Sucrose-density flotation analysis of Triton-insoluble membrane domains isolated from highly purified sheep ventricular sarcolemma revealed the presence of two major 120- and 100-kDa proteins. Both species migrated in two-dimensional isoelectric focussing/SDS gels with an apparent pI of ~4.3, suggesting that they might be related. Microsequence analysis of peptides derived from the 100-kDa protein yielded amino acid sequences with high homology to T-cadherin, a truncated cadherin lacking a cytoplasmic domain. The similarity was confirmed using antibodies to chicken T-cadherin that reacted with both proteins on immunoblots. T-cadherin was released from the detergent-insoluble sarcolemmal fraction by phospholipase C treatment indicating that it is linked to the membrane by a glycophosphotidylinositol anchor. T-cadherin could be ADP-ribosylated by a transferase that was also present in the caveolin-enriched Triton-insoluble fraction. T-cadherin-containing membrane fragments cofractionated on sucrose gradients with caveolin-3, a marker protein for myocyte caveolea. However, immunopurified caveolin-3-containing membranes contained no associated T-cadherin. Immunocytochemical analysis of cultured rat atrial myocytes revealed that T-cadherin and caveolin have related but nonoverlapping staining patterns. These results suggest that T-cadherin is a major glycophostidylinositol-linked protein in cardiac myocytes and that it may be located in plasma membrane “rafts” distinct from but possibly adjacent to caveolea.

Diverse membrane proteins are anchored to the external surface of the plasma membrane by a covalently attached glycolipid tail, frequently a glycophostidylinositol (GPI) moiety (1). Although more than a hundred such proteins have been described including enzymes, adhesion molecules, and receptors, the function of the GPI anchor is uncertain. Whereas enhanced mobility of GPI-anchored proteins might be expected on the basis of the lipid component, a significant fraction of GPI-linked proteins seem paradoxically to exhibit severely restricted mobility within the plasma membrane (1). Recently, it has become apparent that some GPI-modified proteins reside in specific subcompartments (“rafts”) of the plasma membrane that are insoluble in nonionic detergents (2–4). Detergent insolvency is conferred by the presence of high levels of glycosphingolipids and cholesterol in such rafts, characteristics that may also apply to caveolea (4, 5). Caveolea are flask-shaped nonclathrin-coated 50–100-nm vesicles associated with the inner surface of the plasma membrane. First described by Palade (6), they have been subsequently found in many mammalian cell types with fat, endothelial, epithelial, and muscle cells exhibiting the greatest abundance (4). The functions of caveolea are not known with certainty, but they have been proposed to comprise a novel endocytic compartment involved in such functions as “potocytosis” (internalization of small ligands; Ref. 7) as well as transcytosis (movement of material between the apical and basolateral membranes in epithelia; Refs. 3 and 4).

In addition, biochemical studies of putative caveolea isolated from epithelial, endothelial and fibroblastic cell types have suggested an important function for these organelles in signal transduction. Numerous signaling elements appear concentrated in “caveolea” isolated by various methods from different tissues (8–11). However, whether the “Triton-insoluble floatable fraction” (TIFF) prepared from a variety of cells consists principally of caveolea or contains a heterogeneous mixture of membranes is controversial (12, 13). The relationship between GPI-anchored proteins and caveolea has been extensively debated; whereas there may be bona fide physical linkage in some cases, it seems that detergent treatment may artifically promote association in others (14–16) (see “Discussion”).

Microscopic examination of cardiac muscle reveals numerous caveolea associated with the myocyte plasma membrane comprising 25% or more of the total cell surface area (17, 18). Only limited information is available on the biochemical characteristics of cardiac myocyte caveolea or other detergent-insoluble domains of the sarcolemma. We previously showed that it is possible to purify large amounts of sarcolemma from sheep heart using stringent extraction procedures and density gradient centrifugation (19). These membranes are 20–40-fold enriched in the tetrodoxin-insensitive Na+ channel, a specific marker for cardiac plasma membrane, and are therefore useful as a starting material for further fractionation. Here we use nonionic detergent treatment, density-gradient flotation, and immunoprecipitation to investigate caveolea and other detergent-insoluble domains of the cardiac sarcolemma. We find that T-cadherin, a truncated member of the cadherin family of proteins, is a major GPI-linked protein that may be found in cardiac caveolea.
extracellular Ca$$^{2+}$$-dependent adhesion proteins (20), is the major GPI-linked species in the detergent-resistant fraction. This fraction is characterized by morphological entities that resemble caveolae and are enriched in the caveolar-marker protein caveolin. However, immunochemical studies suggest that T-cadherin and caveolin do not copurify and are not colocalized on the surface of cardiac myocytes, indicating that this GPI-linked protein is probably present in noncaveolar rafts at the cell surface.

