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Interactions of intermediate filament protein synemin with dystrophin and utrophin

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Abstract

Synemin is a unique, very large intermediate filament (IF) protein present in all types of muscle cells, which forms heteropolymeric intermediate filaments (IFs) with the major IF proteins desmin and/or vimentin. We show herein that tissue-purified avian synemin directly interacts with both dystrophin and utrophin, and that specific expressed regions of both of the mammalian (human) synemin isoforms (α-synemin and β-synemin) directly interact with specific expressed domains/regions of the dystrophin and utrophin molecules. Mammalian synemin is also shown to colocalize with dystrophin within muscle cell cultures. These results indicate that synemin is an important IF protein in muscle cells that helps fortify the linkage between the peripheral layer of cellular myofibrils and the costameric regions located along the sarcolemma and the sarcolemma region located within the neuromuscular and myotendinous junctions (NMJs and MTJs).

Keywords: Intermediate filaments; Desmin; Synemin; Dystrophin; Utrophin; Protein–protein interactions

All metazoan cells contain a cytoskeleton comprised of three types of filamentous networks, namely actin-containing microfilaments, tubulin-containing microtubules, and the intermediate filament (IF) protein network. The different IF proteins are expressed in a cell-type specific manner and form long, 10-nm diameter intermediate filaments (IFs), which are intermediate between the 6–8-nm diameter microfilaments and the 25-nm diameter microtubules [1,2]. At least 65 different IF proteins have been identified in humans [3,4]. All IF proteins contain three common structural features, namely an N-terminal head domain, a central α-helical rod domain, and a C-terminal tail domain [5]. IFs are known to play a structural role in eukaryotic cells by forming an important part of the cell cytoskeleton and providing mechanical stability to the cells [6]. The desmin-containing IFs in muscle cells are believed to encircle the Z-lines of each integral myofibril, thereby connecting all adjacent myofibrils and linking the Z-lines of the peripheral layer of cellular myofibrils to the sarcolemma at the costameres [7,8].

Synemin is an unusually large, obligate heteropolymeric IF protein that forms IFs with other major IF proteins in muscle cells such as desmin and/or vimentin [9,10]. Avian synemin (predicted molecular mass of 182.2 kDa), which was originally referred to as an intermediate filament-associated protein [9], was first identified as a bona fide IF protein, and subsequently shown to interact with desmin and vimentin [10–12]. Avian

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synemin also interacts with the actin-binding and costameric protein α-actinin, and the costameric protein vinculin [13]. The human synemin gene encodes two splice variants, α-synemin and β-synemin, which differ only in their C-terminal tail domain [14]. α-Synemin (predicted molecular mass of 172.7 kDa) is the larger isoform, containing an extra 312 amino acids located near the end of its long tail domain that are absent in the smaller β-synemin (predicted molecular mass of 140.1 kDa) [14]. Both α- and β-synemins can form heteropolymeric IFs with desmin in muscle cells [14,15]. β-Synemin (also called desmuslin) interacts with the dystrophin-related protein α-dystrobrevin [15], and localizes to the neuromuscular junctions (NMJs) and myotendinous junctions (MTJs) with α-dystrobrevin in skeletal muscle cells [15,16]. These interactions suggest that synemin may be an important protein enabling the desmin/synemin heteropolymeric IFs to link the Z-lines of the peripheral layer of myofibrils to both the costameres located periodically along and subjacent to the sarcolemma, and to the sarcolemma that is present in the NMJs and MTJs in muscle cells [13,16].

Costameres are multi-protein assemblies located subjacent to the sarcolemma in striated muscle cells and aligned with the Z-lines of the nearby peripheral layer of cellular myofibrils [17,18]. It is believed that forces generated inside a muscle cell by the contractile myofibrils are laterally transmitted via their Z-lines to the costameres at the sarcolemma, and ultimately to the extracellular matrix [18]. Dystrophin is a large protein (427 kDa) located primarily at the costameres in muscle cells [19,20]. Utrophin (395 kDa) is an autosomal homologue of dystrophin that is present primarily at the NMJs and MTJs [21]. Dystrophin and utrophin belong to the spectrin superfamily of proteins, which includes spectrin and α-actinin, and share a similar molecular structure containing four well-defined domains [19]. Dystrophin and utrophin are connected to the sarcolemma via their interactions with the dystrophin-associated protein β-dystroglycan [22,23]. The N-terminal domains, in conjunction with the rod domains, of both dystrophin and utrophin contain actin-binding sites, but dystrophin and utrophin differ in their binding affinities to γ-actin filaments [24–27]. In Duchenne Muscular Dystrophy (DMD) the interactions between dystrophin and its binding partners are absent because dystrophin is missing in muscle cells, thus causing the severe phenotype observed in DMD [19,20]. Although much research has been done, there is still no effective cure for DMD [28,29]. A long-term, effective cure for DMD may require knowledge of the exact function/s of dystrophin in muscle cells, which in turn may require elucidation and characterization of all protein interactions involving dystrophin [30].

Protein–protein interactions have recently been demonstrated between components of the IF network in muscle cells and components of the dystrophin-associated protein complex (DAPC) [31]. Two IF proteins, β-synemin and syncoilin, interact with α-dystrobrevin [15,32]. In addition, IFs containing cytokeratin K19 were reported to directly interact with the N-terminal actin-binding domain of dystrophin [33]. Our hypothesis is that the IF protein synemin directly interacts with both dystrophin and utrophin molecules. We show herein, using *in vitro* protein–protein binding assays, that avian synemin and both human synemin isoforms directly bind to dystrophin and utrophin. We have identified specific regions within the human synemin isoforms that interact with specific regions/domains within both the dystrophin and utrophin molecules. These direct interactions of synemin with dystrophin and utrophin may represent critical linkages between the peripheral layer of cellular myofibrils, and both the costameres located along and subjacent to the sarcolemma and the portion of the sarcolemma located within the NMJs and MTJs in skeletal muscle cells.

