Caveolin-3 Directly Interacts with the C-terminal Tail of β-Dystroglycan

IDENTIFICATION OF A CENTRAL WW-LIKE DOMAIN WITHIN CAVEOLIN FAMILY MEMBERS*

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Caveolin-3, the most recently recognized member of the caveolin gene family, is muscle-specific and is found in both cardiac and skeletal muscle, as well as smooth muscle cells. Several independent lines of evidence indicate that caveolin-3 is localized to the sarcolemma, where it associates with the dystrophin-glycoprotein complex. However, it remains unknown which component of the dystrophin complex interacts with caveolin-3. Here, we demonstrate that caveolin-3 directly interacts with β-dystroglycan, an integral membrane component of the dystrophin complex. Our results indicate that caveolin-3 co-localizes, co-fractionates, and co-immunoprecipitates with a fusion protein containing the cytoplasmic tail of β-dystroglycan. In addition, we show that a novel WW-like domain within caveolin-3 directly recognizes the extreme C terminus of β-dystroglycan that contains a PXY motif. As the WW domain of dystrophin recognizes the same site within β-dystroglycan, we also demonstrate that caveolin-3 can effectively block the interaction of dystrophin with β-dystroglycan. In this regard, interaction of caveolin-3 with β-dystroglycan may competitively regulate the recruitment of dystrophin to the sarcolemma. We discuss the possible implications of our findings in the context of Duchenne muscular dystrophy.

Caveolae are 50–100 nm invaginations that represent an appendage or subcompartment of the plasma membrane (1). They are found in most cell types and are localized in close proximity to the plasma membrane (2). Caveolins, a gene family of 21–25-kDa integral membrane proteins, are the principal protein components of caveolae membranes (3, 4).

Caveolins are thought to play an important structural role in the formation of caveolae membranes, by acting as scaffolding proteins to organize and concentrate specific caveolin-interacting lipids and proteins within caveolae microdomains (5–7). To date, the mammalian caveolin gene family consists of caveolins 1, 2, and 3 (2, 8–10).

Caveolin-3, the most recently recognized member of caveolin gene family, is muscle-specific and is found in both cardiac and skeletal muscle, as well as smooth muscle cells (8, 11). The expression of caveolin-3 is induced during the differentiation of skeletal myoblasts; immunolocalization of caveolin-3 in normal skeletal muscle fibers reveals that caveolin-3 is localized to the sarcolemma and co-incident with the localization of dystrophin (11). Biochemically, subcellular fractionation studies indicate that caveolin-3 co-fractionates with members of the dystrophin-associated protein complex (11), although caveolin-3 itself is not a required component for the biogenesis of the dystrophin glycoprotein complex (12). However, it remains unknown which member of the dystrophin-dystroglycan complex interacts with caveolin-3.

Here, we have examined the direct interaction of caveolin-3 with β-dystroglycan (an integral membrane protein component of the complex), using a variety of complementary molecular and genetic approaches. We have localized the caveolin-binding site to the extreme C terminus of β-dystroglycan. As dystrophin is known to bind β-dystroglycan through 15 residues at the C-terminal of β-dystroglycan (13–16), we demonstrate that caveolin-3 may regulate the interaction of β-dystroglycan with dystrophin by competitively recognizing the same site as dystrophin on β-dystroglycan.

EXPERIMENTAL PROCEDURES

Materials—Antibodies and their sources were as follows: anti-caveolin-3 IgG (mouse mAb1 cl 26 (11) was the generous gift of Dr. Roberto

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FIG. 1. Co-localization of caveolin-3 and β-dystroglycan within a single cell. NIH 3T3 cells were co-transfected with the cDNAs encoding caveolin-3 and AP-β-DG. Caveolin-3 expression was detected with a specific mouse mAb (clone 26); β-dystroglycan expression was detected with a rabbit pAb that recognizes the alkaline phosphatase epitope. Bound primary antibodies were visualized by incubation with distinctly tagged fluorescent secondary antibodies (fluorescein-conjugated for caveolin-3 and rhodamine-conjugated for AP-β-DG). Cells expressing both gene products were visualized by confocal laser fluorescence microscopy. Note that caveolin-3 and β-dystroglycan demonstrate significant co-localization at the level of the plasma membrane.

Caveolin-3 Directly Interacts with β-Dystroglycan

Caveolin-3 was shown to interact with β-dystroglycan. This interaction was demonstrated through several experiments:

**Immunofluorescence**—NIH 3T3 cells were grown on coverslips coated with poly-L-lysine (Sigma), fixed in 2% paraformaldehyde, and permeated for caveolin-3 and rhodamine-conjugated for AP-β-DG. Bound primary antibodies were visualized by incubation with specific antibodies directed against caveolin-3 and β-dystroglycan.

