela cells was not a result of decreased number of viable cells, in that the viability of Dmn_4-Hela cells was similar with wildtype Hela cells as measured by trypan blue staining using Guava viacount. No more than 5-8% dead cells were detected in all experiments (data not shown).

To examine the function of synemin in cell migration, the 2-D wound healing assays were performed using Dmn_4-Hela cells and Fl siRNA-Hela cells. Dmn_4-Hela cells exhibited markedly compromised ability in closing the “wound”, whereas Fl siRNA-Hela cells recovered most of the denuded area of the plate 48 hr after wounding (Fig. 6B). In the modified Boyden chamber assays, Fl siRNA-Hela cells exhibited a robust ability in migrating through the porous membranes, whereas traversing of Dmn_4-Hela cells through the membranes were markedly inhibited (Fig. 6, C and D). These results indicated that synemin
promoted the adhesion and migration of Hela cells.

Discussion
We have discovered zyxin as a novel interaction partner for the large type VI IF protein synemin. Using the entire tail domain of human β-synemin in a yeast two-hybrid screening of the human adult skeletal muscle total cDNA library, fifteen positive clones were obtained. Nine of the fifteen positive clones contained cDNA sequences encoding the full-length or truncated human zyxin (Fig. 1A). Subsequent yeast two-hybrid retransformation assays (Fig. 1B), CO-IP assays (Fig. 1C), and in vitro protein-protein interaction assays (Fig. 2 and Fig. 3) indicated that interaction of synemin with zyxin is specific but not resulted from false-positives in the yeast two-hybrid screen.

The binding site for zyxin in human β-synemin was mapped to the C-terminal 332 amino acid region at the end of the tail domain (SNβTII, amino acid residues 922-1253) (Fig. 2, E and F). This is the first evidence that β-synemin contains a binding site for a protein that localizes to actin-membrane interactions and modulates cell-cell and cell-extracellular matrix adhesion. Transient expression of SNβTII within mammalian cells led to a markedly reduced zyxin protein level and loss of zyxin at the FA sites without disruption of other cellular architectures (Fig. 4). This could be that, in cells expressing exogenous SNβTII, zyxin was trapped within the cytoplasm by interacting with SNβTII, and the resulting mislocalization of zyxin would subsequently lead to its degradation. Zyxin has been shown to be able to travel between the nucleus and the FAs within cells, and may therefore mediate communications between these two subcellular compartments (Nix and Beckerle, 1997; Nix et al., 2001). Whether synemin and synemin-containing IFs are involved in regulating translocation of zyxin between the nucleus and the FAs remains to be elucidated.

Our results also indicate that human α-synemin, which contains the 332 amino acid sequence (SNβTII), interacts with zyxin as well (Fig. 2E). We have previously shown that the 312 amino acid insert that is only present within α-synemin (SNTIII) interacts specifically with vinculin and talin, both are cytoskeletal proteins present at actin-membrane interactions, thereby conferring additional functions to α-synemin (Sun et al., 2008b; Sun et al., 2008a).
Notably, SNTIII itself did not bind to zyxin (Fig. 2, E and F). But its presence interfered with the interaction of the SNβTII region with and thereby reduced the affinity of α-synemin for zyxin (Fig. 2E). Except for the additional insert within the tail domain, α-synemin is identical to and seems to share all the potential functions of the smaller β-synemin. However, it is possible that different ratios of α-synemin versus β-synemin would modulate interactions with their protein partners and thereby fulfill specific functions within different cellular contexts. This is reflected by the fact that both α- and β-synemins are differentially expressed in the three types (skeletal, cardiac, and smooth) of muscle cells (Titeux et al., 2001; Xue et al., 2004), as well as by the fact we found in this study that Hela cells, a human cervix epithelial adenocarcinoma cell line, express a very high level of α-synemin but very small amount of β-synemin (Fig. 5, A and B).

