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THE 16kDa SUBUNIT OF VACUOLAR H⁺-ATPase IS A NOVEL SARCOGLYCAN-INTERACTING PROTEIN

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SUMMARY

The sarcoglycan complex in muscle consists of α -, β -, γ - and δ -sarcoglycan and is part of the larger dystrophin-glycoprotein complex (DGC), which is essential for maintaining muscle membrane integrity. Mutations in any of the four sarcoglycans cause limb-girdle muscular dystrophies (LGMD). In this report, we have identified a novel interaction between δ -sarcoglycan and the 16kDa subunit c (16K) of vacuolar H⁺-ATPase. Co-expression studies in heterologous cell system revealed that 16K interacts specifically with δ -sarcoglycan and the highly related γ -sarcoglycan through the transmembrane domains. In cultured C2C12 myotubes, 16K forms a complex with sarcoglycans at the plasma membrane. Loss of sarcoglycans in the sarcoglycan-deficient BIO14.6 hamster destabilizes the DGC and alters the localization of 16K at the sarcolemma. In addition, the steady state level of β_1 -integrin is increased. Recent studies have shown that 16K also interacts directly with β_1 -integrin and our data demonstrated that sarcoglycans, 16K and β_1 -integrin were co-immunoprecipitated together in C2C12 myotubes. Since sarcoglycans have been proposed to participate in bi-directional signaling with integrins, our findings suggest that 16K might mediate the communication between sarcoglycans and integrins and play an important role in the pathogenesis of muscular dystrophy.

INTRODUCTION

Sarcoglycans consists of a family of six single-pass transmembrane proteins, designated α -, β -, γ -, δ -, ε - and ζ -SG [1-7]. γ -, δ - and ζ -SG share significant sequence similarity while α -SG is highly related to ε -SG. In skeletal muscle, α -, β -, γ - and δ -SG are assembled into the sarcoglycan complex via a stepwise pathway [8]. β -SG and δ -SG form a central core structure that is essential for the assembly and targeting of the sarcoglycan complex to the muscle membrane [9;10]. Mutations in the genes encoding any of the four muscle sarcoglycans cause limb-girdle muscular dystrophies (LGMD) type 2C-F, commonly known as sarcoglycanopathies [2-4;11;12]. At the sarcolemma, the sarcoglycan complex is part of the larger dystrophin-glycoprotein complex (DGC), which provides a transmembrane linkage between the actin cytoskeleton and the extracellular matrix (ECM) and is critical for muscle membrane integrity [13]. The other members of the DGC include dystrophin, α/β -dystroglycans, syntrophin, dystrobrevin and sarcospan [14;15].

Currently, the function of sarcoglycans is not completely understood. Genetic ablation of sarcoglycans in animal models causes instability of the dystrophin-mediated transmembrane linkage in muscle [16-19], suggesting that sarcoglycans play a critical role in the stability of the DGC. Supporting this idea, we have previously demonstrated that the β/δ -SG core interacts with dystrophin [20]. Recently, sarcoglycan are also thought to have non-structural functions, such as, bi-directional signaling with integrins. It has been shown that sarcoglycans associate with $\alpha_5\beta_1$ -integrin in rat L6 myocytes [21] and co-localize with $\alpha_7\beta_1$ -integrin to costameres in human muscle fibers [22]. In skeletal muscle, $\alpha_7\beta_1$ -integrin is the primary laminin receptor in the ECM. The proper maintenance of this integrin- mediated transmembrane linkage is critical for muscle cell survival [23]. Mutations in α_7 -integrin have been reported to cause other forms of muscular

dystrophies in human [24] and animal models [25]. In support of the existence of a bi-directional communication between sarcoglycans and integrins, several animal studies have demonstrated a functional redundancy between the two complexes for the maintenance of muscle membrane stability. For example, α_7 -integrin is up-regulated in sarcoglycan-deficient γ -SG-null mice [18]. Increased expression of $\alpha_7\beta_1$ -integrin in muscle can reduce the dystrophic phenotypes in dystrophin-null *mdx* mice [26]. Moreover, double knockout mice in both α_7 -integrin and γ -SG (or dystrophin) develop severe muscle degeneration and cause pre-mature death [18;27;28].

In this study, we searched for novel sarcoglycan-interacting proteins with the hope of gaining insights into the function of the sarcoglycan complex. Recently, a novel interaction between δ -SG and the 16kDa subunit c (16K) of the vacuolar H^+ -ATPase (V-ATPase) has been documented in the protein interaction database from CuraGen Corporation. The V-ATPase is a multi-subunit protein complex that functions as an ATP hydrolysis driven proton pump essential for acidification of intracellular compartments. The complex is commonly found in endosomes and lysosomes [29] but is also present on the cell surface to secrete protons to the extracellular space in other highly specialized cell types, including kidney tubule cells and osteoclasts [30]. We demonstrated that a sub-population of 16K in C2C12 myotubes is localized at the plasma membrane and associates with the sarcoglycan complex via the transmembrane and/or intracellular domains. In addition to co-immunoprecipitate with 16K, sarcoglycans were also shown to co-immunoprecipitate with β_1 -integrin as previously reported by Yoshida et al [21]. In sarcoglycan-deficient BIO14.6 hamsters, loss of the sarcoglycan complex affects the localization of 16K but not the other subunits of V-ATPase in muscle. We also confirmed that the DGC is destabilized [31] and the steady state level of β_1 -integrin is increased. Up-regulation of integrins has been reported in animal models deficient in sarcoglycans and in muscular dystrophy patients

[18;27;28;32]. Previous studies in L6 myoblasts have shown that 16K and β_1 -integrin act coordinately independent of V-ATPase to induce signals controlling cell adhesion [33-35]. In vitro binding assay using epitope-tagged 16K and β_1 -integrin revealed that they can directly bind to each other [33]. Since we have demonstrated that 16K also interacts with the sarcoglycan complex, it raises the possibility that 16K is involved in mediating the communication between sarcoglycans and integrins. The characterization of 16K in muscle and its interaction with the sarcoglycan complex should generate novel insights into the molecular mechanisms underlying muscular dystrophy.

MATERIALS AND METHODS

DNA constructs. Full-length cDNA encoding mouse α -, β -, γ -, or δ -SG was subcloned into the pDEST mammalian expression vector (Invitrogen, Carlsbad, CA) as previously described [10]. HSV-tagged human 16K and the deletion mutant (TM4) were described in a previous study [33].

Primary antibodies. Antibodies used in this study are listed in Supplementary Fig. 1. Rabbit polyclonal antibodies against mouse γ -SG (ANA- γ) and 16K (AW-1) have been previously described in other studies [20;36]. Rabbit polyclonal anti-16K antibody (UGMS-1) was generated by Anaspec (San Diego, CA) using peptide against human 16K sequence ³⁶KSGTGIAAMSVMRPEQ⁵¹ in the extracellular domain.