**Immunoblotting**—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum (19). Cells (17, 18). 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum (19). Cells were transfected using a modified calcium phosphate precipitation method and analyzed 48 h post-transfection. The derivation of a 293T stable cell line overexpressing AP-β-DG was as described previously (15). Briefly, AP-β-DG is a fusion protein carrying the transmembrane and cytoplasmic domain of β-dystroglycan fused to the ectodomain of alkaline phosphatase (AP). The cDNA for caveolin-3 was as previously described (10).

**Cell Culture and Transfection**—NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum (17, 18). 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum (19). Cells were transfected using a modified calcium phosphate precipitation method and analyzed 48 h post-transfection. The derivation of a 293T stable cell line overexpressing AP-β-DG was as described previously (15).

**Immunofluorescence**—NIH 3T3 cells were grown on coverslips coated with poly-L-lysine (Sigma), fixed in 2% paraformaldehyde, and permeabilized in 0.1% Triton X-100. Cells were then immunostained with monoclonal anti-caveolin-3 and polyclonal anti-placental alkaline phosphatase. Bound primary antibodies were visualized with a fluorescein-conjugated anti-mouse antibody and a rhodamine-conjugated anti-rabbit antibody (Jackson ImmunoResearch Inc.). As expected, omission of the primary antibodies prevented immunostaining.

**Preparation of Caveolae-enriched Membrane Fractions**—Transfected 293T cells were scraped into 0.7 ml of Mes-buffered saline (MBS, 25 mM Mes, pH 6.5, 0.15 mM NaCl) containing 1% (v/v) Triton X-100 (19–29). Homogenization was carried out with 10 strokes of a tight fitting Dounce homogenizer. The homogenates were adjusted to 40% sucrose by the addition of 0.7 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homogenate and centrifuged at 44,000 rpm for 16–20 h in a SW60 rotor (Beckman Instruments). A light scattering fraction was separated by SDS-PAGE and subjected to immunoblot analysis.

**Immunoblotting**—Samples were separated by SDS-PAGE under reducing conditions, and transferred to nitrocellulose membranes (Schleicher and Schuell). The protein bands were visualized with Ponceau S (Sigma). Membranes were blocked with 5% low-fat dried milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20). Blots were then incubated at room temperature for 1 h with either anti-caveolin-3 IgG or anti-caveolin-3-IgG, washed in TBST, and incubated with a secondary antibody conjugated with horseradish peroxidase (BD Transduction Laboratories). Bound IgG were detected using a chemiluminescent substrate (Pierce).

**Co-immunoprecipitation Assay**—Co-immunoprecipitation studies were performed essentially as previously described (11). Briefly, 293T cells stably expressing AP-β-DG were transiently transfected with increasing amounts of the caveolin-3 cDNA. Cells were lysed and subjected to immunoprecipitation with specific a mouse monoclonal IgG directed against caveolin-3. After extensive washing, samples were then probed with a mAb directed against β-dystroglycan. Note that caveolin-3 specifically co-immunoprecipitates with β-dystroglycan; no β-dystroglycan was found in the precipitate lacking caveolin-3.
responds to amino acids 3046–3447. Cav-3-WW-like domain (corresponding to amino acids 34–129) and Cav-1-WW-like domain (corresponding to amino acids 61–156) were amplified and subcloned into the BamHI/SalI sites of pGEX-4T vector. All the GST fusion protein constructs were transformed into *Escherichia coli* (BL21 strain; Novagen, Inc.). After induction of expression through addition of 5 mM isopropyl-β-D-galactoside (Sigma), GST fusion proteins were affinity purified on glutathione-agarose beads, using the detergent Sarcosyl for initial solubilization (30).

**GST-β-dystroglycan “Pull-down” Assay**—GST-β-dystroglycan full-length, a variety of deletion mutants and GST alone (bound to glutathione-agarose beads) were extensively washed first with TNET buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) (3 times) and lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octylglucoside), both containing protease inhibitors. SDS-PAGE followed by Coomassie staining was used to determine the approximate molar quantities of the fusion proteins per 100 μl of packed bead volume. Pre-cleared lysates of 293T cells overexpressing caveolin-3 were diluted in buffer A (10 mM Tris, pH 8.0, 0.1% Tween 20) and added to approximately 100 μl of equilibrated bead volume for overnight incubation at 4 °C. After binding, the beads were extensively washed with phosphate-buffered saline (6 times). Finally, the beads were resuspended in 3 x sample buffer and subjected to SDS-PAGE.

**GST-Cav-3 Pull-down Assay**—The pull-down assay using GST alone or GST-Cav-3 fusion proteins was essentially as described above, except that 293T cells stable expressing AP-β-DG cDNA were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 300 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). Pre-cleared lysates were diluted in Tween buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.1% Tween 20, 1 mM dithiothreitol, and protease inhibitors) and added to approximately 100 μl of equilibrated bead volume for overnight incubation at 4 °C.

**Overlay Assay**—Overlay binding assays were performed essentially as described previously, with minor modifications (15). Peptide dot blots (SPOTS; Genosys Biotechnologies) were blocked overnight with blocking buffer supplied by the manufacturer (Genosys, Inc.) and then probed with 125I-labeled GST-Cav-3-WW, GST-Cav-1-WW, or GST alone. Bound protein complexes were visualized by autoradiography.

