Caveolae are small pits on the plasma membrane found in several, if not all, differentiated cells. They are involved in potocytosis, endocytosis, transcytosis, membrane trafficking, and signal transduction. Although caveolin has recently been identified in subcellular fractions from rat liver there is no clear-cut morphologic evidence for the presence of prototypical caveolae on the surface of hepatocytes. In this study the presence of caveolae at the cell surface of hepatocytes was directly shown by rapid-freeze, deep-etching electron microscopy. Moreover, combined deep-etching and immunogold techniques revealed caveolin in caveolae of the dorsal membrane of primary culture hepatocytes. Using reagents that perturb membrane cholesterol and interfere with endocytosis through the caveolae, a caveolae-dependent internalization of cholera toxin B and retinol-binding protein by hepatocytes in primary culture was shown. Finally, immunocytochemical analysis of caveolin in non-parenchymal cells of the rat liver showed its presence in Kupffer and stellate cells, however no caveolin could be detected in endothelial cells. (HEPATOLOGY 2001;33:1259-1269.)

The hepatocyte is a highly polarized epithelial cell with a plasma membrane divided into three main functional domains: the sinusoidal domain, facing the blood and the hepatic endothelial cells; the lateral domain, containing the junctional complexes (e.g., desmosomes and gap junctions); and the canalicular plasma membrane, involved in bile secretion. The sinusoidal domain of the hepatocyte plasma membrane (basolateral) in contact with the blood contains most of the receptors for hormones, growth factors, and metabolites. A functional continuity of this basolateral membrane with the endosomal compartment is mediated by clathrin-coated pits and vesicles, which eventually fuse with the early/sorting endocytic compartment. Although most of receptors involved in endocytosis or transcytosis are located in clathrin-coated pits, alternative ports of entry by nonclathrin-coated pits have been shown in various hepatocyte-derived cell lines.2,3

In biochemical analysis of isolated rat-liver plasma membranes, the presence of caveolin was not considered, mainly because of its low expression in the liver and the lack of appropriate antibodies, but also because such cell-surface structures were believed to be restricted to endothelial cells. However, the expression of caveolin in liver homogenates was shown by Northern4 and by Western blot,5 and the internalization of specific ligands through caveolae has been reported.5-7 Recently, we have shown the presence of caveolin in the endocytic compartment and in a caveole-enriched plasma membrane fraction from rat liver,8 together with differential distribution of signaling molecules.9

The liver is composed of parenchymal, hepatocytes, and nonparenchymal cells, mainly Kupffer, stellate, and endothelial cells. Little is known about the cellular distribution of caveolae in liver cells. Smooth-surfaced caveolae have been described on the perisinusoidal surface of stellate cells,10 and they have also been observed in Kupffer cells.11 Unlike other tissues, liver endothelial cells do not seem to contain a significant amount of caveolar structures, in the plasma membrane, but they were enriched in clathrin-coated pits.12,13

Although clathrin-coated pits and vesicles transport receptors and ligands en route to lysosomes or for transcytosis, caveolae may be alternative endocytic pathways involved in cholesterol transport and signal transduction. They are 50- to 70-nm plasma membrane invaginations enriched in cholesterol and sphingolipids, and they contain caveolin (recently reviewed by Kurzchalia and Parton 1999,14 and Anderson 199815).

The biochemical finding that subcellular fractions from rat liver, derived from plasma membrane and endosomes, contained caveolin prompted us to study the possibility that hepatic cells were endowed with caveolae, like other polarized epithelial cells, e.g., MDCK cells.

