The 16 kDa subunit of vacuolar H⁺-ATPase is a novel sarcoglycan-interacting protein

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Abstract

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 16 kDa 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 β1-integrin is increased. Recent studies have shown that 16K also interacts directly with β1-integrin and our data demonstrated that sarcoglycans, 16K and β1-integrin were 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.

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1. 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 αβ-integrin in rat L6 myocytes [21] and co-localize with αβ-integrin to costameres in human muscle fibers [22].
skeletal muscle, α-7β1-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-γ-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-γ-integrin is up-regulated in sarcoglycan-deficient γ-SG-null mice [18]. Increased expression of α-β1-integrin in muscle can reduce the dystrophic phenotypes in dystrophin-null mdx mice [26]. Moreover, double knockout mice in both α-γ-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 16 kDa 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 immunoprecipitate with 16K, sarcoglycans were also shown to immunoprecipitate with β1-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 β1-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 β1-integrin act coordinately independent of V-ATPase to induce signals controlling cell adhesion [33–35]. In vitro binding assay using epitope-tagged 16K and β1-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.

2. Materials and methods

2.1. DNA constructs

Full-length cDNA encoding mouse αS, βS, γS, 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].

2.2. 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 (KSGTGIAAMSVMRPEQ) in the extracellular domain.

2.3. 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 guidelines 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 (IAUC).

2.4. 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 μg ml⁻¹ streptomycin, and 100 U ml⁻¹ penicillin (Sigma, St. Louis, MO). Cell cultures were incubated at 37 °C with 5% CO2. 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 μg 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 48 h 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 24 h post-transfection and harvested 72 h 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).

2.5. 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'-ACCCGCAAGACATGTCCGAGT and (reverse) 5'-ACGAATAGTCG-GGCGTGCC at annealing temperature of 60 °C.

2.6. Muscle membrane preparation

Mouse skeletal muscle (400 mg) or C2C12 myotubes were homogenized by a Potter-Elvejem homogenizer on ice for 2 min with 4 ml of pyrophosphate buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM MgCl2, 0.5 mM EDTA, and 10% sucrose, pH 7.1). The mixture was centrifuged at 500×g for 10 min at 4 °C to remove cell debris. The supernatant was then centrifuged at 18000×g for 10 min to collect the pellet, which contains crude membrane vesicles. Membrane proteins were extracted from the pellet with 2 ml of TX-100 buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS).

2.7. Co-immunoprecipitation

Muscle membrane fraction or total cell lysate from cultured cells was first pre-cleared with 20 μl of protein G-sepharose beads (Sigma) for 1 h at 4 °C. The
pre-cleared cell lysate was incubated with antibody on ice for 4 h and then with 20 μl of protein G-rosephore beads for 1 h. The immune complex was collected by centrifugation at 10,000×g for 1 min and washed three times with 0.5 ml of PBS with 0.1% Tween20. The final pellet was solubilized in 30 μl 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.

2.8. Immunofluorescent analysis

C2C12 myotubes were fixed in methanol at −20 °C for 10 min and treated with 0.5% Triton X-100 in PBS for 10 min followed by blocking with 20% normal goat serum in PBS. Cells were incubated with primary antibody in blocking buffer overnight at 4 °C and then with secondary antibodies conjugated with Alexa488 or Alexa594 for 1 h. 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 μm. Images were processed with Leica confocal software.

2.9. Immunohistochemistry

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

2.10. Immuno-electron microscopy

Quadriceps femoris muscle from normal mouse was fixed in 0.1% glutaraldehyde and 2% paraformaldehyde in PBS for 30 min. The specimen was further trimmed into 2 mm strips, 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 (365 nm) 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 polymerized with UV light (365 nm) for 5 days at 25 °C. Samples were embedded in fresh Lowicryl K4M medium and examined by Leica SP2 laser scanning microscope (Leica, Exton, PA). Cells were sectioned into serial optical sections of 0.16 μm. Images were processed with Leica confocal software.

3. Results

3.1. 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 16 kDa 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 V_1 domain and an integral membrane V_0 domain [29]. The 16 kDa subunit c (16K) has four transmembrane helices (Supplementary Fig. 2) and forms a hexamer in the V_0 domain.

