Dystrophin Associates With Caveolae of Rat Cardiac Myocytes
Relationship to Dystroglycan

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Abstract—The possibility of an interaction between the cytoskeletal protein dystrophin and cell surface caveolae in the mammalian myocardium was investigated by several techniques. Caveolin (cav)-3–enriched, detergent-insoluble membranes isolated from purified ventricular sarcolemma by density-gradient fractionation were found to contain dystrophin and dystroglycan. Further purification of cav-3–containing membranes by immunoprecipitation using anti–cav-3–coated magnetic beads yielded dystrophin but not always dystroglycan. Electron microscopic analysis of precipitated material revealed caveola-sized vesicular profiles that could be double-labeled with anti-dystrophin and anti–cav-3 antibodies. In contrast, immunoprecipitation of membranes with anti-dystrophin–coated beads yielded both cav-3 and dystroglycan. Electron microscopic analysis of this material showed heterogeneous membrane profiles, some of which could be decorated with anti–cav-3 antibodies. To confirm that dystrophin and cav-3 were closely associated in cardiac myocytes, we verified that dystrophin was also present in immunoprecipitated cav-3–containing membranes from detergent extracts, as well as in sonicated extracts of purified ventricular myocytes. Confocal immunofluorescence microscopy of ventricular and atrial cardiac myocytes showed that the cellular distributions of cav-3 and dystrophin partially overlapped. Immuno–electron micrographs of thin sections of rat atrial myocytes revealed a fraction of dystrophin molecules that are in apparently close apposition to caveolae. These results suggest that a subpopulation of dystrophin molecules interacts with cardiac myocyte caveolae in vivo and that some of the dystrophin is engaged in linking cav-3 with the dystroglycan complex.

Key Words: heart ■ myocyte ■ caveola ■ dystrophin

The flask-shaped plasma membrane–associated vesicles called caveolae are non–clathrin-coated organelles first described by Palade in 1953.† Recently, caveolae have been proposed to be multifunctional organelles that, in diverse cell types, participate in one or more of the following: as sites for concentration and subsequent internalization of small molecules and as the loci of multiple signal-transducing molecules, and as sites of cytoskeletal attachment to the plasma membrane. Presumptive caveolar properties or “markers” include the caveolar coat protein caveolin (cav), an 18- to 24-kDa protein (depending on the isoform), and a high concentration of cholesterol and sphingolipids. Four isoforms of caveolin encoded by 3 distinct genes have so far been identified. Cav-1α, also known as VIP21 or VCAV, is found in a variety of tissues, as is cav-1β, a truncated version also derived from the same gene. Cav-2, the isoform most abundant in fat cells, coexpresses with cav-1 in many nonmuscle tissues, whereas cav-3, or MCAV, is found predominantly if not exclusively in muscle cells. Thus, in the heart, cav-3 is the isoform expressed in cardiac myocytes and vascular smooth muscle cells, whereas the cav-1 isoform is predominantly expressed in nonmuscle cells.

Several laboratories have isolated cav-enriched supramolecular complexes from diverse cell types, using either detergent insolubility or sonication of whole-cell or crude membrane fractions followed by sucrose or OptiPrep (Gibco-BRL) density flotation as a final purification step. However, a number of reports have suggested that density gradient flotation of such preparations is by itself insufficient to obtain caveolae free of contamination with noncaveolar membranes. For example, we previously showed that sucrose density flotation of detergent-insoluble membrane preparations derived from highly purified sheep ventricular sarcolemma yields a diverse population of proteins including cav-1 and cav-3 and the glycosphingosinol (GPI)-linked protein T-cadherin. To determine whether T-cadherin was a bona fide caveolar protein, we purified the cav-enriched...
complexes of cardiac membranes further by immunoprecipitation using magnetic beads coated with an antibody to cav-3. Immunoblot analysis of the material thus bound showed that, whereas a substantial fraction of cav-3 was immunoprecipitated, other detergent-insoluble membrane fragments containing T-cadherin were not precipitated, which indicated that T-cadherin is not directly associated with cardiac caveolae. Dystrophin, a large (430-kDa), rod-shaped protein, is a component of the subsarcolemmal cytoskeleton in both striated and smooth muscle cells and is abundant in cardiac myocytes. Although it is established that dystrophin is implicated in muscular dystrophy, its role in normal cells is incompletely understood. Dystrophin is attached at a cysteine-rich region of its C terminus to the trans-sarcolemmal β-subunit of dystroglycan, a member of the protein complex dystrophin-associated glycoproteins, which link dystrophin to the extracellular matrix. Dystrophin is also associated with other elements of the cytoskeleton through binding to filamentous actin. Dystrophin and dystroglycan have been previously shown to comigrate with cav-3 in sucrose density gradients of crude detergent extracts from skeletal muscle cells, and cav-3 was shown to coimmunoprecipitate with dystrophin. However, cav-3 was subsequently shown not to be a component of the dystroglycan complex. Immuno–electron microscopy (EM) of smooth muscle cells has shown dystrophin to be present in the caveola-rich region of the sarcolemma, but no direct association of dystrophin with caveolae was demonstrated.