Animals. Male BIO14.6 Syrian hamsters and F1-B wild-type controls were purchased from BioBreeders (Watertown, MA). Animals were maintained in the animal facility according to guides outlined by the Association for Assessment and Accreditation of Laboratory Animal Care

International (AAALAC). Animal studies were approved by the Geisinger Clinic Institutional Animal Care and Use Committee (IACUC).

Cell culture and transient transfection. COS-1 cells and mouse C2C12 myoblasts were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Invitrogen), 100 $\mu\text{g}\cdot\text{ml}^{-1}$ streptomycin, and 100U $\cdot\text{ml}^{-1}$ penicillin (Sigma, St. Louis, MO). Cell cultures were incubated at 37 $^{\circ}\text{C}$ with 5% CO_2 . To induce differentiation into myotubes, C2C12 myoblasts were grown to confluency and the media was switched to DMEM supplemented with 2% horse serum (Invitrogen). Plates were pre-coated with 20 $\mu\text{g}\cdot\text{ml}^{-1}$ laminin (Sigma). Sarcoglycan and 16K constructs were transiently transfected into COS-1 cells by Lipofect Amine reagent (Invitrogen) according to the manufacturer's protocol. Transfected cells were harvested 48h post-transfection. 16K construct was transfected into C2C12 myoblasts using Nucleofactor technology developed by Amaxa (Gaithersburg, MD). Transfected C2C12 myoblasts were switched to differentiation media 24h post-transfection and harvested 72h later. Total proteins were extracted in M-PER extraction buffer (Pierce, Rockford, IL) according to the manufacturer's protocol. All extraction buffers used in this study contain 1x protease inhibitor cocktail (Roche, Indianapolis, IN).

RT-PCR. Total RNA was extracted from mouse skeletal muscle, mouse kidney, cultured C2C12 myotubes and rat cultured Schwann cells using the RNeasy Mini Kit from QIAGEN according to the manufacturer's protocol (Valencia, CA). The 16K PCR fragment was generated using primers (forward) 5'-ACCCGCAGACATGTCCGAGT and (reverse) 5'-ACGAATAGTCGGGGCTGC at annealing temperature of 60 $^{\circ}\text{C}$.

Muscle membrane preparation. Mouse skeletal muscle (400mg) or C2C12 myotubes were homogenized by a Potter-Elvehjem homogenizer on ice for 2min with 4ml of pyrophosphate buffer (20mM sodium pyrophosphate, 20mM sodium phosphate, 1mM MgCl₂, 0.5mM EDTA, and 10% sucrose, pH7.1). The mixture was centrifuged at 500g for 10min at 4⁰C to remove cell debris. The supernatant was then centrifuged at 18,000g for 10min to collect the pellet, which contains crude membrane vesicles. Membrane proteins were extracted from the pellet with 2ml of TX-100 buffer (50mM Tris pH8.0, 150mM NaCl, 1% Triton X-100, and 0.1% SDS).

Co-immunoprecipitation. Muscle membrane fraction or total cell lysate from cultured cells was first pre-cleared with 20μl of protein G-sepharose beads (Sigma) for 1h at 4⁰C. The pre-cleared cell lysate was incubated with antibody on ice for 4h and then with 20μl of protein G-sepharose beads for 1h. The immune complex was collected by centrifugation at 10,000g for 1min and washed three times with 0.5ml of PBS with 0.1% Tween20. The final pellet was solubilized in 30μl of protein sample buffer (Invitrogen) and loaded on Novex pre-cast 4-20% SDS-PAGE gels (Invitrogen). Co-immunoprecipitation experiments were performed three times to confirm the results.

Immunofluorescent analysis. C2C12 myotubes were fixed in methanol at -20⁰C for 10min and treated with 0.5% Triton X-100 in PBS for 10min followed by blocking with 20% normal goat serum in PBS. Cells were incubated with primary antibody in blocking buffer overnight at 4⁰C and then with secondary antibodies conjugated with Alexa488 or Alexa594 for 1hr. Slides were mounted with Vectashield medium and examined by Leica SP2 laser scanning microscope

(Leica, Exton, PA). Cells were sectioned into serial optical sections of 0.16 μ m. Images were processed with Leica confocal software.

Immunohistochemistry. Unfixed frozen sections (10 microns) of skeletal muscle were treated with 0.5% Triton X-100 in PBS for 10min followed by blocking with 20% normal goat serum in PBS. Sections were stained with primary antibody diluted in the same buffer for 2h at room temperature followed by washing three times in PBS and incubating with secondary antibodies conjugated with Alexa488 or Alexa594 (Invitrogen) for 1h. After additional three 20min washes in PBS, sections were mounted on coverslips under Vectashield (Vector Laboratories). Slides were examined by Nikon Eclipse E800 inverted fluorescence microscopy (Nikon, Melville, NY) and photographed at 40x magnification using a CCD camera at standard gain and exposure time. Images were processed by MetaMorph Imaging System (Universal Imaging Corporation, Western Chester, PA).

Immunoelectron microscopy. Quadriceps femoris muscle from normal mouse was fixed in 0.1% glutaraldehyde and 2% paraformaldehyde in PBS for 30min. The specimen was further trimmed into 2 mm stripes, washed with cool PBS, dehydrated with cold alcohol and infiltrated with Lowicryl K4M medium (EM Sciences, Hatfield, PA) at -25°C . Samples were embedded in fresh Lowicryl K4M and polymerized with UV light (365nm) for 5 days at -25°C . Ultrathin sections were cut with a diamond knife, mounted on Formvar-coated Nickel grids, incubated overnight at 4°C with anti-16K antibody (UGMS-1), followed by 15-nm gold-conjugated secondary antibody (Ted Pella, Redding, CA). After 4 times wash with PBS, the sections were fixed with 2% glutaraldehyde, and contrasted with uranyl acetate and lead citrate, and analyzed

with a FEI Tecnai-T12 electron microscope operated at 80kv. Digital images were captured with a Gatan 2K camera.

RESULTS

Identification of 16K as a potential sarcoglycan-interacting protein

Recent advances in bioinformatics have led to development of numerous protein interaction databases. In order to identify novel proteins that interact with sarcoglycans, we searched the Drosophila Interaction Database from CuraGen Corporation. This database documented more than 20,000 protein interactions derived from yeast two-hybrid screening of the *Drosophila melanogaster* genome [37]. These interactions were further refined to approximately 5,000 interactions of high confidence by a computational method in which the 16kDa subunit c of the vacuolar H⁺-ATPase (V-ATPase) was listed as a potential candidate to interact with the δ -SG homolog in Drosophila. The V-ATPase complex is an ATP hydrolysis driven proton pump essential for acidification of intracellular compartments. It consists of a peripheral V₁ domain and an integral membrane V₀ domain [29]. The 16kDa subunit c (16K) has four transmembrane helices (Supplementary Fig. 2) and forms a hexamer in the V₀ domain.