Materials and methods

Materials. The full-length mouse cDNA of dystrophin was a gift from Joel Pearlman (Baylor College of Medicine, Houston, TX). The full-length mouse cDNA of utrophin was a gift from Dr. John P. Merlie (Washington University School of Medicine, St. Louis, MO). The full-length human cDNAs for both α- and β-synemin were provided by Dr. Denise Paulin (Université Paris, Paris, France). Reagents for polymerase chain reaction (PCR) amplifications were obtained from Qiagen (Valencia, CA) and/or Bio-Rad (Hercules, CA). All primers used for PCR amplification of the regions/domains of dystrophin, utrophin, and synemin were synthesized at the DNA Facility, Iowa State University (Ames, IA). Restriction enzymes used for cloning were from Bio-Rad. Tissue-purified, full-length avian synemin was prepared in our laboratory as described previously [12]. All other chemical reagents (analytical grade) were obtained from Fisher Scientific (Hanover Park, IL) and/or Sigma (St. Louis, MO).

Synemin polyclonal antibody (pAb) 2856 was prepared as described previously [10]. Dystrophin monoclonal antibody (mAb) VIA4-2 A3 (IgM) was provided by Dr. Kevin P. Campbell's laboratory, desmin mAb was obtained from Sigma, anti-His and anti-GST mAbs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-MBP antiserum was obtained from New England BioLabs (Ipswich, MA). Secondary antibodies for Western blotting, horse-radish peroxidase (HRP)-labeled goat anti-rabbit IgG and goat anti-mouse IgG, were obtained from Sigma. Secondary fluorescent Abs, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgM, Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 546 goat anti-mouse IgG, for immunolabeling of mammalian cells were obtained from Invitrogen (Carlsbad, CA).

Cloning of cDNA constructs of dystrophin, utrophin, and synemin. The full-length mouse dystrophin cDNA was used to PCR-amplify cDNAs encoding eight consecutive dystrophin regions/domains starting with the N-terminal domain (N); five consecutive regions of the long 24 spectrin-like repeat-containing rod (R) domain (R1, R2, R3, R4, and R5); the cysteine-rich domain (CR); and the C-terminal domain (C) (Fig. 1). These cDNAs were then cloned, using standard molecular biology procedures, between *Sal*I and *Not*I sites of the bacterial fusion protein expression vectors pProEX HTb (Life Technologies, Carlsbad, CA) and pGEX-4T2 (Amersham Biosciences, Pittsburgh, PA), to be expressed as N-terminal 6X histidine (His)-tagged fusion proteins and N-terminal glutathione-*S*-transferase (GST)-tagged fusion proteins, respectively. Cloned constructs were transformed into *Escherichia coli* BL21-CodonPlus (DE3) RIL cells (Stratagene, La Jolla, CA) for further expression and purification of the fusion proteins. The full-length mouse utrophin cDNA was used to PCR-amplify cDNAs encoding eight consecutive utrophin regions/domains similar to those in dystrophin (Fig. 1). The cDNAs of the utrophin regions/domains were cloned into vectors as described for dystrophin.

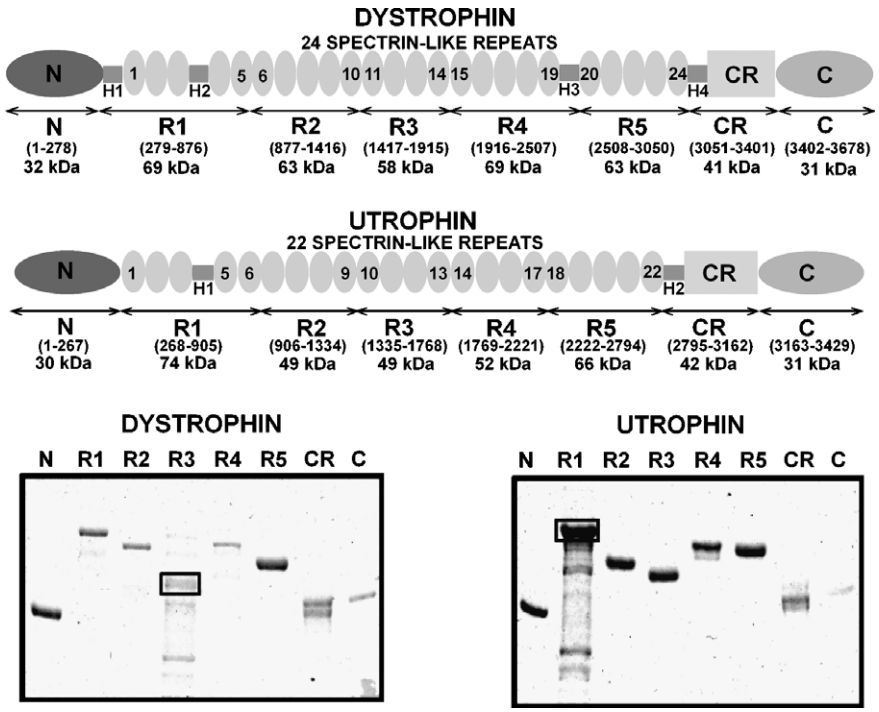

Fig. 1. Dystrophin and utrophin molecules and their fusion protein constructs used in protein–protein interaction assays. Schematic representations of dystrophin and utrophin showing their four well-defined domains: N-terminus, N; rod domain consisting of spectrin-like repeats; cysteine-rich domain, CR; and C-terminus, C are depicted. Also shown are the small proline-rich hinge regions (H) present in the rod domain (H1, H2, H3, and H4). The fusion protein constructs are depicted below the schematics of both proteins. The rod domains (R) of dystrophin and utrophin were divided into five regions containing 4–5 spectrin-like repeats each—R1, R2, R3, R4, and R5. Numbers in parenthesis below each construct refer to amino acid residues in the construct, with the N-terminus of the protein corresponding to residue number 1. Predicted molecular masses (without fusion protein tags) for each construct are also shown below the residue numbers. Coomassie blue-stained SDS–PAGE gels of purified GST-fusion protein constructs of dystrophin and utrophin are shown. Note that the construct R3 of dystrophin (boxed) is a His-tagged fusion protein. The fusion protein construct R1 of utrophin is also boxed to show the purified protein.