To determine whether dystrophin is associated with cav-3 in cardiac myocytes and whether dystroglycan also participates, we studied the relationship between these proteins in mammalian heart muscle cells using immunoprecipitation, immuno-EM, and immunofluorescence microscopy. Our results suggest that a component of dystrophin associates with caveolae in cardiac myocytes and that at least a portion of this dystrophin is simultaneously linked to dystroglycan.

Figure 1. On discontinuous sucrose density gradients, dystrophin coisolates with cav-1 and cav-3 in Triton-insoluble floatable membrane fragments from sheep heart. Sarcolemma-enriched membranes prepared from sheep heart were solubilized by treatment with cold Triton X-100 (0.5% final). They were then made to be 40% with respect to sucrose in 2 mL, layered under a 10% to 30% sucrose gradient, and centrifuged at 140 000g overnight, after which they were fractionated into 12 1-mL fractions. Fractions 1 to 10 contained floatable complexes. Fractions 10 to 12 contained soluble protein. A, Immunoblots of proteins from each fraction of a gradient (equal volumes loaded) shown with a previously reported profile of a similarly produced gradient. Floatable membrane fragments are enriched in cav-1 and cav-3, as well as in dystrophin, an abundant cytoskeletal protein. Also present is a minority portion of the β-dystroglycan resident in the starting sarcolemma-enriched fraction. B, Electron micrograph of membranes in the cav-enriched floatable fractions. Note the presence of electron-lucent and electron-dense vesicular profiles about the size and shape as caveolae, as well as profiles larger than caveolae and/or of inappropriate shape to be caveolae. C, Vesicular fragments the appropriate size and shape of caveolae immunoprecipitated by anti–cav-3–coated magnetic beads. Some profiles have sharply defined circumferences yet are interiorly electron-lucent (long arrow), whereas some are more electron-opaque throughout (short arrow). Bar=200 nm.
T-cadherin, and beads but present in pelletable (100 000 g) material were cav-1, dystrophin in the starting material. Not bound (NBp) to the beads (B), as well as to a significant subset of the material in the starting Triton-treated sarcolemma (S) was found to be by enhanced chemiluminescence amplification. Cav-3 present in the starting Triton-treated sarcolemma (S) was found to bind to the beads (B), as well as to a significant subset of the dystrophin in the starting material. Not bound (NBp) to the beads but present in pelletable (100 000g) material were cav-1, T-cadherin, and β-dystroglycan. Be, Electron micrograph of a thin section of membranes adhered to immunobeads, such as in panel A (ie, to the bound fraction), immunolabeled by rabbit anti–cav-3 polyclonal antibody and mouse anti-dystrophin monoclonal antibodies (Dys1 and Dys2). A few membrane fragments (<5% by rough estimate), apparently not of the appropriate shape or size of caveolae, were observed to be associated with caveolea-like profiles. C, Enlargement (2×) of indicated region of panel B. Initial primary antibody labeling was followed by 5 nm gold–labeled Fc chain-specific anti-rabbit secondary antibody (long arrows) and 10 nm gold–labeled Fc chain-specific anti-mouse secondary antibody (short arrows). D, Bead-captured membranes incubated with equivalent concentrations of rabbit and mouse IgG as a control. E, Enlargement of indicated region of D. Gold particles of both species are specifically (see Table) associated with well-defined vesicular membranes the size of caveolae. Bars=200 nm.