The binding site for synemin in zyxin was mapped to the C-terminal region containing three tandem repeats of the LIM domains (Fig. 2, B and C). The LIM domains are modular protein-binding interfaces mediating protein-protein interactions (Feuerstein et al., 1994; Schmeichel and Beckerle, 1994; Kadrmas and Beckerle, 2004). And, it has been reported that the zyxin LIM domains contain binding sites for several other proteins such as cysteine-rich protein-1 (CRP-1) (Schmeichel and Beckerle, 1994) and p130Cas (Yi et al., 2002). Zyxin has also been implicated to act as an adapter protein that recruits different partners to appropriate subcellular compartments and promote their activity (Kadrmas and Beckerle, 2004). Especially the zyxin LIM domains are able to direct zyxin and Ena/VASP family proteins to FAs (Nix et al., 2001). Thus, synemin may be recruited simultaneously with Ena/VASP family proteins, CRP-1 and p130Cas by the zyxin LIM domains to the FAs and participate in actin remodeling and FA dynamics within cells. As a member of the Cas family, p130Cas is a cytoskeletal mechanosensor protein that regulates integrin signaling cascades and cell adhesion/cell motility (Defilippi et al., 2006). Upon mechanical extension by physiological forces, p130Cas is phosphorylated by src family kinase and thereby activates downstream signaling events (Sawada et al., 2006). Recruitment of synemin and p130Cas by zyxin may help in relaying mechanical forces from adhesion sites to the IF cytoskeletons within the cells, and thereby assist in cell adhesion and migration. How interactions of synemin isoforms with zyxin as well as those proteins binding to the zyxin LIM domains are regulated within living
cells awaits further exploration.

We have also revealed novel functions for protein synemin in promoting cell adhesion and cell motility by RNAi studies (Fig. 6). These functions may be partially reflected by the evidence that synemin is able to interact with several proteins present in adhesion sites, such as vinculin, talin, and zyxin. Both vinculin and talin have been shown to regulate cell adhesion and migration (Critchley, 2004; Hu et al., 2007; Le Clainche and Carlier, 2008). And, zyxin has also been shown to be important in regulating actin filament assembly, with loss of zyxin within mice fibroblasts resulted in increased cell motility (Hoffman et al., 2006). Different ratios of α- and β-synemin may also modulate interactions with zyxin, vinculin, and talin, and thereby regulating cell adhesion and migration. Thus, a very high amount of α-synemin expressed within Hela cells could be beneficial for the invasiveness of these cancer cells. Indeed, another mammalian type VI IF protein nestin shows similar functions in enhancing prostate cancer cell migration and metastasis (Kleeberger et al., 2007).

Identification of interaction of synemin isoforms with zyxin broadened our understanding in the cellular functions of synemin. Synemin may link the synemin-containing heteropolymeric IFs to FA dynamics via interactions with zyxin, vinculin, and talin. As a result, synemin, like nestin, plays essential roles in cell adhesion and migration, and may contribute to cancer cell metastasis.