16K interacts with δ -SG and γ -SG through the transmembrane and intracellular domains

To confirm their interaction, we co-expressed δ -SG and 16K in COS-1 cells and assessed their ability for association. Although the sarcoglycan complex exists as a hetero-tetramer in muscle, the interaction between a particular sarcoglycan subunit and other proteins can be demonstrated by in vitro systems without the presence of other sarcoglycan subunits. For example, we have previously documented the interaction between γ -sarcoglycan and filamin 2

using an *in vitro* cell-free translation system [38]. We have also used this heterologous expression system to investigate the interaction of sarcoglycan subunits during the assembly process [10;20]. The construct encoding 16K contains a C-terminal HSV-tag (16K-HSV) to facilitate identification. As shown in Fig. 1A, the association between the HSV-tagged 16K and δ -SG was demonstrated by their co-immunoprecipitation using an anti-HSV antibody. Similar result was obtained when 16K-HSV was co-expressed with the highly related γ -SG, which shares 52% sequence identity to δ -SG (Fig. 1B). Neither β -SG nor α -SG was co-immunoprecipitated with 16K-HSV when co-expressed individually with 16K (Fig. 1C-D). These findings suggest that the interaction of 16K with δ -SG and γ -SG is specific and not the result of non-specific interaction between hydrophobic transmembrane helices.

Our previous study has shown that the extracellular domain is essential for the interaction of sarcoglycan subunits [20]. To determine whether this region is required for interacting with 16K, we co-expressed 16K-HSV with the K57X δ -SG mutant, which deletes the entire extracellular domain but retains the transmembrane helix and intracellular tail. This truncated δ -SG mutant was previously shown not to interact with other sarcoglycan subunits in co-expression study [20]. As shown in Fig. 1E, the K57X δ -SG mutant was still able to co-immunoprecipitate with 16K-HSV, indicating that the transmembrane and intracellular domains of δ -SG are sufficient to interact with 16K. To examine whether the transmembrane domain of 16K is involved in interacting with δ -SG, we generated a truncated deletion in 16K (TM4-HSV) lacking the highly conserved 4th transmembrane domain and the short cytoplasmic tail consisting of 6 amino acids (Supplementary Fig. 2). The C-terminal HSV-tagged TM4 mutant was not co-immunoprecipitated with δ -SG when they were co-transfected together into COS-1 cells (Fig. 1F). Since the TM4-HSV mutant is incorporated into hexamers with endogenous 16K [39], the

lack of binding of this mutant to δ -SG is likely not due to altering the structure of 16K to the extent that it is incapable of interacting with other proteins.

Given that sarcoglycans exist as tetramers in muscle, it is important to demonstrate that their interaction with 16K occurs under physiological environment where sarcoglycans form a stable complex. To explore this issue, we transfected 16K-HSV into C2C12 myoblasts and examined its interaction with endogenous sarcoglycans during myotube formation. During terminal differentiation, myoblasts are fused into myotubes expressing characteristic muscle proteins, including sarcoglycans. As shown in Fig. 1G, an anti- γ -SG antibody was able to co-immunoprecipitate the recombinant 16K-HSV with endogenous α -, β -, γ - and δ -SG from 3-day-old C2C12 myotubes. This anti- γ -SG antibody was previously shown to immunoprecipitate α -, β -, γ - and δ -SG as a stable complex from muscle lysate [20].

Expression of 16K in muscle

The expression of 16K in muscle was first determined by RT-PCR. 16K transcripts were detected in skeletal muscle and cultured C2C12 myotubes as well as non-muscle cells, such as kidney and Schwann cells (Fig. 2A). This is consistent with the broad pattern of 16K expression in many tissues. To confirm the expression of 16K protein in muscle, we generated a rabbit polyclonal antibody (UGMS-1) against its N-terminal extracellular domain. On Western blot, both anti-HSV and anti-16K antibodies recognized the HSV-tagged 16K expressed in COS-1 cells (Fig. 2B, lane 2-3, arrow). When membrane fractions of C2C12 myotubes were probed with the anti-16K antibody, a specific immuno-reactive band at 16kDa was observed (lane 5, arrowhead), in agreement with its expected size. Western blot using the anti-16K antibody

showed that 16K is abundantly expressed in brain and kidney (data not shown). Northern blot analysis also reported a similar pattern of tissue distribution for 16K in a previous study [40].

Sarcoglycans associate with endogenous 16K in C2C12 myotubes

To verify the association of sarcoglycans with 16K in muscle, we determined whether endogenous 16K can be co-immunoprecipitated with sarcoglycans from cultured C2C12 myotubes. As shown in Fig. 3, 16K was co-immunoprecipitated with the sarcoglycan complex using the anti- γ -SG antibody, thus confirming the findings in co-expression studies. To eliminate the possibility of non-specific binding of 16K to the antibody, we showed that the anti- γ -SG antibody did not immunoprecipitate recombinant 16K-HSV expressed in COS-1 cells or endogenous 16K from kidney extract where γ -SG is not present (Supplementary Fig. 3). Interestingly, the subunit F of V-ATPase was not co-immunoprecipitated with sarcoglycans and 16K. Other members of the DGC, including syntrophin, dystrobrevin, β -dystroglycan and dystrophin, were also not detected in the immune complex, indicating that the interaction between sarcoglycans and 16K occurs independent of other DGC members. As a negative control, caveolin-3 was not co-immunoprecipitated with sarcoglycans and 16K (Fig. 3). Since caveolin-3 is a lipid raft component and do not interact directly with the DGC, this ruled out the possibility that the association of sarcoglycans with 16K is due to non-specific protein aggregation.

Localization of 16K to the plasma membrane

Given the plasma membrane localization of sarcoglycans, their interaction with 16K implies that 16K should be present on the cell surface of C2C12 myotubes. When C2C12

myotubes were co-stained with antibodies against 16K and γ -SG, both 16K and γ -SG were found to co-localize to the plasma membrane by confocal microscopy (Fig. 4A-C). Interestingly, 16K staining was punctate and discontinuous along the plasma membrane (arrows in A) whereas the membrane staining of γ -SG from the same optical section was uniform (B). When different optical sections were analyzed, distinct staining of 16K was also observed in the cytoplasm (arrowheads in D), most likely representing the V-ATPase complex in the intracellular compartments. C2C12 myotubes were also treated with membrane impermeable NHS-LC-biotin and 16K was shown to be labeled with biotin (data not shown), thus confirming the presence of 16K on the cell surface.

We then examined the sub-cellular localization of 16K in muscle fibers. When cross-sections from mouse skeletal muscle were co-stained with antibodies against 16K and γ -SG, 16K was shown to co-localize with γ -SG at the sarcolemma (Fig. 5A-C). Labeling of 16K was also found in the cytoplasm (A). Confocal microscopy revealed that the intensity of 16K labeling varies in the muscle membrane and appears to concentrate in localized regions (arrows in D), in contrast to the continuous sarcolemmal staining displayed by sarcoglycans (Fig. 5E). This pattern of staining is similar to what we observed for 16K in the plasma membrane of C2C12 myotubes. Using a different anti-16K antibody (AW-1) directed against the N-terminal cytoplasmic domain, similar patterns of sarcolemmal and cytoplasmic staining were detected for 16K in muscle cross-sections (Fig. 5F). Moreover, immunoelectron microscopy studies also showed specific labeling of 16K at the sarcolemma (Fig. 6A-B, arrows) and in the intracellular space within myofibrils (Fig. 6C, arrowhead). Together, these results suggest that there are two pools of 16K in muscle: one at the sarcolemma associated with sarcoglycans and the other within the cytoplasm.