In the present study, rapid freeze deep-etching and immuno-electron microscopy were used to show the presence of caveolae in hepatocytes in primary culture and to characterize them morphologically. We also found that internalization of cholera toxin subunit B (CT) and retinol binding protein (RBP) was impaired by drugs or agents reported to bind or alter cholesterol-rich rafts, such as caveolae, in isolated hepatocytes.
MATERIALS AND METHODS

Antibodies

The rabbit and mouse anti-caveolin antibodies and the anti-β-adaptin were from Transduction Laboratories (Lexington, KY); a mouse anti-caveolin, used for cryo-immuno-electron microscopy, was from Zymed Laboratories (South San Francisco, CA), the mouse anti-actin was from ICN (Costa Mesa, CA), and the anti-ASGP receptor was prepared in our laboratory; the mouse anti-pIgR was kindly donated by Dr. K.E. Mostov (University of California, San Francisco, CA); the monoclonal anti-βlg120 antibody (clone GM10) was provided by K. Siddle and J. Hutton (University of Cambridge, UK); the monoclonal anti-clathrin X22 was donated by Dr. Frances Brodsky (University of California, San Francisco, CA); the monoclonal mouse anti-MRC OX43 was used as an endothelial-cell marker (Harlan Bioproducts, Madison, WI); and a monoclonal mouse anti-CD11b (clone WT.3.), FITC conjugated (Calbiochem-Novabiochem Corporation, Darmstadt, Germany), was used as a Kupffer cell marker. Finally, fluorescein conjugated (FITC and Cy3) antibodies were from Jackson ImmunoResearch (West Grove, PA) and gold-coupled secondary antibodies for immun-electro microscopy were from British BioCell International Ltd. (Cardiff, UK).

Animals

Male Sprague-Dawley rats weighing 200 to 250 g were kept under a controlled lighting schedule with a 12-hour dark period. All animals received humane care in compliance with institutional guidelines. Food and water were available ad libitum.

Liver Cell Isolation and Culture

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated from a young male Sprague-Dawley rat using a sterile method.\textsuperscript{16} The portal vein was cannulated, and the liver was perfused in a nonrecirculating manner with buffered, warm Hanks' balanced salt solution (HBSS) saturated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} gas, with 0.5\text{mmol/L EGTA at a rate of 20 ml/min for 10 minutes.} The liver was excised from the animal while perfusion continued. Next, liver was dissociated gently with a glass rod after recirculating perfusion with warm HBSS containing type IV collagenase (Sigma, Madrid, Spain) (0.035\%) and 4 mmol/L CaCl\textsubscript{2}. Cell suspension was filtered through gauze with cold Kreb's Henseleit medium and allowed to settle for 10 minutes. Cells were then decanted, resuspended in Kreb's, and washed 3 times. The obtained pellet containing hepatocytes was resuspended in medium DMEM/HAM F12 (Sigma) 10% FCS and counted. Trypan blue exclusion showed cell viability greater than 95%. Cells were plated on 60-mm culture dishes at a density of 3 × 10\textsuperscript{6} cells per dish. Cultured cells were incubated at 37°C in a humidified 95% O\textsubscript{2} atmosphere for 12 to 24 hours.

Isolation and Culture of NPCs. Isolation of rat stellate cells was performed as described in Geerts et al.\textsuperscript{17} After isolation of rat stellate cells, these were seeded in cell-culture dishes and glass coverslips in Iscove's medium supplemented with 0% nonessential amino acids, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 10% FCS, and antibiotics.

Isolation of liver Kupffer and endothelial cells was performed as described in Pertofi et al.\textsuperscript{16} The interface at 25% to 50% of the Percoll gradient, containing mainly Kupffer and endothelial cells, was seeded on cell-culture plates and incubated for 30 minutes at 37°C. Purified Kupffer-cell preparations were obtained by the rapid adherence of these cells (30 minutes) to a glass or plastic surface. Kupffer cells were cultured for 1 day in RPMI 1640 medium supplemented with 2 mmol/L glutamine 5% FCS and antibiotics. Nonattached cells (enriched in endothelial cells) after this period were seeded on fibronectin covered cell-culture dishes or glass coverslips in RPMI 1640 supplemented with heparin 8 U/mL, 15% N-sucrose, 15% bovine calf serum and antibiotics, and cultured for 1 week.

