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Characterization of Membrane Rafts Isolated From Rat Sertoli Cell Cultures: Caveolin and Flotillin-1 Content

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ABSTRACT: Membrane rafts from Sertoli cell cultures were isolated as detergent-insoluble glycosphingolipid–enriched (DIG) fractions on the basis of their enriched content of glycosphingolipids and cholesterol and the resulting insolubility in 1% Triton X-100 and their low buoyant density. Because lipid rafts have been implicated in numerous cell functions, including cell signaling and sites for actin/membrane attachment, studies were initiated to characterize Sertoli cell rafts. This study reports the distribution of the raft structural proteins, caveolin and flotillin-1, implicated in raft microdomain organization. Methods employed included the immunoblotting of cell lysates and detergent-insoluble glycosphingolipid–enriched (DIG) fractions, the immunofluorescent microscopy of peritubular myoid cell (PMC) cultures and cryostat-sectioned testis, and the immunohistochemical staining of paraffin-embedded sections following microwave antigen retrieval techniques. Sertoli cells and Sertoli DIG fractions were found to lack the common raft-associated protein, caveolin, a marker protein for caveolae, but they are enriched in the 48-kd protein, flotillin-1, a protein also implicated in raft formation, cell signaling, and cell motility. Since the primary cell contaminant of Sertoli cell cultures is the PMC, these cells, along with spermatogenic cell fraction (SPGC), were also examined for caveolin and flotillin-1 content. The PMCs contained significant concentrations of both caveolin and flotillin-1. PMCs in culture exhibited a punctate caveolin staining pattern at the cell surface characteristic of a caveolar location. These data support the idea that the pinocytotic vesicles observed in PMCs are caveolae. PMCs also show a perinuclear location for caveolin characteristic of a Golgi location. Cryostat sections of rat testis showed a marked concentration of caveolin in the PMCs. The PMC location of caveolin was also confirmed by the immunohistochemical staining of sections from paraffin-embedded rat testis following microwave antigen retrieval techniques. Similar experiments showed a more ubiquitous, stage-specific distribution of flotillin-1 among testicular cell types.

Key words: Peritubular myoid cells, detergent-insoluble glycosphingolipid–enriched fractions.

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Lipid rafts are membrane microdomains enriched in cholesterol and a complex mixture of glycosphingolipids. These lipid microdomains function in roles such as membrane trafficking, cell morphogenesis, and cell signaling (reviewed in Harder and Simons, 1997; Simons and Ikonen, 1997; Kasahara and Sanai, 1999; Kurzchalia and Parton, 1999; Brown and London, 2000; Razani et al, 2002). Lipid rafts possess a greater hydrophobicity than the surrounding membrane and thus may be isolated on the basis of their insolubility in a 1% Triton X-100 solution at 4°C and their buoyant density. When isolated in this manner, membrane microdomains (rafts) are referred to by a variety of names; in this study, we use detergent-insoluble glycosphingolipid–enriched (DIG) fractions (Parton and Simons, 1995).

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DIG Membrane Fractions—Associated Structural Proteins

DIG fractions are frequently related to cellular structures called caveolae and contain a significant enrichment in the protein caveolin (Sargiacomo et al, 1993), a marker protein for these cellular structures (Rothberg et al, 1992). Caveolae are 50- to 100-nm-diameter, flask-shaped invaginations of the plasma membrane that are most abundant in terminally differentiated cell types such as adipocytes, endothelial cells, smooth muscle cells, and fibroblasts (Fan et al, 1983; Scherer et al, 1994, 1996) and are believed to play roles in both signaling and transport (Lisanti et al, 1994; Couet et al, 1997). The caveolin family of proteins, which consists of the widely expressed caveolin-1 and caveolin-2 and the muscle-specific caveolin-3, has been shown to play both structural and regulatory roles in caveolae (Way and Parton, 1995; Scherer et al, 1996, 1997; Song et al, 1996a,b; Couet et al, 1997). Caveolin-1 isoforms are the most extensively characterized; they consist of a single peptide chain with a long, 33-amino acid, hydrophobic stretch that corresponds to the

membrane anchor domain (Das et al, 1999). In vivo, caveolin-1 has both N-terminal and C-terminal domains facing the cytosol (Dupree et al, 1993). In addition, caveolin-1 contains a tyrosine-14 phosphorylation site and a scaffolding domain that associates with other proteins and regulates signaling functions (Li et al, 1995, 1996; Garcia-Cardena et al, 1996). Caveolin-2 and caveolin-3 share the membrane-spanning portion with caveolin-1, but they lack the scaffolding domain necessary for regulatory functions (Way and Parton, 1995; Scherer et al, 1996). Although caveolin is considered a marker protein for caveolae, plasmalemmal vesicles resembling caveolae have been noted in cells that lack detectable caveolin expression. Also, some rafts exist as flat membrane microdomains, and evidence is mounting that suggests that a multiplicity of raft domains exist within most plasma membranes (Fra et al, 1994; Gorodinsky and Harris, 1995; Schnitzer et al, 1995; Bickel et al, 1997; Waugh et al, 1998, 1999; Maxfield, 2002).

A second family of proteins, the flotillin family, may also have a structural role in raft formation. Two isoforms of flotillin have been characterized, the 45-kd flotillin-1 and the 42-kd flotillin-2 (Bickel et al, 1997; Galbiati et al, 1998; Volonte et al, 1999). Flotillins are found in both cell types that contain and that lack caveolin expression (Bickel et al, 1997). Flotillin-1 possesses 2 hydrophobic regions that may interact with the plasma membrane; however, the conformation assumed by flotillin-1 is still under debate (Bickel et al, 1997; Volonte et al, 1999). Flotillin-2 shares structural homology with flotillin-1, although it lacks the N-terminal domain contained by flotillin-1 (Volonte et al, 1999).