Figure 2. Dystrophin coimmunoprecipitates with rat heart caveolae in the presence of anti–cav-3–coated magnetic beads. A, An aliquot of the starting material (S) consists of Triton-solubilized sarcolemma-enriched membranes prepared from whole rat ventricle. Starting-material membranes were incubated with anti–cav-3–coated magnetic beads. Bound membranes (B) were separated from nonbound material by a magnet. Non-bound pelletable material, NBp, was collected by centrifugation at 100 000g for 1 hour. Proteins from equal aliquots of each fraction were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membrane filters were immunoblotted with primary antibodies against cav-3 and cav-1 (Transduction Laboratories), T-cadherin, dystroglycan, dystrophin, and horse-radish peroxidase–conjugated secondary antibodies. Detection was by enhanced chemiluminescence amplification. Cav-3 present in the starting Triton-treated sarcolemma (S) was found to bind to the beads (B), as well as to a significant subset of the dystrophin in the starting material. Not bound (NBp) to the beads but present in pelletable (100 000g) material were cav-1, T-cadherin, and β-dystroglycan. B, Electron micrograph of a thin section of membranes adhered to immunobeads, such as in panel A (ie, to the bound fraction), immunolabeled by rabbit anti–cav-3 polyclonal antibody and mouse anti-dystrophin monoclonal antibodies (Dys1 and Dys2). A few membrane fragments (<5% by rough estimate), apparently not of the appropriate shape or size of caveolae, were observed to be associated with caveolea-like profiles. C, Enlargement (2×) of indicated region of panel B. Initial primary antibody labeling was followed by 5 nm gold–labeled Fc chain-specific anti-rabbit secondary antibody (long arrows) and 10 nm gold–labeled Fc chain-specific anti-mouse secondary antibody (short arrows). D, Bead-captured membranes incubated with equivalent concentrations of rabbit and mouse IgG as a control. E, Enlargement of indicated region of D. Gold particles of both species are specifically (see Table) associated with well-defined vesicular membranes the size of caveolae. Bars=200 nm.

Preparation of cav-Enriched Membrane Fragments From Sheep and Rat Whole Ventricles
To produce membranes enriched in plasmalemma from sheep and rat whole ventricles, we used our previously reported approach, which is based on the method of Jones.26 Triton-insoluble membrane fragments were prepared and isolated from these preparations as previously described.19

Preparation of cav-Enriched Membrane Fragments From Rat Heart Isolated Ventricular Myocytes
Rat ventricular cells were dispersed by collagenase treatment of whole heart via perfusion on the Langendorff cannula using MEM (Gibco-BRL). Viable myocytes were further purified by Percoll density gradient centrifugation.27 By visual count, >95% of the cells were found to be myocytes. Cav-enriched membranes were prepared by detergent dissolution or by sonication.

Detergent Extraction
Myocytes were incubated with 0.5% Triton X-100 detergent on ice.

Extraction by Sonication
Cav-3–enriched membranes were also prepared from acutely isolated myocytes by a detergent-free procedure based on methods developed by Lasley et al.28 One portion of the samples prepared as described above was reserved as starting material, and an aliquot was subjected to immunoprecipitation as described below.

Immunopurification of Membranes Containing cav-3
Membrane fragments, prepared from whole ventricle or from isolated myocytes as described above, were incubated with anti–cav-3–coupled magnetic beads to separate bound from nonbound (NB) material. Pelletable nonbound material (NBp) was collected by centrifugation at 100 000g for 1 hour. NBp and some bound material were dissolved in sample buffer for subsequent SDS-PAGE. Proteins in an equivalent volume of starting incubation material and, in some cases, nonbound nonpelletable material were precipitated with trichloroacetic acid before dissolution in sample buffer. Some bound material was incubated with primary antibodies and colloidal gold-labeled secondary antibodies and then fixed and prepared for viewing by EM.

Immunoenzyme-EM
Rat atria were thin sectioned and processed for immune-EM as described by Chang et al.29 Magnetic beads with captured cav-3–containing membranes (above) were incubated at 4°C with polyclonal anti–cav-3 and monoclonal anti-dystrophin (Dys1 and Dys2) antibodies or, as a control, with equivalent concentrations of rabbit and mouse IgGs, decorated with gold-labeled Fc fragment–specific anti-rabbit and anti-mouse secondary antibodies, fixed with 3% glutaraldehyde, postfixed with 0.8% osmium tetroxide, treated with 1% tannic acid, dehydrated, and embedded in Epon for subsequent thin sectioning.