Electron-Microscopy Procedures. Cell monolayers on petri dishes were fixed at room temperature in 2% glutaraldehyde in 0.1 mol/L phosphate buffer for 1 hour. Cells were scraped and collected into 0.1 mol/L phosphate buffer containing 2% of paraformaldehyde. After 3 rinses in 0.1 mol/L phosphate buffer, the pellets were postfixed in 1% OsO\textsubscript{4} in 0.1 mol/L phosphate buffer. Finally, samples were embedded in Epon.

For cryo-immuno-electron microscopy, livers of young adult Sprague-Dawley rats were fixed by perfusion with 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 mol/L phosphate buffer. After cryoprotection in 2.1 mol/L sucrose, livers were frozen in liquid nitrogen, and ultrathin sections were cut using a Reichert Ultracut S cryo-ultramicrotome equipped with a cryochamber attachment (Leica, Heidelberg, Germany). Cryo-sections were immunolabeled as described elsewhere.\textsuperscript{19} After labeling with a monoclonal anti-caveolin-1 antibody (2.5 μg/mL) (Zymed) and a secondary anti-mouse conjugated to gold to 15 nm, sections were rinsed in distilled water and embedded in 1.8% methyl cellulose 0.3% uranyl acetate.\textsuperscript{20} In some experiments, cryo-sections were also labeled with anti-clathrin X22 antibody (1:40).

For cryo-substitution, hepatocytes in primary culture were grown on transwell filters. Small pieces of the filters were cryofixed by projection against a copper block cooled in liquid nitrogen (−196°C) using a Cryo-Block (Leica) as described.\textsuperscript{21} Freeze-substitution was performed in a home-made cryosystem,\textsuperscript{22} using acetone containing 0.5% of uranyl acetate, for 3 days at −90°C. On the fourth day, the temperature was slowly increased, 5°C/h, to −30°C. At this temperature samples were rinsed in acetone and then infiltrated and embedded in Lowicryl HM20. Ultrathin sections were picked up on Formvar-coated gold. For immunogold localization, samples were blocked with 2% ovalbumin for 30 minutes and incubated at room temperature for 1 hour with monoclonal anti-caveolin antibody from Zymed (2.5 μg/mL). Washes were performed with PBS prior to adding goat anti-mouse conjugated to 15 nm colloidal gold for 45 minutes at room temperature. Finally, samples were washed and contrasted with 2% uranyl acetate for 30 minutes.

Freeze-Fixation, Freeze-Drying Electron Microscopy. Dorsal plasma membranes of rat hepatocytes in primary culture were obtained by a sandwich technique. Cell membranes on coverslips were prepared by overlaying a TESPA-coated coverslip onto the upper surface of primary cultured hepatocytes, applying gentle pressure, and removing the coverslip with adherent membranes, all at 4°C.\textsuperscript{23,24} Ventral membranes were obtained by a lysis-squirt technique. Briefly, cells grown on coverslips were chilled with cold PBS, lysed with high-salt buffer (30 mmol/L KCl, 70 mmol/L HEPES, 70 mmol/L MgCl\textsubscript{2}, 3 mmol/L EGTA, pH 7.5) diluted 1:3 in distilled water, and squirited by a stream of buffer (30 mmol/L HEPES, 70 mmol/L KCl, 5 mmol/L MgCl\textsubscript{2}, 3 mmol/L EGTA, pH 7.5). For immunogold localization, membranes were chilled to 4°C and fixed for 20 minutes with 3.7% paraformaldehyde in 0.1 mol/L phosphate buffer and rinsed in buffer containing 20 mmol/L HEPES, 100 mmol/L KCl, 5 mmol/L MgCl\textsubscript{2}, 3 mmol/L EGTA, pH 6.8 before incubation with primary antibody for 1 hour at 4°C. After washing, membranes were incubated with secondary antibody conjugated to gold. Then samples were washed and fixed with 2.5% glutaraldehyde before processing as for rapid-freezing and deep-etching electron microscopy. Briefly, coverslips were cryofixed by projection against a copper block as described earlier. The frozen samples were stored at −196°C in liquid nitrogen until subsequent use. Samples were freeze-dried and coated with platinum and carbon using a freeze-etching unit (model BAF-060, BAL-TEC, Liechtenstein). A rotary shadowing of the exposed surface was made by evaporating 1 nm platinum-carbon at an angle of 24° above the horizontal, followed by 10 nm of carbon evaporated at a 75° angle.\textsuperscript{24} The replica was separated from the coverslip by immersion in full-strength hydrofluoric acid, washed twice in distilled water, and digested with 5% sodium hypochlorite for 5 to 10 minutes. Finally, the replicas were washed several times in distilled water, broken into small pieces, and picked up on Formvar-coated copper grids for electron microscopy. When immunolabeling was carried out, the replicas were only washed in distilled water. All electron micrographs were obtained on a Hitachi HU-600, operating...
at 75 kV. The photographic negatives were digitalized without contrast-reversing and treated by the IMAT program (Alejandro DiGior- gio, Serveis Científico Tècnics, Universitat de Barcelona). As controls for single immunostaining, incubation with the second antibody only was accomplished. For double labeling, controls using only 1 primary antibody, and the respective secondary antibodies were performed to establish that the colocalization was not the result of recognition of the same primary antibody. In both cases, the labeling was specific, as no signal was obtained (data not shown).