Based on the previously reported preparation of the fusion protein constructs of avian synemin [13], the full-length human α- and β-synemin cDNAs [14] were used to PCR-amplify the synemin rod domain (Rod), six regions of the long C-terminal tail (T) domain of α-synemin (TIA, TIB, TIIA, TIIB, TIII, and TIV), and the TII region of β-synemin (Fig. 2). As for dystrophin, these were cloned into the vector pGEX-4T2 for expression and purification as GST-fusion proteins. The synemin rod domain and α-synemin TIIB were also cloned into pMAL-c2X vector (New England BioLabs) for expression and purification as maltose-binding protein (MBP)-fusion proteins. All cloned constructs were checked for accuracy by sequencing at the DNA Facility, Iowa State University.

Expression and purification of fusion protein constructs. All the His-tagged, GST-tagged, and MBP-tagged fusion proteins of dystrophin, utrophin, and synemin were expressed in *E. coli* cells by induction with isopropyl-β-D-thiogalactopyranoside (IPTG). His-tagged fusion proteins were purified using nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen, Inc.), GST-fusion proteins were purified using glutathione–agarose resin (Sigma), and MBP-fusion proteins were purified using amylose resin (New England BioLabs) according to standard protocols supplied by the affinity resin manufacturers. Purified fusion proteins were quantified by using Bio-Rad protein assay reagent (Bio-Rad) or BCA protein assay reagent (Pierce Biotechnology, Rockford, IL).

Blot overlay assays. Blot overlay assays were performed as previously described [10,13] with slight modifications. Equal amounts of all His-tagged and/or purified GST-fusion proteins [50 or 100 pico-moles (pmoles)] of dystrophin or utrophin were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% or 10% polyacrylamide gel. The proteins were then electrophoretically transferred onto a nitrocellulose membrane (Amersham Biosciences) that was subsequently blocked by incubation in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder at 4 °C overnight. The blots were incubated with 10 μg/mL of either purified avian synemin (for His-fusion proteins of dystrophin or utrophin) or each of the purified human synemin fusion proteins (for GST-fusion proteins of dystrophin or utrophin) in PBS containing 0.1% (v/v) Tween 20 and 1% (w/v) non-fat milk powder at room temperature for 2 h, and then washed thoroughly with several fresh changes of PBS containing 0.1% Tween 20. A control blot was treated identically, but incubated with buffer alone. Western blot analysis was done to detect any protein interactions with synemin pAb 2856 or with anti-MBP antiserum (in the case of overlays using MBP-synemin fusion proteins), diluted 1:25,000 or 1:50,000 in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder. Protein interactions were detected by chemiluminescence using ECL reagent (Amersham Biosciences).

GST pull-down assays. Each of the purified GST-fusion proteins (100 pmoles) of dystrophin or of utrophin was separately bound to pre-equilibrated glutathione agarose beads (100 μL of 50% slurry) in GST pull-down (GPD) buffer (20 mM Tris–HCl, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.2% v/v Triton X-100, and 0.2% v/v NP-40, pH 7.5) at 4 °C for 4–6 h. Simultaneously, purified MBP-fusion proteins of synemin were pre-cleared by mixing them with an excess of glutathione–agarose beads. After washing the beads that had been loaded with GST-fusion proteins of dystrophin or utrophin, the pre-cleared MBP-fusion proteins (~100 pmoles) of synemin were mixed with them in excess GPD buffer at 4 °C overnight. The beads were then washed thoroughly with GPD buffer, and proteins were extracted in standard SDS–PAGE loading buffer by boiling the beads. The extracted proteins were subjected to SDS–PAGE, and Western blot analysis was carried out with anti-MBP antiserum to determine if the MBP-fusion proteins of synemin bound to any of the

