Caveolin-3 is not an integral component of the dystrophin glycoprotein complex

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Abstract The dystrophin-glycoprotein complex is a multisubunit protein complex that spans the muscle plasma membrane (sarcolemma) and forms a link between the intracellular cytoskeleton and the extracellular matrix. Caveolin-3, the muscle specific form of caveolin, is also a major structural and regulatory integral membrane protein found at the sarcolemma. Oligomers of caveolin-3 form the structural framework for small membrane pockets known as caveolae. We directly examined whether caveolin-3 is an integral component of the dystrophin-glycoprotein complex by examining four common biochemical and cellular properties of proteins integrally bound to the dystrophin-glycoprotein complex. We found that caveolin-3 deenriches with partial purification of the dystrophin-glycoprotein complex although a small amount of caveolin-3 is present. Sucrose gradient fractionation and laminin affinity chromatography completely separate this residual caveolin-3 from the core components of the dystrophin-glycoprotein complex. We also show that caveolin-3 expression at the sarcolemma is not reduced in patients with primary mutations in either dystrophin or the sarcoglycans. This data demonstrates that localization of caveolin-3 to the sarcolemma occurs independently of the dystrophin-glycoprotein complex and that caveolin-3 is not an integral component of the dystrophin-glycoprotein complex.

Key words: Muscle; Sarcolemma; Dystrophin-glycoprotein complex; Caveolin; Sarcospan

1. Introduction

The dystrophin-glycoprotein complex (DGC) consists of both peripheral and integral membrane proteins and spans the skeletal muscle plasma membrane known as the sarcolemma. Purification of the DGC from rabbit skeletal muscle has led to the identification of many of its constituent proteins and has provided insights into the function of this large oligomeric complex [1–4]. In addition to dystrophin, the DGC is composed of α/β-dystroglycan, the sarcoglycans (α, β, γ, and δ subunits), the syntrophins, and a 25-kDa hydrophobic protein known as sarcospan [1–3,5]. Disruption of the DGC eventually progresses to muscle cell necrosis, as evidenced by the dystrophic muscle phenotypes that result from defects in several of the DGC components (for reviews, see [6,7]).

The DGC is known to be essential for normal muscle physiology, although its precise role remains to be determined. One function ascribed to the dystrophin-glycoprotein complex is to confer stability to the sarcolemma, protecting it from damage that might otherwise result from shear forces generated during repeated contraction of muscle fibers [8,9]. While this is indeed likely to be true, the organization of the DGC into distinct subcomplexes [10] suggests that the complex may contribute in multiple ways to muscle cell function. One possibility is that the link between the extracellular matrix and the cell interior provides a framework for signal transduction across the muscle cell membrane. α-Dystroglycan is structurally positioned for such a role as it binds laminin-2 in the extracellular matrix and is attached to the membrane by its association with β-dystroglycan and the sarcoglycans [11–13].

Caveolin-3, a 21–24-kDa integral membrane protein, is also an important structural and regulatory component of the sarcolemma [14–18]. Oligomers of caveolin form the scaffold of caveolae pockets, which are non-clathrin coated membrane invaginations (for reviews, see [19,20]). Caveolae are thought to concentrate signaling events located at or near the surface of the plasma membrane of most cell types, including skeletal muscle [21–25]. Immunoprecipitation and subcellular fractionation studies from differentiated C2C12 mouse myotubes suggest that dystrophin may be associated with caveolin-3 [26]. These experiments, however, do not probe for high affinity interactions and are not criteria for examining integral components of the dystrophin-glycoprotein complex. Thus, we undertook to determine whether caveolin-3 is an integral component of the dystrophin-glycoprotein complex.

2. Materials and methods

2.1. Protein preparations

The DGC was extracted with 1% digitonin from rabbit skeletal muscle KCl-washed microsomes [27] and then purified by sWGA affinity chromatography followed by ion exchange on a DEAE column, as described previously [1,2,28].