Materials and Methods
An antibody to the N-terminal residues 2 to 18 of the rat cav-3 sequence (QCB) was made commercially. Polyclonal and monoclonal antibodies to cav-1 and cav-3 were obtained from Transduction Laboratories. Polyclonal antibodies to rat T-cadherin were provided by Dr Barbara Ranscht (Burnham Institute, La Jolla, Calif). A polyclonal antibody to dystrophin was the gift of Dr Timothy Byers (Indiana University School of Medicine, Indianapolis, Ind); 2 monoclonal antibodies to dystrophin (Dys1 and Dys2) and a monoclonal antibody to β-dystroglycan were purchased from Vector Laboratories. Anti-rabbit and anti-mouse IgG Fc-specific gold-labeled antibodies were purchased from E-Y Laboratories. Tosylated magnetic polystyrene beads were obtained from Dynal Corp.

A, An aliquot of the starting material (S) consists of Triton-solubilized sarcolemma-enriched membranes prepared from whole rat ventricle. Starting-material membranes were incubated with anti–cav-3–coated magnetic beads. Bound membranes (B) were separated from nonbound material by a magnet. Non-bound pelletable material, NBp, was collected by centrifugation at 100 000g for 1 hour. Proteins from equal aliquots of each fraction were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membrane filters were immunoblotted with primary antibodies against cav-3 and cav-1 (Transduction Laboratories), T-cadherin, dystroglycan, dystrophin, and horse-radish peroxidase–conjugated secondary antibodies. Detection was by enhanced chemiluminescence amplification. Cav-3 present in the starting Triton-treated sarcolemma (S) was found to bind to the beads (B), as well as to a significant subset of the dystrophin in the starting material. Not bound (NBp) to the beads but present in pelletable (100 000g) material were cav-1, T-cadherin, and β-dystroglycan. B, Electron micrograph of a thin section of membranes adhered to immunobeads, such as in panel A (ie, to the bound fraction), immunolabeled by rabbit anti–cav-3 polyclonal antibody and mouse anti-dystrophin monoclonal antibodies (Dys1 and Dys2). A few membrane fragments (<5% by rough estimate), apparently not of the appropriate shape or size of caveolae, were observed to be associated with caveolea-like profiles. C, Enlargement (2×) of indicated region of panel B. Initial primary antibody labeling was followed by 5 nm gold–labeled Fc chain-specific anti-rabbit secondary antibody (long arrows) and 10 nm gold–labeled Fc chain-specific anti-mouse secondary antibody (short arrows). D, Bead-captured membranes incubated with equivalent concentrations of rabbit and mouse IgG as a control. E, Enlargement of indicated region of D. Gold particles of both species are specifically (see Table) associated with well-defined vesicular membranes the size of caveolae. Bars=200 nm.
**Coprecipitation of Dystrophin With Immunopurified Cardiac Caveolae**

We previously reported that Triton X-100-insoluble floatable membrane fragments (the TIFF or T-CAV fractions) obtained from plasmalemmal preparations of sheep heart ventricle isolated by sucrose density gradient centrifugation are enriched in cav-1 and cav-3, as well as in a variety of other proteins including T-cadherin, the major GPI-linked protein in sarcolemma from sheep heart. However, immunoprecipitation of cav-3-containing membranes by anti-cav-3–coated magnetic beads did not coprecipitate T-cadherin or cav-1, each of which remained in other detergent-insoluble membrane fragments. These results suggested that the TIFF fraction is heterogeneous.

Analysis of the same TIFF fraction revealed the presence of substantial dystrophin and of a small fraction of the dystroglycan originally present in the plasmalemmal preparation, as well as cav-1 and cav-3 (Figure 1A). As visualized by EM of thin-sectioned pellets, the TIFF fraction from heart contains a heterogeneous mixture of membranes that includes electron-lucent and electron-opaque vesicles of about the same profile size as that of caveolae in situ (50 to 100 nm). Vesicles of larger mean profile size were also seen (Figure 1B). When these detergent-insoluble membranes were subjected to immunoprecipitation by anti-cav-3–coated magnetic beads, vesicles having the approximate profile size of caveolae were captured (Figure 1C). Many of these captured vesicles were electron-lucent, thus resembling vesicles immunoprecipitated by Waugh et al using anti–cav-1 antibodies from extracts of sonicated A431 cells. Other vesicles were electron-opaque, resembling some of the anti–cav-1–immunoprecipitated membranes from rat lung seen by Stan et al.