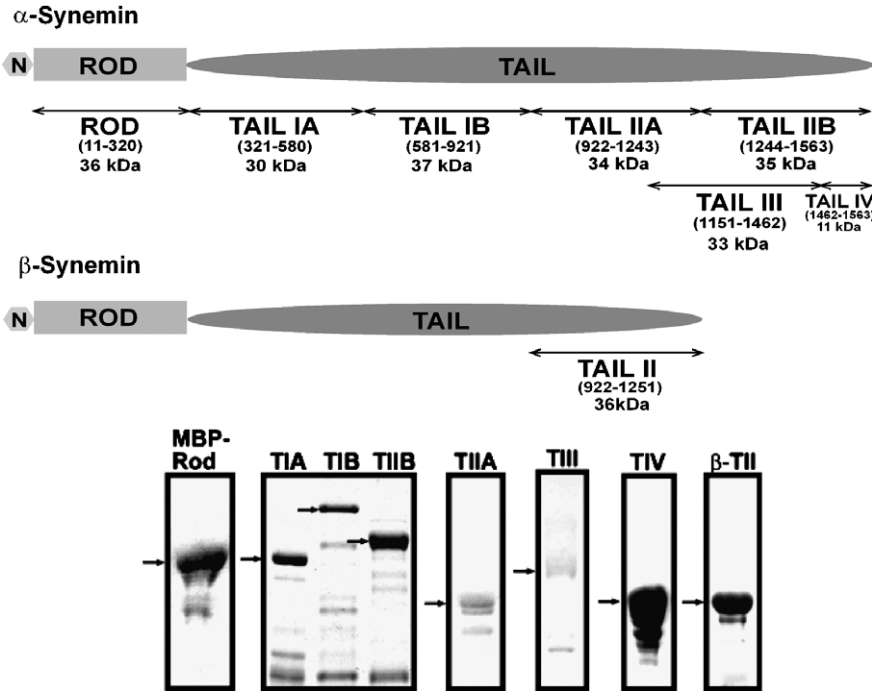

Fig. 2. Human synemin molecules and their fusion protein constructs used in the protein–protein interaction assays. Schematic representations of both α-synemin and β-synemin molecules showing their three domains: N-terminus, N; rod domain, Rod; and tail domain, Tail are depicted. The fusion protein constructs are depicted below the schematics. The tail domain (T) of α-synemin was divided into six regions—TIA, TIB, TIIA, TIIB, TIII, and TIV. The tail domain of β-synemin, which is 312 a.a. smaller than the tail domain of α-synemin, contains TIA, TIB, and TII. Numbers in parenthesis below each construct refer to amino acid residues in the construct, with the N-terminus of the protein corresponding to residue number 1. Predicted molecular masses (without fusion protein tags) for each construct are shown below the residue numbers. Coomassie blue-stained SDS–PAGE gels (on separate gels) of purified GST-fusion protein constructs (MBP-fusion protein for synemin rod) of the synemin molecules are shown. Small arrows point to the correct fusion protein constructs on the gels.

GST-fusion proteins of dystrophin or utrophin. GST protein was also used in the assay as a negative control.

Immunofluorescent labeling of A-10 cells. A-10 cells (American Type Culture Collection, Manassas, VA), derived from embryonic rat aorta smooth muscle, were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) (all reagents from Mediatech, Herndon, VA). For immunolabeling, the cells were grown on collagen-coated coverslips until ~70% confluency. The cells were then washed with PBS and fixed with 2% (v/v) formaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 3 min at 25 °C. After thoroughly washing with PBS, the cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 7–8 min at room temperature. The cells were again thoroughly washed with PBS, and blocked with 5% (w/v) bovine serum albumin (BSA) in PBS at 4 °C overnight. The fixed, permeabilized, and blocked cells on coverslips were then incubated with anti-synemin 2856 pAb plus anti-dystrophin VIA4-2 A3 mAb, or with anti-synemin 2856 pAb plus anti-desmin mAb diluted in PBS with 5% BSA for 2 h at 25 °C. As a negative control, cells were also incubated in PBS with 5% BSA without any Abs. After washing, the cells were incubated with the appropriate fluorescent secondary antibodies for 90 min at 25 °C. The negative controls were also incubated with each of the secondary Abs corresponding to those used for synemin and dystrophin, or synemin and desmin. The coverslips were then thoroughly washed with PBS mounted onto glass slides with Immuno Fluore mounting media (ICN Biomedicals, Irvine, CA), and observed on a Zeiss Photomicroscope III at the Iowa State University College of Agriculture Microscopy Facility. The images were captured by a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI), attached to the microscope and prepared by using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Results

Cloning, expression, and purification of fusion protein constructs

Because dystrophin and utrophin are very large proteins, they were divided into smaller, non-overlapping, consecutive regions/domains in order to test direct and specific interactions of dystrophin and utrophin with synemin. Dystrophin regions/domains include the N-terminus (N), five different consecutive regions containing 4 or 5 repeats each of the long, 24 spectrin-like repeat-containing rod (R) domain (R1, R2, R3, R4, and R5), the cysteine-rich domain (CR), and the C-terminus (C) (Fig. 1). The cDNAs corresponding to each of these regions/domains of dystrophin were bacterially expressed as either His- or GST-tagged fusion proteins. Because utrophin is similar in general structure to dystrophin, it was divided in the same way, and different regions/domains of utrophin were also expressed as either His- or GST-tagged fusion proteins (Fig. 1). Purified GST-fusion proteins of dystrophin and utrophin are shown in Fig. 1 on Coomassie blue-stained SDS–PAGE gels. All the His-fusion proteins and purified GST-fusion proteins of dystrophin and utrophin were then used for *in vitro* protein–protein interaction studies.

In order to map interactions of specific regions of synemin with the different regions/domains of both dystrophin and utrophin, the human synemin proteins were also divided into smaller pieces. Human α-synemin regions include the rod domain (Rod) and six different regions of the long tail (T) domain (TIA, TIB, TIIA, TIIB, TIII, and TIV) (Fig. 2). Human β-synemin is identical to human α-synemin, except for a 312 a.a. stretch in the C-terminal tail region (TIII) found in α-synemin [14]. Therefore, a separate tail region (TII) specific to β-synemin was also prepared (Fig. 2). The cDNAs corresponding to each of these regions of the human synemin isoforms were expressed and purified as GST-fusion proteins. The rod domain of synemin was expressed and purified as an MBP-fusion protein because the GST-fusion protein was not very soluble. Purified MBP-fusion protein of the rod domain and purified GST-fusion proteins of both human synemins are shown in Fig. 2 on Coomassie blue-stained SDS–PAGE gels. Purified MBP-fusion protein of the rod domain and all GST-fusion proteins of human synemin isoforms were then used for *in vitro* protein–protein interaction studies.