Although it is known that caveolins and flotillins may interact in vitro through hetero-oligomerization (Lisanti et al, 1993; Scherer et al, 1997; Volonte et al, 1999), the mechanism by which these proteins facilitate vesicle formation has not been characterized. It has been shown, however, that the transfection of cells lacking caveolae either with caveolin-1 or flotillin-1 is sufficient to form plasmalemmal vesicles (Lipardi et al, 1998; Volonte et al, 1999). It is also believed that flotillins plays a structural role in vesicle formation in cells lacking caveolin expression, such as neuronal cells (Bickel et al, 1997; Volonte et al, 1999). Other studies, however, have implicated flotillin-2 in filopodia formation in neuronal cells that lack caveolae (Lang et al, 1998; Hazarika et al, 1999). In these cases, flotillin-2 is thought to localize to noncaveolar rafts rich in glycosylphosphatidylinositol (GPI)-anchored cell adhesion proteins (Lang et al, 1998).

Rafts in Sertoli Cells

DIG fractions have been isolated from Sertoli cells in culture, and labeling studies suggest that these fractions arise from the apical portion of the Sertoli cell (Fortna et al, 1999). The location of these rafts in vivo remains unknown. Ultrastructural studies of Sertoli cells have shown the presence of endocytotic vesicles in the apical cytoplasm, described as C-shaped, which later form multivesicular bodies (Nagano and Suzuki, 1978; Morales et al, 1985). Little is known about these vesicles. Alternatively, Sertoli rafts may exist in close contact with the developing spermatogenic (SPGC) cell in the adlumenal compartment. Because of the role of rafts in cell signaling (Lisanti et al, 1994; Simons and Ikonen, 1997), these microdomains warrant further investigation since they may play a role in Sertoli cell/SPGC communication.

Peritubular myoid cells (PMCs) are the major contaminant in Sertoli cell primary cultures; thus, this study also examined PMC lysates. Early studies (Lacy and Rotblat, 1960; Leeson and Leeson, 1963; Ross, 1967) of PMCs using transmission electron microscopy clearly identified a population of contractile cells possessing flask-shaped, pinocytotic vesicles on both surfaces of the cell. These vesicles structurally resemble caveolae, and we recently reported that PMCs contain caveolin distributed in a punctate pattern characteristic of caveolae. DIG fractions isolated from these PMCs were significantly enriched in caveolin (Shubert et al, 2001).

Little is known about the composition of Sertoli cell DIG fractions, and to our knowledge, the only previous study of Sertoli DIG fractions (Fortna et al, 1999) reported the concentration of a novel GPI-anchored form of the copper-binding protein ceruloplasmin in these fractions. In this study, we investigated the distribution of the structural proteins caveolin and flotillin-1 in Sertoli cell DIG fractions. The impact of primary Sertoli cell culture contaminants and peritubular myoid and SPGC on DIG fraction content was also evaluated.

Methods

Cell Cultures

Sertoli cell cultures were established from the testes of 19- to 21-day-old male Sprague-Dawley rats (Hilltop Animals Inc, Scotsdale, Pa) according to an established procedure (Page et al, 1990). Sertoli cells isolated from 15 rat pups were plated into six 225-cm2 culture flasks and maintained at 33°C in Dulbecco modified Eagle with Ham F-12 (DME/F-12) (1:1) medium. After 24 hours, the medium was supplemented with testosterone (10 nM), estradiol (10 nM), hydrocortisone (1 nM), insulin (2 μg/mL), epidermal growth factor (10 ng/mL), transferrin (5 μg/mL), and retinol (50 ng/mL). Sertoli cell cultures were maintained for 4 days before use and were subjected to a hypotonic shock 24 hours before use. To monitor Sertoli culture purity, all cultures were visually inspected prior to use; contamination was less than 10%. Plating density was critically monitored since low-density plating allows the proliferation of PMCs in monolayer gaps, and

high-density plating promotes the proliferation of PMCs around Sertoli clumps, resulting in mound/ball formation. Cultures with these contamination problems were discarded.

PMC cultures were obtained by pelleting the supernatant of the first collagenase/dispase digestion of the seminiferous tubules during Sertoli cell isolation. The pellet from one isolation (15 pups) was suspended in 25 mL of DME/F-12 (1:1) supplemented with 10% newborn calf serum and allowed to sediment for approximately 10 minutes to remove any cell clumps; then, the upper 20 mL of supernatant was plated in a 75-cm2 flask. After 24 hours, the medium was changed, and after 4 days, the cells were passed, split 1:10, grown to confluence, passed again, and harvested as second-pass PMCs. The identity of these cells as PMCs, fraction purity greater than 95%, was based on phase-contrast morphology and immunofluorescent staining using antismooth muscle α-actin (Sigma Chemical Co, St Louis, Mo) (Tung and Fritz, 1990).

Madin-Darby canine kidney (MDCK) and NIH-3T3 cell cultures were maintained in DME/F-12 (1:1) with 10% bovine calf serum and were grown to confluence prior to lysate preparation. Preelutriation SPGC fractions were prepared as previously described (Nyquist and Holt, 1986).

Isolation of Detergent-Insoluble Microdomains

Sertoli cell DIG fractions were isolated by a modification of the method described by Sargiacomo et al (1993). Four to six 225-cm2 flasks of primary Sertoli cells were used per isolation. All procedures were carried out at 4°C. The cultures were washed with cold PBS, extracted by scraping off the cells in 1 mL/flask of ice-cold 1% Triton X-100 in morpholinoethanesulfonic acid–buffered saline (MBS; 25 mM 2-[N-morpholino]ethanesulfonic acid, pH 6.5, 0.15 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF]), and homogenized with 10 passes of a Potter-Elvehjem tissue homogenizer (Daigger, Vernon Hill, Ill). The homogenate was adjusted to 40% sucrose and divided into 6 clear 5-mL ultracentrifuge tubes and overlaid with a 25%/15%/5% discontinuous sucrose gradient in MBS. Gradients were centrifuged for 18 hours at 46 000 rpm in an SW55Ti rotor (Beckman Instruments Inc, Palo Alto, Calif).The DIG fraction appeared as a light-scattering band at the 25%/15% sucrose interface. This insoluble material was collected, diluted in MBS, and pelleted by centrifugation for 45 minutes at 46 000 rpm in an SW55Ti rotor. Pellets were resuspended in 100 μL of phosphate-buffered saline (PBS; 0.1 mM sodium phosphate, pH 7.2, and 0.15 M NaCl) containing 0.2 mM PMSF. A portion of the DIG fraction was saved for protein quantification using the method of Lowry et al (1951), and the remainder was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the addition of an equal volume of sample preparatory buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue), which was followed by heating at 95°C for 3–5 minutes.