**EXPERIMENTAL PROCEDURES**

**Materials—**Polyclonal anti-caveolin-1 and -3 antibodies were obtained from Transduction Laboratories (Lexington, KY). A polyclonal antibody to caveolin-3 was produced in rabbits to residues 3–19 of the rat caveolin-3 sequence (21). Production of antibody and affinity purification of IgG were carried out by QCB (Hopkinson, MA). The production and characterization of T-cadherin antibodies are described elsewhere (22). I$$^{32}$$P]ATP and I$$^{32}$$P]NAD were from NEN Life Science Products. Bacterial phosphatidylinositol-specific phospholipase C (PP-PLC) was obtained from Boehringer-Mannheim.

Preparation of Cardiac Sarcolemma and Triton-insoluble Fraction—Sarcolemma was purified from adult sheep ventricle as described previously (19). In brief, defatted tissue was subjected to homogenization in a high salt (0.6 M KCl) buffer with a Tekmar T185 shaft followed by low-speed (600 × g, 10 min) centrifugation to produce a crude pellet. The pellet was then subjected to further homogenization in the same high salt buffer and centrifuged at 14,000 × g for 20 min to produce a medium-speed pellet. This step was repeated twice (except that a low salt buffer was employed) and, in the second round, 10,000 × g for 10 min sufficient to precipitate the crude microsomes (designated the M fraction). The pellet was resuspended in 10 ml HEPES-Tris buffer (pH 7.4) containing 42% sucrose, 0.4 M KCl, 20 mM Na pyrophosphate, 1 mM MgCl$$\text{2}$, and layered under a discontinuous sucrose gradient with steps of 0, 10, and 25% sucrose made in the same buffer. After overnight centrifugation at 140,000 × g the fraction above the 25% sucrose layer (called the M fraction) was collected, diluted 4-fold with water, and pelleted onto a 42% sucrose cushion by centrifugation at 100,000 × g for 1 h. Collected material, designated the SL fraction and equivalent in properties to the sarcolemma-enriched fraction described previously (19), was aliquoted and stored at −70 °C. All solutions contained aprotinin (1 μg/ml), benzamidine (0.5 mM), leupeptin (1 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM), o-phenanthroline (1.25 mM), pepstatin (0.1 μg/ml), EDTA (2 mM), and TATTA (1 mM) as protease inhibitors.

A detergent-insoluble fraction was prepared from the SL as follows. The SL fraction was diluted 1:1 with 50 mM MES (pH 6.5) containing 0.15 M NaCl plus 1% Triton X-100 and the protease inhibitors indicated above (MBS); the mixture was incubated with gentle shaking at 4 °C for 30 min. The preparation was then made 40% with respect to sucrose and layered under an 8–30% sucrose gradient in (MBS) that was then centrifuged at 140,000 × g for 16 h in a Type 641 rotor (Sorvall) at 4 °C. The gradient was fractionated into 1-ml fractions, and the peak of detergent-insoluble membrane fragments (centered around 15% sucrose) pooled, diluted with water, and pelleted by centrifugation at 20,000 × g for 30 min or directly precipitated with 5% (final) trichloroacetic acid. We refer to this fraction as Triton-insoluble caveolin-rich membranes (T-CAV). The TFF fraction was prepared from cultured primary endothelial cells (either bovine or rat aorta; kindly provided by Dr. M. E. O'Donnell, University of California, Davis) by a procedure very similar to that previously described (23).

Immunopurification of Caveolin-3-containing Membranes—Tosylated polystyrene magnetic beads (Dynal, Lake Success, NY) were coated with antibody according to manufacturers instructions. Briefly, beads were incubated at 37 °C overnight on a rotator with goat anti-rabbit (FC chain specific) secondary antibody in 0.1 M borate buffer (pH 9.5). After washing, the coated beads were further incubated at 4 °C overnight with anti-caveolin-3 antibody in phosphate-buffered saline containing 0.1% bovine serum albumin. Cardiac SL (0.5 ml), prepared as described above, was taken from the 25% layer of the sucrose gradient and diluted 1:2 with 0.75% Triton X-100 in 50 mM HEPES buffer (pH 7.4) plus protease inhibitors. Half the sample was reserved as starting material and the other half was added 100 μl of antibody-coated magnetic beads and 80 μl of 1% bovine serum albumin. Following overnight rotation at 4 °C, the beads were washed 4 times with phosphate-buffered saline, bovine serum albumin, 150 mM NaCl (10 min/wash) to separate bound from nonbound material. Pelletable nonbound material was collected by centrifugation at 100,000 × g for 1 h. Pellets were raised in 1 ml of phosphate-buffered saline, and the proteins in the starting material, and nonbound pellets were precipitated with 5% trichloroacetic acid, followed by solubilization of the washed pellet in gel sample buffer and analysis by immunoblotting.