2.2. DGC sucrose gradients

DGC fractions from the DEAE column were pooled and concentrated to 800 μl. The samples were applied to a 5–30% sucrose gradient and centrifuged with a Beckman VTi65.1 vertical rotor at $200\,000 \times g$ for 90 min at 4°C [2,28]. The gradients were fractionated into 800-μl fractions, and 80 μl of each fraction was resolved on 3–15% SDS-polyacrylamide gradient gels and transferred to nitrocellulose [29].

2.3. Immunoblot analysis

Immunoblot staining was performed as previously described [27]. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Millipore) by the method of Towbin et al. [29]. Nitrocellulose transfers were blocked in TBS/blotto (20 mM Tris, 100 mM

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Abbreviations: Cav-3, caveolin-3; DGC, dystrophin-glycoprotein complex; DG, dystroglycan; SG, sarcoglycan

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NaCl, pH 7.5, 3% non-fat dry milk) and subsequently incubated for 1 h with primary antibodies to DGC (goat 20, 1:500 [30]), caveolin-3 (Transduction Labs, monoclonal IgG, 1:2000 [26]), or sarcospan (rabbit 217, 1:20 [5]). Antibodies to the DGC were generated by immunizing a goat with the DGC purified from rabbit skeletal membranes, as described previously. The serum contains antibodies to all of the DGC components, except sarcospan. We generated antibodies to sarcospan by injecting an N-terminal KLH-coupled peptide into rabbits, as described previously [5]. The blots were washed with TBS/blotto and incubated for 1 h with the appropriate horseradish peroxidase conjugated secondary antibodies. The blots were developed using either chloronaphthol (for goat 20 and sarcospan blots) or enhanced chemiluminescence (for caveolin-3 blots, Pierce).

2.4. Laminin affinity chromatography

Mouse EHS sarcoma laminin (a kind gift from Dr. Hynda K. Kleinman at the National Institutes of Health) was coupled to CNBr-activated Sepharose-4B (Pharmacia), as described previously [31]. DGC (0.4 ml) was incubated at 4°C overnight with 0.1 ml of laminin Sepharose in the presence of 5 mM Tris-HCl, pH 7.4, 130 mM NaCl, 0.1% digitonin, 1 mM $CaCl_2$. The laminin-Sepharose was centrifuged to collect the supernatants (voids), washed extensively, and eluted with the same volume of 10 mM EDTA buffer. Samples from the DGC, void, and eluate (100 μl each) were electrophoresed on a 3–12% SDS-PAGE and transferred to nitrocellulose.

2.5. Immunofluorescence

Immunofluorescence on muscle biopsies from C57/BL wild type and *mdx* mice as well as control human and several Duchenne muscular dystrophy (DMD) patients was accomplished as described [5]. Muscle sections were incubated with antibodies to dystrophin and caveolin-3 (Transduction Labs, 1:1000) for 1 h at room temperature. After 1 h, the sections were washed with TBS and incubated with the appropriate Cy3-conjugated secondary antibodies (1:250) for 1 h. The sections were observed under a Bio-Rad MRC-600 laser scanning confocal microscope and the digitized images were captured under identical conditions.

3. Results and discussion

Integral components of the dystrophin-glycoprotein complex can be defined by four biochemical and cellular criteria. First, purification of the dystrophin-glycoprotein complex from skeletal muscle membranes enriches proteins that are associated in a complex with dystrophin. Purification of this complex is achieved by sWGA affinity chromatography followed by ion exchange chromatography. A second characteristic of integral DGC proteins is their migration as a complex during high speed centrifugation through sucrose gradients. Only proteins that bind with high affinity and specificity will be retained with dystrophin during sucrose gradient fractionation. Previous experiments have demonstrated that the DGC migrates as an 18S complex in sucrose gradients [32]. Third, the core components of the DGC can be isolated by laminin affinity chromatography. α-Dystroglycan, an extracellular component of the DGC, is a high affinity receptor for laminin [11,12,31,33] and thus binds tightly to laminin conjugated to Sepharose. By virtue of this interaction, all members of the complex are retained during laminin affinity chromatography. And lastly, integral components of the DGC depend on the integrity of the complex for proper localization to the sarcolemma. If dystrophin is absent, as in the case of DMD patients, the entire DGC is absent from the sarcolemma. We used these distinguishing criteria to directly determine whether caveolin-3 is an integral component of the DGC.