Preparation of Cell/Tissue Lysates

For the preparation of cell lysates (Sertoli, PMC, NIH-3T3, and MDCK), cultures were washed twice with cold PBS. To each flask, 1 mL of lysis buffer (1% SDS, 1.0 mM sodium vanadate, 10 mM Tris, pH 7.4, and 0.2 mM PMSF) was added. Lysed cells were scraped and heated at 95°C for 5 minutes. For tissue lysates, samples were obtained from an adult male Sprague-Dawley rat. Tissues were washed twice with cold PBS, and 1 mL of lysis buffer per gram of tissue was added. Tissue was homogenized in a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY) for 1 minute. Lysates were sonicated briefly to decrease viscosity, insoluble material was removed by centrifugation, and a protease inhibitor cocktail (General Use, 20 μL/mL, Sigma) and PMSF (0.2 mM) were added. Samples of all lysates were removed for protein quantification by the method of Lowry et al (1951), and the remainder was prepared for SDS-PAGE as described above for DIG fractions. All protein samples were stored at −80°C until use.

Western Blotting

Samples were separated by SDS-PAGE (Laemmli, 1970) using 12% (flotillin and caveolin) or 10% (total protein) acrylamide gels and transferred to nitrocellulose. Transfer was monitored by Ponceau S staining. Blots were blocked for 1 hour in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and 5% nonfat dried milk (TBST-MILK). All antibodies were diluted in TBST-MILK, and incubations were carried out at room temperature. All secondary antibodies were horseradish peroxidase (HRP) conjugates, and all blots were visualized by enhanced chemiluminescence (ECL). For flotillin-1 detection, blots were incubated in a mouse antiflotillin-1 monoclonal antibody (F65020; Transduction Laboratories, Newington, NH) at a 1:250 dilution for 1 hour, and goat anti-mouse immunoglobulin G (IgG) secondary antibody (Bio-Rad Laboratories, Richmond, Calif) was used at a 1:1000 dilution for 30 minutes. For caveolin detection, blots were incubated in a rabbit anticaveolin polyclonal antibody (C13630; Transduction) at a 1:1000 dilution for 1 hour, and a goat anti-rabbit IgG secondary antibody (Bio-Rad) was used at a 1:1000 dilution for 30 minutes. The immunogen for production of the antiflotillin-1 antibody (F65020) was the C-terminus of mouse flotillin-1; it recognizes a 48-kd protein and does not cross-react with flotillin-2. The immunogen used for production of the anticaveolin antibody (C13630) was the N-terminus of human caveolin-1. This antibody recognizes both the α and β forms of caveolin-1 and cross-reacts with caveolin-2.

Immunofluorescence Microscopy

PMCs were plated on 18-mm coverslips and maintained for several days in DME/F-12 medium containing 10% newborn calf serum prior to fixation. Coverslip cultures were fixed for 10 minutes in a 0.1-M phosphate buffer, pH 6.8, containing 4% paraformaldehyde, and washed with phosphate buffer. Cells were permeabilized with a 2-minute incubation in phosphate buffer containing 0.05% Triton X-100 and then washed in phosphate buffer.

Coverslips were blocked for 20 minutes in phosphate buffer containing 5% bovine serum albumin (BSA). Caveolin labeling experiments were conducted using polyclonal anticaveolin-1 antibody (C13630; Transduction) at a 1:100 dilution in phosphate buffer containing 1% BSA. Incubations in primary antibody were carried out for 30 minutes at room temperature and were followed by a second block for 20 minutes in 10% goat serum in 0.1 M phosphate buffer, pH 7.2. Fluorescein isothiocyanate

(FITC)-conjugated donkey anti-rabbit IgG antibody (Calbiochem, San Diego, Calif) and rhodamine-conjugated donkey anti-mouse IgG antibody (Chemicon, Temecula, Calif) were used at a 1:100 dilution in phosphate buffer containing 1% BSA. Coverslips were washed once in water and mounted with Fluormount-G containing 2.5% 1,4-diazobicyclo(2,2,2)-octane. Slides were visualized by epifluorescence and confocal microscopy on a Nikon (Melville, NY) Eclipse E800 microscope, and images were processed using Simple PCI imaging software (Compix Inc, Cranberry Township, Pa). The anti-Golgi marker (GM130) antibody was a gift from Dr Carolyn Machamer.

Preparation and Staining of Tissue Sections

Rat testes were perfusion fixed with 4% paraformaldehyde in a 0.05-M phosphate buffer, pH 7.4, and were then immersion fixed for 24 hours. Paraffin-embedded sections were irradiated in the microwave at 800 W for 4–7 minutes to retrieve antigenicity. Sections were cooled, placed into PBS for 10 minutes, incubated in Immunopure Peroxidase Suppressor (Pierce Biotechnology Inc, Rockford, Ill) for 10 minutes, and blocked for 20 minutes in 20% goat serum in PBS. For caveolin staining, sections were incubated for 1 hour in a polyclonal anticaveolin antibody (C13630; Transduction) at a 1:500 dilution, which was followed by a 45-minute incubation in a biotin-conjugated anti-rabbit antibody (Chemicon). For flotillin-1 staining, sections were incubated for 1 hour with an antiflotillin-1 (F65020; Transduction) at a 1:75 dilution, which was followed by a 45-minute incubation in a biotin-conjugated anti-mouse antibody (Chemicon). Sections were then washed and incubated with streptavidin HRP conjugate (Zymed Laboratories Inc, South San Francisco, Calif) at a 1:150 dilution, and staining was visualized using the aminoethyl carbazole (AEC) substrate. Sections were counterstained with Mayer hematoxylin.

