# Characterization of aquaporin-4 in muscle and muscular dystrophy

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ABSTRACT **Aquaporins are a growing family of transmembrane proteins that transport water and, in some cases, glycerol and urea across cellular membranes. Aquaporin-4 (AQP4) is enriched at the sarcolemma of skeletal muscle and may play a role in accommodating the rapid changes in cell volume and hydrostatic forces that occur during contraction in order to prevent damage to the sarcolemma. Recent evidence has shown that AQP4 is absent in dystrophin-deficient *mdx* mice, suggesting that AQP4 associates with dystrophin and has a role in the dystrophic process. To examine the relationship between aquaporins and muscle disease, and between aquaporins and dystrophin, we have investigated aquaporin expression in various mouse models of muscular dystrophy and cardiomyopathy before and after the onset of pathology. We find that AQP4 is expressed in prenecrotic mdx muscle despite the absence of dystrophin and that AQP4 is lost after the onset of muscle degeneration. Analysis of various dystrophin transgenic mice reveals that AQP4 is lost even when the dystrophin–glycoprotein complex is present, suggesting that loss of AQP4 is not directly resulting from loss of the DGC. AQP4 was also lost in muscular dystrophies caused by primary mutations in the sarcoglycan genes. Taken together, our data demonstrate that AQP4 loss in skeletal muscle correlates with muscular dystrophy and is a common feature of pathogenesis.–Crosbie, R. H., Dovico, S. A., Flanagan, J. D., Chamberlain, J. S., Ownby, C. L., Campbell, K. P. Characterization of aquaporin-4 in muscle and muscular dystrophy. *FASEB J.* 16, 943–949 (2002)**

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MUSCULAR DYSTROPHIES REPRESENT a group of progressive muscle disorders characterized by extensive muscle wasting and weakness. Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene that result in loss of dystrophin, a peripheral membrane protein localized to the sarcolemma through its association with the dystrophin–glycoprotein complex (DGC) (1–5). The DGC components consist of the sarcoglycan–sarcospan subcomplex, α-/β-dystroglycan, the syntrophins, and dystrobrevin (reviewed in ref 6). The sarcoglycans are composed of α-, β-, γ-, and δ-sarcoglycan, which are transmembrane glycoproteins (reviewed in ref 6). Sarcospan, a four-transmembrane protein with tetraspanin-like characteristics, is tightly associated with the sarcoglycans (7, 8). Dystrophin binds to filamentous actin and the intracellular portion of β-dystroglycan (10–13). α-Dystroglycan is a high-affinity receptor for several extracellular matrix components, including laminin and perlecan (4, 9–13). Dystrobrevin is a cytosolic protein associated with the syntrophins and dystrophin (18–21). Together, the DGC functions to structurally link the extracellular matrix with the intracellular actin network (14). In DMD, loss of dystrophin results in loss of the entire DGC complex, which renders the muscle plasma membrane susceptible to contraction-induced damage (14, 15).

Mutations in α-, β-, γ-, or δ-sarcoglycan result in autosomal recessive limb girdle muscular dystrophy (AR-LGMD) types 2D, 2E, 2C, and 2F (24–32). Loss of one sarcoglycan generally results in absence of the entire sarcoglycan–sarcospan subcomplex, although cases of partial sarcoglycan loss have been reported (16). Mouse models for DMD and AR-LGMD have shed light on some of the pathogenetic mechanisms that contribute to muscular dystrophy. For instance, comparisons of the α-, β-, and δ-sarcoglycan knockout mice have revealed that β- and δ-sarcoglycan null mice have a higher incidence of cardiomyopathy than do α-sarcoglycan knockout mice (17–19). Cardiomyopathy likely develops from microvascular constrictions of the blood vessels in δ- and β-sarcoglycan knockouts due to the loss

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of the sarcoglycan–sarcospan complex within the smooth muscle vasculature (18, 19).

To determine factors that contribute to disease pathogenesis, much effort has been made to identify proteins that are affected during muscle degeneration. Recent studies have shown that aquaporin-4 (AQP4) is absent in the *mdx* mouse, a model for DMD (20, 21). AQP4 is a mercury-insensitive water channel that regulates flux of $H_2O$ molecules across the membrane bilayer in response to changes in osmolarity within a cell (22). AQP4 is preferentially expressed in fast twitch muscle, which has higher water permeability than that of slow twitch fibers (20). AQP4 forms oligomers at the plasma membrane, which are observed as orthogonally aggregated particles (OAPs) of 6–7 nm in freeze fracture electron microscopy (23, 24). OAP structures are nearly absent in membranes of DMD patients and the *mdx* mouse (25), which is consistent with loss of AQP4 from dystrophin-deficient muscle.

Aquaporins may play an important role in regulating the rapid changes in myofiber volume during muscle contraction (26, 27). Muscle cells accommodate large changes in hydrostatic forces as well as the intracellular generation of osmotically active solutes from lactate production and metabolism of creatine phosphate (28). In the present study, we investigate the expression of AQP4 in muscle and in several animal models of muscular dystrophy. We report that AQP4 is lost in dystrophin- and sarcoglycan-deficient animal models of muscular dystrophy. Furthermore, we provide evidence that reduced AQP4 levels are a common feature of muscle pathology regardless of the primary cause of muscle degeneration.