**Immunoblotting and Immunocytochemistry—**Standard immunoblotting procedures were used. Following electrophoretic transfer nitrocellulose sheets were blocked in 5% nonfat dried milk in Tris-buffered saline plus 0.5% Tween-20 then incubated overnight at 4 °C in rabbit anti-T-cadherin antisemur (1:500). Secondary development was with peroxidase-coupled goat anti-rabbit IgG and immunoreactive bands were detected by enhanced chemiluminescence. Similar procedures were used with other antibodies. For immunocytochemistry, primary cultures of rat atrial myocytes were prepared by procedures described elsewhere (24). After 6–9 days in culture, cells on coverslips were fixed in 4% paraformaldehyde and blocked in 1% bovine serum albumin. They were then exposed to polyclonal anti-T-cadherin and monoclonal anti-caveolin-3 antibodies followed by development with fluorescein isothiocyanate- and rhodamine-coupled secondary antibodies. Cells were visualized in a confocal fluorescence microscope (Nikon Instruments, Middleton, WI) and images from the same plane were digitized and analyzed using Metamorph software (Universal Imaging Corp., West Chester, PA).

**Electron Microscopy—**T-CAV membranes were pelleted in a microfuge (18,000 × g for 1 h) and were resuspended by trituration in 50 mM NaPO$$\text{4}$ buffer (pH 7.2); some aliquots were then treated with 80 mM NaCl. The suspension was made 2% TCA and washed several times with 0.1 M Na acetate and incubated at room temperature for 10 min. After washing in NaPO$$\text{4}$ buffer, some grids were incubated in 0.01% saponin on ice for 10 min to promote antibody accessibility. Then 2 μl of these suspensions were deposited on formvar-carbon-coated 400-mesh nickel grids and allowed to air dry. Immunolabeling was carried out with anti-caveolin antibodies followed by goat anti-rabbit IgG coupled to 5 nm colloidal gold. The grids were then washed in distilled water, dried, and viewed and a Hitachi 600 electron microscope.

**Two-dimensional Electrophoresis and Microsequencing—**Two-dimensional isoelectric-focusing (IEF)/SDS-PAGE was conducted in a standard manner (25). The pH gradient was determined by pH measurement of segments cut from blank gels run in parallel with samples. For microsequence analysis, after completion of the second dimension proteins were electrophototransferred to polyvinylidene difluoride membrane in a CAPS buffer (pH 11). The position of bands of interest was determined by India ink staining; these were excised, washed in methanol, dried, and then subjected to both N-terminal and internal microsequencing after proteolytic cleavage and HPLC (J. Fernandez and S. Mische, Protein Sequencing Facility, Rockefeller University).

**Phosphorylation and ADP-ribosylation of Membranes—**ADP-ribosylation of membranes was carried out by incubating T-CAV membranes (40 μg) in the presence of I$$^{32}$$P[NAD (5 μl; 1000 cpm/pmol) for 15 min at 37 °C in a buffer containing 50 mM HEPES (pH 7.4), 1 mM EGTA plus 0.5 mM dithioerythritol. At completion of the reaction, the membranes were pelleted to remove free label; proteins were then resolved by IEF/SDS-PAGE and autoradiography as described above.

**PLC Treatment of Membranes—**To label T-cadherin, 20 μg T-CAV membranes were isolated as described above and phosphorylated in the presence of I$$^{32}$$P[ATP (10 μM)] (I$$^{32}$$P[ATP] 200 cpm/μl) for 5 min at 37 °C in a buffer containing 50 mM HEPES (pH 7.4), 5 mM MgSO$$\text{4}$, 1 mM EGTA, and 0.5 mM dithioerythritol. The membranes were pelleted to remove free label then subjected to PI-PLC treatment using a bacterial enzyme (Bacillus cereus; 10 units/ml, 37 °C, 30 min). Bound and free material was separated by centrifugation (30,000 × g for 30 min) and analyzed by SDS-PAGE, autoradiography, and scintillation counting.