Results

Earlier studies of membrane rafts, DIG fractions, isolated from cultured Sertoli cells reported the presence of a GPI-anchored form of the ferroxidase ceruloplasmin (Fortna et al, 1999). Using the isolation methods described in this study, the proteins contained in Sertoli cell DIG fractions account for approximately 0.45% plus or minus 0.05% (n = 6) of total Sertoli cell proteins. In this study, we sought to characterize the structural protein (caveolin and flotillin) content of these DIG fractions. To obtain a profile of total DIG fraction proteins, SDS-PAGE was performed on Sertoli cell DIG samples, which were then stained with Coomassie blue. The protein-banding pattern suggests the presence of approximately 9 major proteins, one of which is the 135-kd GPI-anchored ceruloplasmin, along with other less abundant protein bands. In addition, multiple DIG isolations were analyzed by SDS-PAGE, which was followed by silver staining. These results suggested a reproducible protein profile of DIG fractions (data not shown). A representative SDS-PAGE protein profile is shown in Figure 1.

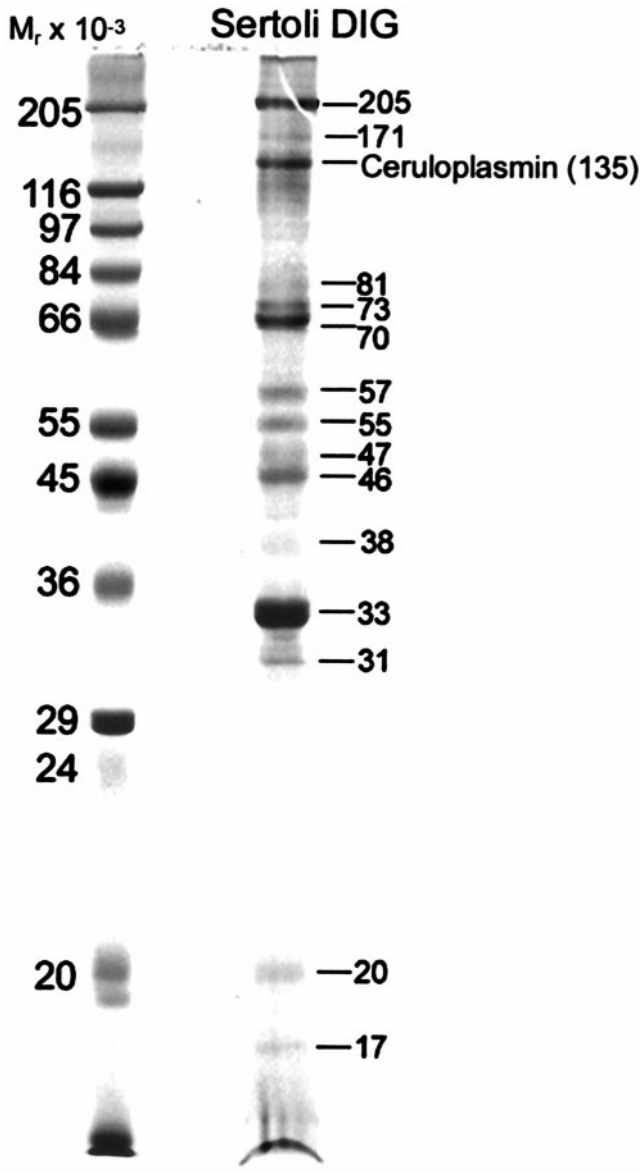

Figure 1. Protein profile of Sertoli cell detergent-insoluble glycosphingolipid–enriched (DIG) fraction. Triton X-100–insoluble (DIG) fractions were obtained from Sertoli cell cultures (20 μg of total protein) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. Lane 1 contains molecular-weight standards (Sigma Marker, wide range, 10 μL). Lane 2 contains the Sertoli DIG fraction. A prominent ceruloplasmin band (135 kd) and 8 other major bands are noted. Several moderate-to-light bands can also be seen.

Membrane rafts, detergent-insoluble membrane microdomains, are frequently found to be enriched in the 21-kd protein caveolin, a marker protein for caveolae. For this reason, we explored the distribution of caveolin in testicular lysates, Sertoli cell lysates, and Sertoli cell DIG fractions. SDS-PAGE and Western blotting with a polyclonal anticaveolin-1 antibody suggested the absence of