Distribution of 16K at the sarcolemma is altered in sarcoglycan-deficient hamsters

Within the DGC, the sarcoglycan complex is preferentially associated with sarcospan, which belongs to the tetraspan superfamily of transmembrane proteins [41]. Primary sarcoglycan mutations in LGMD patients often leads to instability of sarcospan at the sarcolemma [42]. To investigate whether the absence of sarcoglycans has an adverse effect on 16K, which also has four transmembrane helices, we analyzed the expression and localization of 16K in sarcoglycan-deficient BIO14.6 hamster, a widely used animal model for muscular dystrophy and cardiomyopathy. The BIO14.6 hamster has a deletion in the δ -SG gene [43;44]. The loss of δ -SG leads to concomitant reduction of other sarcoglycan subunits and instability of the DGC in muscle [16;17;31]. Immunofluorescent analysis of muscle cross-sections confirmed the absence of the sarcoglycan complex in BIO14.6 hamsters as demonstrated by near absent staining for α -SG at the sarcolemma (Fig. 7B). Meanwhile, we noticed distinct accumulation of 16K at the sarcolemma of BIO14.6 hamsters compared to that of controls (Fig. 7D and F, arrows). In contrast, the staining pattern of subunits F and G2 of the V-ATPase complex did not change significantly (Fig. 7G-J). These results indicate that loss of sarcoglycans specifically affects the 16K subunit in muscle and raise the possibility that the interaction between sarcoglycans and 16K does not involve the V-ATPase complex. Interestingly, Western blot analysis showed that the steady state levels of 16K in both control and BIO14.6 muscle membranes were not noticeably different (Fig. 8A). The levels of dystrophin and dystroglycans were reduced in the mutant animals, consistent with the critical role of sarcoglycans in the stability of the DGC.

Up-regulation of β_1 -integrin in the absence of the sarcoglycan complex

Previous reports have demonstrated the up-regulation of α_7 -integrin in the absence of sarcoglycans [18] or the DGC [32]. Consistent with these findings, we have observed an increased level of β_1 -intergrin in BIO14.6 hamsters. The immunofluorescent staining for β_1 -intergrin at the sarcolemma was more intense in BIO14.6 hamsters (Fig. 7L). The increased expression of β_1 -intergrin was confirmed by Western blots (Fig. 8A). Thus, our data provide additional evidence to support the proposed role of $\alpha_7\beta_1$ -integrin in compensating the sarcoglycan complex for the maintenance of the transmembrane linkage in skeletal muscle. In light of these results, it is important to mention that 16K can also bind directly to β_1 -integrin [33-35]. This raises the possibility of a higher-order association among 16K, sarcoglycans and integrins. To explore this issue, we performed co-immunoprecipitation of C2C12 myotube membrane fractions using an anti- β_1 -intergrin antibody and showed that 16K, α -SG, β -SG, γ -SG, and δ -SG were co-immunoprecipitated together with β_1 -intergrin (Fig. 8B).

DISCUSSION

In order to elucidate the function of the sarcoglycan complex in muscle, we searched for novel sarcoglycan-interacting proteins. In the present study, we reported a novel interaction between sarcoglycans and the 16kDa subunit c of the vacuolar H^+ -ATPase through database searching. Our results demonstrated that 16K interacts specifically with δ -SG and the highly related γ -SG through the transmembrane and/or intracellular domains. Although the sarcoglycan complex is part of the larger DGC, its interaction with 16K in C2C12 myotubes does not appear to require other critical members of the DGC. The absence of other DGC components in the immune complex is most likely due to the possibility that the anti- γ -SG antibody used in this study selectively dissociates sarcoglycans from other DGC components during

immunoprecipitation [20]. Yoshida et al has also shown that the sarcoglycan complex can be dissociated from other DGC components under certain conditions, such as in the presence of n-octyl β -D-glucoside [45]. In addition, there are several lines of evidence to suggest that the interaction between sarcoglycans and 16K is also independent of V-ATPase. Previous studies have reported that 16K can form structures outside V-ATPase, including gap junction complex in *Drosophila* [46] and neurotransmitter release channel [47]. In this study, the subunit F of V-ATPase, which is in direct contact with 16K in the V-ATPase complex, was not co-immunoprecipitated with sarcoglycans. In addition, localization studies revealed the existence of a sub-population of 16K at sarcolemma where it associates with sarcoglycans. Subcellular fractionation experiments also showed that δ -SG was enriched with 16K but not the subunit F in the sarcolemmal fractions (data not shown). Interestingly, 16K staining along the plasma membrane of C2C12 myotubes is discontinuous and does not completely co-localize with sarcoglycans, indicating that 16K is absent in areas where sarcoglycans are present. As a result, sarcoglycans are not always complexed with 16K and this might account for the observation that the anti- γ -SG antibody did not efficiently immunoprecipitate endogenous 16K in C2C12 myotubes. Finally, loss of the sarcoglycan complex in BIO14.6 hamsters specifically disrupts the localization of 16K at the sarcolemma but has no effect on the other subunits of V-ATPase. Other proteins known to interact with sarcoglycans, such as sarcospan and filamin C, also display altered localization patterns in sarcoglycan-deficient animal models [38;41]. The abnormal localization and expression of these sarcoglycan-interacting proteins might contribute, at least in part, to the cellular defects underlying LGMD.

Although sarcoglycans are critical for the stability of the DGC, recent studies have suggested that sarcoglycans might communicate with integrins in mediating cell adhesion to the

ECM [21]. It has been proposed that up-regulation of $\alpha_7\beta_1$ -integrin might be a compensatory mechanism for the disruption of the DGC-mediated transmembrane linkage in muscle [18;26-28]. Consistent with this idea, α_7 -integrin has been shown to up-regulate in the absence of sarcoglycans or the DGC [18;32]. The level of β_1 -integrin is also increased in the skeletal muscle of sarcoglycan-deficient BIO14.6 hamsters as reported in the current study. Since 16K can interact with β_1 -integrin [33-35] as well as δ -SG/ γ -SG as documented in this study, we propose that 16K might play an important role in mediating the communication between sarcoglycans and integrins. It is conceivable that these molecules could form a tertiary complex at the sarcolemma where both sarcoglycan- and integrin-mediated transmembrane linkages interact together with laminin in the ECM to strengthen muscle cell adhesion and to withstand larger mechanical force. In support of this hypothesis, a previous study has documented the co-localization of $\alpha_7\beta_1$ -integrin and sarcoglycans in costamere [22;48], which is sub-membrane specialization along Z disk essential for force transduction from myofibrils to sarcolemma [49]. In L6 myotubes, α -sarcoglycan, $\alpha_5\beta_1$ -integrin and vinculin exhibited overlapping distribution at the plasma membrane, especially at focal adhesion-like spotty structures [21]. Immunofluorescent analysis also revealed that 16K and β_1 -integrin were co-localized in focal adhesions [33]. Consistent with these findings, we demonstrated that sarcoglycans, 16K and β_1 -integrin were co-immunoprecipitated together in C2C12 myotubes.