Interactions of synemin with dystrophin and utrophin

Blot overlay assays were used to test interactions between full-length avian synemin, and specific regions/domains of dystrophin and utrophin as described in the methods. Avian synemin bound to His-fusion proteins R4 and CR of dystrophin, and weakly to R3 (Fig. 3B). An identical control blot overlaid with only buffer solution showed no binding (result not shown). Avian synemin bound significantly to His-fusion protein CR and weakly to the N-terminal domain of utrophin (Fig. 3D). An identical control blot overlaid with only buffer solution showed no binding (result not shown). These results indicate that avian synemin directly interacts with specific regions of the dystrophin and utrophin proteins.

Because of the potential implications of interactions between synemin and both dystrophin and utrophin for DMD research, fusion protein constructs of human α- and β-synemin were utilized for the remainder of the experiments. Avian synemin molecule is homologous to human α-synemin, but is only 37% identical in amino acid sequence to α-synemin [14]. The rod domains of avian synemin and α-synemin are 46% homologous, and the long C-terminal tail domains are only 40% homologous [14]. Thus, binding of the two human synemin molecules to dystrophin and utrophin was expected to be somewhat different than that obtained with avian synemin.

Blot overlay assays were employed to test interactions between specific regions/domains of dystrophin and specific fragments of human synemin molecules as described in Materials and methods. All of the purified GST-fusion proteins of dystrophin on blots were overlaid, in separate experiments, with each of the purified GST-fusion proteins of human synemins. One of the blots was overlaid with purified MBP-fusion protein of the synemin rod domain. GST-α-synemin TIIB interacted moderately with

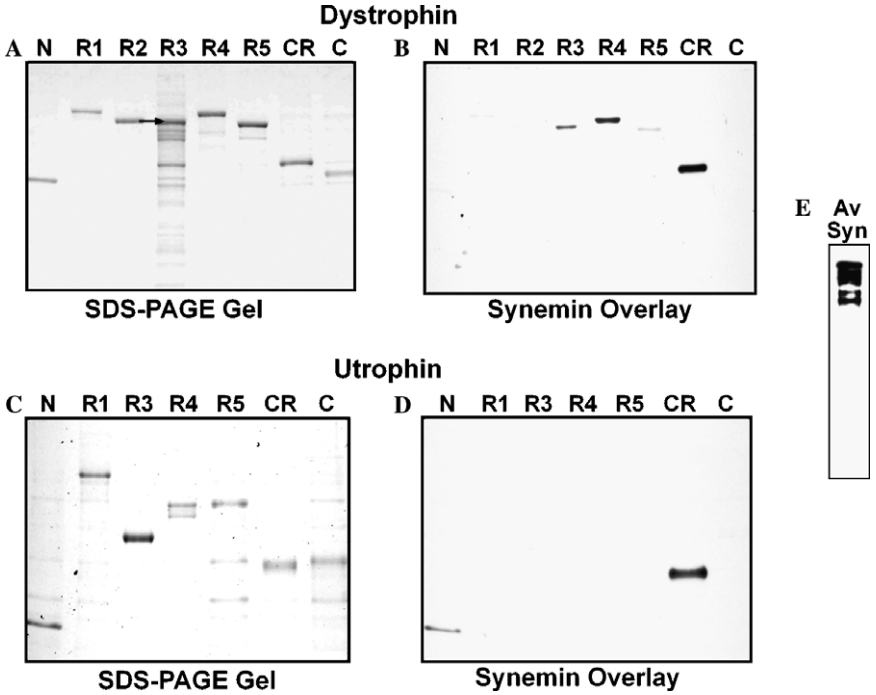

Fig. 3. Blot overlay analysis of interactions of purified avian synemin with bacterially expressed His-tagged fusion proteins of dystrophin and utrophin. (A) Coomassie blue-stained SDS–PAGE of His-tagged fusion proteins (bacterial cell lysates) of dystrophin (arrow points to R3), and (C) Coomassie blue-stained SDS–PAGE of His-tagged fusion proteins (bacterial cell lysates) of utrophin. (B) His-tagged fusion proteins of dystrophin, as shown in (A), and (D) His-tagged fusion proteins of utrophin, as shown in (C), on blots were overlaid with purified full-length avian synemin separately. The blots were then probed with anti-synemin 2856 pAb. Avian synemin showed no binding to utrophin R2 (not shown). (E) Avian synemin (Av Syn) is recognized by the anti-synemin 2856 pAb in a Western blot. Control blots for both assays were treated identically, but overlaid with buffer only (not shown).

GST-dystrophin CR (Fig. 4A), and the MBP-synemin rod interacted moderately with GST-dystrophin R3 (Fig. 5A). In all the blot overlay assay experiments, identical control blots were overlaid with only buffer solution. The fusion proteins on the control blots did not show any binding to the pAb itself (Figs. 4B and 5B). Purified GST protein was also included on the blots as a negative control. A control blot of all purified GST-fusion proteins of dystrophin overlaid with purified MBP protein showed no binding (result not shown). All the blot overlay assays were performed in buffer with approximately physiological pH (7.4) and ionic strength (0.17). The results of all the blot overlay assays (each repeated at least three times) with all the GST-fusion proteins of dystrophin overlaid with each of the synemin fusion proteins showed that the synemin rod domain interacted with the rod domain region R3 of dystrophin, and α-synemin TIIB interacted with the CR region of dystrophin. None of the other regions of the human synemin molecules showed any binding to the regions/domains within dystrophin (results not shown). These findings indicated that both the human synemin proteins interacted with dystrophin.