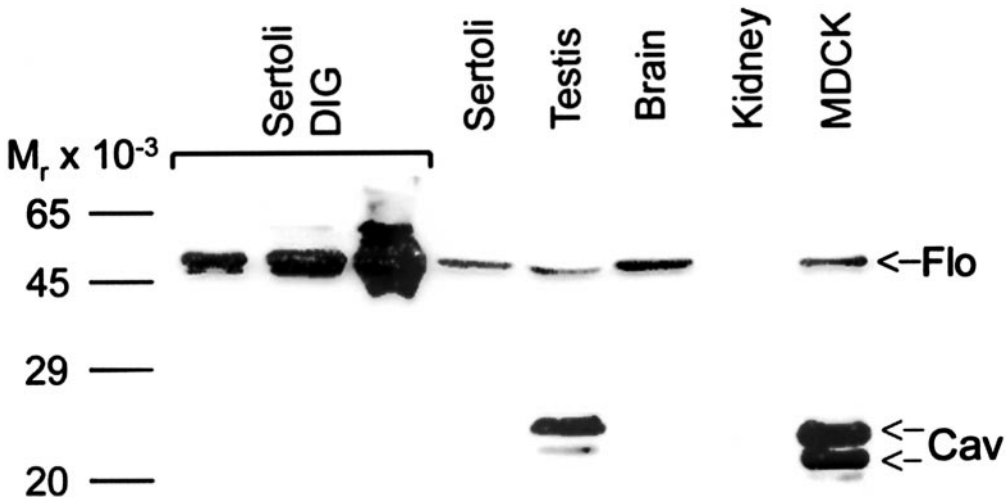

Figure 2. Distribution of flotillin and caveolin in the Sertoli cell lysate and detergent-insoluble glycosphingolipid–enriched (DIG) fraction. Sertoli DIG fractions, Sertoli cell lysate, testis lysate, brain lysate (positive control for flotillin), kidney lysate (negative control), and Madin-Darby canine kidney (MDCK) DIG fractions (positive control for caveolin) were probed with polyclonal anticaveolin-1 antibody and monoclonal antiflotillin-1 antibody following Western blotting. Lanes 1–3 contain Sertoli DIG fractions loaded at 0.5, 1, and 5 μg of total protein, respectively. Lanes 4–7 contain lysates loaded at 20 μg of total protein. Lane 8 contains a DIG fraction isolated from MDCK cells (5 μg of total protein). Flotillin-1, the 48-kd band, is present in the Sertoli cell and is greatly enriched in the DIG fraction. Caveolin, the 2 bands at 21 and 23 kd, although present in the testis, are absent from Sertoli cells. Increased exposure showed trace amounts of caveolin in the Sertoli lysate and in the Sertoli DIG fraction in lane 5 and the Sertoli lysate in lane 4 (data not shown).

caveolins in Sertoli cells and Sertoli cell DIG fractions (Figure 2). To probe more rigorously for low levels of caveolin in Sertoli cell DIG fractions, exposure of the Western blot was increased. These results suggested the presence of trace amounts of caveolin in the heavily loaded Sertoli DIG sample in lane 3 and in the Sertoli cell lysate in lane 4 (data not shown). These bands migrated at a position on the gel identical to that of the positive caveolin control, MDCK cell lysate.

To determine whether this low level of caveolin was endogenous to the Sertoli cell or whether it was the result of the low levels of PMCs traditionally found as the major contaminant in primary Sertoli cell cultures, experiments were conducted to evaluate the caveolin content of the PMCs. PMC cultures were established and evaluated by immunofluorescent staining for smooth muscle α-actin (Figure 3A). On the basis of antismooth muscle α-actin staining, the PMC cultures were exceptionally homogeneous, although there was evidence of a phenotypic change to a more contractile cell with time in culture. These cells were also double-stained for caveolin and smooth muscle actin (Figure 3B and C). Caveolin clearly exhibited a punctate image along the cell surface as would be expected for a caveolar distribution. Caveolin was also heavily concentrated in the perinuclear region of the cell consistent with a Golgi location. The smooth muscle α-actin, in addition to forming the large stress fibers, was concentrated along the plasma membrane at the leading edge of the PMC.

Other testicular cells were also examined for caveolin content. Lysates were prepared and examined for the presence of caveolin-1 by SDS-PAGE, Western blotting, and immunostaining using the same polyclonal anticaveolin-1 antibody (Figure 4). Among the testicular cell lysates examined, only the PMC lysates and the whole-testis fractions showed prominent caveolin bands.

A cryostat-sectioned rat testis was also examined by immunofluorescent microscopy using the same anticaveolin-1 antibody (Figure 3D). The peritubular location of the immunofluorescence is consistent with caveolin localization within the PMCs. These results confirm the presence of caveolin in testicular cells, a result that conflicts with earlier reports of an absence of caveolin messenger RNA in the testis (Scherer et al, 1994) and suggests that PMCs contribute significantly to the caveolin content of whole-testis homogenates.

In addition to the peripheral ring of fluorescence around each seminiferous tubule, contributed by the PMCs, other spots of fluorescence were observed within the epithelium of the tubule, and the distribution and intensity of spots varied among tubules. Since caveolin is also endogenous to Golgi apparatus membranes and since both Sertoli and SPGC cells contain large quantities of Golgi membranes, cryostat sections of rat testis were subjected to a double stain with anticaveolin-1 and anti-GM130 (Golgi-specific) antibodies. No co-localization was apparent (Figure 3E), thus indicating that these fluorescent spots did not likely come from Golgi membranes. Nor was co-localization observed with developing acrosomes, although the immunofluorescent staining of sper-