When detergent-resistant membranes prepared from whole rat ventricle were subjected to immunoprecipitation using anti–cav-3–coated magnetic beads, a major portion of the cav-3 and a significant portion of the dystrophin were captured (Figure 2A). In agreement with our previous results, detergent-insoluble membranes containing cav-1 or T-cadherin were not retained by the beads. In 3 of 6 experiments no detectable β-dystroglycan was immunoprecipitated (Figure 2A). In 3 of 6 experiments a small portion of the β-dystroglycan was immunoprecipitated (data not shown). The bead-captured vesicles could be codecorated with anti–cav-3 and anti-dystrophin antibodies (Figures 2B through 2E). In view of the background associated with double-label EM studies of this type, quantification of electron micrographs was attempted by carefully counting the numbers of vesicles that immunostained for either epitope and for both epitopes together. These studies confirmed that caveola-sized vesicles were significantly immunolabeled with antibodies to both cav-3 and dystrophin (Table). These results suggest that we are isolating a fraction that is enriched in bona fide cardiac caveolae and that some of the cellular dystrophin is bound to these organelles. The interaction between dystrophin and caveolae is sufficiently strong to survive washes of both low (10 mmol/L HEPES-EDTA) and high (0.6 mol/L KCl) ionic strength.

In correlative experiments, we examined whether anti–dystrophin–coated beads could precipitate membranes that were immunoreactive toward cav-3. This turned out to be the case, as shown in Figure 3A, and such beads captured dystroglycan but virtually no cav-1 or T-cadherin. Examination of precipitated membranes by EM revealed a heterogeneous mixture of fragments, some of which could be decorated by anti–cav-3 antibodies (Figures 3B through
These results again suggest that a fraction of dystrophin is associated with a membranous organelle that contains cav-3.

**Dystrophin Is Present in cav-Enriched Membranes Prepared by Either Detergent Extraction or Sonication of Isolated Cardiac Ventricular Myocytes**

Sarcolemma-enriched membrane preparations isolated from mammalian whole ventricle contain membranes derived from all cell types present in the heart, including vascular smooth muscle cells, which, like cardiac myocytes, also contain caveola-associated cav-3. To substantiate further that dystrophin associates with cav-3 in cardiac myocytes, we first enzymatically dispersed the hearts into their component cells and then isolated the myocytes by Percoll density gradient centrifugation. The resultant, highly (>95%) cardiomyocyte-enriched preparations were examined by confocal immunofluorescence microscopy, which confirmed that both cav-3 and dystrophin were present in close association with the cardiac myocyte sarcolemma (data not shown). Membrane fragments were prepared from these cells by homogenization in low- and high-ionic-strength buffers similar in manner to our approach to whole ventricle. Immunoisolation of cav-3-containing membranes from detergent extracts of these membranes resulted in coprecipitation of dystrophin (Figure 4A). As with the caveolar material prepared from whole ventricle, in some experiments such as those shown in Figure 2, dystroglycan was not associated with the dystrophin-cav-3 complexes captured by the beads. Dystrophin and dystroglycan not associated with caveolae were in soluble form, not pelletable by centrifugation at 100 000g.