In light of these results, it is important to mention that 16K is involved in regulating the expression and glycosylation of β_1 -integrin in a V-ATPase-independent manner [34]. It has been shown that the assembly of integrins is dependent on their interaction with the chaperon protein calnexin in ER [50] and that overexpression of 16K in human fibrosarcoma HT180 cells down-regulates surface expression of β_1 -integrin through inhibition of β_1 -integrin binding to calnexin

[35]. As shown in this study, the disruption of the sarcoglycan complex in BIO14.6 hamsters causes mis-localization of 16K at the sarcolemma although the cellular mechanism remains to be determined. It is tempting to speculate that there would be less 16K available to bind to β_1 -integrin such that more β_1 -integrin can associate with calnexin, leading to its increased expression on the cell surface. This could allow sarcoglycans to communicate and influence integrins through 16K. In this respect, 16K functions as a shuttle molecule in transmitting cellular information between the sarcoglycan and integrin complexes.

16K could also participate in other essential cellular functions. For example, the V_0 domain is thought to play a direct role in membrane fusion independent of the proton pumping mechanism [51]. In situ hybridization in E17 and E20 rat embryos has demonstrated that the expression of 16K mRNA becomes more restricted to the myoblasts that are in the process of fusion [52]. In addition, 16K forms a complex with the E5 oncoprotein of papillomaviruses and has been proposed to mediate the ability of E5 to cause ligand-independent activation of the PDGF- β receptor [39]. It is possible that 16K might recruit membrane-bound growth factors and signaling molecules and facilitate assembly of signaling complexes by tethering these molecules to sarcoglycans. Thus, mis-localization of 16K could potentially disrupt signaling pathways essential for muscle cell function and survival.

In summary, the identification of 16K as a novel sarcoglycan-interacting protein should generate novel insights into the pathogenesis of muscular dystrophy. Since 16K-null mice are embryonic lethal [53], a conditional knockout of 16K is necessary to address the precise function of 16K in muscle and to elucidate the functional significance of its interaction with sarcoglycans and $\alpha_7\beta_1$ -integrin. This would provide new directions into the development of new pharmacological agents for compensating sarcoglycan function in muscular dystrophy.

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FIGURE LEGENDS

Figure 1. Co-expression of HSV-tagged 16K and sarcoglycans. COS-1 cells were co-transfected with (A) 16K-HSV and δ -SG, (B) 16K-HSV and γ -SG, (C) 16K-HSV and β -SG, (D) 16K-HSV and α -SG, (E) 16K-HSV and K57X δ -SG mutant, or (F) TM4-HSV mutant and δ -SG. (G) C2C12 myoblasts were transfected with 16K-HSV followed by differentiation into myotubes. Cell lysate was co-immunoprecipitated using anti-HSV antibody (lane 2, A-F), anti-M5 antibody as negative control (lane 3, A-F), or anti- γ -SG (ANA- γ) antibody (lane 4, G). The immune complex was analyzed by Western blots using antibodies against specific sarcoglycan, HSV-tag, and caveolin-3 as negative control. Lane 1, total cell lysate (10 μ g, 25% input); Lane 2-3, co-immunoprecipitated product (40 μ g); Lane 4, co-immunoprecipitated product (200 μ g).

Figure 2. Expression of 16K in muscle. (A) Detection of 16K transcripts by RT-PCR in kidney (lane 1), skeletal muscle (lane 2), C2C12 myotubes (lane 3), and Schwann cells (lane 4). (B) Detection of recombinant 16K-HSV in COS-1 cells (lane 1-3), and endogenous 16K in C2C12 myotubes (lane 4-5) by Western blots using pre-immune serum (lane 1, 4), anti-16K (UGMS-1) antibody (lane 2, 5), or anti-HSV antibody (lane 3). Note that 16K-HSV has a MW of 22kDa (arrow) due to the HSV-tag. Endogenous 16K has a MW of 16kDa (arrowhead).

Figure 3. Association of 16K with sarcoglycans in cultured myotubes. Membrane fractions prepared from mouse C2C12 myotubes were incubated with anti- γ -SG (ANA- γ) antibody (lane 2) or pre-immune serum (lane 3). The co-immunoprecipitated product was identified by Western blots using antibodies against 16K, sarcoglycans, subunit F of V-ATPase (V-ATP F), syntrophin,

dystrobrevin, β -dystroglycan, dystrophin, and caveolin-3. Lane 1, membrane fraction (20 μ g, 20% input); Lane 2-3, co-immunoprecipitated product (100 μ g).

Figure 4. Localization of 16K to the plasma membrane of C2C12 myotubes. Cultured myotubes were co-stained with antibodies against 16K (UGMS-1) and γ -SG (NCL-g-SARC) and analyzed by confocal microscopy. Confocal images demonstrating plasma membrane staining of 16K (A, arrows) and γ -SG (B) were taken from the same optical section. Co-localization of 16K and γ -SG at the plasma membrane is demonstrated by yellow color (C, arrows). A different optical section is shown to reveal cytoplasmic staining of 16K (D, arrowheads). Bar, 20 μ m.

Figure 5. Localization of 16K at the sarcolemma. Frozen cross-sections of mouse skeletal muscle were co-stained with antibodies against 16K and sarcoglycans. (A) Cross-section was stained with antibody against 16K (UGMS-1). (B) Identical cross-section was co-stained with antibody against γ -SG (NCL-g-SARC). (C) Merged image of panels A and B. Co-localization of 16K and γ -SG at the sarcolemma is demonstrated by yellow color. (D) Cross-section was stained with antibody against 16K (UGMS-1). (E) Identical cross-section was co-stained with antibody against α -SG (NCL-a-SARC). Images from panel D and E were analyzed by confocal microscopy. (F) Cross-section was stained with a different anti-16K antibody (AW-1). Note the presence of cytoplasmic staining for 16K in panels A, D, and F. Bar, 25 μ m in A-C, F; 18 μ m in D-E.

Figure 6. Ultrastructural analysis of 16K localization in muscle fibers. Mouse skeletal muscle was labeled with anti-16K (UGMS-1) antibody and analyzed by electron microscopy. Labeling

of 16K at the sarcolemma is demonstrated by the presence of 15-nm gold particles (A-B, arrows). Note that 16K labeling is also observed in the intracellular space within myofibrils (C, arrowhead). Mt denotes mitochondria. ECM denotes extracellular matrix. Dashed lines represent the boundary between plasma membrane and ECM. Bar, 0.4 μ m in A, 0.15 μ m in B, 0.25 μ m in C.