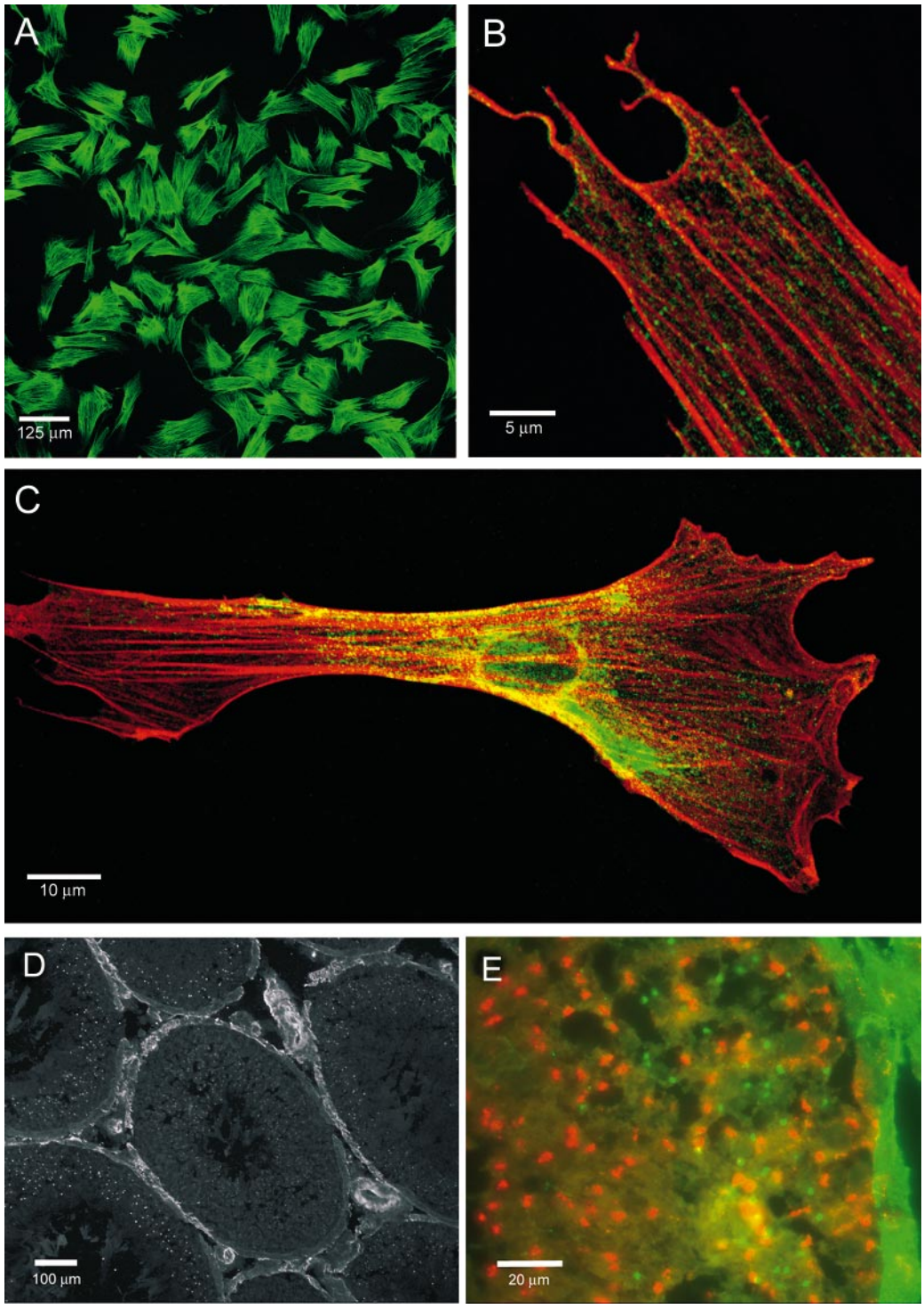

Figure 3. Immunofluorescent localization of caveolin and smooth muscle α-actin on peritubular myoid cell (PMC) cultures and on cryostat sections of whole-rat testis. Second-pass PMCs were examined by confocal laser immunofluorescent microscopy **(Panel A)**. The parallel actin bundles characteristic of PMCs are seen after staining with antismooth muscle α-actin (Sigma) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Excellent purity of PMC cultures was obtained. PMCs **(Panels B, C)** were additionally probed with a rabbit polyclonal anticaveolin antibody (Transduction) and a Cy5-conjugated secondary antibody. They show the punctate distribution of caveolin characteristic of a caveolar location. Caveolin is also concentrated in the perinuclear region characteristic of a Golgi location. The abundant smooth muscle actin stress fibers of PMCs are also observed, as is the actin accumulation at the leading edge of the cell. In cryostat sections of the whole testis, the PMCs around the periphery of the tubules avidly stain using the polyclonal anticaveolin antibody (Transduction) **(Panel D)**. PMCs that were double-stained with a polyclonal anticaveolin antibody (with FITC-conjugated secondary) and a monoclonal anti-Golgi marker GM130 antibody (with rhodamine-conjugated secondary) showed no co-localization **(Panel E)**. Image was photographed using false color dual imaging.

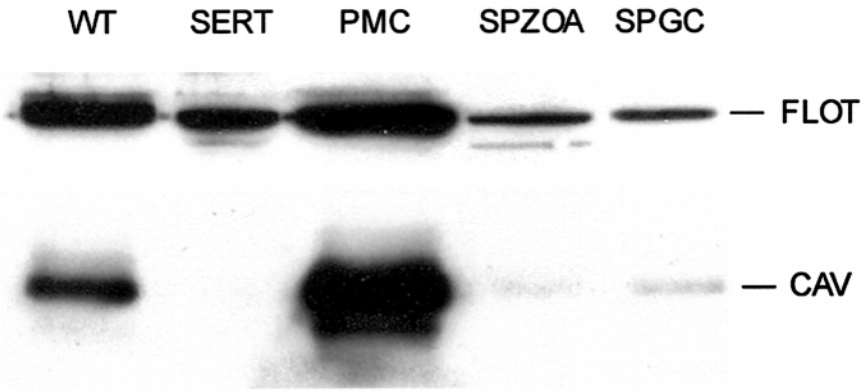

Figure 4. Distribution of caveolin and flotillin-1 among lysates of testicular cells. Lysates of whole testis (WT), Sertoli cell cultures (SERT), peritubular myoid cells (PMCs), washed epididymal spermatozoa (SPZOA), and nonflagellated spermatogenic cell fractions (SPGCs) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, immunostaining, and enhanced chemiluminescence (ECL). All lanes were loaded with 10 μg of protein. Caveolin stains were observed in the WT and PMC fractions. Flotillin-1 was abundant in both the Sertoli cells and PMCs, but lesser quantities were also seen in the SPGC and SPZOA.

matozoal (SPZOA) smears under identical conditions yielded false-positive caveolin reactions. The identity of these spots is under continued investigation.