**β-Dystroglycan Peptide Pull-down Assay**—293T cells were transiently transfected with the cDNA encoding caveolin-3. Forty-eight
Caveolin-3 family members contain a central WW-like domain. A, caveolin family members. The protein sequences of vertebrate caveolins identified to date are shown. Note the positions of the conserved aromatic residues (Trp, Tyr, or Phe) and proline (Pro) that are required to form a WW domain.

Our results indicate that caveolin-3 and β-dystroglycan dem-

FIG. 5. Caveolin family members contain a central WW-like domain. A, caveolin family members. The protein sequences of vertebrate caveolins identified to date are shown. Note the positions of the conserved aromatic residues (Trp, Tyr, or Phe) and proline (Pro) that are characteristic of known WW domains. The important residues are boxed and the consensus is indicated in bold. W-X-Y-n-X-f(−)₁₈-W-X-(−)₈-W-X-P. B, caveolin-2 lacks certain critical residues that are required to form a WW-like domain. B, other WW-like domains. WW-like sequences have also been identified in the PDGF-β receptor and related receptors (32) and in CD45-AP (33). An alignment of caveolin-3 with the WW-like regions of PDGF-β receptor, CD45-AP, and YAP is shown and highlights the conserved Trp, aromatic (Φ), and Pro residues.

hours post-transfection, cells were collected into 1 ml of lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 60 mM octylglucoside), containing protease inhibitors. After pre-clearing, biotinylated β-dystroglycan-derived peptides (either WT or the AA mutant) were added pre-bound to streptavidin-agarose beads. After incubation for 4 h at 4°C, the beads were extensively washed with phosphate-buffered saline (6 times). Finally, the beads were resuspended in 3 x sample buffer, separated by SDS-PAGE (12% acrylamide), and transferred to nitrocellulose membranes. Blots were probed with a mouse mAb directed against the N terminus of caveolin-3 (11). To generate streptavidin beads containing pre-bound biotinylated peptides, the beads (50 µl) were incubated overnight with a 1-ml solution containing ~15 µg/ml of peptide dissolved in lysis buffer.

Dystrophin Competition Assay—A 10-cm plate of confluent cells overexpressing AP-β-DG and four 10-cm plates overexpressing caveolin-3 were lysed in Buffer B (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM sodium deoxycholate, 1% Nonidet P-40, and protease inhibitors). Clarified AP-β-DG cell lysates were diluted in Tween buffer and incubated with glutathione-Sepharose immobilized GST-dystrophin fusion protein, glutathione-Sepharose immobilized GST, or with glutathione-Sepharose alone. Sample were then treated with 0.75 ml of cell lysate from 293T cells transfected with caveolin-3 cDNA, or with an equal volume of lysis buffer. Following an overnight incubation at 4°C, glutathione-Sepharose conjugates were pelleted by centrifugation and washed extensively in phosphate-buffered saline. Complexes were disrupted by boiling in 3 x Laemmli buffer. Equal volumes of each sample were subjected to SDS-PAGE as described above.

WW-ligand Peptide Competition—293T cells were transiently transfected with the cDNAs encoding full-length caveolin-3 and β-dystroglycan. Forty-eight hours post-transfection, cells were lysed in IP buffer containing protease inhibitors. After pre-clearing, antibodies directed against caveolin-3 and a given peptide competitor (200 µg/ml) were added to the cell lysates. After incubation for 3 h at 4°C, the immunoprecipitates were washed 3 times with IP buffer, the samples were separated by SDS-PAGE, and transferred to nitrocellulose membranes. Blots were probed with antibodies directed against β-dystroglycan.

RESULTS

Caveolin-3 and β-Dystroglycan Interact in Vivo—Caveolin-3 and β-dystroglycan are known to localize to the sarcolemma and both coincide with the distribution of dystrophin (11, 31). To examine a possible association between caveolin-3 and β-dystroglycan, we used a heterologous expression system. NIH 3T3 cells were co-transfected with the cDNAs encoding caveolin-3 and alkaline phosphatase-tagged β-dystroglycan (AP-β-DG) and subjected to immunostaining. Caveolin-3 expression was detected with a specific mouse monoclonal Ab (clone 26); β-dystroglycan expression was detected with a rabbit polyclonal Ab that recognized the alkaline phosphatase epitope. Cells expressing both gene products were visualized by imaging with confocal laser fluorescence microscopy (Fig. 1). Our results indicate that caveolin-3 and β-dystroglycan demonstrate significant co-localization at the level of the plasma membrane.