**Internalization Assays With Cholera Toxin-Gold**

To study the binding and the internalization of cholera toxin-gold by caveolae, a pre-embedding procedure was performed. Briefly, hepatocytes cultured on 12-well cell-culture plates for 20 hours were washed once with DMEM/25 mmol/L HEPES containing 0.1% BSA at 4°C and then incubated for 2 hours with cholera toxin-gold (15 μg/ml) at 4°C. After several washes, the medium was replaced by prewarmed fresh medium to induce the internalization for 10 minutes. Finally, cells were fixed with 2.5% glutaraldehyde – 2% paraformaldehyde in phosphate buffer for 1 hour at room temperature. Fixed cells were scrapped and prepared for electron microscopy as described earlier.

**Other Procedures**

**SDS-PAGE and Western Blotting.** SDS/PAGE of proteins was performed in 10% polyacrylamide, as described by Laemmli. For Western blotting, polypeptides were transferred electrophoretically at 60 V for 90 minutes to immobilon-P transfer membranes (Millipore Ibérica, Madrid, Spain), and antigens were identified using specific antibodies diluted in TBS (Tris-buffered saline) containing 0.5% powdered skimmed milk, and finally the reaction product was detected using the ECL system (Amersham Ibérica, Madrid, Spain). Image analysis of Western-bLOTS and band quantitation was performed with a Bio-Image system (Millipore). The protein content of the samples was measured by the method of Bradford using BSA as standard.

**Conjugation of Asialofetuin and RBP to FITC.** Asialofetuin (Sigma, Madrid, Spain) was diluted (10 mg/ml) in 50 mmol/L sodium carbonate buffer pH 9.2, and RBP (Sigma) (2.5 mg/ml) in 0.2 mol/L borate buffer pH 9.0 and then mixed end-over-end with fluorescein isothiocyanate (Sigma) dissolved (10 mg/ml) in ethanol for 2 hours in the dark at room temperature. The conjugates were transferred to a PBS pre-equilibrated PD-10 column to separate FITC-conjugated asialofetuin from free FITC.

**Conjugation of Low-Density Lipoprotein to DiI.** Human low-density lipoprotein (LDL) was isolated and dialyzed in 0.15 mol/L NaCl at 4°C, was brought to a concentration of 2 mg/ml in PBS 0.5% BSA at 37°C, and mixed with 1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine (DiI) (Molecular Probes, Eugene, OR) (30 mg/ml in DMSO) at a conjugation ratio of 0.3 mg DiI/2 mg LDL for 8 to 10 hours at 37°C. The solution was brought to a density of 1,063 mg/ml with KBr and centrifuged at 36,000 rpm for 20 hours in a Beckman (Palo Alto, CA) SW 50.1. The top supernatant was collected and dialyzed in 0.15 mol/L NaCl at 4°C for 1 to 2 days.