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Figure 1. Zyxin was identified to interact with the β-synemin tail domain in the yeast two-hybrid screen of a human skeletal muscle cDNA library. (A) Schematic diagram showing human zyxin molecule as well as the zyxin regions from the nine positive clones resulted from the yeast two-hybrid screening. Only 8 clones are listed because two of the nine positive clones were identical (AD-Zyx 322-572). The clone containing the shortest zyxin region (AD-Zyx 375-572) represents the C-terminal three tandem repeats of the LIM domains. (B) Phenotypes of the yeast two-hybrid retransformation assays. Each AD-Zyx construct were cotransformed again with DB-SNβT into the MaV203 yeast strains and were tested with the three reporter gene systems (LacZ, His3, and Ura3) of the ProQuest yeast two-hybrid system. They all exhibited phenotypes similar to the positive control, whereas the self activation controls of the bait only (DB-SNβT plus AD vector) and the prey only (AD-Zyx 1-572 plus DB vector) showed negative phenotypes. (C) Co-immunoprecipitation of synemin with zyxin. The experiments were performed as described in the “Materials and Methods” section. Synemin was specifically precipitated along with zyxin from Hela cell lysates using anti-human zyxin mAb immobilized on Protein A/G Plus beads. Synemin was not precipitated using immobilized anti-GAPDH mAb, or the Protein A/G Plus beads only, incubated with Hela cell lysates. Note that α-synemin was highly expressed in Hela cells.
Figure 2. Mapping the binding site within synemin and zyxin. (A-C) Synemin binds to the zyxin LIM domains. (A) Schematic diagram showing regions of human zyxin molecule and regions (Zyx 1-572, Zyx 82-572, Zyx 197-572, Zyx 375-572, and Zyx 1-383) used in the mapping studies. (B) Yeast two-hybrid β-galactosidase liquid culture assays testing the interaction of each zyxin region in the AD vector with DB-SNβT. The five controls (A-E) from the ProQuest yeast two-hybrid system were included as the standards of the interaction strength. The cotransformant of empty DB vector plus AD vector served as a negative control. The values of β-galactosidase unit represent the mean ± s.d. from triplicates of the experiments. (C) FLAG immunoprecipitation assays. The experiments were performed as described in the "Materials and Methods" section. Both GST tagged Zyx 1-572 and Zyx 375-572, but not GST-Zyx 1-383, were precipitated by FLAG-SNβT. (D-F) Zyxin binds to the C-terminal 332 amino acid sequence (SNβTII) within the β-synemin tail domain. (D) Schematic showing of human α- and β-synemin molecules as well as each region used in the yeast two-hybrid β-galactosidase liquid culture assays. The entire tail domain of β-synemin (SNβT, a.a. residues 322-1253), the three sub-regions of SNβT (SNTIa, a.a. residues 321-579; SNTIb, a.a. residues 580-920; and SNTβII, a.a. residues 922-1253), the 312 amino acid insert present only in α-synemin (SNTIII, a.a. residues 1153-1464), and the C-terminal region of α-synemin tail (SNαTII, a.a. residues 922-1565) were cloned into the yeast two-hybrid DB vectors.(E) Yeast two-hybrid β-galactosidase liquid culture assays testing the interaction of each synemin region in DB vector with AD-Zyx 82-572. The co-transformants of empty DB vector plus AD vector and of DB-SNβT plus AD-zyx 82-572 served as the negative and positive control, respectively. The values of β-galactosidase unit represent the mean ± s.d. from triplicates of the experiments. Note that both SNTβII and SNTαII showed interaction with Zyx 82-572 as compared with the positive control. (F) Each FLAG-tagged synemin fusion protein was incubated with purified GST-Zyx 1-572 in the GST pull-down assays. GST-Zyx 1-572 precipitated only FLAG-SNβTII but not FLAG-SNTIa, FLAG-SNTIb, and FLAG-SNTIII. GST alone incubated with FLAG-SNβTII served as a negative control.