**RESULTS**

Isolation of Triton-insoluble Membranes from Cardiac Sarcolemma and Analysis of Protein Composition—When purified sheep sarcolemma was solubilized with Triton X-100 (0.5%) and fractionated by flotation in a 10–30% sucrose gradient, a minor protein peak centered around 15% sucrose (fraction 4) was obtained. Only ~3–8% of the total sarcolemmal protein appeared in these lighter fractions; the remainder of the pro-
tein was either solubilized (fractions 10–12) or pelleted to the bottom of the tube (presumptive cytoskeleton) (Fig. 1A). The floatable fractions (called T-CAV) were pooled and analyzed by SDS-PAGE alongside samples from the M and SL fractions (Fig. 1B). Several differences in protein composition were noted; some proteins that were prominent in the SL were depleted in T-CAV membranes (Fig. 1B, arrowheads). On the other hand, two proteins of ~120 and ~100 kDa were enriched in T-CAV (Fig. 1B, open arrows). Comparison of the same fractions by immunoblotting with polyclonal anti-caveolin-1 and -3 antibodies revealed that both proteins were enriched in the T-CAV fraction (Fig. 1C; see also Fig. 1A). The two isoforms of caveolin with apparent sizes of 24 and 20 kDa, respectively, exhibited ~9-fold enrichment in T-CAV membranes over the SL fraction. Elsewhere, we confirm that the caveolin-1 isoform resides in nonmuscle cells of the heart, whereas caveolin-3 resides only in myocytes (24, 26, 27). It therefore seems likely that the sheep heart T-CAV fraction is derived from both myo-

![Figure 1](http://www.jbc.org/)

**Fig. 1. Preparation and characterization of the T-CAV fraction from sheep myocardial sarcolemma.** A, upper panel, total protein profile of sucrose gradient fractions. Purified sheep sarcolemma was solubilized by Triton X-100 (0.5% final) treatment and made 40% with respect to sucrose. This material was layered under a 10–30% sucrose gradient and spun at 100,000 × g overnight prior to fractionation (1-ml fractions). Fractions 10 and 11 correspond to the soluble fraction. Lower panel, immunoblot of proteins from each fraction (equal volumes loaded) labeled with either anti-caveolin-1 or -3 (Cav 1 and Cav 3) antibodies. B, comparison of total protein profile (Coomassie Blue staining) of M, SL, and T-CAV fractions (12 μg of protein) from sheep sarcolemma. Proteins were separated by SDS-8% PAGE. Arrowheads indicate the 120- and 100-kDa proteins enriched in T-CAV, whereas the open arrows indicate other proteins that are less abundant in this fraction. C, caveolin isoforms are both enriched in the T-CAV fraction. M, SL, and T-CAV membranes (12 μg of protein) were separated by SDS-11% PAGE, transferred to nitrocellulose, then probed with anti-caveolin-1 or -3 antibodies followed by ECL detection. Enrichment factors were 9.3 (Cav 1) and 8.8 (Cav 3) based on densitometry of this fluorogram. D, electron microscopy analysis of the T-CAV fraction. T-CAV membranes were deposited on nickel 400-mesh grids and negatively stained with uranyl acetate. Upper panel, vesicular membranes of diverse sizes, many in the range of 50–100 nm, are evident (arrows); lower panel, membranes were treated with octylglucoside prior to fixation and with saponin prior to staining. Inset, immunogold staining of this preparation with anti-caveolin-3 antibodies. Note lattice structures of vesicular geometry with some smaller fragments and reactivity of these structures with antibody. Bar = 0.1 μm.
were separated as in weight markers (205, 116, 97, 66, and 43 kDa). The prominent 120- and 100-kDa proteins (gradient from 4–7.5 in the first dimension and by 7.5% polyacrylamide similarity with chick T-cadherin. The sequences of two major peptides are shown aligned with homologous sequences from chick T-cadherin-2 (22). Peptide 1 has 69% identity and 84% similarity, whereas peptide 2 has 62% identity and 79% similarity with Coomassie Blue; both species migrated with a pI 4.3 in the IEF dimension. Shown in the right lane is molecular weight markers (205, 116, 97, 66, and 43 kDa). Panel B, T-CAV proteins were separated as in panel A then transferred to polyvinylidene difluoride membranes; the 100-kDa protein was located by staining and subjected to proteolysis, reverse phase-HPLC, and microsequencing. The sequences of two major peptides are shown aligned with homologous sequences from chick T-cadherin-2 (22). Peptide 1 has 69% identity and 84% similarity, whereas peptide 2 has 62% identity and 79% similarity with chick T-cadherin.