We next examined the subcellular distribution of caveolin-3 and β-dystroglycan. To separate membranes enriched in caveolin-3 from the bulk of cellular membranes and cytosolic pro-
proteins, an established equilibrium sucrose density gradient system was utilized (19–29). In this fractionation scheme, immunoblotting with anti-caveolin-3 IgG can be used to track the position of caveolae-derived membranes within these bottom-loaded sucrose gradients. Fig. 2 shows that 90–95% of caveolin-3 (fractions 4 and 5) is separated from the bulk of cellular proteins. We also examined the distribution of \( \beta \)-dystroglycan in these sucrose density gradients. Fig. 2 demonstrates that 20–25% of \( \beta \)-dystroglycan co-fractionates with caveolin-3.

To further investigate whether caveolin-3 and \( \beta \)-dystroglycan are physically associated, co-immunoprecipitation experiments were performed. Lysates from 293T cells overexpressing both proteins were immunoprecipitated with anti-caveolin-3 IgG. As a negative control, a lysate from cells overexpressing only \( \beta \)-dystroglycan was used. Immunoprecipitates were then subjected to immunoblot analysis with anti-\( \beta \)-dystroglycan specific IgG. Note that only GST-caveolin-3 bound \( \beta \)-dystroglycan; no binding was observed with GST alone or with the same reaction performed with beads alone. C, as in panel B, except the \( \beta \)-dystroglycan binding activity of GST-Cav-3-(34–129) and GST-Cav-2-(46–141) were compared. Note that the homologous region of caveolin-2 shows little or no binding activity; it also lacks the WW domain consensus. See Fig. 5A.

**Fig. 6. Binding of \( \beta \)-dystroglycan to the WW-like domain of caveolin-3.** A, schematic representation of full-length caveolin-3 and a GST construct containing the WW-like domain of caveolin-3 (residues 34–129). B, lysates from a 293T stable cell line overexpressing \( \beta \)-dystroglycan were incubated with affinity purified GST alone or GST-Cav-3-WW-(34–129) immobilized on glutathione-agarose beads. After extensive washing, the beads were resuspended in 3 \times \) sample buffer, boiled, and subjected to immunoblot analysis with anti-\( \beta \)-dystroglycan specific IgG. Note that only GST-caveolin-3 bound \( \beta \)-dystroglycan; no binding was observed with GST alone or with the same reaction performed with beads alone. C, as in panel B, except the \( \beta \)-dystroglycan binding activity of GST-Cav-3-(34–129) and GST-Cav-2-(46–141) were compared. Note that the homologous region of caveolin-2 shows little or no binding activity; it also lacks the WW domain consensus. See Fig. 5A.
Identification of a Central WW-like Domain in Caveolin-3—As the extreme C-terminal domain of β-dystroglycan serves as the dystrophin anchoring site (13, 14), and as this interaction occurs via the WW domain of dystrophin (15, 16), we searched for a putative WW domain within the caveolin-3 protein sequence. We found two tryptophan (Trp) residues separated by 29 amino acids; interestingly, these Trp residues are highly conserved among other caveolin family members. In addition, the second tryptophan (Trp) is followed by a highly conserved proline (Pro) residue. A similar sequence arrangement is found in the WW domains of other proteins. Fig. 5A shows an alignment of the caveolin family members and highlights the conserved Trp, Tyr, and Pro residues. Note that caveolin-2 lacks certain critical residues that are required to form a WW domain.

Other WW-like sequences have been identified in the PDGF-β receptor and related receptors (32), and in CD45-AP (33). Fig. 5B shows an alignment of caveolin-3 with the WW-like regions of the PDGF-β receptor, CD45-AP, and YAP and highlights the conserved Trp, aromatic (Φ), and Pro residues. In the case of the mPDGF-β receptor, the WW-like sequence was shown to bind proline-rich ligands. It is possible that the WW domain, like the SH3 module, could “tolerate” insertions and maintain the basic core fold that mediates interaction with proline-rich ligands (34, 35).

Interestingly, the region of caveolins that contains the central WW-like domain has been previously proposed to function as a putative membrane-spanning domain. However, this proposal was based solely on primary sequence predictions (36–38). Several independent lines of evidence now suggest that this region of caveolins does not function as a membrane anchor. For example, this region of caveolins shows little or no affinity for membranes in vitro or in vivo (39, 40), while other adjacent regions experimentally confer membrane attachment (39, 40). In addition, the putative membrane spanning domains of caveolins 1 and 2 interact with each other to form hetero-oligomeric complexes that contain both caveolins 1 and 2 (41). Thus, this provides evidence that this putative membrane spanning segment can function as a domain that mediates protein-protein interactions (41). In support of this notion, a GST fusion protein containing the putative membrane spanning domain of caveolin-1 is soluble, does not aggregate in

Fig. 7. Fine mapping of the region of β-dystroglycan that interacts with the WW-like domain of caveolins 1 and 3. A, sequence and description of the 7 different peptides synthesized directly on SPOTs membranes. B, these SPOTs membranes were probed with 125I-labeled GST-Cav-3-WW, GST-Cav-1-WW, or with GST alone. Note that the WW-like domains of both caveolin-3 and caveolin-1 recognize the C-terminal 12 amino acids of β-dystroglycan that corresponds to the ligand of the WW domain of dystrophin. No binding activity was observed with GST alone.