As a first test for association of caveolin-3 with the DGC, we examined partially purified DGC for the presence of caveolin-3. Immunoblots of purified DGC stained with DGC antibodies show a clear enrichment of these purified proteins relative to the levels in rabbit skeletal muscle membranes (Fig. 1). Identical immunoblots illustrate that caveolin-3 does not enrich in DGC prepared from rabbit membranes (Fig. 1). Dystrophin is enriched by approximately 10-fold during purification of the DGC, while caveolin-3 is reduced by 3-fold. A small amount of caveolin-3 is present in the partially purified DGC and we performed several experiments in order to determine the specificity of this association.

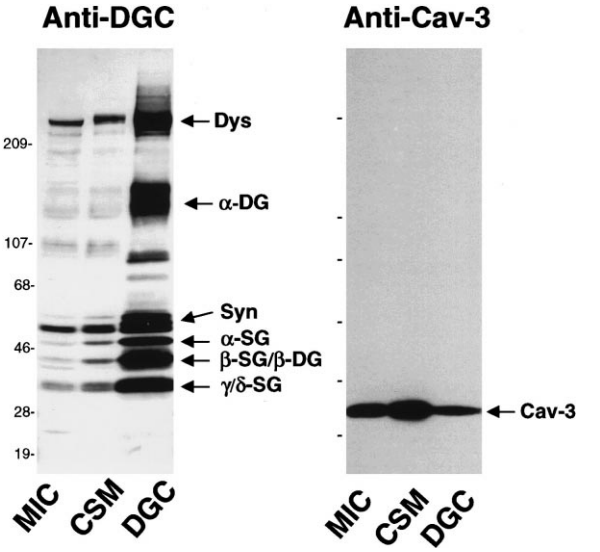

Fig. 1. Caveolin-3 is not a component of the dystrophin glycoprotein complex (DGC) isolated from rabbit skeletal muscle. Caveolin-3 does not enrich with the dystrophin-glycoprotein complex. Rabbit skeletal muscle KCl-washed membranes (MIC), crude surface membranes (CSM), and purified dystrophin-glycoprotein complex (DGC) were electrophoretically separated on 3–15% SDS polyacrylamide gels and transferred to nitrocellulose. Nitrocellulose transfers were separately stained with antibodies against DGC (left panel) or monoclonal antibodies against caveolin-3 (right panel). Molecular size standards are indicated on the left of each panel ($\times 10^3$ Da).

The tight association of the DGC components is illustrated by centrifugation of the DGC through sucrose density gradients. Non-specifically bound proteins are efficiently separated from the core components of the DGC by this method. Proteins from the sucrose gradient fractions were separated by SDS-PAGE. The resultant polyacrylamide gels, immunoblotted with DGC antibodies, illustrate that the DGC peaks in fractions 8–10 (Fig. 2A). Western blotting of these same fractions with caveolin-3 antibodies demonstrates that caveolin-3 begins to sediment in fraction 12 and peaks in fraction 15 (Fig. 2A). Caveolin-3 migrates in the later fractions due to its self assembly into higher order oligomers. These results from sucrose gradient centrifugation indicate that caveolin-3 is not an integral component of the DGC and is not tightly bound to this complex.