3.2. 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 immunoprecipitated with 16K-HSV when co-expressed individually with 16K (Fig. 1C–D). These findings suggest that

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**Fig. 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 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 μg, 25% input); lanes 2–3, immunoprecipitated product (40 μg); lane 4, immunoprecipitated product (200 μg).
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 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 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 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].

3.3. 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. Western blot, both anti-HSV and anti-16K antibodies recognized the HSV-tagged 16K expressed in COS-1 cells (Fig. 2B, lanes 2–3, arrow). When membrane fractions of C2C12 myotubes were probed with the anti-16K antibody, a specific immuno-reactive band at 16 kDa 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].

3.4. 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 immunoprecipitated with sarcoglycans from cultured C2C12 myotubes. As shown in Fig. 3, 16K was immunoprecipitated with anti-γ-SG antibody (Fig. 3A).

Fig. 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 (lanes 1–3), and endogenous 16K in C2C12 myotubes (lanes 4–5) by Western blots using pre-immune serum (lanes 1, 4), anti-16K (UGMS-1) antibody (lanes 2, 5), or anti-HSV antibody (lane 3). Note that 16K-HSV has a MW of 22 kDa (arrow) due to the HSV-tag. Endogenous 16K has a MW of 16 kDa (arrowhead).

Fig. 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 μg, 20% input); lanes 2–3, immunoprecipitated product (100 μg).
with the sarcoglycan complex using the anti-\(\gamma\)-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-\(\gamma\)-SG antibody did not immunoprecipitate recombinant 16K-HSV expressed in COS-1 cells or endogenous 16K from kidney extract where \(\gamma\)-SG is not present (Supplementary Fig. 3). Interestingly, the subunit F of V-ATPase was not immunoprecipitated with sarcoglycans and 16K. Other members of the DGC, including syntrophin, dystrobrevin, \(\beta\)-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 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.

3.5. 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 \(\gamma\)-SG, both 16K and \(\gamma\)-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 \(\gamma\)-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 cyto-
plasmic domain, similar patterns of sarcolemmal and cytoplas-
mic 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 asso-
ciated with sarcoglycans and the other within the cytoplasm.

3.6. 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 sarco-
glycan 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]. Immunofluo-
rescent analysis of muscle cross-sections confirmed the absence
of the sarcoglycan complex in BIO14.6 hamsters as demon-
strated 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.

Fig. 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 μm in A, 0.15 μm in B, 0.25 μm in C.
3.7. Up-regulation of $\beta_1$-integrin in the absence of the sarcoglycan complex

Previous reports have demonstrated the up-regulation of $\alpha_7$-integrin in the absence of sarcoglycans [18] or the DGC [32]. Consistent with these findings, we have observed an increased level of $\beta_1$-integrin in BIO14.6 hamsters. The immunofluorescent staining for $\beta_1$-integrin at the sarcolemma was more intense in BIO14.6 hamsters (Fig. 7L). The increased expression of $\beta_1$-integrin 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 $\beta_1$-integrin [33–35]. This raises the possibility of a higher-order association among $\alpha$-$\beta$-SG, $\gamma$-SG, and $\delta$-SG. To explore this issue, we performed co-immunoprecipitation of C2C12 myotube membrane fractions using an anti-$\beta_1$-integrin antibody and showed that 16K, $\alpha$-SG, $\beta$-SG, $\gamma$-SG, and $\delta$-SG were immunoprecipitated together with $\beta_1$-integrin (Fig. 8B).

### 4. 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 16 kDa 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 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 sarcosomal 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 α7β1-integrin might be a compensatory mechanism for the disruption of the DGC-mediated transmembrane linkage in muscle [18,26–28]. Consistent with this idea, α7-integrin has been shown to up-regulate in the absence of sarcoglycans or the DGC [18,32]. The level of β1-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 β1-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 αβ3-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, αβ3-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 β1-integrin were co-localized in focal adhesions [33]. Consistent with these findings, we demonstrated that sarcoglycans, 16K and β1-integrin were 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 β1-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 β1-integrin through inhibition of β1-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 β1-integrin such that more β1-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 V0 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 αβ3-integrin. This would provide new directions into the development of new pharmacological...
agents for compensating sarcoglycan function in muscular dystrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2007.01.014.

References