Fig. 8. Recognition of β-dystroglycan by the WW-like domain of caveolin-3 is dependent on the PPXY motif. A, sequence and description of the 2 different biotinylated peptides used in the pull-down assay. B, 293T cells were transfected with the cDNA encoding caveolin-3. Lysates were then prepared and incubated with biotinylated peptides pre-bound to streptavidin beads. After washing, these precipitates were subjected to Western blot analysis with antibodies directed against caveolin-3. Note that the wild-type β-dystroglycan-peptide containing the PPXY motif (Biot-βDG-WT) effectively pulls down caveolin-3. In contrast, a mutant of the same β-dystroglycan-peptide lacking the PPXY motif (Biot-βDG-AA) shows little or no binding to caveolin-3. Similarly, streptavidin beads alone showed no caveolin-3 binding.
solution, and shows no affinity for membranes in vitro (5, 39). In addition, this putative membrane spanning segment is 32–33 amino acids, which is much longer than most transmembrane domains (18–22 amino acids) (3, 4). Primary sequence prediction programs indicate that this region is predicted to assume a \( \beta \)-sheet conformation (not shown), rather than the typical \( \alpha \)-helical conformation of traditional transmembrane domains. A predicted \( \beta \)-sheet conformation is more consistent with a WW domain-like structure.

To test the hypothesis that this caveolin-3 WW-like domain recognizes \( \beta \)-dystroglycan, we constructed a caveolin-3 GST fusion protein containing this WW-like domain (residues 34–129). As a critical control for these studies, we compared the binding of caveolin-3 to GST alone and agarose beads alone to rule out any possible nonspecific binding. As substrate for these binding experiments, lysates from cells overexpressing \( \beta \)-dystroglycan were utilized. Binding of AP-\( \beta \)-DG was detected by Western blotting using a mAb against \( \beta \)-dystroglycan. Fig. 6, A and B, shows that the GST-caveolin-3 containing the WW-like domain specifically bound \( \beta \)-dystroglycan. In contrast, the homologous region of caveolin-2, which does not conform to the WW consensus (Fig. 5A), fails to bind \( \beta \)-dystroglycan (Fig. 6C).

Fine Mapping of the \( \beta \)-Dystroglycan Region which Interacts with the WW-like Domain of Caveolin-3—The dystrophin-binding site on \( \beta \)-dystroglycan has been localized to the C-terminal 15 amino acids of \( \beta \)-dystroglycan (13, 14). Thus, we next investigated whether the caveolin-3 WW-like domain is able to recognize the same C-terminal 15 amino acids of \( \beta \)-dystroglycan. A series of 12 amino acid peptides were directly synthesized on SPOTs membranes. These peptides included a number of known WW domain ligands and the \( \beta \)-dystroglycan ligand for the WW domain of dystrophin. Fig. 7A shows the sequence and description of these peptides. The SPOTs membranes were then probed with \( ^{125} \)I-labeled GST-Cav-3-WW, GST-Cav-1-WW, or GST alone. Fig. 7B shows that the WW-like domains of caveolin-1 and caveolin-3 are both capable of binding only SPOT number 5 (TPYRSPPPYVPP) which is the \( \beta \)-dystroglycan ligand (residues 884–895) for the WW domain of dystrophin. Importantly, no nonspecific binding was observed with GST alone. These results suggest that caveolin-3 and dystrophin are able to bind the same site on \( \beta \)-dystroglycan.

FIG. 9. A C-terminal truncation mutant of \( \beta \)-dystroglycan (AP-\( \beta \)-DG-\( \Delta \)PPXY) that lacks the PPXY motif does not interact with caveolin-3. A, sequence and description of the 2 different AP-\( \beta \)-DG constructs. WT, wild-type; \( \Delta \)PPXY, lacking the extreme C terminus. B, recombinant expression of AP-\( \beta \)-DG-WT and AP-\( \beta \)-DG-\( \Delta \)PPXY. 293T cell lysates were subjected to immunoblot analysis with a pAb directed against the AP-epitope. C, lysates from 293T cells transiently co-expressing AP-\( \beta \)-DG (WT or \( \Delta \)PPXY) and caveolin-3 were immunoprecipitated with anti-caveolin-3 IgG. As negative controls, lysates from cells overexpressing AP-\( \beta \)-DG alone were also utilized. Immunoprecipitates were then subjected to immunoblot analysis with a mAb directed against the AP epitope. Note that caveolin-3 specifically co-immunoprecipitates with AP-\( \beta \)-DG-WT, while little or no binding is observed with AP-\( \beta \)-DG-\( \Delta \)PPXY.