Figure 3. Biacore surface plasmon resonance analyzing affinity of the interaction between SNβT and zyxin. (A) Reference subtracted sensorgrams showing interaction of purified recombinant zyxin with SNβT. Purified GST-Zyx 1-572 was immobilized on a CM5 sensorchip as the ligand. Increasing concentrations of FLAG-SNβT (250 nM, 500 nM, 1000 nM, 2000 nM, and 3000 nM) were injected as the analyte over the sensorchip with an association time of 180 seconds and a dissociation time of 100 seconds. Note that the interaction reached saturation at high concentrations (2000 nM and 3000 nM) of FLAG-SNβT injected. RU denotes response units. (B) Affinity of the interaction was calculated based upon resulted RUs from two separated experiments. The $K_d$ value for the interaction was estimated at $5.68 \times 10^{-8}$ M.
Figure 4. Transient expression of SNβTII in mammalian cells led to loss of zyxin at the FA sites. (A-R) Transient expression of EGFP alone or EGFP-SNβTII within Hela cells. Cells were double labeled with anti-GFP pAb in green and with each appropriate mAb or reagent to the corresponding endogenous protein in red as indicated. (F) and (L) represent the boxed area in (E) and (K), respectively. Asterisks in (E), (F), (K), and (L) indicate GFP positive cells. Note that EGFP-SNβTII positive cells exhibited significantly reduced level of zyxin (F) and VASP (L) at the FA sites in comparison with surrounding cells. EGFP alone did not block zyxin to the FAs (A-C). The normal architectures of the GFP positive cells were not disrupted as indicated by labeling FAs (G-I), IFs (M-O), and F-actin (P-R) with vinculin, vimentin, and phalloidin, respectively. (S-V) Transient expression of EGFP-SNβTII within A-10 cells. Arrows indicate the EGFP-SNβTII positive cells. Localization of zyxin, but not vinculin, at the FA sites was also significantly reduced. (X) Western blotting showing significantly decreased zyxin, but not vinculin, protein level in EGFP-SNβTII positive Hela cells. Approximately 10,000 cells were collected by flow cytometry, lysed, and subjected to SDS-PAGE and Western blotting. Wildtype Hela cells without transfection served as the control. (W) RT-PCR showing similar mRNA level of zyxin and vinculin in both EGFP-SNβTII positive Hela cells and wildtype Hela cells.
Figure 5. Loss of synemin did not alter the protein level and localization of zyxin within Hela cells. (A-C) Characterization of synemin expression within Hela cells. (A) Western blotting showing that α-synemin was highly expressed in Hela cells, whereas both α- and β-synemin were expressed at a relatively similar level in A-10 and UTSMC cells. (B) RT-PCR showing that α-synemin mRNA was highly expressed within Hela cells. (C) Double labeling of synemin (green) and vimentin (red) with anti-synemin 2856 pAb and anti-vimentin mAb V9 showed that synemin co-localized with vimentin in filamentous structures within Hela cells. (D-F) Knockdown synemin expression within Hela cells using siRNA. (D) Quantitative PCR analyzing synemin mRNA levels within Hela cells transfected with siRNAs. ∼87% synemin mRNA was knockdown within Hela cells treated with HS_Dmn_4_HP (Dmn_4) siRNA for 72 hr. (E) Western blotting showing synemin (Syn) knockdown within Hela cells treated with Dmn_4 siRNA for 72 hr. Wildtype Hela cells (Wt), Hela cells treated with transfection reagent only (mock), and Hela cells transfected with Alexa Fluor 488 labeled negative control siRNA (Fl siRNA) served as controls. Expression levels of α-tubulin (α-Tub) served as a household protein standard. Knockdown of synemin within Hela cells did not change the protein levels of both vinculin (Vin) and zyxin (Zyx). (F) Knockdown of synemin did not change the filamentous organization of vimentin and the localization patterns of zyxin within Dmn_4 siRNA treated Hela cells.
Figure 6. Synemin promotes cell adhesion and cell motility. (A) Hela cells with synemin knockdown (Dmn_4) exhibited compromised adhesion to the culture plates when compared with Hela cells transfected with Fl siRNA (*, p = 0.024). (B) Dmn_4 siRNA transfected Hela cells exhibited markedly compromised healing ability in the wound healing assays 48 hr after wounding. Representative photos from three independent experiments are shown. Bar, 100 μm. (C) Much fewer cells traversed the porous membrane in the modified Boyden chamber assays when Hela cells were treated with Dmn_4 siRNA than Hela cells treated with Fl siRNA did. Black dots in the images were those 8 μm pores on the membrane. Bar, 100 μm. (D) Number of cells that traversed the uncoated membranes in the modified Boyden chamber assays. HPF, high-power field.
GENERAL CONCLUSIONS