α-Dystroglycan has been shown to bind laminin-2 in the extracellular matrix in a Ca^{2+} dependent manner. We used laminin Sepharose affinity chromatography to test whether caveolin-3 is specifically associated with the DGC. The partially purified DGC was incubated with laminin conjugated Sepharose. The void was collected from the laminin-Sepharose by centrifugation and the Sepharose was subsequently washed, and eluted with 10 mM EDTA. Treatment with EDTA chelates calcium, causing release of bound α-dystro-

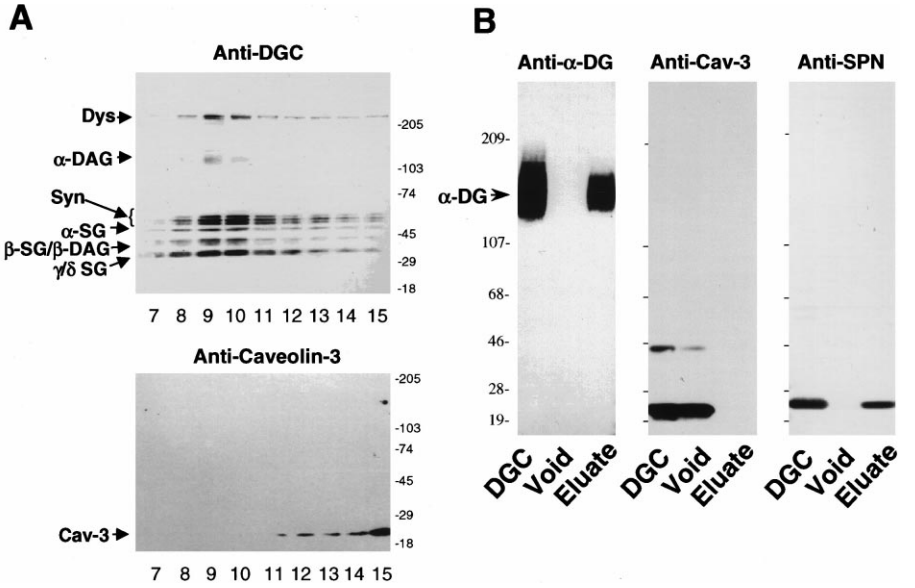

Fig. 2. Dissociation of caveolin-3 from the DGC. A: Caveolin-3 separates from the DGC during sucrose gradient centrifugation. DGC purified from rabbit skeletal muscle membranes was centrifuged through sucrose gradients. DGC proteins in fractions 7–15 from the sucrose gradient were electrophoresed on 3–15% SDS-polyacrylamide gels. Nitrocellulose transfers of identical samples were stained with DGC and caveolin-3 antibodies. B: Purification of the DGC with laminin Sepharose removes caveolin-3. Purified DGC (input) was incubated with laminin Sepharose. The void (void) and eluate (eluate) protein samples were separated by SDS-PAGE and transferred to nitrocellulose. Identical blots were stained with α-dystroglycan, caveolin-3, and sarcospan antibodies. Molecular size standards are indicated on each panel ($\times 10^3$ Da).

glycan from the laminin-Sepharose. The starting material (DGC), void (void), and eluate (eluate) protein samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to α-dystroglycan, caveolin-3, and sarcospan (Fig. 2B). Caveolin-3 did not adhere to the laminin Sepharose and was found only in the 'flow through' or void of the column (Fig. 2B). Both α-dystroglycan and sarcospan bound tightly to the laminin column and were exclusively present in the eluate. The DGC does not completely elute from the laminin sepharose, which accounts for the small differences in the amount of α-dystroglycan in DGC and eluate. Thus, caveolin-3 was completely separated from the DGC by this method.

As another probe for the possible interaction between caveolin-3 and the DGC, we examined caveolin-3 expression in muscle from DMD patients and the murine model for this disease, the *mdx* mouse. We examined several DMD patients with primary mutations in dystrophin, resulting in loss of the entire dystrophin protein. Indirect immunofluorescence assays with antibodies against dystrophin's N and C termini and the central rod domain verify that dystrophin is completely absent from the DMD sarcolemma (Fig. 3). Without dystrophin, the other members of the complex are reduced, perhaps the result of premature protein degradation, improper assembly of the complex, or aberrant transportation to the sarcolemma. We show that caveolin-3 is present at the sarcolemma in normal and DMD muscle (Fig. 3). In addition, we found positive caveolin-3 staining at the sarcolemma in muscle from several