## MATERIALS AND METHODS

### Animal models

Wild-type (C57BL/10) and *mdx* (C57BL/10ScSn) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Dystrophin transgenic mice have been described (29–33). Male F1B and BIO 14.6 cardiomyopathic hamsters were obtained from BioBreeders (Fitchburg, MA). Generation and characterization of α- (Sgca null), δ- (Sgcd null), and β-SG-deficient mice (Sgcb null) have been reported before (17–19). All mice used in experiments were between 6 and 15 wk of age unless otherwise indicated. All animals were maintained at the University of Iowa Animal Care Unit in accordance with animal usage guidelines.

### Antibodies and immunoblotting

Polyclonal antibodies to AQP4 were purchased from Alpha Diagnostics (AQP41, San Antonio, TX). Affinity-purified polyclonal aquaporin antibodies were also purchased from Chemicon (AB3068, Temecula, CA). Affinity-purified antibodies to SSPN (Rabbit 236) have been described (7). KCl-washed skeletal muscle microsomes were prepared from adult mice, as described previously (7). Protein samples (75 μg/lane) were resolved under reducing conditions by 3–15% SDS-PAGE and transferred to PVDF (Immobilon-P) membranes (Millipore Corp., Bedford, MA). PVDF membranes were probed with anti-AQP4 antibodies (Chemicon) at a dilution 1:200. After incubation with primary antibody, blots were probed with HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence (Supersignal; Pierce Chemical Co., Rockford, IL).

### Immunofluorescence

Transverse muscle cryosections (7 μm) were analyzed by immunofluorescence as described (7). Aquaporin antibodies were incubated at a dilution of 1:500 with mouse muscle cryosections. After washing with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.4), the sections were incubated with either Cy3- or FITC-conjugated secondary antibodies at a dilution of 1:250 (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. For staining of neuromuscular junctions, samples were simultaneously incubated with fluorescein-conjugated α-bungarotoxin (1:1000; Molecular Probes, Eugene, OR). After washing with TBS, the slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a Bio-Rad MRC-600 laser scanning confocal microscope (Hercules, CA). Digitized images were captured under identical conditions.

### Recombinant adenovirus injections

Preparation of the δ-SG recombinant adenovirus and intramuscular injections were performed as described previously (34). Briefly, $10^9$ viral particles in 100 μl of normal saline were injected into the quadriceps femoris of 3-wk-old BIO 14.6 hamsters after the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL) at a calculated dose of 75 mg/kg. Quadriceps muscle was collected 2 wk after the injection.

## RESULTS

The aquaporins constitute a family of channels that transport water molecules across a cellular membrane. Aquaporins have six transmembrane regions that traverse the plasma membrane and two functional loops, which are also embedded in the bilayer (reviewed in ref 35). Eleven aquaporin proteins have been identified from a variety of mammalian tissues. Previous reports demonstrating that AQP4 is absent in dystrophin-deficient skeletal muscle (20, 36, 37) prompted our use of other murine models to investigate the fate of AQP4 during muscular dystrophy. Dystrophic muscle is characterized by elevated $Ca^{2+}$ levels, sarcolemma damage, central nucleation, and a large variation in fiber size (**Fig. 1**).

### Membrane localization of AQP4 in multiple models of dystrophin deficiency

Chamberlain and colleagues have shown that transgenic expression of various dystrophin isoforms in muscle can restore membrane localization of the DGC and subsequently prevent onset of muscular dystrophy. The transgenes, which were individually expressed on an *mdx* background, encode truncated dystrophin products (29, 31, 33, 38). Muscle from the transgenic

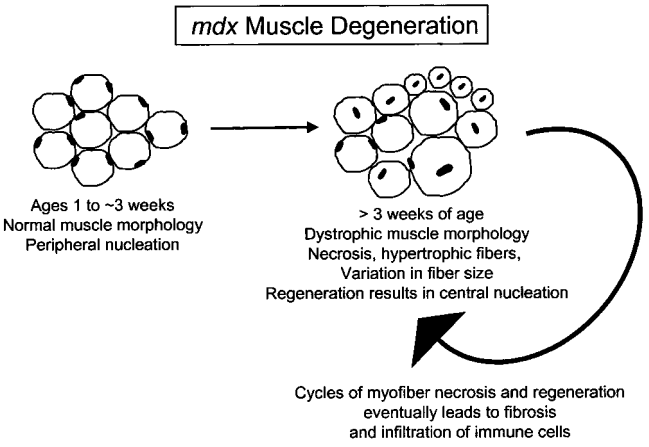


**Figure 1.** Muscle degeneration in *mdx* mice. Schematic representation of transverse skeletal muscle cross sections from dystrophin-deficient *mdx* mice that possess a premature stop codon within exon 23 of the dystrophin gene. From birth to ~3 wk of age, *mdx* muscle appears histologically normal although the DGC complex is absent (40). Myofibers are of uniform size with nuclei located at the subsarcolemma. At 3 wk of age, myofibers exhibit an increase in intracellular $Ca^{2+}$ as the first signs of myofiber necrosis and membrane damage appear. Fibers show a greater variation in size with many hypertrophic fibers. Regeneration results in new myofibers with nuclei located in the center of the cell. Cycles of necrosis and regeneration eventually lead to fibrosis and infiltration of immune cells.