sarcolemma to immunoblot analysis (Fig. 3). Antibodies to chicken T-cadherin recognized proteins of 120 and 100 kDa in the M, SL, and T-CAV fractions with a substantial enrichment (5.6-fold) in the T-CAV fraction over SL. In an attempt to determine if T-cadherin- and caveolin-containing membranes can be distinguished on the basis of density, the T-CAV fraction was pooled and run on a second sucrose gradient of slightly different composition (5–30%). T-cadherin and caveolin immunoreactivity again comigrated, suggesting either that both proteins were associated with the same membrane fragments or that different membrane fragments had similar densities (see below). Because of the cell type heterogeneity of the myocardium, and the likelihood that T-CAV contains membrane material from myocyte and nonmyocyte sources, we wished to determine if T-cadherin is present in endothelial cells. However, immunoblotting of crude particulate fractions or gradient-purified TFF from cultured primary bovine or rat aortic endothelial cells with anti-T-cadherin antibodies proved negative (data not shown). Thus T-cadherin is probably derived from cardiac sarcolemma not from contaminating endothelial membranes.

**T-cadherin Is a Myocyte Protein and Is Present in Membrane Domains Distinct from Those Containing Caveolin—**As indicated above, the T-CAV fraction is likely a mixture of detergent-resistant membranes. To assess whether T-cadherin was associated with presumptive cardiac caveoleae, we subjected this fraction to further purification using anti-caveolin-3 N-terminal antibodies. Using a procedure analogous to that of Stan et al. (13), we coated magnetic beads with anti-caveolin-3 IgG then incubated them with the T-CAV fraction overnight at 4 °C. Bound membranes were separated magnetically from nonbound material and the latter collected by centrifugation. Equal aliquots of these fractions were then analyzed for caveolin-3 and T-cadherin expression. As shown in Fig. 4, a substantial amount of caveolin-3 was precipitated by this procedure, whereas all of the T-cadherin remained in the nonbound (but pelletable) fraction. This procedure was specific as no caveolin-1 was associated with the anti-caveolin-3 beads. In addition, vesicular structures could be visualized when the beads were analyzed by transmission electron microscopy (data not shown).

Fig. 3. The 120- and 100-kDa proteins react with anti-T-cadherin antibodies and comigrate with caveolin in sucrose gradients. M, SL, and T-CAV fractions (12 µg of protein) were separated by SDS-PAGE and probed with specific antibodies to T-cadherin (A). Two proteins that comigrated with the 120- and 100-kDa species identified by staining gels were labeled. The enrichment of these two proteins in the T-CAV fraction was 5.6-fold based on densitometry of this fluorogram. T-CAV membranes purified as described under “Experimental Procedures” were subjected to a second sucrose gradient (5–30%) (B). The gradient was fractionated and the positions of T-cadherin (T-cad) and caveolin in fractions 1–12 (bottom) were determined by immunoblot following SDS-PAGE. Note that the T-CAV fractions appear lower in the gradient than in Fig. 1 due to the difference in sucrose density.

In neurons, T-cadherin is comprised of 120-kDa (precursor) and mature 100-kDa forms (20). To assess whether the 120- and 100-kDa proteins of sheep sarcolemma are immunologically similar to T-cadherin and are enriched in T-CAV, we subjected fractions from sucrose gradients of detergent-treated
These results were corroborated by immunocytochemical localization of T-cadherin and caveolin-3 on the surface of cultured adult rat atrial myocytes (Fig. 5). Both antibodies abundantly stained the cell surface of these cells. Image analysis of double-labeled cells revealed a similar pattern of labeling, but little colocalization was apparent when pixel counts were compared by image analysis (e.g. in Fig. 5 the overlap is ~13%). These results suggest that T-cadherin and caveolin-3 are not colocalized at the cell surface but may occupy adjacent regions of the plasma membrane. The same images also revealed a significant amount of perinuclear staining with both antibodies that may reflect a pool of these proteins at or near the Golgi apparatus.

**T-cadherin Is Tethered to the Cardiac Membrane by a GPI Anchor**—Since T-cadherin is a GPI-linked protein in neurons (20, 29) we tested whether the same can be said of the cardiac sarcolemmal protein. To provide a convenient label for T-cadherin quantitation we phosphorylated T-CAV preparations in the presence of cAMP; an endogenous cAMP-dependent protein kinase was found in the T-CAV fraction that efficiently phosphorylated several proteins including T-cadherin (data not shown). 32P-labeled membranes were then incubated with bacterial PIPLC, which specifically cleaves the GPI anchor between the glycerol backbone and the phosphate group (1). Protein staining and autoradiography showed that a substantial amount of both T-cadherin isoforms was released by this treatment, indicating that these proteins are linked to the membrane by a GPI anchor (Fig. 6).