FIG. 10. Caveolin-3 and dystrophin compete for binding to the C-terminal tail of \( \beta \)-dystroglycan. Lysates from a stable line overexpressing \( \beta \)-dystroglycan were incubated overnight with affinity purified GST-dystrophin (residues 3046–3447) or GST alone immobilized on glutathione-agarose beads. In a parallel experiment, a cell lysate from 293T cells overexpressing caveolin-3 was mixed with the lysate from the stable line overexpressing \( \beta \)-dystroglycan. This mixed lysate was then incubated overnight with affinity purified GST-dystrophin or GST alone immobilized on glutathione-agarose beads. Note that the presence of caveolin-3 disrupts the binding of dystrophin to \( \beta \)-dystroglycan. These results are as predicted and suggest that caveolin-3 competes with dystrophin for the same or an overlapping binding site on \( \beta \)-dystroglycan.
Immunoprecipitation experiments were performed. Lysates from cells overexpressing AP-β-dystroglycan specifically co-immunoprecipitates with AP-β-dystrophin-WW domain, lysates from cells overexpressing AP-β-dystroglycan-peptide lacking the PPXY motif (TPYRSAAAAVPP) shows little or no binding to caveolin-3. Thus, the PPXY motif and its surrounding sequence is necessary for caveolin-3 binding in vitro.

To further investigate the requirement for the PPXY motif, we next generated a C-terminal truncation mutant of β-dystroglycan (AP-β-DG-ΔPPXY) that lacks the PPXY motif (Fig. 9, A and B). To assess its possible interaction with caveolin-3, co-immunoprecipitation experiments were performed. Lysates from 293T cells transiently co-expressing AP-β-DG (WT or ΔPPXY) and caveolin-3 were immunoprecipitated with anti-caveolin-3 IgG. As negative controls, lysates from cells overexpressing AP-β-DG alone were also utilized. Immunoprecipitates were then subjected to immunoblot analysis with a pAb directed against the AP-epitope. Fig. 9C shows that caveolin-3 specifically co-immunoprecipitates with AP-β-DG-WT, while little or no binding is observed with AP-β-DG-ΔPPXY. Taken together, these results suggest that the PPXY motif is critical for the interaction of β-dystroglycan with caveolin-3.

**Competition of Caveolin-3 and Dystrophin for Binding to β-Dystroglycan**—Since caveolin-3 and dystrophin are able to bind the same site on β-dystroglycan, we next wanted to test the hypothesis that they interact competitively with β-dystroglycan. Lysates from cells overexpressing AP-β-DG were prepared and incubated with the GST-dystrophin-WW domain fusion protein. In a parallel experiment, cell lysates from cells overexpressing β-dystroglycan were pre-mixed with lysates from cells overexpressing caveolin-3. The mixture was then added to beads containing the GST-dystrophin-WW domain fusion protein. As negative controls, reactions with GST alone and beads alone were performed.

Fig. 10 shows that in the absence of caveolin-3, the GST-dystrophin-WW domain is able to pull-down β-dystroglycan. In contrast, the presence of caveolin-3 interferes with the interaction between β-dystroglycan and dystrophin, such that β-dystroglycan binding to GST-dystrophin-WW is no longer detected.

**Is Binding of the Caveolin-3 WW-like Domain to β-Dystroglycan Affected by Phosphorylation of the PPXY Motif?**—It has been recently shown that phosphorylation of the tyrosine in the PPXY motif of β-dystroglycan blocks its recognition by the WW domains of both utrophin (42, 43) and dystrophin (16). Thus, we next examined if tyrosine phosphorylation of the motif might also block the recognition of this motif by the WW-like domain of caveolin-3.

Interestingly, Fig. 11 shows that the WW domain of caveolin-3 recognizes the wild-type unphosphorylated peptide (TPYRSPPPYPVPP) and two different phosphorylated forms of the same peptide (TP(pY)RSPPPYPVPP or TPYRSPPP(pY)YPVPP) equally well. Thus, unlike the binding of utrophin and dystrophin to β-dystroglycan, recognition by caveolin-3 is tyrosine phosphorylation independent.

**Deletion Mutagenesis of the Cav-3 WW-like Domain**—In order to define a minimal functional region of the caveolin-3 WW domain, we used a mutagenesis approach. A series of eight GST-Cav-3 WW deletion mutants were generated and are shown schematically in Fig. 12A. These mutants were then used as the substrate for binding to β-dystroglycan.

Note that, as predicted, a central region from residues 64 to 114 appears most critical for binding (Fig. 12B). This region also contains the W-Φ-Φ-W-P consensus sequence. In addition, our results suggest that the caveolin-3 scaffolding domain does not participate in β-dystroglycan binding, as four different GST-Cav-3 fusion proteins (residues 34–99, 34–84, 34–74, and 55–74) containing the full-length scaffolding domain (residues 55–74) show no binding activity (Fig. 12C).

To further investigate a possible role for the caveolin-3 scaffolding domain in β-dystroglycan binding, we next used a second independent approach, i.e. peptide competition. 293T cells were doubly transfected with cDNAs encoding full-length caveolin-3 and β-dystroglycan. Lysates from these cells were prepared and subjected to immunoprecipitation with anti-caveolin-3 IgG. Co-immunoprecipitation of β-dystroglycan was then detected by Western blotting.