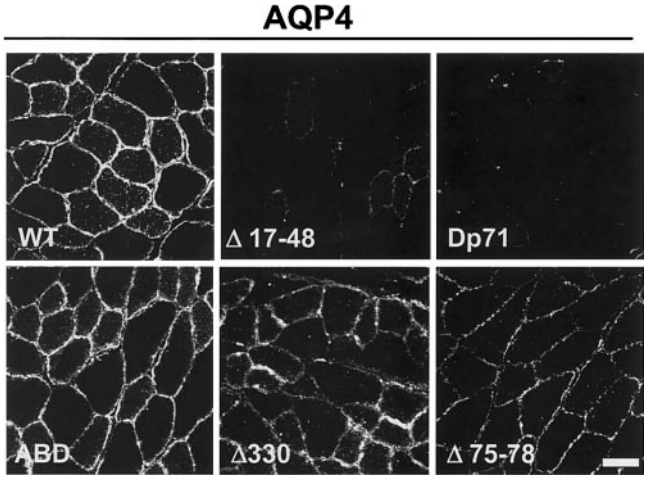


**Figure 2.** Restoration of AQP4 by dystrophin transgenic products. Various truncated and internally deleted dystrophin transgenes were expressed on an *mdx* background and previously analyzed for their ability to rescue the *mdx* phenotype. Skeletal muscle cryosections from adult 'Becker' (Δ17–48), Δ1–62 (Dp71), ΔABD (ABD), Δ71–74 (Δ330), and Δ75–78 mice were stained with AQP4 antibodies and visualized by indirect immunofluorescence. Muscle pathology is present in Dp71 and Δ17–48 transgenic mice, as previously documented (29, 31, 33, 38). Bar, 50 μm.

mice was previously evaluated for its morphology and for the ability of the transgene to restore sarcolemma localization of the DGC (29, 31, 33, 38). We detected AQP4 expression in skeletal muscle cross sections by indirect immunofluorescence staining with AQP4 antibodies and found normal levels of expression in muscle from adult Δ4–7 (ΔABD), Δ71−74 (Δ330), and Δ75–78 mice (**Fig. 2**). Dystrophin transgenes in these mice restored membrane localization of the DGC and prevented symptoms associated with muscle degeneration. However, AQP4 was dramatically reduced in muscle from Δ1–62 (Dp71) and Δ17–48 (Becker) mice (Fig. 2). The DGC complex is present in Δ1–62 (Dp71) and Δ17–48 mice, suggesting that loss of APQ4 is not directly resulting from loss of the DGC. Rather, the absence of AQP4 correlates with even mild dystrophy (29, 31, 33, 38).

### AQP4 is expressed in young, prenecrotic *mdx* mice

To distinguish whether loss of AQP4 from transgenic mice was the result of direct interaction with dystrophin or a consequence of muscle pathology, we examined muscle from *mdx* mice at various stages of muscle degeneration. The dystrophic process in dystrophin-deficient *mdx* mice is evident at 3 wk of age (Fig. 1). During the prenecrotic stage (from birth to ~3 wk), muscle histology is normal despite the loss of dystrophin (39). The cycles of myofiber necrosis and regeneration as well as increased membrane fragility are evident in *mdx* mice just after 3 wk. It is not clear why normal muscle morphology exists until this time, given that the DGC is absent in *mdx* mice from birth (40). Quadriceps muscle from wild-type and *mdx* mice were examined for expression of AQP4. We found that prenecrotic *mdx* mice ages 1 and 2 wk had normal immunostaining of AQP4 at the sarcolemma (**Fig. 3**), despite absence of the DGC. Expression of AQP4 in 3-wk-old *mdx* muscle is heterogeneous, as seen by the absence of AQP4 in some, but not all myofibers. Older

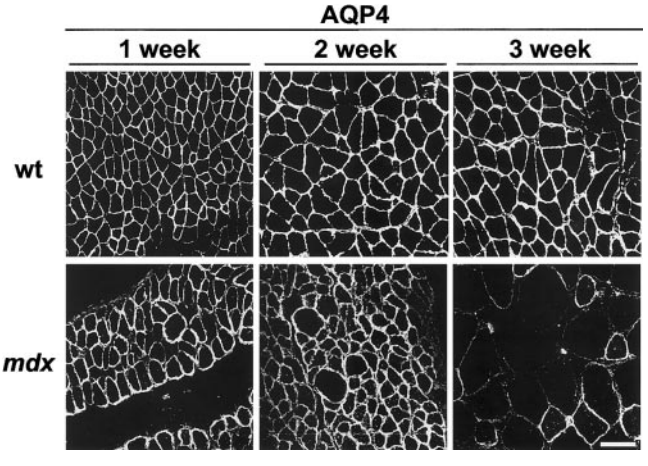


**Figure 3.** AQP4 membrane localization is normal in young *mdx* mice. Sections from quadriceps femoris muscles of wild-type and *mdx* mice at ages 1, 2, and 3 wk were stained with AQP4 antibodies. AQP4 staining was visualized by indirect immunofluorescence with Cy3-conjugated secondary antibodies. AQP4 expression decreased in *mdx* muscle with the onset of muscle degeneration at 3 wk of age. Sarcolemma localization of AQP4 was not affected by age in wild-type muscle. Bar, 50 μm.

(>12 wk) mice completely lacked sarcolemma expression of AQP4. Wild-type mice of all ages had normal, high levels of AQP4 expression (Fig. 3).