**T-cadherin Is ADP-ribosylated by an Endogenous Transferase in the T-CAV Fraction**—An ADP-monoribosyltransferase has been identified in skeletal muscle sarcolemma (30, 31) and has been proposed to be GPI-linked and to ribosylate the adhesion protein integrin-α7 in this tissue (32). RNA hybridization analysis indicated that an mRNA with a similar molecular size is present in heart (30). We therefore searched for ADP-ribosyltransferase activity in cardiac sarcolemma by analysis of labeling patterns after addition of [32P]NAD to membrane fractions. Reactions carried out in the isolated cardiac SL fraction gave rise to insignificant incorporation. When the T-CAV fraction was analyzed under similar conditions several proteins became labeled, of which T-cadherin appeared to be the major substrate as determined by IEF/SDS-PAGE (Fig. 7); other proteins of 50 and 35 kDa also became radiolabeled under these conditions. These results indicate that an ADP-ribosyltransferase is concentrated in the T-CAV fraction and that T-cadherin is its major substrate in this fraction.

**FIG. 4.** T-cadherin is not found in immunopurified caveolin-3 enriched membranes. T-CAV membranes were subjected to immunoprecipitation with magnetic beads coated with anti-caveolin-3 IgG. Bound membranes (B) were separated from nonbound (NB) material by collection of the beads with a magnet followed by washing. Nonbound membranes were pelleted by ultracentrifugation. S, an aliquot of T-CAV membranes equivalent to the amount exposed to the beads. Note that no T-cadherin immunoreactivity appears in the bound fraction.

**FIG. 5.** Immunocytochemical analysis of T-cadherin and caveolin in cultured rat atrial myocytes. Atrial myocytes were prepared from young adult rats and cultured as described previously. After fixation, coverslips were incubated with both polyclonal rabbit anti-T-cadherin antibodies and monoclonal mouse anti-caveolin-3 antibodies. After development with the appropriate secondary antibodies, cells were visualized in a confocal microscope for caveolin (A) and T-cadherin (B) reactivity. Merging of the images (C) showed very limited colocalization (yellow) of the two antigens. Pixel analysis using Metamorph software yielded a total of 4541 pixels (split almost evenly between green and red) of which 569 (12.5%) were common to both images.
were incubated in the presence of \[ ^{32}P \]NAD for 15 min at 37 °C. 

Isolated T-CAV membranes (20 mg) were pelleted to remove label, resuspended, and divided into two aliquots. One aliquot was left untreated while the other was subjected to hydrolysis with PI-PLC. Pellet (P) and supernatant (S) samples were analyzed separately by SDS-7.5% PAGE followed by autoradiography. In this experiment 35% of the 120- and 100-kDa T-cadherin bands were cleaved from the membrane by the enzyme as determined by scintillation counting. Lower panel, Coomassie blue-stained (CBB) lanes of equivalent samples (only the region corresponding to the ~100–130 kDa proteins is shown). Two other prominent proteins of 68 and 33 kDa of unknown identity were released from the membrane by this treatment (data not shown).

**DISCUSSION**

Cadherins comprise a family of Ca-binding, homophilic adhesion proteins important in development and maintenance of various tissues (33). For example, N-cadherin is important during skeletal muscle development during which it accumulates at the neuromuscular junction (34). Similarly, N-cadherin appears early in cardiac development and is localized at adherens junctions in the adult heart (35). In the present study we show that two major species of 120 and 100 kDa found in adult cardiac sarcolemma comprise the related protein, T-cadherin. Previous studies identified this protein as an atypical truncated member of the cadherin family found at high levels in the nervous system (20). In contrast to other family members, T-cadherin lacks a cytoplasmic domain and transmembrane segment. Instead, it is linked to the plasma membrane via a GPI anchor in certain classes of peripheral neurons (20). T-cadherin transcripts were previously shown to be present in both skeletal muscle and heart (22); the protein is present on the surface of skeletal muscle cells (36), but its localization in heart has not been investigated. We used specific anti-T-cadherin antibodies to verify the identity of the cardiac proteins and to analyze its distribution in sarcolemma and cultured atrial myocytes. As in neurons (20), T-cadherin is present in sheep cardiac sarcolemma as both 120 and 100 kDa proteins. In neurons these two species appear to bear a precursor-product relationship to each other, but whether both isoforms are functional is still unknown. We also found that T-cadherin, as in neurons (20), is linked to the cardiac sarcolemma by a GPI anchor, since it could be released from the membrane by a specific PI-PLC.