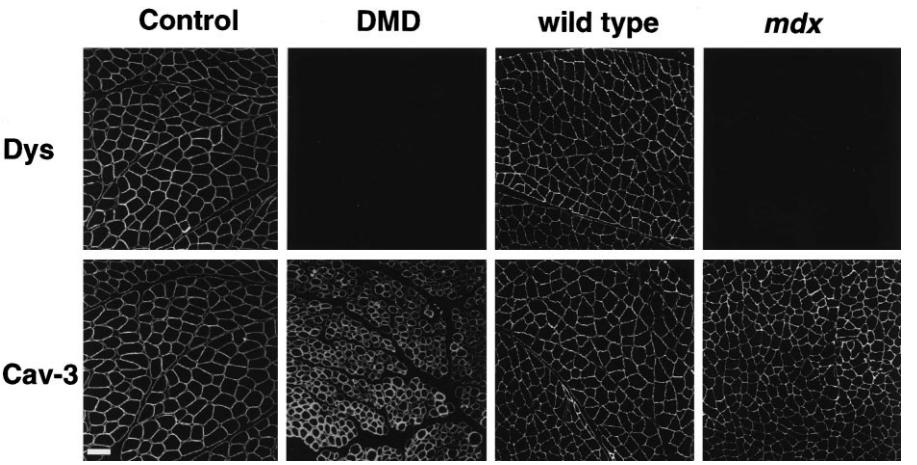

Fig. 3. Sarcolemma localization of caveolin-3 is independent of the DGC. Immunohistochemical analysis of skeletal muscle from normal human control (control), DMD (DMD), wild type mouse (wild type) and *mdx* mouse (*mdx*). Transverse cryosections were labeled by indirect immunofluorescence with antibodies against dystrophin and caveolin-3. Absence of dystrophin in the DMD patient was confirmed with three separate antibodies (not shown). Bar, 100 μm.

limb-girdle muscular dystrophy patients with primary mutations in the sarcoglycans (data not shown). We also examined caveolin-3 expression in the *mdx* mouse, which has a premature stop codon in the dystrophin gene resulting in absence of the protein. We show that caveolin-3 is present in skeletal muscle of these mice (Fig. 3). These data provide strong evidence that caveolin-3 localizes to the sarcolemma independently of dystrophin.

Taken together, our results indicate that caveolin-3 is not an integral part of the dystrophin-glycoprotein complex, although small quantities of caveolin-3 can be detected in partially purified DGC preparations. Sucrose gradient fractionation and laminin affinity purification separate the integral components of the DGC from caveolin-3, demonstrating that this residual caveolin-3 is not tightly bound to the DGC. Our data do not rule out the possibility that caveolin-3 might interact with the DGC or that the DGC may be found in caveolae. Several membrane associated proteins (i.e. actin, nNOS, and laminin) interact with the DGC at the sarcolemma, but are not integral components of this complex. We suspect that the DGC is assembled as a complex in the endoplasmic reticulum prior to translocation to the sarcolemma. This may account for the absence of the complex when one of the components is missing, as in the case of DMD. It is also feasible to speculate that caveolin-3 interacts transiently with the DGC. Such interactions could play a regulatory function and would be consistent with the suggestion that defective caveolin-3 might be involved in the pathophysiology of muscular dystrophy. This latter suggestion is based on observed abnormalities in caveolae size and distribution in patients with Duchenne muscular dystrophy [34–37]. There are many examples of muscle diseases which are caused by primary defects in proteins that are not integral components of the DGC (i.e. emerin, calpain) and our data do not exclude the possibility that defects in caveolin-3 may give rise to a muscular dystrophy phenotype.