### AQP4 in sarcoglycan-deficient models of muscle damage

Mice with targeted deletions in the SG genes were used to determine whether AQP4 expression is affected in sarcoglycan-deficient models of limb girdle muscular dystrophy. Mutations within α-, β-, γ-, and δ-sarcoglycan are responsible for causing autosomal recessive limb girdle muscular dystrophy types 2D, 2E, 2C, and 2F (24–32). We specifically examined muscle from adult (>8 wk) α-, β-, and δ-SG null mice (Scga, Sgcb, and Sgcd), which lack the SG–SSPN subcomplex (8, 17–19). AQP4 expression is severely diminished in each of these mice over 8 wk of age (**Fig. 4*A***). Identical to young, prenecrotic *mdx* mice, young (<3 wk) sarcoglycan-deficient mice exhibit normal expression of AQP4 (data not shown). As further demonstration of the loss of AQP4 from the sarcolemma of sarcoglycan-deficient muscle, we immunoblotted KCl-washed membranes prepared from skeletal muscle of adult wild-type (wt), *mdx*, Sgca, and Sgcd null mice. AQP4 is present in wt samples but absent in Sgca and Sgcd membranes (Fig. 4*B*). Skeletal muscle membranes from *mdx* mice are shown for comparison.

The BIO 14.6 hamster represents another model for δ-SG-deficient limb girdle muscular dystrophy-2F with cardiomyopathy (41, 42). A large deletion of the δ-SG gene (43, 44) causes selective loss of the entire SG–SSPN subcomplex from skeletal muscle of the BIO 14.6 hamster (16, 17, 42, 45, 46). Expression levels of β-DG and dystrophin are not altered by loss of the SG–SSPN complex (42, 46). We now show that AQP4 expression is also absent from the BIO 14.6 sarcolemma in adult hamsters (**Fig. 5**). AQP4 expression is maintained within the BIO 14.6 muscle sarcolemma after delivery of an adenovirus encoding δ-SG (Fig. 5). Injection of the δ-SG adenovirus into young BIO 14.6 hamsters maintains expression of the SG–SSPN subcomplex and prevents the pathogenesis of muscular dystrophy (8, 34).

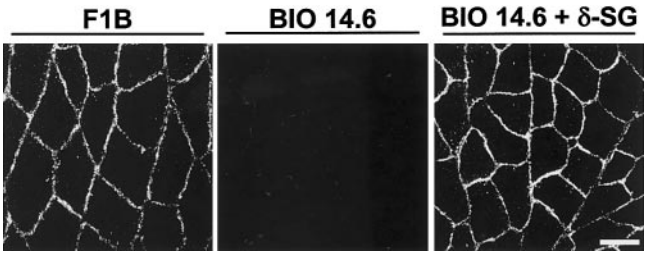


**Figure 5.** Restoration of AQP4 expression in sarcoglycan-deficient muscle. Loss of aquaporin from the δ-sarcoglycan-deficient BIO 14.6 hamster can be restored with recombinant δ-SG adenovirus. Quadriceps muscle of the BIO 14.6 hamster was injected with $10^9$ particles of δ-SG adenovirus particles (BIO 14.6+γ-SG). Tissue was harvested 7 days postinjection. Muscle cryosections from these injected animals as well as from the F1B and BIO 14.6 (uninjected) hamsters were examined for aquaporin expression by indirect immunofluorescence with AQP4 antibodies. Bar, 50 μm.

### AQP4 is not localized to the neuromuscular junction

In our survey of AQP4 expression in skeletal muscle, we discovered that AQP4 was only expressed extrasynaptically. To visualize neuromuscular junctions (NMJs), cryosections from the quadriceps femoris of wild-type mice were stained with fluorescein α-bungarotoxin, which selectively binds to acetylcholine receptors at the NMJ (**Fig. 6**). By indirect immunofluorescence, we showed that AQP4 is expressed at the sarcolemma but not at the NMJ (Fig. 6). In fact, we *never* observed AQP4

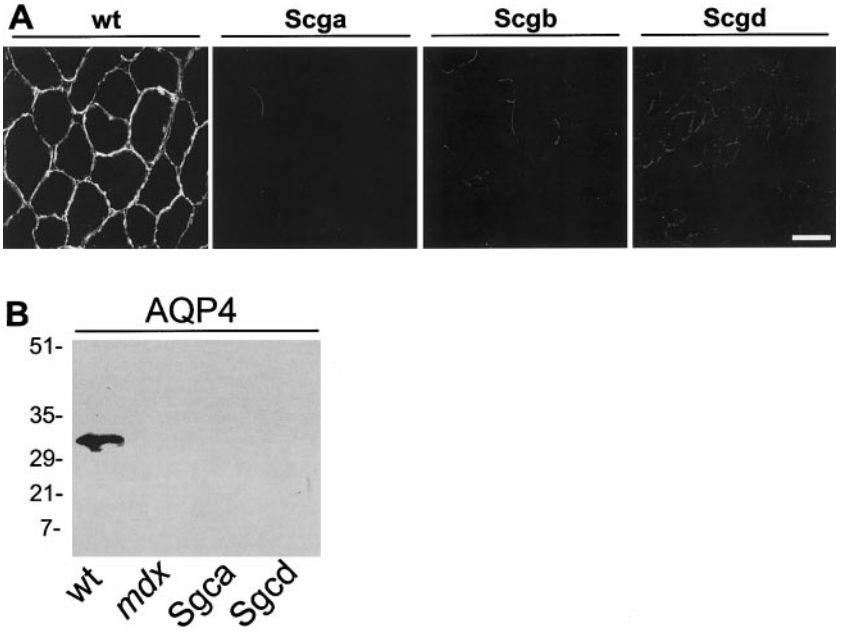


**Figure 4.** AQP4 localization sarcoglycan-deficient muscular dystrophy. *A)* Cryosections from quadriceps of adult wild-type (wt), α-, β-, and δ-SG null mice (Scga, Scgb, Scgd) were stained with antibodies to AQP4. Sections were visualized by indirect immunofluorescence with Cy3-conjugated secondary antibodies. *B)* Skeletal muscle membranes were prepared from adult wild-type (wt), *mdx*, α-, and δ-SG null mice were analyzed by 3–15% SDS-PAGE and immunoblotted using antibodies against AQP4. AQP4 migrates at ~32 kDa. The level of AQP4 expression is dramatically reduced in the sarcoglycan and dystrophin-deficient muscle. Molecular weights are indicated on the left ($\times 10^3$ Da).