The presence of T-cadherin in a detergent-insoluble membrane fraction is in agreement with previous findings on several other GPI-linked proteins in numerous tissues (2, 3, 7). It appears that such extracellularly-disposed lipid-linked proteins partition into domains of the membrane termed rafts that are rich in cholesterol and sphingolipids and are resistant to nonionic detergents (4). In the present study these membrane fragments comigrated on sucrose density gradients with putative caveolar membranes, distinguished by the presence of the protein caveolin. Previous studies of cardiac tissue reveal that caveolae are abundant in the sarcolemma (17, 18) and are morphologically similar to those identified in diverse cells and tissues. The present work demonstrates that structures physically resembling caveolae are also found in the T-CAV fraction derived from purified sarcolemma. A controversy has arisen with respect to the homogeneity of the TIFF fraction, equivalent to the T-CAV fraction studied here, because different membrane structures may be Triton-insoluble and these may display similar densities or the detergent may promote artifactual fusion between insoluble domains that are not normally in contact (12, 14, 16). Indeed, TIFF fractions containing abundant GPI-linked proteins can be obtained from cells that lack caveolin or morphologically identifiable caveolae (37, 38), and direct separation of caveolar from noncaveolar membranes in lung has shown that GPI-linked proteins are abundant only in the noncaveolar material (39). Thus in the present experiments we suspected that comigration of T-cadherin-containing membranes with those containing caveolin might well be due to the equivalent density of two or more types of Triton-insoluble membranes present in the T-CAV fraction.

We resolved this issue by (a) immunopurifying caveolin-3-containing membranes from T-CAV and testing for bound T-cadherin, and (b) immunolocalizing T-cadherin and caveolin-3 on the surface of cardiac myocytes. Both approaches indicate that T-cadherin and caveolin-3 (as a marker for caveolae) reside in distinct membrane domains of the cardiac sarcolemma. T-cadherin did not coprecipitate with caveolin-3-rich membranes isolated on antibody-coated beads, and immunocytochemical analysis showed that the surface distribution of T-cadherin and caveolin-3 is distinct. Nevertheless, inspection of confocal immunofluorescence images suggests that the overall
staining patterns of T-cadherin and caveolin are similar. It is conceivable that these two proteins occupy adjacent compartments in the membrane with T-cadherin residing in a “pericellular” domain enriched in GPI-linked proteins as has been previously hypothesized (4, 39). Another GPI-linked protein that may be present in this domain is an ADP-ribose transferase enzyme capable of modifying T-cadherin. A comparable enzyme ADP ribosylates another extracellular matrix protein, α7 integrin, in skeletal muscle (32). The significance of extracellular ADP-ribosylation is unclear (see Ref. 40 for review) and it will be interesting to test whether T-cadherin functions like Ca2+ binding are affected by this post-translational modification.

What is the function of T-cadherin in the myocardium? Like other members of the cadherin family, T-cadherin can act as a homophilic Ca2+-dependent adhesion protein, but it exhibits a weaker interaction than its relatives (29). Developmental studies suggest that the presence of T-cadherin on the surface of certain motor neurons may provide negative axon guidance cues (36, 41). Studies in chick indicate that T-cadherin is expressed late (post-myoblast fusion) in skeletal muscle development; in mature muscle it appears to be widely distributed on the surface but excluded from the neuromuscular junction (36). Such a distribution is complementary to that of N-cadherin, the other major cadherin isomorph expressed in skeletal muscle (35). This has led to the hypothesis that T-cadherin expression might also be a negative determinant of synaptogenesis in skeletal muscle and that it may act as a barrier to motor neuron sprouting (36). Expression studies in epithelial cells also reveal a differential distribution of T- and N-cadherin (42). Whether this complementary expression pattern extends to adult cardiac tissue is currently under investigation. It is conceivable that T-cadherin plays a role in synaptic placement during cardiac development as suggested for motor neurons and skeletal muscle, but as the protein remains abundant in the adult it may prove to have additional functions. A more speculative possibility is that T-cadherin could play a role as a low affinity extracellular Ca2+ “sponge” leading to the accumulation of localized high [Ca2+] on the surface of the plasma membrane. Such extracellular Ca2+ pools could be important in cardiac function. For example, there is evidence that atrial granule secretion occurs in the vicinity of caveolae (43), and caveolae themselves reportedly contain Ca2+ pumps (44); the T-cadherin/Ca2+ pool may then provide Ca2+ for the secretory apparatus in this region of the sarcolemma.