**Preparation of Cholera Toxin-B-Gold.** Colloidal gold (17 nm) was prepared as described by Slot and Geuze. The pH of the solution was adjusted to 6.9. The cholera toxgin-gold was stabilized with BSA to a concentration of 0.1% and then washed twice by centrifugation. Immediately before use, the gold was washed once more and resus- pended in medium or PBS containing 0.1% BSA.

**Ligands Internalization and Immunofluorescence.** Hepatocytes on glass coverslips were incubated for 30 minutes with HEPES-modified DMEM 1% BSA and treated with 5 μg/ml filipin (Sigma) for 1 hour or with 1.5% cycloexodrin (Sigma) for 30 minutes. Control and treated cells were allowed to uptake cholera toxin subunit B-FITC conjugated (8 μg/ml) (Sigma), asialofetuin-FITC or DiI-LDL (20-50 μg/ml) for 10 minutes with the presence of the drugs. Cells were quickly rinsed and incubated in the absence of labeled ligands, and with or without the presence of drugs in the medium for different periods. Cells were fixed with 3.7% paraformaldehyde, washed in PBS, and mounted in Mowiol (Calbiochem, La Jolla, CA).

Cells grown on glass coverslips were fixed in 3.7% paraformaldehyde for 10 minutes at room temperature and then permeabilized for 15 minutes in PBS, 1% BSA, 0.1% saponin. After 3 washes in PBS, cells were incubated for 1 hour at 37°C in a humidified atmosphere with primary antibody in PBS 0.1% BSA, 0.1% saponin. Coverslips were then washed 3 times in PBS and incubated for 1 hour at 37°C with corresponding secondary antibodies in PBS 0.1% BSA, 0.1% saponin. After 3 washes in PBS, samples were mounted on glass slides with Mowiol and examined under a confocal microscope. For the detection of CD11b in Kupffer cells, fixation was carried out with acetone (2 minutes at −20°C), and no secondary antibody was needed as mouse anti-CD11b was conjugated to FITC.

The measure of internalization of fluorescence-ligands in control and treated hepatocytes were digitalized using an MC80 camera. For all these experiments, the same settings of the camera and system were used. Surface fluorescence and internalization were measured with KS100 Kontron Imaging System software. For this purpose, the periphery of cells was manually defined, and the mean cell-associated fluorescence intensity and the area of the cells were determined. The integrated optical density of the cellular staining was then determined, and the mean was calculated. The results are the average of at least 3 separate experiments, and the values are expressed as a percentage of the maximum signal-detected: control cells. For each experiment a minimum of 100 cells of control and treated groups were analyzed.

**Measurement of Endocytosis of 125I-LCT and 125I-ASF.** Hepatocytes were washed briefly in DMEM-HEPES incubated with and without CD (1.5%) (2-hydroxypropylβ-cyclodextrin) for 15 minutes at 37°C or potassium depleted for the inhibition of clathrin-dependent internalization. Asialofetuin (125I-ASF) were then added to the cells and incubated for 30 minutes at 0°C in DMEM-HEPES 1% BSA or simplified medium (140 mmol/L NaCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 5.5 mmol/L glucose, 0.1% BSA, 20 mmol/L HEPES, pH 7.3) for potassium-depleted cells. After washes in cold medium, cells were incubated at 37°C for 30 minutes with and without CD or simplified medium. Control for the K+ depletion was made by incubating the cells in 10 mmol/L KCl. Endocytosed 125I-LCT and 125I-ASF were measured and calculated as the percentage of total cell-associated CT and ASF. Surface-bound CT was measured as the amount of CT that could be released by low pH after 3 rapid washes in medium at 0°C. The cells were then incubated at 0°C for 5 minutes with low pH buffer (0.5 mol/L NaCl and 0.2 mol/L acetic acid, pH 2.5), followed by 1 rapid wash in medium. Surface-bound ASF was measured as the detection of ASF that could be released after 3 rapid washes in medium at 0°C and then incubated at 0°C for 5 minutes with 5 mmol/L EGTA in medium. After a quick wash with medium, endocytosed ligands were measured as the amount of CT and ASF that could not be removed by these treatments. The results are the average of at least 3 separate experiments and the values are expressed as a percentage of the maximum detected signal: control cells.