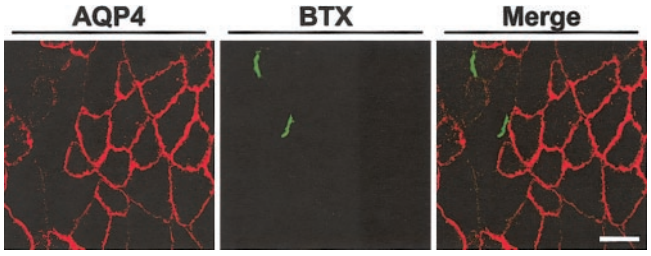


**Figure 6.** AQP4 is excluded from the neuromuscular junction. Analysis of aquaporins at the neuromuscular junction of adult wild-type murine muscle. AQP4 was visualized by indirect immunofluorescence with Cy3-conjugated secondary antibodies (AQP4, red) and synaptic sites were identified by fluorescein-α-bungarotoxin staining (BTX, green). Merged image (overlapping sites are yellow) is shown in the right panel. AQP4 is not localized to the NMJ and is clearly excluded from synaptic regions. Bar, 50 μm.

at the sarcolemma adjacent to innervated regions of myofibers.

Enrichment at the NMJ is a common characteristic of proteins associated with the dystrophin–glycoprotein complex. At the NMJ, dystrophin is replaced by a structurally and functionally similar protein, utrophin (47–52). The demonstration that AQP4 is not found in NMJs is a clear indicator that AQP4 is not associated with the utrophin–glycoprotein complex. Furthermore, our data suggest that therapeutic up-regulation of utrophin in dystrophin-deficient muscle would not restore AQP4 to the sarcolemma membrane.

## DISCUSSION

We show that AQP4 is deficient in six distinct animal models of muscular dystrophy. Our data demonstrate that loss of AQP4 in dystrophic muscle is a consequence of muscle pathology. This is emphasized by the normal expression of AQP4 in young, prenecrotic dystrophin-deficient and sarcoglycan-deficient muscle, despite the absence of syntrophins and other DGC components (Fig. 3; data not shown). It is not clear whether loss of aquaporin during muscle degeneration contributes to the progress of disease pathogenesis. We also find normal expression of AQP4 in nNOS null mice (53) and sarcospan-deficient mice (54), which have normal DGC expression and muscle morphology (data not shown). A recent report demonstrates that protein and mRNA levels of AQP4 are diminished in denervated muscle, suggesting that innervation may also influence AQP4 expression (55). Hind limb suspension increased AQP4 expression in rat soleus, suggesting that AQP4 levels may be determined by the glycolytic capabilities of muscle fibers (37).

It is interesting to speculate on the possible roles of AQP4 within distinct muscle substructures. Measurements of force generation and osmostic water permeability of EDL muscles from AQP4$^{-/-}$ and *mdx* mice suggest that AQP4 null mice are physiologically normal (24). The authors conclude that AQP4 does not play an important role in regulating water homeostasis in either normal or dystrophic muscle (24). Compensation for aquaporin function by an undetermined water channel may at least partially explain results from the AQP4 null mice. However, results from AQP4$^{-/-}$ mice do not agree with the well-established fragility and osmotic instability of the dystrophin-deficient sarcolemma (14, 15, 56). Aquaporins are poised to play an important role in physiological water homeostasis in muscle, given that myofibers must rapidly respond to changes in cell volume during muscle contraction. Our data clearly demonstrate that loss of aquaporins is a common feature of skeletal muscle pathology. However, it is unclear whether loss of aquaporins in muscular dystrophy contributes to muscle pathology. This could be tested directly by generating *mdx*:AQP4 double null mice. Young double mutant mice (<3 wk of age) lacking both dystrophin and AQP4 may exhibit earlier symptoms of muscular dystrophy, indicating that loss of AQP4 contributes to muscle pathology. Recent analysis of skeletal muscle has demonstrated that AQP4 is enriched in type IIA and B (fast) myofibers and that type IIB fibers are the first fiber type to exhibit reduction of AQP4 in *mdx* muscle (36, 37). Type IIB fibers are preferentially affected in DMD patients (57). The authors reported that mRNA levels of AQP4 are not reduced in dystrophin-deficient muscle, suggesting that loss of AQP4 protein occurs post-transcriptionally (36, 37).

Yokota and colleagues have reported that AQP4 is absent in skeletal muscle and perivascular astrocyte end feet of α1-syntrophin null mice, indicating that this syntrophin isoform plays a role in either targeting or stabilizing AQP4 at the sarcolemma (58). Syntrophins interact directly with dystrophin and mediate the attachment of dystrobrevin and nNOS to the DGC complex (59, 60). Recently, Neely and colleagues demonstrated that AQP4 could be immunoprecipitated with syntrophin and β-dystroglycan from chemically cross-linked brain and skeletal muscle lysates (61). Syntrophin expression is normal in sarcoglycan-deficient mice and the BIO14.6 hamster (data not shown). Muscle from the α1-syntrophin knockout mice appears normal and does not display any signs of muscle damage (62).