Figure 7. Localization of 16K in sarcoglycan-deficient BIO14.6 hamsters. Skeletal muscle cross-sections of wild-type controls (**A, C, E, G, I, K**) and δ -SG^{-/-} (BIO14.6) hamsters (**B, D, F, H, J, L**) were stained with antibodies against α -SG (A-B), 16K (C-F), V-ATP F (G-H), V-ATP G2 (I-J), and β_1 -integrin (7E2) (K-L). Panels C-D and E-F represent muscle biopsy taken from tricep brachii and quadriceps femoris of two different hamsters, respectively. Note that 16K in δ -SG^{-/-} hamsters forms distinct aggregates at the sarcolemma (arrows in D and F). Five hamsters, ranging from 12 weeks to 36 weeks old, were analyzed and representative pictures are shown. Bar, 35 μ m.

Figure 8. Expression of 16K and β_1 -integrin in sarcoglycan-deficient BIO14.6 hamsters. **(A)** Muscle membranes (40 μ g) from wild-type controls (lane 1) and δ -SG^{-/-} (BIO14.6) hamsters (lane 2) were analyzed by Western blots using antibodies against 16K, β_1 -integrin, δ -SG, dystrophin, α -/ β -dystroglycans, sarcoglycans and caveolin-3 (negative control). Note that γ -SG was not shown here since the anti- γ -SG antibodies did not recognize hamster γ -SG. **(B)** Association of β_1 -integrin with 16K and sarcoglycans. Membrane fractions of C2C12 myotubes (200 μ g) were incubated with anti- β_1 -integrin (610467) antibody. The co-immunoprecipitated product (lane 3) was identified by Western blots using antibodies against 16K, sarcoglycans and β_1 -integrin. Pre-immune serum (lane 4) was used as a negative control for co-immunoprecipitation.

Supplementary Figure 1. List of antibodies used in the study. mAb, monoclonal; pAb, polyclonal.

Supplementary Figure 2. Sequence alignment of the 16kDa subunit c of vacuolar H⁺-ATPase from *Drosophila melanogaster* and human. Non-identical amino acids between the two species are underlined. The 16kDa subunit c (16K) is a tetraspan membrane protein and the four transmembrane domains are represented by $\alpha 1$ - $\alpha 4$ (boxed).

Supplementary Figure 3. Specificity of the ANA- γ -SG antibody. **(A)** Recombinant 16K-HSV was expressed in COS-1 cells and co-immunoprecipitated with anti- γ -SG (ANA- γ) antibody. Lane 1, total cell lysate (10 μ g, 25% input); Lane 2, co-immunoprecipitated product (40 μ g). **(B)** Kidney extract was co-immunoprecipitated with anti- γ -SG (ANA- γ) antibody. Lane 1, total kidney extract (50 μ g, 50% input); Lane 2, co-immunoprecipitated product (100 μ g). 16K was detected by anti-16K (UGMS-1) antibody on Western blots. Note that the ANA- γ antibody did not immunoprecipitate 16K.