**RESULTS**

**Biochemical Detection of Caveolin in Rat Liver, in Isolated Hepatocytes, and in NPCs.** Homogenates from rat liver and from primary cultured hepatocytes were used to demonstrate the presence of caveolin by Western blotting using a specific anti-caveolin antibody. Figure 1A shows the comparison and differential expression of caveolin, β-adaptin and asialoglycoprotein receptor (ASGP-R) in lung and liver homogenates as well as in hepatocytes in primary culture (HPC). Anti-actin was used as control of protein loading. Figure 1B shows a representative Western blotting comparing the amount of caveolin in hepatocytes, NPC crude cellular fraction, and in isolated Kupffer and stellate cells.
Isolated cells were also analyzed by immunocytochemistry with a confocal microscopy. Figure 2 shows the pattern of caveolin by confocal microscopy. Note that hepatocytes (Fig. 2A) and Kupffer cells (Fig. 2C) have caveolin located at the plasma membrane; in stellate cells caveolin was mainly intracellular, punctate, or concentrated in the Golgi region (Fig. 2E). No caveolin was observed in endothelial cells (Fig. 2G, arrows). Figure 2G and H show a double labeling of endothelial enriched fraction, which also contained some stellate cells, with anti-caveolin (Fig. 2G) and anti-MRC-OX43 (Fig. 2H) antibodies; note that no caveolin can be observed in endothelial cells (arrows), labeled with anti-MRC-OX43 antibody (endothelial cell marker). Contaminant stellate cells were positively labeled with anti-caveolin (arrowheads).

Electron Microscopy Analysis of Hepatocyte Cell Surface. We first followed a morphologic approach to define the existence and the location of caveolae in these two systems: intact rat liver and primary cultured hepatocytes. Figure 3 shows ultrathin cryosections of intact rat liver with caveolae-like structures or vesicles beneath the sinusoidal plasma membrane, among microvilli (Fig. 3A, arrows). When these sections were used for immunodetection with a polyclonal anti-caveolin antibody, followed by an anti-rabbit IgG-conjugated to 10 nm gold, it can be observed that these caveolae were specifically labeled with gold (Fig. 3B).

Ultrathin cryosections from intact livers, immunolabeled with anti-clathrin or with anti-caveolin antibodies, were used for a morphometric analysis to assess the distribution of clathrin (and clathrin-coated pits/vesicles) and caveolin (and caveolae or intracellular caveolin) in the sinusoidal (basolateral) and the canalicular (apical) plasma membrane domains. Data shown in Table 1 clearly indicated that, whereas clathrin and/or clathrin-coated structures were observed in similar proportion in the basolateral as well as in the canalicular plasma membranes, no caveolae could be found in the canalicular plasma membrane, and the amount of caveolin detected in the basolateral domain was 86% compared with the 14% found in the canalicular membranes.

Isolated hepatocytes were cryofixed and prepared for immuno-electron detection by freeze-substitution and embedding in Lowicryl HM20. Ultrathin sections were labeled with a monoclonal anti-caveolin antibody followed by an anti-mouse conjugated to gold (15 nm). Figure 4A shows a caveola decorated with the anti-caveolin on its protoplasmic surface. As an additional direct evidence for the presence of caveolae in the hepatocyte we used cholera toxin B-gold as a highly specific probe shown to bind the GM1 in caveolae.32-34 Cholera toxin-gold was incubated at 4°C for 2 hours, and then hepatocytes were warmed and internalization was proceeded for 10 minutes at 37°C. Figure 4B and C show the specific
location of cholera toxin-gold in caveolae at the cell surface or in caveolae-derived membrane structures.