Taken together, our data demonstrate that AQP4 is lost from dystrophic muscle as a secondary consequence of muscle pathology. The molecular event that triggers the reduction of aquaporins in muscle disease is unknown, but we speculate that it is an early event in pathogenesis. For instance, aquaporins may be very sensitive to increases in intracellular $Ca^{+2}$ levels, which may activate proteases leading to premature aquaporin protein degradation. Understanding the mechanisms of muscle pathogenesis will provide insights for therapies aimed at blocking or preventing the onset of degeneration. FJ

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## REFERENCES

1. Campbell, K. P., and Kahl, S. D. (1989) Association of dystrophin and an integral membrane glycoprotein. *Nature (London)* **338,** 259–262
2. Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990) Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature (London)* **345,** 315–319
3. Yoshida, M., and Ozawa, E. (1990) Glycoprotein complex anchoring dystrophin to sarcolemma. *J. Biochem.* **108,** 748–752
4. Ervasti, J. M., and Campbell, K. P. (1991) Membrane organization of the dystrophin–glycoprotein complex. *Cell* **66,** 1121–1131
5. Ervasti, J. M., Kahl, S. D., and Campbell, K. P. (1991) Purification of dystrophin from skeletal muscle. *J. Biol. Chem.* **266,** 9161–9165
6. Lim, L. E., and Campbell, K. P. (1998) The sarcoglycan complex in limb-girdle muscular dystrophy. *Neurology* **11,** 443–452
7. Crosbie, R. H., Heighway, J., Venzke, D. P., Lee, J. C., and Campbell, K. P. (1997) Sarcospan: the 25kDa transmembrane component of the dystrophin–glycoprotein complex. *J. Biol. Chem.* **272,** 31221–31224
8. Crosbie, R. H., Lebakken, C. S., Holt, K. H., Venzke, D. P., Straub, V., Lee, J. C., Grady, R. M., Chamberlain, J. S., Sanes, J. R., and Campbell, K. P. (1999) Membrane targeting and stabilization of sarcospan is mediated by the sarcoglycan subcomplex. *J. Cell Biol.* **145,** 153–165
9. Ervasti, J. M., and Campbell, K. P. (1993) A role for the dystrophin–glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122,** 809–823
10. Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y., and Ozawa, E. (1994) Molecular organization at the glycoprotein-complex-binding site of dystrophin. Three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. *Eur. J. Biochem.* **220,** 283–292
11. Jung, D., Yang, B., Meyer, J., Chamberlain, J. S., and Campbell, K. P. (1995) Identification and characterization of the dystrophin anchoring site on beta-dystroglycan. *J. Biol. Chem.* **270,** 27305–27310
12. Montanaro, F., Lindenbaum, M., and Carbonetto, S. (1999) alpha-Dystroglycan is a laminin receptor involved in the extracellular matrix assembly on myotubes and muscle cell viability. *J. Cell Biol.* **145,** 1325–1340
13. Talts, J. F., Andac, Z., Gohring, W., Brancaccio, A., and Timpl, R. (1999) Binding of the G-domain of laminin alpha1 and alpha2 chains and perlecan to heparin, sulfatides, alpha-dystroglycan and several extracellular matrix proteins. *EMBO J.* **18,** 863–870
14. Weller, B., Karpati, G., and Carpenter, S. (1990) Dystrophin-deficient *mdx* muscle fibers are preferentially vulnerable to necrosis induced by experimental lengthening contractions. *J. Neurol. Sci.* **100,** 9–13
15. Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M., and Sweeney, H. L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. USA* **90,** 3710–3714
16. Crosbie, R. H., Lim, L. E., Moore, S. A., Hirano, M., Hays, A. P., Maybaum, S. W., Collin, H., Dovico, S. A., Stolle, C. A., Fardeau, M., Tome, F. M., and Campbell, K. P. (2000) Molecular and genetic characterization of sarcospan: insights into sarcoglycan–sarcospan interactions. *Hum. Mol. Genet.* **9,** 2019–2027