Abbreviations List

DG; dystroglycan

DGC; dystrophin-glycoprotein complex

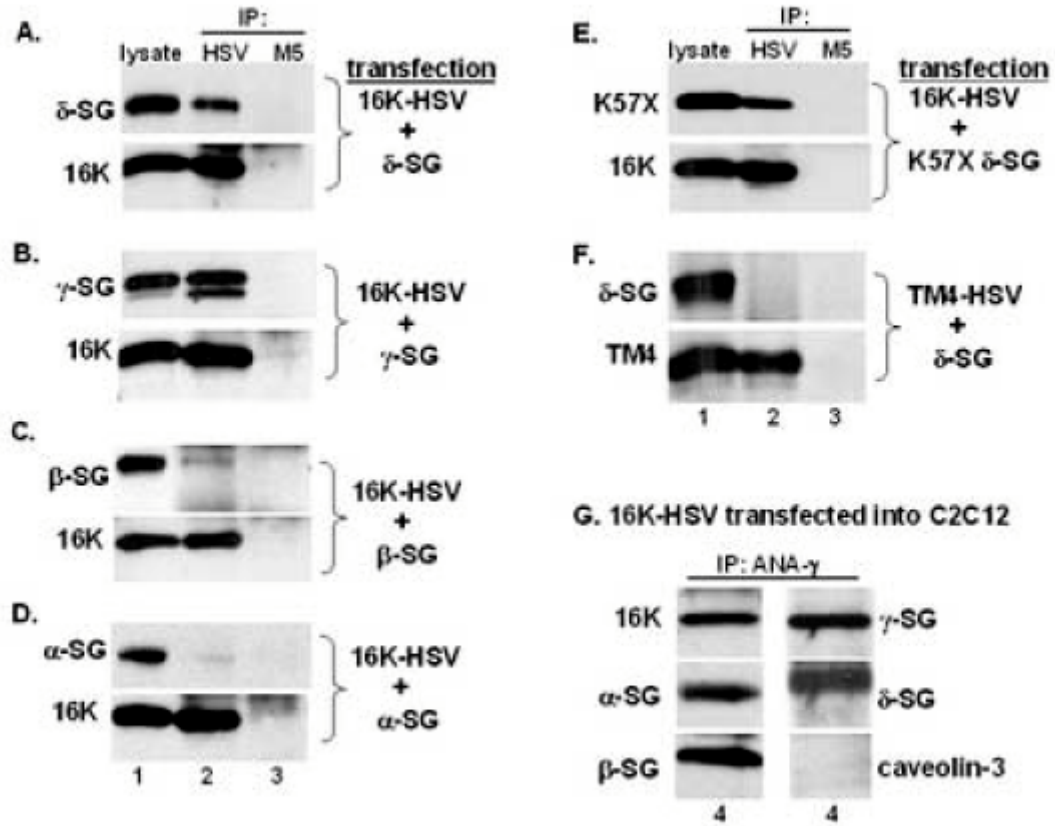
ECM; extracellular matrix

LGMD; limb-girdle muscular dystrophy

SG; sarcoglycan

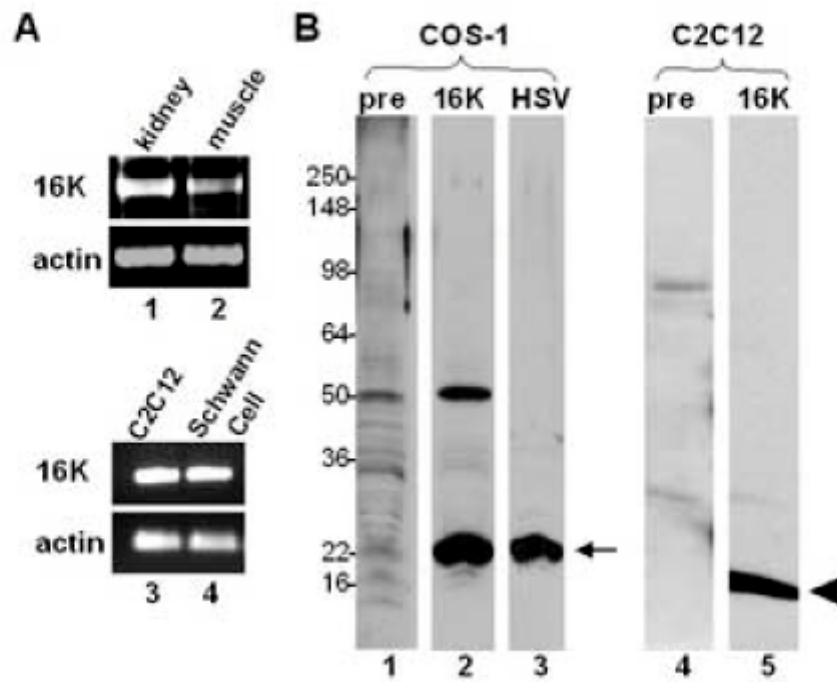
V-ATPase; vacuolar H⁺-ATPase

Fig. 1



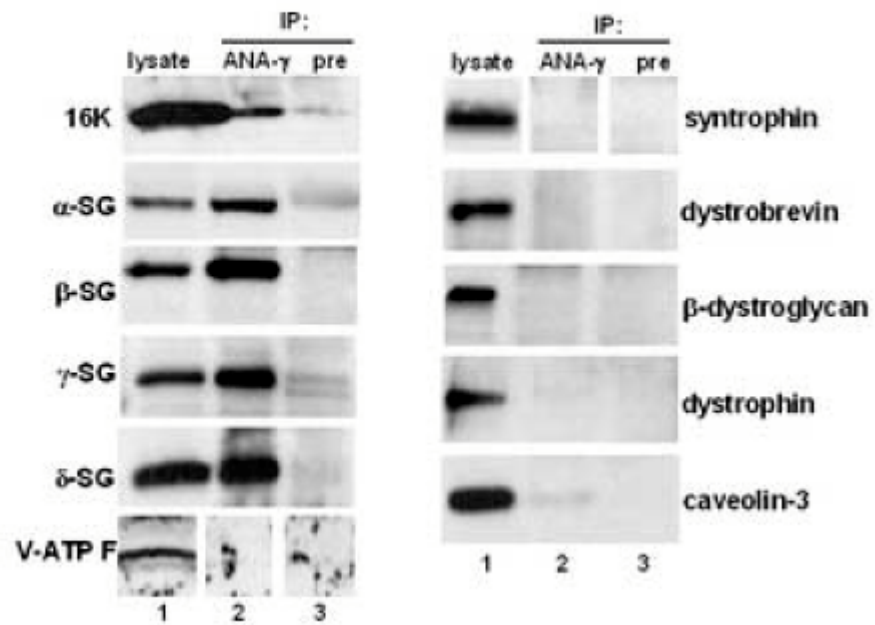
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Fig. 2



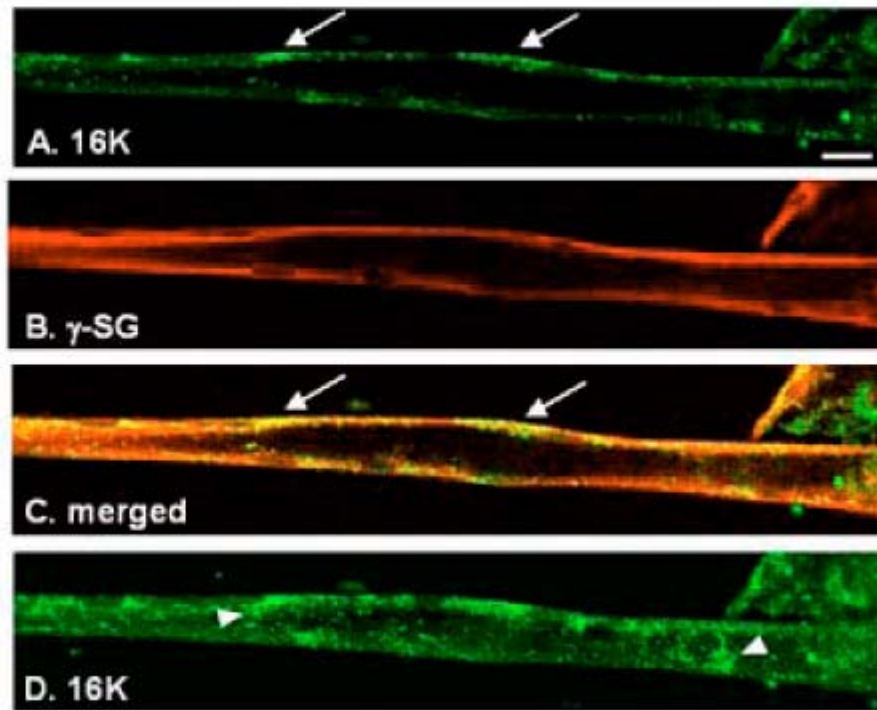
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Fig. 3



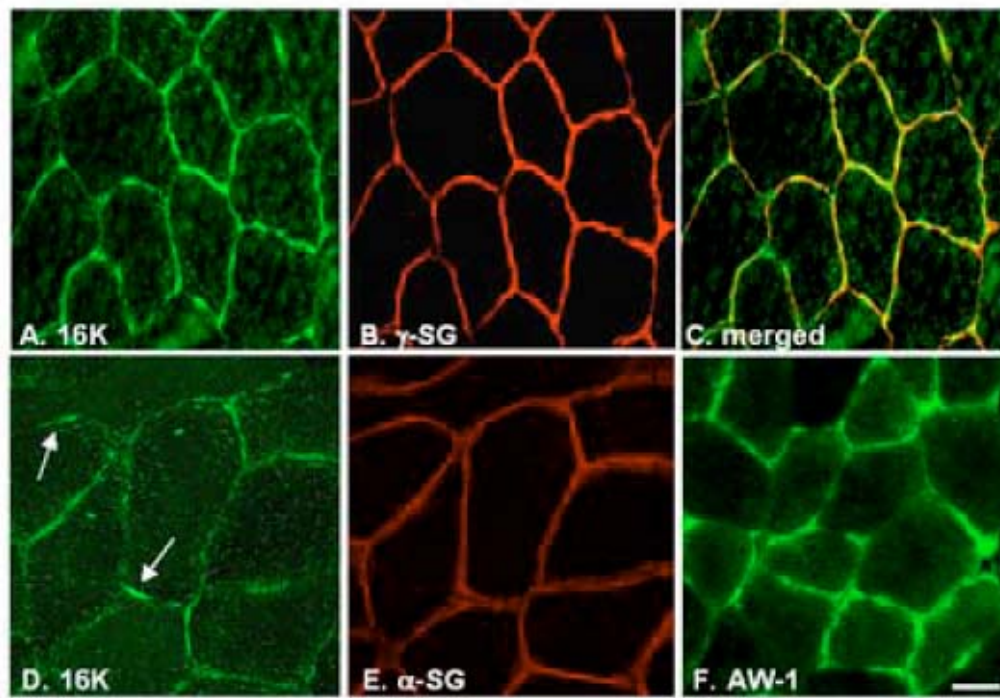
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Fig. 4