Prior to immunoprecipitation, peptides were added to these lysates. Fig. 13A shows that when a peptide encoding a known ligand (THETEKDLHEKMEDV; from Gqα) to the caveolin scaffolding domain (44) was added, little or no effect was observed. In contrast, when a peptide encoding the WW domain ligand from β-dystroglycan (TPYRSPPPYPVPP) was added, co-immunoprecipitation of β-dystroglycan with caveolin-3 was blocked (Fig. 13B). Importantly, a mutant of the same β-dystroglycan-peptide lacking the PPXY motif (TPYRSAAAAVPP)
did not prevent co-immunoprecipitation of β-dystroglycan with caveolin-3. These results are consistent with the idea that the caveolin-3 scaffolding domain does not participate in β-dystroglycan binding in vivo. In addition, they suggest that the WW domain within full-length caveolin-3 can functionally recognize the C-terminal tail of β-dystroglycan in vivo.

DISCUSSION

Dystrophin is the protein product of the Duchenne muscular dystrophy (DMD) gene (45, 46) and is tightly associated with the sarcolemmal membrane (31). Dystrophin forms a complex with a series of specific dystrophin-associated glycoproteins, termed DAGs. One major component of this complex is dystroglycan (47).

Dystroglycan provides a continuous link between laminin-2 in the extracellular matrix and dystrophin that is attached to the intracellular cytoskeleton (48). Dystroglycan begins as a precursor protein that is proteolytically cleaved into two interacting subunits, α- and β-dystroglycan (49). α-Dystroglycan is a heavily glycosylated extrinsic membrane protein that interacts directly with laminin-2; in contrast, β-dystroglycan is an integral membrane glycoprotein that binds tightly to dystrophin. The dystrophin-anchoring site on β-dystroglycan is localized to the extreme C terminus of β-dystroglycan at amino acids 880–895 (13, 14).

Recent studies have shown that α-dystroglycan can function as an agrin receptor, suggesting that it may play a role in neuromuscular synapse formation (50, 51). In addition, several lines of evidence suggest that β-dystroglycan is part of a membrane-anchored signal transduction complex that interacts with the Src homology 3 domain of Grb-2. Grb-2 is an adaptor protein that helps to initiate the Ras-MAP kinase signal transduction cascade and is involved in controlling cytoskeletal organization (52). Disruption of the dystrophin-glycoprotein complexes.

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### Table: Caveolin-3 WW-like Domain Binding Activity

| Mutant | Binding Activity |
|--------|-----------------|
| 34-129 | +               |
| 49-129 | +               |
| 64-129 | +               |
| 79-129 | +               |
| 34-114 | -               |
| 34-99  | -               |
| 34-84  | -               |
| 34-74  | -               |
| 55-74  | -               |

FIG. 12. **Deletion mutagenesis of the caveolin-3 WW-like domain.** A, to define a minimal functional region of the caveolin-3 WW domain, we used a mutagenesis approach. A series of eight GST-Cav-3 WW deletion mutants were generated and are shown schematically. These mutants were then used as the substrate for binding to β-dystroglycan, as detailed in the legend of Fig. 6. B, note that, as predicted, a central region from residues 64 to 114 appears most critical for binding. C, note that GST-Cav-3 fusion proteins (residues 34–74 and 55–74) containing the full-length caveolin scaffolding domain (residues 55–74) show no binding activity.
plex underlies the molecular pathogenesis of a variety of forms of muscular dystrophy. This suggests that the extracellular matrix-cytoskeletal linkage is critical for maintaining the structural integrity of the sarcolemma (55).

DMD is one of the most common and severe muscle disorders caused by a deficiency of dystrophin. Several morphological observations seemingly implicate muscle cell caveolae in the pathogenesis of DMD: (i) dystrophin has been localized to plasma membrane caveolae in smooth muscle cells using immunoelectron microscopy techniques (54) and (ii) another electron microscopy study demonstrates that skeletal muscle caveolae undergo characteristic changes in size, number, and their distribution in patients with DMD, but not in other forms of neuronally based muscular dystrophies examined (55). In accordance with an increased number of caveolae in DMD patients, recent studies have shown that caveolin-3 protein levels are dramatically up-regulated in mdx mice and in patients with DMD (56, 57). These results suggest that up-regulation of caveolin-3 may contribute to the pathogenesis of DMD.