17. Duclos, F., Straub, V., Moore, S. A., Venzke, D. P., Hrstka, R. F., Crosbie, R. H., Durbeej, M., Lebakken, C. S., Ettinger, A. J., van der Meulen, J., Holt. K. H., Lim, L., Sanes, J. R., Davidson, B. L., Faulkner, J. A., Williamson, R., and Campbell, K. P. ( 1998) Progressive muscular dystrophy in α-sarcoglycan deficient mice. *J. Cell Biol.* **142,** 1461–1471
18. Coral-Vazquez, R., Cohn, R. D., Moore, S. A., Hill, J. A., Weiss, R. M., Davisson, R. L., Straub, V., Barresi, R., Bansal, D., Hrstka, R. F., Williamson, R., and Campbell, K. P. (1999) Disruption of the sarcoglycan–sarcospan complex in vascular smooth muscle: a novel mechanism for cardiomyopathy and muscular dystrophy. *Cell* **98,** 465–474
19. Durbeej, M., Cohn, R. D., Hrstka, R. F., Moore, S. A., Allamand, V., Davidson, B. L., Williamson, R. A., and Campbell, K. P. (2000) Disruption of the beta-sarcoglycan gene reveals pathogenetic complexity of limb-girdle muscular dystrophy type 2E. *Mol. Cell* **5,** 141–151
20. Frigeri, A., Nicchia, G. P., Verbavatz, J. M., Valenti, G., and Svelto, M. (1998) Expression of aquaporin-4 in fast-twitch fibers of mammalian skeletal muscle. *J. Clin. Invest.* **102,** 695–703
21. Liu, J. W., Wakayama, Y., Inoue, M., Shibuya, S., Kojima, H., Jimi, T., and Oniki, H. (1999) Immunocytochemical studies of aquaporin 4 in the skeletal muscle of mdx mouse. *J. Neurol. Sci.* **164,** 24–28
22. Hasegawa, H., Ma, T., Skach, W., Matthay, M. A., and Verkman, A. S. (1994) Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues. *J. Biol. Chem.* **269,** 5497–5500
23. Frigeri, A., Gropper, M. A., Turck, C. W., and Verkman, A. S. (1995) Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. *Proc. Natl. Acad. Sci. USA* **92,** 4328–4331
24. Yang, B., J. M. Verbavatz, Y. Song, L. Vetrivel, G. Manley, W. M. Kao, T. Ma, and, A. S. Verkman (2000) Skeletal muscle function and water permeability in aquaporin-4 deficient mice. *Am. J. Physiol.* **278,** C1108–C1115
25. Wakayama, Y., Jimi, T., Misugi, N., Kumagai, T., Miyake, S., Shibuya, S., and Miike, T. (1989) Dystrophin immunostaining and freeze-fracture studies of muscles of patients with early stage amyotrophic lateral sclerosis and Duchenne muscular dystrophy. *J. Neurol. Sci.* **91,** 191–205
26. van Beaumont, W., Strand, J. C., Petrofsky, J. S., Hipskind, S. G., and Greenleaf, J. E. (1973) Changes in total plasma content of electrolytes and proteins with maximal exercise. *J. Cell. Physiol.* **34,** 102–106
27. Kowalchuk, J. M., Heigenhauser, G. J., Lindinger, M. I., Sutton, J. R., and Jones, N. L. (1988) Factors influencing hydrogen ion concentration in muscle after intense exercise. *J. Cell. Physiol.* **65,** 2080–2089
28. Lindinger, M. I., Spriet, L. L., Hultman, E., Putman, T., McKelvie, R. S., Lands, L. C., Jones, N. L., and Heigenhauser, G. J. (1994) Plasma volume and ion regulation during exercise after low- and high- carbohydrate diets. *Am. J. Physiol.* **266,** R1896–R1906
29. Cox, G. A., Sunada, Y., Campbell, K. P., and Chamberlain, J. S. (1994) Dp71 can restore the dystrophin-associated glycoprotein complex in muscle but fails to prevent dystrophy. *Nature Genet.* **8,** 333–339
30. Phelps, S. F., Hauser, M. A., Cole, N. M., Rafael, J. A., Hinkle, R. T., Faulkner, J. A., and Chamberlain, J. S. (1995) Expression of full-length and truncated dystrophin mini-genes in transgenic *mdx* mice. *Hum. Mol. Genet.* **4,** 1251–1258
31. Rafael, J. A., Cox, G. A., Corrado, K., Jung, D., Campbell, K. P., and Chamberlain, J. S. (1996) Forced expression of dystrophin deletion constructs reveals structure–function correlations. *J. Cell Biol.* **134,** 93–102
32. Corrado, K., Rafael, J. A., Mills, P. L., Cole, N. M., Faulkner, J. A., Wang, K., and Chamberlain, J. S. (1996) Transgenic *mdx* mice expressing dystrophin with a deletion in the actin-binding domain display a 'mild Becker' *phenotype. J. Cell Biol.* **134,** 873–884