The overall goal of the research presented in this dissertation is to extend our understanding of the cellular functions of the mammalian synemin isoforms. By identifying and studying interactions of synemin isoforms with their specific protein partners, the exact role of synemin within mammalian cells could be gradually clarified. And, these efforts should provide useful information for future investigations on this large unique type VI intermediate filament protein.

Although previous results from our lab showed that avian synemin interacts with vinculin, whether the two major mammalian synemin isoforms interact with vinculin and its muscle specific isoform metavinculin has been unclear. In addition, a recent study demonstrated co-precipitation of human synemin with talin from hepatic stellate cell extracts and co-localization of synemin with talin within cultured cells. However, evidence of a direct interaction of mammalian synemin with talin has still been missing. In order to study direct interactions of mammalian synemins with vinculin/metavinculin and talin, several different approaches testing protein-protein interactions were conducted using bacterially expressed and purified recombinant synemin regions as well as purified recombinant regions from vinculin/metavinculin and talin. These studies discovered that the 312 amino acid insert (SNTIII) present only within α-synemin exhibited specific interactions with both the vinculin/metavinculin tail domain and the talin rod domain in both blot overlay assays and GST pull-down assays, whereas regions of β-synemin did not. Both the vinculin tail domain and the talin rod domain exhibited saturated interaction with SNTIII with similar affinities in ELISA solid phase binding assays. Furthermore, it was shown that vinculin and talin interact with SNTIII in a competitive manner in the ELISA solid phase binding assays, suggesting they do not bind to synemin simultaneously in vivo.

Interactions of SNTIII with both vinculin and talin were further supported by evidence that EGFP tagged SNTIII demonstrated specific co-localization with both vinculin and talin in the focal adhesions within transfected mammalian cells. In contrast, β-synemin regions showed distinct localization patterns, but without specific localization at focal adhesion sites. These results in toto indicate that α-synemin, but not β-synemin, is the isoform that interacts
specifically with vinculin/metavinculin and talin. Interestingly, analysis of the primary sequence of the SNTIII region indicates that it is composed of seven ~39 amino acid tandem repeats, with each repeat containing a specific S/T-X-R-Q/H motif. This unique primary sequence arrangement, and possibly of the corresponding unique secondary and tertiary structures, of SNTIII may be the underlying basis for its specific interactions with both vinculin and talin.

In order to extend our understanding of the cellular function of the smaller β-synemin, novel interactions of β-synemin with its protein partners were discovered by yeast two-hybrid screening assays. By using the entire β-synemin tail domain (SNβT) as the bait in screening against a human adult skeletal muscle total cDNA library, the LIM domain protein zyxin was pulled out as one of the β-synemin interacting partners. Mapping the respective binding site within these two molecules using β-galactosidase liquid culture assays indicated that synemin binds specifically to the LIM domains of zyxin, whereas the zyxin binding site within the β-synemin molecule was located within the C-terminal ~300 amino acid (SNβTII) sequence at the end of the β-synemin tail domain. Because α-synemin shares with β-synemin the same sequence containing the binding site for zyxin, it is therefore not surprising to observe interaction of α-synemin with zyxin as well. However, the presence of the unique 312 amino acid insert within α-synemin may interfere with the interaction, as reflected by a lower affinity of the α-synemin tail domain for zyxin than that of the β-synemin tail domain in the β-galactosidase liquid culture assays. In vitro protein-protein interaction assays, such as GST pull-down assays and surface plasmon resonance assays, provided further support for a direct interaction of the β-synemin tail domain with zyxin. In addition, synemin co-precipitated with zyxin from mammalian cell extracts. When expressed within mammalian cells, EGFP-tagged SNβTII led to a markedly reduced protein level of zyxin and the loss of zyxin localization at the focal adhesion sites. These results suggest synemin and zyxin associate with each other in vivo. Zyxin has been implicated as being essential in modulating actin cytoskeletons at actin-membrane interactions as well as in cell adhesion and migration. Thus, synemin may link the heteropolymeric intermediate filaments to these cellular processes within synemin-expressing cells. Indeed, novel functions of synemin in
cell adhesion and cell motility is presented in this dissertation. RNAi studies knocking down synemin expression within Hela cells, a human cervix epithelial adenocarcinoma cell line, led to markedly compromised cell adhesion and migration.

Taken *in toto*, the research presented in this dissertation demonstrates that mammalian synemins interact differentially with the cytoskeletal proteins vinculin/metavinculin, talin, and zyxin, all of which are present in cell-cell and/or cell-substratum adhesion sites, and are essential in regulating cell adhesion and migration. Thus, it is our hypothesis that synemin plays important roles in linking the heteropolymeric intermediate filaments to these adhesion sites within synemin-expressing cells. Different ratios of $\alpha$- versus $\beta$-synemin may modulate the interactions of mammalian synemins with their protein partners, and thereby regulate the strength of the linkages between the synemin-containing heteropolymeric intermediate filaments and the adhesion sites in mammalian muscle cells, and more generally, in all mammalian cells expressing synemin.
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