Because we were concerned about possible molecular rearrangement induced by detergent extraction, we applied an alternate approach to caveolar isolation. Acutely isolated cells suspended in an isotonic sucrose buffer were homogenized with a polytetrafluoroethylene (Teflon) pestle, and a plasmalemma-enriched fraction was isolated by Percoll density gradient centrifugation. The resulting membranes were sonicated, and a cav-enriched fraction was isolated by OptiPrep gradient centrifugation. Immunoisolation of cav-3-containing membranes from these sonicated extracts resulted in coprecipitation of dystrophin (Figure 4B). Under these milder preparative conditions, precipitation of dystrophin from rat heart. We believe this is a result of low avidity due to species differences. B, Electron micrograph of a thin section of membranes adhered to immunobeads such as in panel A (ie, to the bound fraction) immunolabeled by rabbit anti–cav-3 polyclonal antibody or an equivalent concentration of rabbit IgG as a control (C). This initial labeling was followed by 5 nm gold-labeled Fc chain-specific anti-rabbit secondary antibody (arrows). Gold particles are specifically (see Table) associated with well-defined vesicular membranes the size of caveolae. D, Membranes bound to beads and labeled as in panel B with anti–cav-3 antibody. This is one of numerous examples found in the examined bead profiles of membrane fragments (arrowhead), none of which is of the appropriate size and/or shape to be caveolae (arrows). Bar = 200 nm.
dystroglycan was seen to coprecipitate with cav-3 in all experiments. Examination of precipitated sonicated membranes by EM revealed vesicles similar in size and appearance to those prepared with detergent (Figure 4C). These results support the notion that dystrophin associates with cav-3-containing membranes in cardiac myocytes. These results also emphasize that the relative association of cav-3, dystrophin, and dystroglycan in cellular fragments is dependent on conditions of preparation.

**Association of Dystrophin With Caveolae in Rat Atrial Tissue**

The above data suggest that a fraction of dystrophin is somehow linked to caveolae from rat ventricle. Because the status of dystrophin in rat atrial tissue has not been probed in depth, we investigated the disposition of this protein in frozen sections of atria from mature rats that lack or have only vestigial T-tubules.32 We confirmed by confocal immunofluorescence that cav-3 labeling in rat atria is limited to the surface of the cells (Figures 5A and 5C), whereas dystrophin appears both at the cell surface and in the cell interior (Figure 5B). Dystroglycan labeling was also limited to the cell surface (Figure 5D).

To assess the relationship between dystrophin, dystroglycan, and morphologically identified caveolae, we immunostained thin sections of rat atria with anti-dystrophin and anti-dystroglycan antibodies, followed by exposure to gold-labeled secondary antibodies and examination by EM (Figure 6A). Some dystrophin labeling was found to be in close proximity to optically well-resolved myocyte caveolae, which suggests that this dystrophin may reside in or in close apposition to caveolae of atrial myocytes. That the distribution of dystroglycan labeling was distinct from that of dystrophin was suggested by the finding that dystroglycan was localized closer to the cell surface sarcolemma, as might be expected for a transmembrane, laminin-binding protein (Figure 6B).

**Discussion**

For about 10 years it has been widely assumed that numerous proteins are present within or closely associated with caveolae; this assumption is based on studies of crude...
Figure 6. Electron microscopic localization of dystrophin and dystroglycan in rat atrial myocytes. Rat atria, fixed overnight in 3% paraformaldehyde, were sectioned with a Vibratome (Technical Products, Int'l), labeled with anti-dystrophin polyclonal antibody (A) or anti-β-dystroglycan monoclonal antibody (B), exposed to gold-labeled secondary antibody, fixed with osmium tetroxide, embedded in Epon, and thin sectioned. Overnight paraformaldehyde fixation preserved structural integrity well enough that caveolae (short arrows) could be seen in cross section of cardiomyocytes, while the antigenicity of the probed-for proteins was sufficiently retained to allow for labeling. A, Some dystrophin (arrowheads) is localized well within a caveolar diameter (50 to 100 nmol/L), above and at the circumference of the caveolae. Asterisk denotes a region where the sarcolemmal profile is outside the plane of the section. B, In contrast, β-dystroglycan, although likewise in the vicinity of caveolae (arrowheads), is more closely localized to the plasmalemma (long arrow). Bars=100 nm.

Triton-insoluble as well as sonication-impervious floatable fragments (TIFFs and SIFFs), some of which were isolated from whole cells. It is now clear that such fractions are heterogeneous and that the presence of a protein in a TIFF or SIFF light membrane fraction is insufficient to conclude that it is within or bound to caveolae. One approach to this problem is to purify caveolae further by immunoprecipitation. Others have shown that this procedure can separate caveolae from noncaveolar membranes in lung endothelial cells and A431 cells. Indeed, in previous work, we showed that the major GPI-linked protein in the mammalian myocardium, T-cadherin, is present in the TIFF fraction but can be readily separated from caveolae by immunoprecipitation with anti-cav-3 beads. In this report we apply a similar methodology to show that dystrophin in mammalian heart muscle is reproducibly associated with cardiac myocyte caveolae. This conclusion is further supported by electron microscopic observations both on immunopurified membranes and in situ heart muscle preparations.