Rat testes were also paraffin embedded, sectioned, subjected to antigen retrieval techniques, and stained for caveolin content (Figure 5A and B). Reaction product was seen clearly only within the PMCs of the seminiferous tubules. Within the interstitium, the walls of the blood vessels also showed a strong positive reaction for caveolin. Control sections not subjected to microwave antigen retrieval showed little deposition of reaction product. Unfortunately, the antigen retrieval process negatively affected section quality.

Recently, another purported structural protein, the 48-kd flotillin-1, was also shown to be enriched in both caveolar and noncaveolar rafts, where it has been implicated in raft organization. Therefore, Sertoli cell lysates and Sertoli cell DIG fractions were examined for the presence of flotillin-1. This protein, in contrast to caveolin, appears to be present in the Sertoli cell and highly localized in the DIG fraction (Figure 2). The content of flotillin-1 in the Sertoli whole-cell lysate (lane 4) was similar to that noted in brain whole-cell lysate (lane 6). Flotillin-1 distribution was also examined among a number of testicular cell lysates (Figure 4). Flotillin-1 was abundant in both Sertoli cells and PMCs, but lesser quantities were also seen in the SPGC and SPZOA fractions (Figures 2 and 4). Although flotillin-1 is abundantly present in PMC and Sertoli cell cultures, its concentration varies depending on culture conditions.

Rat testes were also paraffin embedded, sectioned, subjected to microwave antigen retrieval techniques, and stained for flotillin-1 content. Flotillin-1 concentration within the seminiferous tubules was clearly stage-dependent, with intense labeling observed around the spermatid heads prior to spermiation (Figure 5D and E). Reaction product began to accumulate within the adlumenal compartment of the seminiferous tubules during stages 5/6. Less intense deposition of reaction product was also observed in the basal compartment of the seminiferous tubules in the late spermatogonia and early spermatocytes. For reasons not understood, little-to-no reaction product was visible in the PMCs. Without microwave antigen retrieval, no reaction product was visible for flotillin-1 (Figure 5C).

Discussion

This study examines the distribution of the membrane raft structural proteins flotillin-1 and caveolin within Sertoli cells and Sertoli cell rafts as well as in other testicular cell types. Our findings indicate that Sertoli DIG fractions, although they lack significant caveolin, are highly enriched in the protein flotillin-1. Similar caveolin-independent DIG fractions (rafts) have been found in lung endothelial cells, lymphocytes, and brain neuronal cells (Fra et al, 1994; Gorodinsky and Harris, 1995; Schnitzer et al, 1995; Bickel et al, 1997). Thus, the presence of noncaveolar rafts is not unique to the Sertoli cell. Very little, however, is known about the organization of noncaveolar rafts or their function in any of the cells investigated to date.

A major issue raised by this study concerns the functional roles these Sertoli cell rafts play. Cell signaling is the function most widely attributed to both caveolae and noncaveolar rafts. We have, however, demonstrated that Sertoli DIG fractions lack caveolin, the membrane protein often responsible for interacting directly with membrane microdomain signaling components and thus regulating activity (Li et al, 1995, 1996; Mastick et al, 1995; Mastick and Saltiel, 1997). Since our studies have shown that flo-

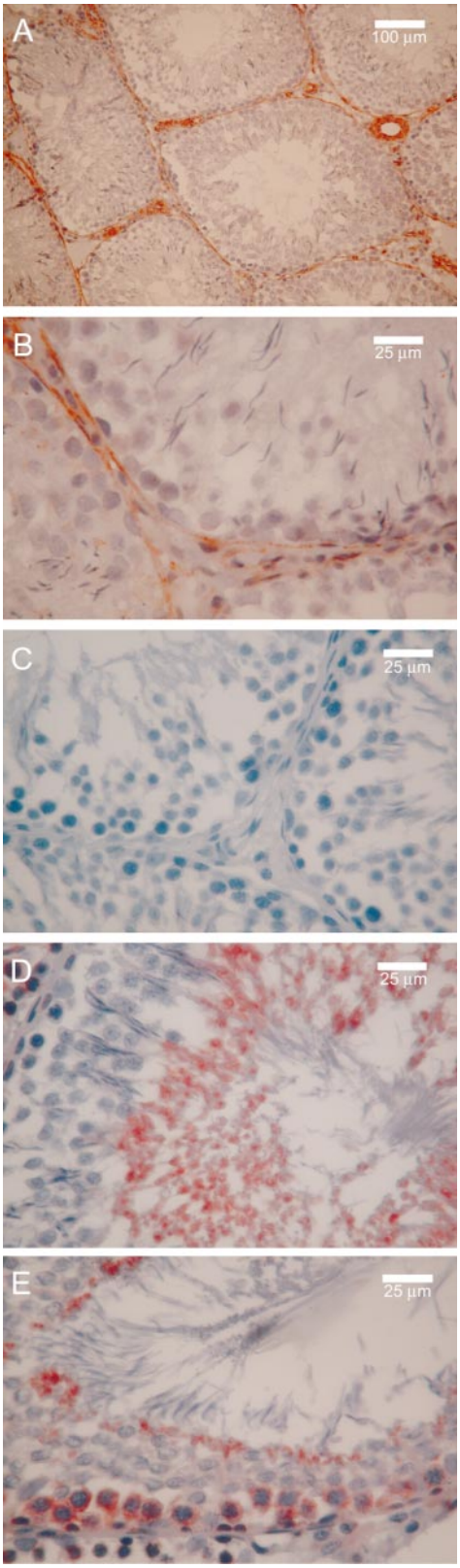

Figure 5. Distribution of caveolin and flotillin in paraffin-embedded sections of rat testis. After antigen retrieval, caveolin and flotillin were visualized using anticaveolin and antiflotillin-1 antibodies (Transduction), biotinylated secondary antibodies (Chemicon), streptavidin horseradish peroxidase (HRP) conjugate, and aminoethyl carbazole (AEC) (red) substrate (Zymed). Caveolin localization was specific to the peritubular myoid cells (PMCs) of the seminiferous tubules. Within the interstitium, the walls of the blood vessels also showed intense reaction product **(Panels A, B)**. Flotillin-1 distribution was stage-specific, with intense labeling observed around the spermatid heads prior to spermiation **(Panel C)**. Reaction product was also observed in the basal compartment of the seminiferous tubules with significant quantities of label present in late spermatogonia and early spermatocytes **(Panel D)**. Without the antigen retrieval process, no flotillin-1 reaction product was visible **(Panel E)**.

tillin-1 is a major structural protein of the Sertoli DIG fraction and since only limited data exist relative to flotillin's involvement in cell signaling, a more careful examination of flotillin's function in the testis may be instructive to the cell biology community at large. Of possible relevance is the very recent report of Baumann et al (2000) showing the involvement of flotillin-1 containing lipid rafts in insulin action.