Blot overlay assays were also done with all of the purified GST-fusion proteins of utrophin overlaid with each of the purified GST-fusion proteins of synemin and with the purified MBP-synemin rod domain. GST-α-synemin TIIB interacted with GST-utrophin R1 and GST-utrophin CR (Fig. 4C). MBP-synemin rod interacted with GST-utrophin R3 (Fig. 5C). All the same controls and conditions were employed for these blot overlay assay experiments (Figs. 4D and 5D), as with those done with the dystrophin fusion proteins. The results of all the blot overlay assays (each repeated at least three times) with all the GST-fusion proteins of utrophin overlaid with each of the synemin fusion proteins showed that the synemin rod domain interacted with the rod domain region R3 of

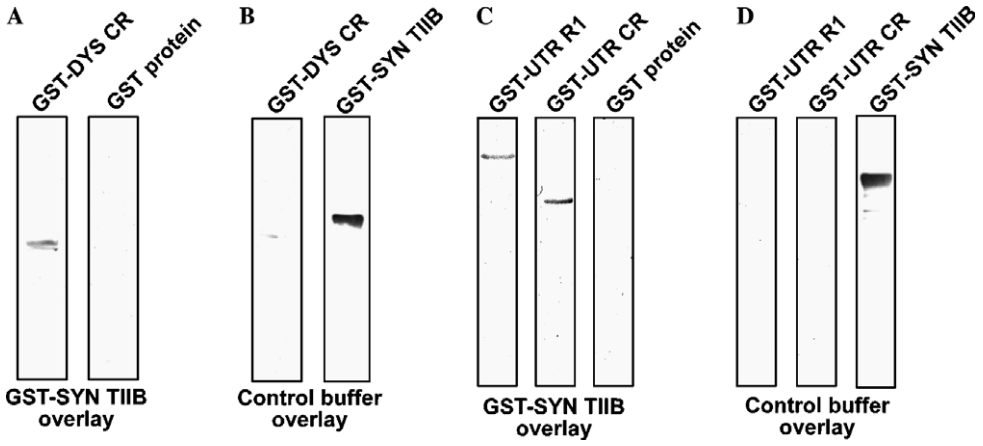

Fig. 4. Blot overlay analysis of interactions of GST-α-synemin TIIB with GST-fusion proteins of dystrophin and utrophin. (A) Purified GST-fusion proteins of dystrophin and (C) purified GST-fusion proteins of utrophin on blots were overlaid with purified GST-α-synemin TIIB protein separately. The blots were then probed with anti-synemin pAb 2856. The GST protein was used as a negative control. (B,D) Identical control blots were overlaid with buffer only and probed with anti-synemin pAb 2856. The pAb recognized GST-α-synemin TIIB (GST-SYN TIIB) (positive control).

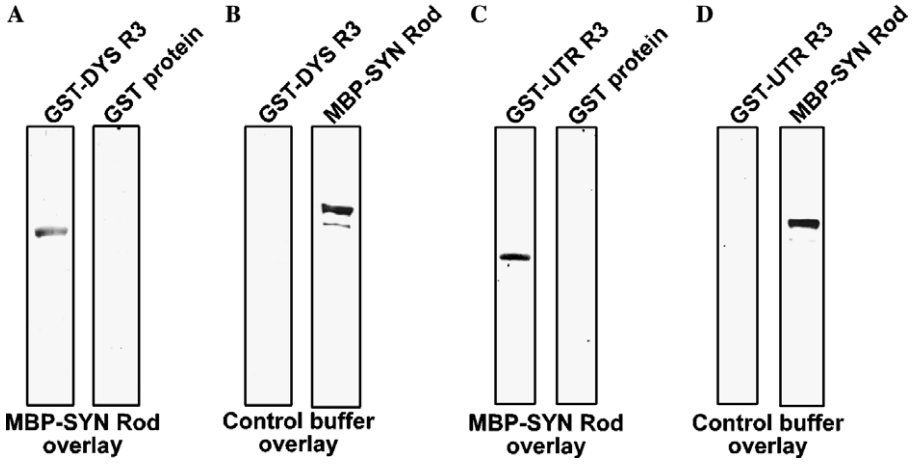

Fig. 5. Blot overlay analysis of interactions of MBP-synemin rod with GST-fusion proteins of dystrophin and utrophin. (A) Purified GST-fusion proteins of dystrophin and (C) purified GST-fusion proteins of utrophin on blots were overlaid with purified MBP-synemin rod protein separately. The blots were then probed with anti-MBP antiserum. The GST protein was used as a negative control. (B,D) Identical control blots were overlaid with buffer only and probed with anti-MBP antiserum. The antiserum recognized MBP-synemin rod (positive control).

utrophin, and α-synemin TIIB interacted with the rod domain region R1 and the CR region of utrophin. None of the other regions of the human synemin molecules showed any binding to the regions/domains within utrophin (results not shown). These findings indicated that both the human synemin proteins interacted with utrophin. These results also showed that there were more binding regions between α-synemin and utrophin (three) than between α-synemin and dystrophin (two).

The cysteine-rich regions of both dystrophin and utrophin are very homologous and also share a common binding partner, β-dystroglycan. It has been shown that β-dystroglycan binds more strongly to dystrophin than to utrophin [23]. Blot overlay assays were done to evaluate whether the binding of α-synemin TIIB was the same, or different, for dystrophin and utrophin. Equal amounts of GST-dystrophin CR and GST-utrophin CR on the blot were overlaid with GST-α-synemin TIIB, and it bound more strongly to GST-utrophin CR than to GST-dystrophin CR (Fig. 6). This result showed that, similar to the β-dystroglycan binding, there were differences in binding of α-synemin to the dystrophin and utrophin molecules.