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REFERENCES
1. Ferguson, M. J. A., and Williams, A. F. (1988) Annu. Rev. Biochem. 57, 285–320
2. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544
3. Parton, R. G. (1996) Curr. Opin. Cell Biol. 8, 542–548
4. Harder, T., and Simons, K. (1997) Curr. Opin. Cell Biol. 9, 534–542
5. Schroeder, R., London, E., and Brown, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12130–12134
6. Palade, G. (1953) J. Appl. Physiol. 24, 1424 (abstr.)
7. Anderson, R. G. W. (1993) Curr. Opin. Cell Biol. 5, 647–652
8. Lisanti, M. P., Scherer, P. E., Tang, Z., and Sargiacomo, M. (1994) Trends Cell Biol. 4, 231–235
9. Smart, E. J., Foster, D. C., Ying, Y., Kamen, B. A., and Anderson, R. G. W. (1993) J. Neurosci. Res. 38, 15160–15165
10. Mayor, S., and Maxfield, F. R. (1995) J. Cell Biol. 129, 271–280
11. Liu, P., Ying, Y., Ko, Y.-G., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 15776–15782
12. Kurchatova, T. V., Hartmann, E. and Dupree, P. (1995) Trends Cell Biol. 3, 187–189
13. Stan, R.-V., Roberts, W. G., Predescu, D., Ibida, K., Sauvan, L., Ghetsu, L., and Palade, G. E. (1997) Mol. Biol. Cell 8, 585–605
14. Mayor, S., Rothberg, K. G., and Maxfield, F. R. (1994) Science 264, 1948–1951
15. Mayor, S., and Maxfield, F. R. (1995) Mol. Biol. Cell 6, 929–944
16. Fujimoto, T. (1996) J. Histochem. Cytochem. 44, 929–941
17. Levin, E. R., and Page, E. (1980) Circ. Res. 46, 244–255
18. Kordylewski, L., Goings, G., and Page, E. (1993) Circ. Res. 73, 135–146
19. Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K., and Ogawa, K. (1992) J. Biol. Chem. 267, 6556–6563
20. Ranscht, B., and Dours-Zimmerman, M. T. (1991) Neuron 7, 391–402
21. Way, M., and Parton, R. G. (1995) FEBS Lett. 376, 108–112
22. Sacristan, M. P., Vestal, D. J., Dours-Zimmerman, M. T., and B. Ranscht (1993) J. Neurosci. Res. 36, 864–868
23. Lisanti, M. P., Scherer, P. E., Vidigalriene, J., Tang, Z., Hemanowski-Vosatka, A., Tu, Y., Cook, R. P., and Sargiacomo, M. (1994) J. Cell Biol. 126, 111–126
24. Doyle, D. D., Ambler, S. K., Upshaw-Early, J., Bastuwrous, A., Goings, G. E., and Page, E. (1997) Circ. Res. 81, 86–91
25. Fukuhara, H., Nairn, A. C., Muldoon, L. L., and Villereal, M. L. (1987) J. Biol. Chem. 262, 9785–9792
26. Song, K. S., Scherer, P. E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chao, C., Kohla, D. S., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 15160–15165
27. Parton, R. G., Way, M., Zorzi, N., and Stang, E. (1997) J. Cell Biol. 136, 157–154
28. Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K., and Ogawa, K. (1992) J. Cell Biol. 119, 1507–1513
29. Vestal, D. J., and Ranscht, B. (1992) J. Biol. Chem. 119, 451–461
30. Zolkiewska, A., Nightingale, M. S., and Moss, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11352–11356
31. Klebl, B. M., Matsushita, S., and Pette, D. (1994) FEBS Lett. 342, 66–70
32. Zolkiewska, A., and Moss, J. (1993) J. Biol. Chem. 268, 25273–25276
33. Takeichi, M. (1995) Curr. Opin. Cell Biol. 7, 619–627
34. Cifuentes-Diaz, C., Nicole, M., Goudon, D., Rieger, F., and Mege, R. M. (1994) Development 120, 1–11
35. Goni, A., Bom, Q., and Geiger, B. (1992) Development 114, 173–183
36. Palfrey, H. C., Kohtz, D. S., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 34273–34276
37. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) J. Biol. Chem. 269, 30745–30748
38. Mirre, C., Monlaurez, L., Garcia, M., Delgrossi, M. H., and LeBivic, A. (1996) J. Physiol. 271, C887–C894
39. Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) Science 269, 1455–1459
40. Okazaki, I., and Moss, J. (1996) Adv. Pharmacol. 35, 247–280
41. Ranscht, B., Miller, J., and Ranscht, B. (1996) Development 121, 3163–3171
42. Keller, E., and Ranscht, B. (1996) J. Biol. Chem. 271, 30061–30067
43. Fujimoto, T. (1993) J. Cell Biol. 120, 1147–1157
T-cadherin Is a Major Glycophosphoinositol-anchored Protein Associated with Noncaveolar Detergent-insoluble Domains of the Cardiac Sarcolemma

Donald D. Doyle, Gwendolyn E. Goings, Judy Upshaw-Earley, Ernest Page, Barbara Ranscht and H. Clive Palfrey

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