The flotillin family of proteins currently consists of 2 isoforms. Flotillin-1, also known as reggie-2, is a protein originally characterized in fish retinal neurons that has been shown to have analogous forms in the rat. In addition, flotillin-2, also called reggie-1, has been described (Lang et al, 1998). Lisanti's research group suggests that flotillin-2 is homologous to epidermal surface antigen (ESA) (Bickel et al, 1997; Volonte et al, 1999), although subsequent studies have demonstrated significant differences between ESA and flotillin-2 (Hazarika et al, 1999). Flotillin-1 has been implicated in caveolar organization along with caveolin (Bickel et al, 1997; Volonte et al, 1999), yet recent studies suggest alternative functions (Lang et al, 1998; Hazarika et al, 1999). Flotillin-2 is thought to play a role in filopodia formation in fish retinal cells and in neuronal cells (Lang et al, 1998; Hazarika et al, 1999). In addition, in studies of fish retinal cells, flotillins segregate into patches of GPI-anchored cell adhesion proteins at the leading edge of filopodia formation in a cell type that lacks observable caveolae (Lang et al, 1998).

The stage-specific accumulation of flotillin-1 in the adlumenal compartment of the seminiferous tubules prior to spermiation raises some major questions. Unfortunately, the resolution available from microwave retrieval on paraffin-embedded sections (at least in our hands) does not provide adequate resolution to determine the precise cellular location of flotillin-1. Immunohistochemical studies offering better resolution need to be conducted. Additionally, work to locate the Sertoli cell rafts in situ would provide important information, as would the identification of other proteins present within these rafts. It is clear, however, that flotillin-1 possesses a more ubiquitous distribution among the testicular cell types than does caveolin.

Flotillin is also known to associate with endocytotic vesicles. Endocytotic vesicles have been described by several authors on the apical surface of the Sertoli cell (Nagano and Suzuki, 1978; Morales et al, 1985); however, to the best of our knowledge, no biochemical analyses of these vesicles have been completed. In neuronal cells, flotillin has been reported to reside in endocytotic vesicles slightly larger than the characteristic caveolin containing caveolae (Volonte et al, 1999), whereas flotillin has also been reported to reside in flat, nonvesicular microdomains (Lang et al, 1998). Immunohistochemical localization studies of flotillin in seminiferous tubule cross sections, as well as ultrastructural studies using immunogold labeling and electron microscopy, need to be conducted.

Although considerable evidence exists for the involvement of caveolae and other membrane microdomains in cell signaling, other possible roles need to be considered. Flotillin-dependent microdomains may have a role in the vesicular transport of materials between Sertoli and SPGC. Also, since members of the flotillin family of proteins have been reported to be involved in filopodia formation (Hazarika et al, 1999), it is possible that flotillin-associated microdomains play a role in the morphogenetic movements of the Sertoli and SPGC cells within the seminiferous tubule. Not only does extensive transport of materials between cells occur at cell-cell contact points, but extensive cytoskeletal networks immediately underlie the plasma membrane of the Sertoli cell. The extensive forces necessary to facilitate the massive remodeling of cell shape that is an integral part of spermatogenesis may be mediated through some of these specialized membrane microdomains. If the latter suggestion is correct, it is likely that cytoskeletal proteins would be physically associated with flotillin. If this is the case, flotillin-associated microdomains may also represent centers of contact between SPGC and Sertoli cells. Studies of possible physical interactions between flotillin and other proteins of the DIG fractions could be instructive.

The observation of abundant caveolin present in the PMC has been shown by the immunoblotting of PMC lysates, the immunofluorescent microscopy of whole-testis cryosections, and the immunohistochemical staining of paraffin-embedded sections of whole testis following microwave antigen retrieval techniques. This, along with previous data of Shubert et al (2001) showing a punctate distribution of caveolin in PMC cultures and an enrichment of caveolin in PMC DIG fractions, strongly supports the idea that the micropinocytotic vesicles observed by early microscopists (Lacy and Rotblat, 1960; Leeson and Leeson, 1963; Ross, 1967) are caveolae. Attention now needs to be focused on functional studies of these vesicles.

The distribution of caveolin observed in whole-testis sections yielded some unidentified foci of fluorescence within the seminiferous tubules. The origin of this fluorescence remains unknown, but this study suggests that the Golgi membranes are not the source. Since Sertoli cell cultures, isolated nonflagellated SPGC fractions, and washed epididymal spermatozoa all lack caveolin, the source of this fluorescence must reside either in a Sertoli cell component lost with time in culture or in a later developmental stage of the SPGC. Although we have been unable to co-localize these fluorescent foci with the acrosomes of developing spermatids, we have noted an artifactual staining of acrosomes on washed epididymal spermatozoa after immunofluorescent staining for caveolin.

In conclusion, with the recognition and isolation of specialized microdomains, rafts, on the Sertoli cell surface, the reproductive cell biologist will possess an additional tool for the study of cell signaling, and perhaps other interactions, between various cells of the seminiferous tubule. The data reported in this study also argue for the necessity of using Sertoli cell cultures as free as possible from peritubular myoid and SPGC contamination when preparing Sertoli cell DIG fractions.

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