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References

[1] Campbell, K.P. and Kahl, S.D. (1989) Nature 338, 259–262.
[2] Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) Nature 345, 315–319.
[3] Yoshida, M. and Ozawa, E. (1990) J. Biochem. 108, 748–752.
[4] Ervasti, J.M. and Campbell, K.P. (1991) Cell 66, 1121–1131.
[5] Crosbie, R.H., Heighway, J., Venzke, D.P., Lee, J.C. and Campbell, K.P. (1997) J. Biol. Chem. 272, 31221–31224.
[6] Campbell, K.P. (1995) Cell 80, 675–679.
[7] Straub, V. and Campbell, K.P. (1997) Curr. Opin. Neurol. 10, 168–175.
[8] Menke, A. and Jockusch, H. (1995) J. Cell Sci. 108, 727–733.
[9] Pasternak, C., Wong, S. and Elson, E.L. (1995) J. Cell Biol. 128, 355–361.
[10] Yoshida, M., Suzuki, A., Yamamoto, H., Noguchi, S., Mizuno, Y. and Ozawa, E. (1994) Eur. J. Biochem. 222, 1055–1061.
[11] Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W. and Campbell, K.P. (1992) Nature 355, 696–702.
[12] Ervasti, J.M. and Campbell, K.P. (1993) J. Cell Biol. 122, 809–823.
[13] Gee, S.H., Blacher, R.W., Douville, P.J., Provost, P.R., Yurchenco, P.D. and Carbonetto, S. (1993) J. Biol. Chem. 268, 14972–14980.
[14] Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y., Glenney, J.R. and Anderson, R.G.W. (1992) Cell 68, 673–682.
[15] Glenney, J.R. (1992) FEBS Lett. 314, 45–48.
[16] Dupree, P., Parton, R.G., Raposo, G., Kurzchalia, T.V. and Simons, K. (1993) EMBO J. 12, 1597–1605.
[17] Way, M. and Parton, R.G. (1995) FEBS Lett. 376, 108–112.
[18] Tang, Z. et al. (1996) J. Biol. Chem. 271, 2255–2261.
[19] Lisanti, M.P., Scherer, P.E., Tang, Z., Kubler, E., Koleske, A.J. and Sargiacomo, M. (1995) Dev. Biol. 6, 47–58.
[20] Lisanti, M.P., Scherer, P.E., Tang, Z. and Sargiacomo, M. (1994) Trends Cell Biol. 4, 231–235.
[21] Severs, N.J. (1988) J. Cell Sci. 90, 341–348.
[22] Simionescu, N., Simionescu, M. and Palade, G.E. (1975) J. Cell Biol. 64, 586–607.
[23] Anderson, R.G.W. (1993) Proc. Natl. Acad. Sci. USA 90, 10909–10913.
[24] Lisanti, M.P., Scherer, P.E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.H., Cook, R.F. and Sargiacomo, M. (1994) J. Cell Biol. 126, 111–126.
[25] Lisanti, M.P., Tang, Z., Scherer, P.E., Kubler, E., Koleske, A.J. and Sargiacomo, M. (1995) Mol. Membr. Biol. 12, 121–124.
[26] Song, K.S. et al. (1996) J. Biol. Chem. 271, 15160–15165.
[27] Ohlendieck, K., Ervasti, J.M., Snook, J.B. and Campbell, K.P. (1991) J. Cell Biol. 112, 135–148.
[28] Ervasti, J.M., Kahl, S.D. and Campbell, K.P. (1991) J. Biol. Chem. 266, 9161–9165.
[29] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[30] Jung, D. et al. (1996) J. Biol. Chem. 271, 32321–32329.
[31] Yamada, H., Shimizu, T., Tanaka, T., Campbell, K.P. and Matsumura, K. (1994) FEBS Lett. 352, 49–53.
[32] Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) Nature 345, 315–319.
[33] Yamada, H. et al. (1996) J. Neurochem. 66, 1518–1524.
[34] Bonilla, E., Fischbeck, K. and Schotland, D.L. (1981) Am. J. Pathol. 104, 167–173.
[35] Peluchetti, D., Mora, M., Protti, A. and Cornelio, F. (1985) Neurology 6, 928–930.
[36] Bonilla, E., Fischbeck, K.H. and Schotland, D.L. (1983) Neurology 33, 1346–1347.
[37] Costello, B.R. and Shafiq (1979) Muscle Nerve 2, 191–201.