33. Rafael, J. A., Sunada, Y., Cole, N. M., Campbell, K. P., Faulkner, J. A., and Chamberlain, J. S. (1994) Prevention of dystrophic pathology in *mdx* mice by a truncated dystrophin isoform. *Hum. Mol. Genet.* **3,** 1725–1733
34. Holt, K. H., Lim, L. E., Straub, V., Venzke, D. P., Duclos, F., Anderson, R. D., Davidson, B. L., and Campbell, K. P. (1998) Functional rescue of the sarcoglycan complex in the Bio 14.6 hamster using δ-sarcoglycan gene transfer. *Mol. Cell* **1,** 841–848
35. Borgnia, M., Nielsen, S., Engel, A., and Agre, P. (1999) Cellular and molecular biology of the aquaporin water channels. *Annu. Rev. Biochem.* **68,** 425–458
36. Frigeri, A., Nicchia, G. P., Nico, B., Quondamatteo, F., Herken, R., Roncali, L., and Svelto, M. (2001) Aquaporin-4 deficiency in skeletal muscle and brain of dystrophic *mdx* mice. *FASEB J.* **15,** 90–98
37. Frigeri, A., Nicchia, G. P., Desaphy, J. F., Pierno, S., De Luca, A., Camerino, D. C., and Svelto, M. (2001) Muscle loading modulates aquaporin-4 expression in skeletal muscle. *FASEB J.* **15,** 1282–1284
38. Greenberg, D. S., Sunada, Y., Campbell, K. P., Yaffe, D., and Nudel, U. (1994) Exogenous Dp71 restores the levels of dystrophin associated proteins but does not alleviate muscle damage in mdx mice. *Nature Genet.* **8,** 340–344
39. McArdle, A., Edwards, R. H., and Jackson, M. J. (1994) Time course of changes in plasma membrane permeability in the dystrophin-deficient *mdx* mouse. *Muscle Nerve* **17,** 1378–1384
40. Ohlendieck, K., and Campbell, K. P. (1991) Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J. Cell Biol.* **115,** 1685–1694
41. Iwata, Y., Nakamura, H., Mizuno, Y., Yoshida, M., Ozawa, E., and Shigekawa, M. (1993) Defective association of dystrophin with sarcolemmal glycoproteins in the cardiomyopathic hamster heart. *FEBS Lett.* **329,** 227–231
42. Roberds, S. L., Ervasti, J. M., Anderson, R. D., Ohlendieck, K., Kahl, S. D., Zoloto, D., and Campbell, K. P. (1993) Disruption of the dystrophin–glycoprotein complex in the cardiomyopathic hamster. *J. Biol. Chem.* **268,** 11496–11499
43. Nigro, V., Okazaki, Y., Belsito, A., Piluso, G., Matsuda, Y., Politano, L., Nigro, G., Ventura, C., Abbondanza, C., Molinari, A. M., Acampora, D., Nishimura, M., Hayashizaki, Y., and Puca, G. A. (1997) Identification of the Syrian hamster cardiomyopathy gene. *Hum. Mol. Genet.* **6,** 601–607
44. Sakamoto, A., Ono, K., Abe, M., Jasmin, G., Eki, T., Murakami, Y., Masaki, T., Yoyo-oka, T., and Hanaoka, F. (1997) Both hypertrophic and dilated cardiomyopathies are caused by mutation of the same gene, delta-sarcoglycan, in hamster: an animal model of disrupted dystrophin-associated glycoprotein complex. *Proc. Natl. Acad. Sci. USA* **94,** 13873–13878
45. Mizuno, Y., Noguchi, S., Yamamoto, H., Yoshida, M., Nonaka, I., Hirai, S., and Ozawa, E. (1995) Sarcoglycan complex is selectively lost in dystrophic hamster muscle. *Am. J. Pathol.* **146,** 530–536
46. Holt, K. H., and Campbell, K. P. (1998) Assembly of the sarcoglycan complex: insights for LGMD. *J. Biol. Chem.* **273,** 34667–34670
47. Khurana, T. S., Watkins, S. C., Chafey, P., Chelly, J., Tome, F. M., Fardeau, M., Kaplan, J. C., and Kunkel, L. M. (1991) Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. *Neuromusc. Disorders* **1,** 185–194
48. Nguyen, T. M., Ellis, J. M., Love, D. R., Davies, K. E., Gatter, K. C., Dickson, G., and Morris, G. E. (1991) Localization of the DMDL gene-encoded dystrophin-related protein using a panel of nineteen monoclonal antibodies: presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles, and in proliferating brain cell lines. *J. Cell Biol.* **115,** 1695–1700
49. Ohlendieck, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J., and Campbell, K. P. (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* **7,** 499–508
50. Pons, F., Augier, N., Leger, J. O., Robert, A., Tome, F. M., Fardeau, M., Voit, T., Nicholson, L. V., Mornet, D., and Leger, J. J. (1991) A homologue of dystrophin is expressed at the neuromuscular junctions of normal individuals and DMD patients, and of normal and mdx mice. Immunological evidence. *FEBS Lett.* **282,** 161–165
51. Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D., and Campbell, K. P. (1992) Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. *Nature (London)* **360,** 588–591
52. Karpati, G., Carpenter, S., Morris, G. E., Davies, K. E., Guerin, C., and Holland, P. (1993) Localization and quantitation of the chromosome 6-encoded dystrophin-related protein in normal and pathological human muscle. *J. Neuropathol. Exp. Neurol.* **52,** 119–128
55. Jimi, T., Wakayama, Y., Murahashi, M., Shibuya, S., Inoue, M., Hara, H., Matsuzaki, Y., and Uemura, N. (2000) Aquaporin 4: lack of mRNA expression in the rat regenerating muscle fiber under denervation. *Neurosci. Lett.* **291,** 93–96
56. Menke, A., and Jockusch, H. (1991) Decreased osmotic stability of dystrophin-less muscle cells from the *mdx* mouse. *Nature (London)* **349,** 69–71
57. Webster, C., Silberstein, L., Hays, A. P., and Blau, H. M. (1988) Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. *Cell* **52,** 503–513
58. Yokota, T., Y. Miyagoe, Y. Hosaka, K. Tsukita, S. Kameya, S. Shibuya, R. Matsuda, Y. Wakayama, and, S. Takeda (2000) Aquaporin-4 is absent at the sarcolemma and perivascular astrocyte endfeet in a1-syntrophin knockout mice. *Proc. Jpn. Acad.* **76,** 22–27
59. Yang, B., Ibraghimov-Beskrovnaya, O., Moomaw, C. R., Slaughter, C. A., and Campbell, K. P. (1994) Heterogeneity of the 59-kDa dystrophin-associated protein revealed by cDNA cloning and expression. *J. Biol. Chem.* **269,** 6040–6044
60. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) SH3 domain-mediated interaction of dystroglycan and Grb2. *J. Biol. Chem.* **270,** 11711–11714
61. Neely, J. D., Amiry-Moghaddam, M., Ottersen, O. P., Froehner, S. C., Agre, P., and Adams, M. E. (2001) Syntrophin-dependent expression and localization of Aquaporin-4 water channel protein. *Proc. Natl. Acad. Sci. USA* **98,** 14108–14113
62. Kameya, S., Miyagoe, Y., Nonaka, I., Ikemoto, T., Endo, M., Hanaoka, K., Nabeshima, Y., and Takeda, S. (1999) Alpha1-syntrophin gene disruption results in the absence of neuronal-type nitric-oxide synthase at the sarcolemma but does not induce muscle degeneration. *J. Biol. Chem.* **274,** 2193–2200

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