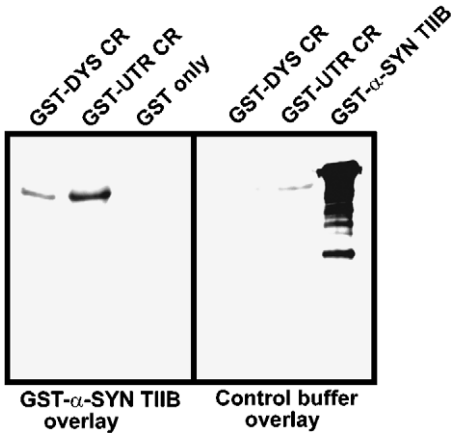

Fig. 6. Blot overlay analysis of the comparison of interactions of GST-dystrophin CR and GST-utrophin CR proteins with GST-α-synemin TIIB. Equal amounts of purified GST-dystrophin CR and GST-utrophin CR on the blot were overlaid with purified GST-α-synemin TIIB protein as shown in the left panel of the figure. The blot was then probed with anti-synemin pAb 2856. The GST protein was used as a negative control. An identical control blot as shown in the right panel was overlaid with buffer only and probed with anti-synemin pAb 2856. The pAb recognized GST-α-synemin TIIB (positive control).

GST pull-down assays were performed as described in Materials and methods in order to compare them with the interactions observed in the blot overlay assays. Each of the purified GST-dystrophin fusion proteins was used to pull down purified MBP-synemin rod. Only GST-dystrophin R3 was able to pull down the MBP-synemin rod (Fig. 7A), which confirmed the result obtained in the blot overlay assay (Fig. 5A). A control consisting of GST protein alone did not bind to MBP-synemin rod. A control GST pull-down assay of the GST-dystrophin fusion proteins with MBP protein alone did not show any binding (result not shown). Each of the purified GST-utrophin fusion proteins was used to pull down purified MBP-synemin rod. Only GST-utrophin R3 was able to pull down the MBP-synemin rod (Fig. 7B), confirming the result observed for the blot overlay assay (Fig. 5C). The same controls used for the dystrophin fusion proteins were also employed in the GST pull-down assays of the utrophin fusion proteins. Each of the purified dystrophin fusion proteins, and each of the purified utrophin fusion proteins were used to pull down purified MBP-α-synemin TIIB. Only GST-utrophin R1 was able to pull down MBP-α-synemin TIIB (Fig. 7C). This confirmed the interaction of utrophin R1 with α-synemin TIIB observed in the blot overlay assay (Fig. 4C). Results observed with the GST pull-down assays confirmed all of the specific interactions detected by the blot overlay assays between regions of synemin (rod and α-synemin TIIB), and regions/domains of

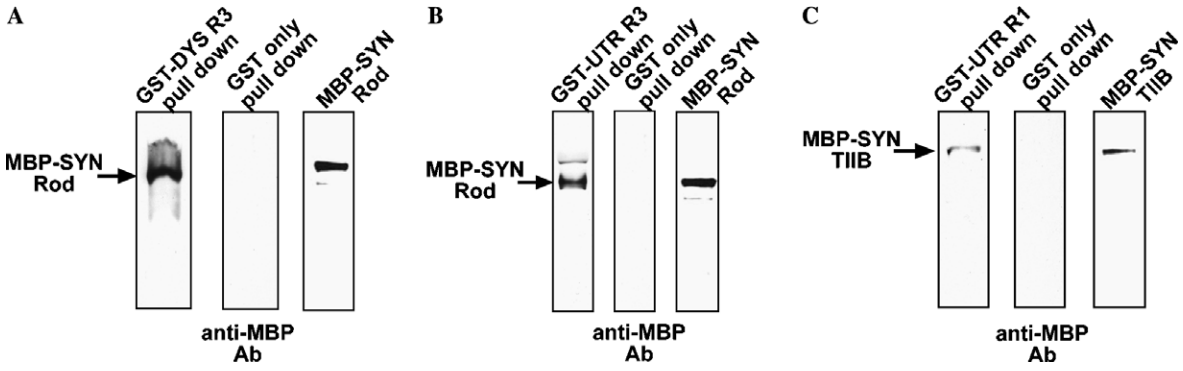

Fig. 7. GST pull-down analysis of the interactions of GST-dystrophin and GST-utrophin fusion proteins with MBP-synemin rod and MBP-α-synemin TIIB. (A,B) Purified GST-fusion proteins of dystrophin and utrophin were bound to glutathione–agarose beads and then mixed with MBP-synemin rod separately. The proteins were then extracted from the beads and Western blotted with anti-MBP antiserum. The GST protein was used as a negative control. The anti-MBP antiserum recognized MBP-synemin rod (MBP-SYN Rod) (positive control). (C) Purified GST-fusion proteins of utrophin were bound to glutathione agarose beads and then mixed with MBP-α-synemin TIIB. The proteins were then extracted from the beads and Western blotted with anti-MBP Ab. The GST protein was used as a negative control. The anti-MBP antiserum recognized MBP-α-synemin TIIB (MBP-SYN TIIB) (positive control).