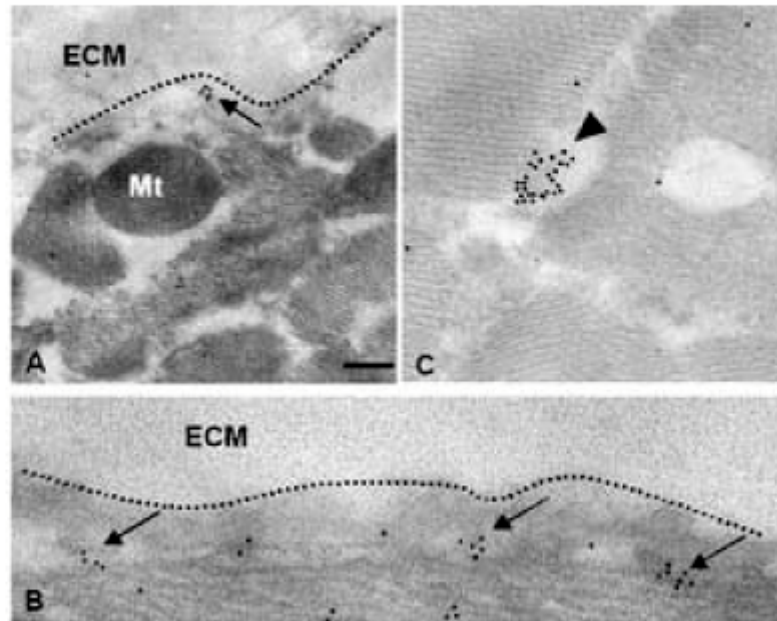
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Fig. 5



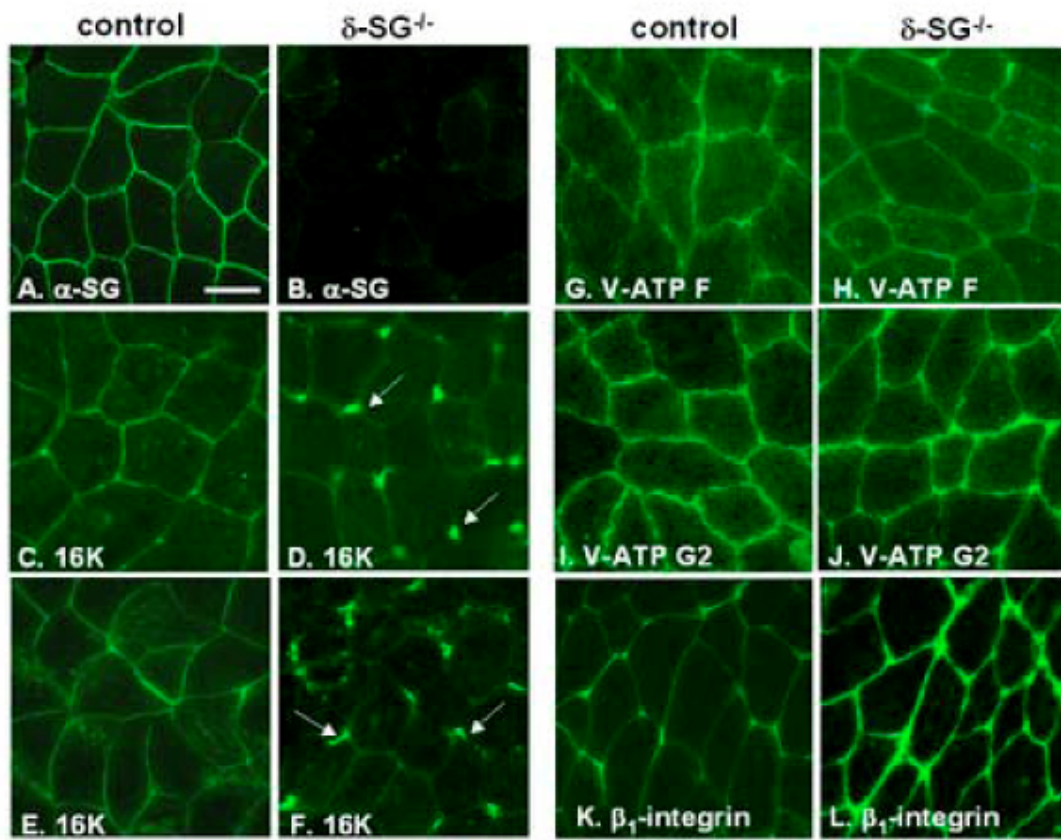
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Fig. 6



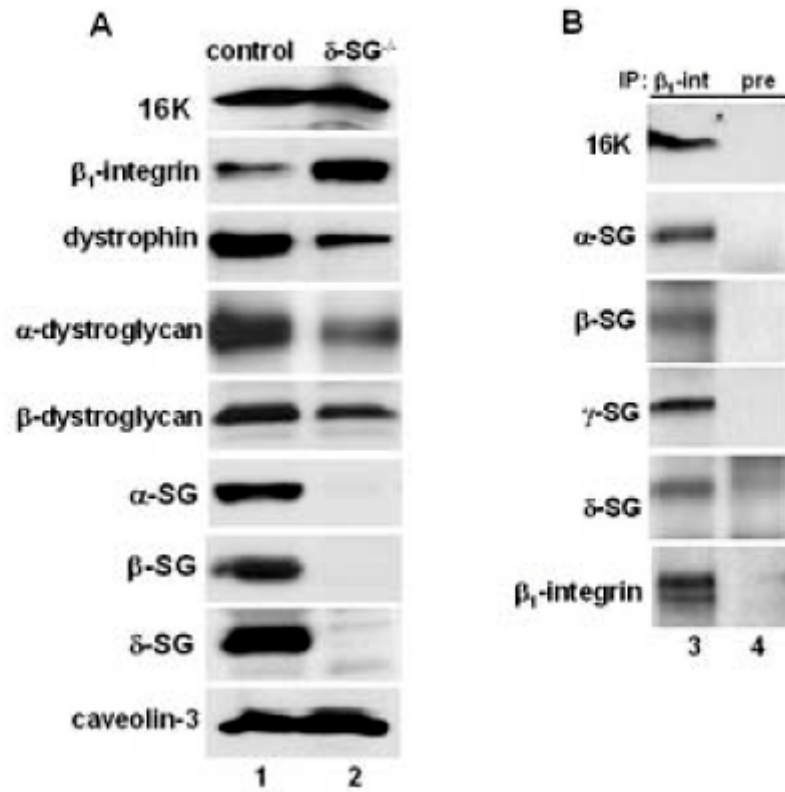
ACCL

Fig. 7



A

Fig. 8



ACU