Previous co-localization, co-immunoprecipitation, and co-fractionation studies have suggested that caveolin-3 is associated with the dystrophin-glycoprotein complex (11). However, under certain conditions caveolin-3 can be physically separated from the dystrophin-glycoprotein complex (12). Here, we have studied the relationship between caveolin-3 and the dystrophin-glycoprotein complex. We examined the direct interaction of caveolin-3 with an integral membrane component of the dystrophin-glycoprotein complex (12). Here, we have studied the relationship between caveolin-3 and the dystrophin-glycoprotein complex. We examined the direct interaction of caveolin-3 with an integral membrane component of the dystrophin-glycoprotein complex, namely β-dystroglycan. Using co-localization, co-fractionation, and co-immunoprecipitation experiments, we demonstrate that caveolin-3 may interact with the dystrophin-glycoprotein complex through the integral membrane protein β-dystroglycan.

We have mapped the caveolin-binding domain on β-dystroglycan to the extreme C terminus of β-dystroglycan. Surprisingly, dystrophin is known to bind β-dystroglycan through 15 residues at the C-terminal of β-dystroglycan (13, 14). Interestingly, the dystrophin/β-dystroglycan interaction occurs primarily through the WW domain of dystrophin (15, 16). The WW domain is a small domain of 38–40 semiconserved amino acids that is widely distributed among various structural, regulatory, and signaling proteins (58, 59). The WW domain is named for the two highly conserved tryptophan (W) residues spaced 20–22 amino acids apart. Various WW domains have been implicated in mediating protein-protein interactions by binding to peptide sequences containing Pro-rich motifs, such as PPXY (60, 61).

Thus, we searched for the presence of a conserved domain which is similar to the WW domain in the caveolin-3 protein. We found two highly conserved tryptophan residues (Trp) separated by 29 residues and followed by a highly conserved proline (Pro). We next constructed a caveolin GST fusion protein carrying this WW-like domain. We demonstrated that this

FIG. 13. The β-dystroglycan WW domain ligand prevents the interaction of full-length caveolin-3 with β-dystroglycan. To further investigate the mechanism of the interaction of caveolin-3 with β-dystroglycan, we used a peptide competition approach. 293T cells were doubly transfected with cDNAs encoding full-length caveolin-3 and β-dystroglycan. Lysates from these cells were prepared and subjected to immunoprecipitation with anti-caveolin-3 IgG. Co-immunoprecipitation of β-dystroglycan was then detected by Western blotting. Prior to immunoprecipitation, peptides were added to these lysates. A, caveolin-scaffolding domain ligand. Note that when a peptide encoding a known ligand (THFTFKDLHFKMFDV; from G$_{o_{i2}}$) for the caveolin scaffolding domain is added, little or no effect is observed. B, WW domain ligand. Note that when a peptide encoding the WW domain ligand from β-dystroglycan (TPYRSPPPYVPP) is added, co-immunoprecipitation of β-dystroglycan with caveolin-3 is blocked. Importantly, a mutant of the same β-dystroglycan-peptide lacking the PPXY motif (TPYRAAAAVPP) does not prevent co-immunoprecipitation of β-dystroglycan with caveolin-3.

FIG. 14. Schematic diagram summarizing the interaction of caveolin-3 with β-dystroglycan. In this report, we show that a WW-like domain within caveolin-3 recognizes the extreme C terminus of β-dystroglycan that contains a PPXY motif. Note that this WW-like domain is located between two distinct domains that have been experimentally shown to attach caveolins to the membrane (40). We have previously termed these functional domains N-MAD (N-terminal membrane attachment domain) and C-MAD (C-terminal membrane attachment domain) (40).
central portion of the caveolin-3 protein is sufficient to mediate β-dystroglycan binding and that this binding is dependent on the PPXY motif in β-dystroglycan. We show that the WW-like domain within caveolin-3 competently recognizes the same site as dystrophin on β-dystroglycan. We also demonstrate that the presence of caveolin-3 is able to disrupt the interaction between β-dystroglycan and dystrophin. These results are summarized schematically in Fig. 14.

In this regard, the interaction of caveolin-3 with β-dystroglycan may competitively regulate the interaction and recruitment of dystrophin to the sarcolemma. As such, the interaction between caveolin-3 and β-dystroglycan may play a key role in stabilizing and regulating the activity of the sarcolemmal membrane. Thus, understanding the competitive interaction of caveolin-3 and dystrophin with β-dystroglycan provides further insight into the structure and the molecular organization of the dystrophin-glycoprotein complex and may be of vital importance in elucidating the pathogenesis of a number of different forms of muscular dystrophy.

Recently, we have created transgenic mice that overexpress caveolin-3 (62). Analysis of skeletal muscle tissue from these mice reveals DMD-like myopathic changes and down-regulation of dystrophin as expected to prevent the interaction of dystrophin with β-dystroglycan. Inhibition of the dystrophin/β-dystroglycan interaction is known to result in degradation of the dystrophin complex and is the molecular basis for Duchenne muscular dystrophy. Thus, these results are consistent with the idea that caveolin-3 competes for binding to β-dystroglycan in vivo.

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Caveolin-3 Directly Interacts with the C-terminal Tail of β-Dystroglycan: IDENTIFICATION OF A CENTRAL WW-LIKE DOMAIN WITHIN CAVEOLIN FAMILY MEMBERS
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