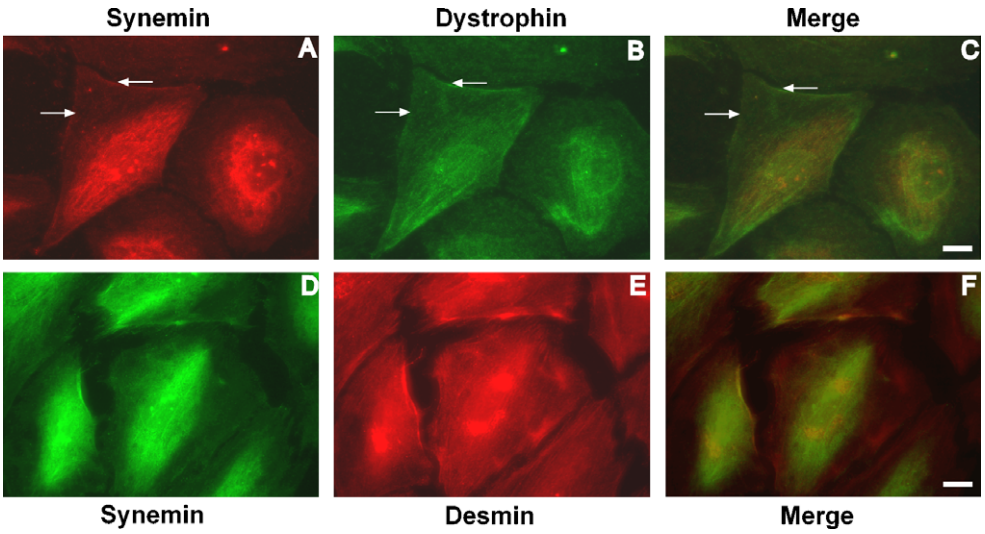

Fig. 8. Colocalization of synemin and dystrophin in rat smooth muscle A-10 cells. Panels on the left (A,D) show immunofluorescence labeling of synemin with synemin pAb 2856. Panels in the middle show immunofluorescence labeling of dystrophin (B) with dystrophin mAb VIA4-2 A3 and of desmin (E) with desmin mAb. Panels on the right (C,F) are merged images of (A,B) and (D,E), respectively. The arrows in (A–C) point to the same position, and show that synemin colocalizes with dystrophin at the cell membrane in A-10 cells. Also as shown in (D–F) synemin colocalizes with desmin (the major IF protein) in A-10 cells (positive control). Bar, 20 μm.

dystrophin (R3) and utrophin (R1 and R3), with the exception of the interaction between α-synemin TIIB and the CR regions of dystrophin and utrophin.

Immunofluorescence colocalization of synemin and dystrophin in cultured muscle cells

The localization of synemin and dystrophin was studied *in vivo* in rat aorta (A-10) cells by indirect immunofluorescence microscopy. The A-10 cells were double-immunolabeled for synemin and dystrophin with anti-synemin pAb 2856 and anti-dystrophin mAb VIA4-2 A3. Synemin colocalized with dystrophin at the cell edges (cell membranes) (Fig. 8A–C). Control cells immunolabeled with only the secondary antibodies did not show any specific staining pattern (result not shown). Because synemin forms heteropolymeric IFs with desmin in muscle cells [8,13], a positive control was included in which the A-10 cells were double-immunolabeled for both synemin and desmin using anti-synemin pAb 2856 and anti-desmin mAb. Synemin colocalized with desmin in the A-10 cells as expected (Fig. 8D–F). These results show that synemin clearly is colocalized with dystrophin *in vivo* in the muscle cell cultures, and are in concert with the binding results observed between synemin and dystrophin in the *in vitro* protein–protein interaction assays.

Discussion

We demonstrate herein for the first time that synemin interacts directly with regions/domains within both dystrophin and utrophin. The unique IF protein synemin is known to form obligate heteropolymeric IFs with the major IF proteins desmin and/or vimentin in muscle cells [10,12,14]. These IFs have been shown to encircle and connect the myofibrillar Z-lines of all the adjacent myofibrils in a muscle cell, and to connect the Z-lines of the peripheral layer of myofibrils to the sarcolemma at the costameres [7]. It was previously shown that avian synemin interacts directly with the actin-binding protein α-actinin and with the costameric protein vinculin [10,13]. It was reported that human β-synemin, which interacts directly with the dystrophin-associated protein α-dystrobrevin [15], is localized to the costameres, NMJs, and MTJs in skeletal muscle cells [16].

We show herein (Fig. 3) that tissue-purified avian muscle synemin binds directly to specific regions/domains within both dystrophin and utrophin molecules. The common rod domain of the α- and β-synemins interacts with specific spectrin-like repeat regions within the long rod domains of both dystrophin (R3) and utrophin (R3) (Figs. 5A and C, 7A and B). The basic spectrin-like repeat region of dystrophin (R3) that reportedly binds to actin [25] is the same region that binds to the synemin rod domain. The rod domain of β-synemin (desmuslin) also interacts with the dystrophin-associated protein, α-dystrobrevin [15]. Thus, the rod domain of synemin has binding regions for dystrophin and for one of the dystrophin-associated proteins. The C-terminal region (TIIB) of the tail domain of α-synemin interacts with the cysteine-rich domains of both dystrophin and utrophin (Fig. 4A and C), with the CR domain of utrophin binding more strongly than the CR domain of dystrophin (Fig. 6). This is somewhat analogous to the different binding affinities of the dystrophin and utrophin CR domains for β-dystroglycan [23]. The C-terminal region (TIIB) of the tail domain of α-synemin also binds to the spectrin-like repeat region (R1) within the rod domain of utrophin (Figs. 5C and 7C). This suggests that the binding of α-synemin and utrophin may be complex in nature.

α-Synemin has two binding sites for both dystrophin and utrophin, whereas β-synemin has only one, i.e., the rod domain.

Costameres, NMJs, and MTJs are sites of multi-protein assemblies located subjacent to the sarcolemma. Direct interactions of the IF protein synemin with both dystrophin and utrophin shown herein help connect the peripheral layer of cellular myofibrils to the costameric region located along and subjacent to the sarcolemma, and to the sarcolemma located in the NMJs and MTJs. Thus, these interactions may play an important role in maintaining the overall cytoskeletal integrity of the muscle cells.

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