The principal association of dystrophin with the plasma membrane of muscle cells is thought to be via its link to the dystroglycan complex, a link mediated by the C-terminal cysteine-rich domain. Indeed, when immunoprecipitation was performed with anti-dystrophin antibodies, β-dystroglycan was found in the complex along with cav-3 (Figure 3A). In addition, apparently noncaveolar membrane fragments were observed to be attached to caveolar profiles in micrographs of precipitated caveolae (Figure 2B). Such results suggest that dystrophin can bind simultaneously to the dystroglycan complex and to caveolae. This conclusion is qualified, however, by the heterogeneity of the membrane fragments precipitated by antidystrophin antibodies (Figure 3D) and by the failure to find significant dystroglycan in anti-cav-3 immunoprecipitates in some experiments (Figure 2A). Nevertheless, although we are not excluding other interpretations, we think it likely that the vigorous disruption of rat ventricle necessary to isolate and immunopurify cardiac myocyte caveolae is capable of producing several populations of dystrophin-containing complexes, including dystrophin bound to caveolae but not bound to dystroglycan; dystrophin bound to dystroglycan; and, in some cases, all 3 components together in a complex. Previous studies on skeletal muscle suggested that dystrophin and dystroglycan appear together with cav-3 in the crude TIFF fraction. Although cav-3 was also shown to coimmunoprecipitate with dystrophin, it was subsequently shown that dystroglycan-containing complexes, which include dystrophin, can be isolated independently of cav-3. Our data remain consistent with the dual hypotheses that, in heart muscle, β-dystroglycan does not reside in caveolae, whereas some dystrophin links caveolae to β-dystroglycan and thereby to other dystrophin-binding proteins present in the cardiac dystroglycan complex. Our data do not exclude the possibility that in situ, either permanently or intermittently, some dystrophin may be bound to caveolae and not to dystroglycan, or to dystroglycan and not to caveolae. Cav-3 and dystrophin may associate by direct binding or indirectly through mutual binding to a third entity. The region of the dystrophin molecule involved in such an interaction remains to be determined; the protein also associates with actin, probably through sequences located in the spectrin-like repeat domain. The roles of other domains, such as the N-terminal, are less well understood. Interestingly, filamin binding to cav-1 has recently been implicated in interaction between caveolae and actin, which occurs in 3T3 fibroblasts and T4.5 trophoblasts. Filamin 2, a muscle-specific isoform, has been shown to interact with the dystrophin-glycoprotein complex of skeletal muscle cells.

Several proteins have been shown to be associated with cardiac myocyte caveolae. Endothelial NO synthase has been shown to be targeted to caveolae of adult mammalian ventricular myocytes. Endothelin has been shown to induce the translocation of protein kinase C isoforms to
caveolae of neonatal ventricular myocytes, where members of the extracellular signal–regulated receptor kinases (ERKs) were shown to reside.35 Muscarinic M2 receptors display agonist-induced translocation to caveolae of adult rat ventricular myocytes.30 Neuregulin receptor erbB4 has been localized to caveolae of neonatal ventricular myocytes.40 Natriuretic peptide receptor type B has been reported.34,43 It has been proposed that recruitment of mono-
carboxylic acid transporters (MCT1)2 have been immunohistochemically localized to myocyte caveolae in situ. Most recently, activated adenosine A1 receptors have been shown to translocate out of adult rat ventricular myocyte caveolae on agonist binding.28 Here we report the first association of a cytoskeletal protein with caveolae of heart myocytes.

Although the functional significance of the dystrophin-caveolar association in heart muscle remains to be clarified, it may be relevant that a correlation between mutations in cav-3 and clinically observed muscular dystrophies has recently been reported.34,43 It has been proposed that recruitment of proteins to caveolae, possibly by the direct binding of some of these proteins to cav-3,35 may play a role in caveolar function.14 Whether the association of dystrophin with caveolae in cardiac myocytes is static and serves a structural role or if it is dynamic and serves a functional role or roles is an important question for further study.

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