Interestingly, a survey of the hepatocyte cell surface in primary culture showed significant differences between dorsal and ventral plasma membranes, and a detailed analysis of a number of sections indicated a polarized distribution of clathrin-coated pits at the ventral surface and caveolae-like structures at the dorsal membranes. These topological features were not resolved in a previous immunofluorescence analysis of isolated hepatocytes, where staining with anti-clathrin,25 or with anti-caveolin antibodies8 revealed an almost homogeneous staining around the cell surface.

To examine the fine morphology of these specific microdomains involved in the endocytosis, we used the rapid-freeze, deep-etch technique. Figure 5 shows the comparison of thin sections and rapid-freeze, deep-etch views of the dorsal (Fig. 5A and C) and ventral (Fig. 5B and D) inner surfaces of a hepatocyte. The most striking observation was that, whereas the ventral membrane contained many clathrin-coated pits, sheets, and almost-formed vesicles, the dorsal cell surface had smooth invaginations, some with a characteristic striated coat, flask-shaped morphology, size, and uniform curvature (Fig. 5C, arrows) of caveolae.

Confirmation of thin-section labeling was obtained by applying the immuno-gold technique to ventral and dorsal membranes obtained by lysis-squirming and sandwich technique, respectively, from hepatocytes in primary culture at 12 hours after plating. Figure 6A shows the freeze-dried replica of the protoplasmic surface of a hepatocyte dorsal membrane immunogold labeled for caveolin (10 nm). Caveolin immunogold was almost restricted to areas rich in smooth vesicles. In Fig. 6B, some caveolae show a striated coat and gold label on the cytoplasmic face.

Because the polymeric immunoglobulin receptor (pIgR) is exclusively located in the clathrin-coated pits of the hepatocyte plasma membrane, we double-labeled the ventral membranes with anti-caveolin (15 nm) and anti-pIgR (10 nm) and then processed them for rapid-freeze, deep etching (Fig. 6C and D). Arrows indicate labeling with anti-caveolin (15 nm) scattered on irregular structures that lack clathrin lattices; arrowheads indicate clathrin-coated pits and sheets labeled with anti-pIgR (10 nm).

In this study, rat hepatocytes were obtained by liver perfusion and plated for 12 to 16 hours. Such primary culture hepatocytes show two plasma membrane surfaces with different functions. The substratum adherent plasma membrane
contains different types of adhesion structures (e.g., focal contacts), and it participates in secretion or uptake of ligands, etc. This cell surface is called the ventral membrane. The top surface is referred to as the dorsal membrane. The analysis of these hepatocytes, by biochemical or morphologic means, showed that no polarization occurred.37

Caveolin-Dependent Endocytosis in Hepatic Cells. To analyze the functionality of caveolae in hepatocytes, we tested the internalization of ligands considered specific to caveolae. CT and RBP have been described such that liver- or hepatic-derived cell lines can be used as specific ligands of the entry through the caveolae. Two different approaches were undertaken: (1) ligands were coupled with fluorescent tags to visualize their internalization by confocal microscopy and (2) selective disruption of caveolae or inhibition of clathrin-dependent entry and subsequent quantification of the internalized radiolabeled or fluorescence-labeled ligands.

Figure 7A shows the internalization of CT coupled to FITC in isolated hepatocytes at different times. After incubation for 10 minutes at 37°C (0 minute chase), labeling was mainly associated with the cell surface. However, after 20 or 30 minutes chase (37°C) CT-FITC was concentrated underneath the plasma membrane and in the perinuclear (Golgi) region, though some labeling remained at the cell surface. The internalization of RBP-FITC was also analyzed in hepatocytes; after 30 minutes RBP-FITC was detected in intracellular punctate structures, most probably early and late endosomes (Fig. 7D); these structures did not colocalize with the prelysosomal (lysosomal) marker lgp120 (Fig. 7E).

When the internalization of RBP was compared with other ligands that enter via clathrin-coated pits, such as asialofetuin (ASF-FITC), it can be observed that, though both ligands shared peripheral vesicular structures, ASF appeared to be more efficiently internalized than RBP (see the high degree of colocalization with prelysosomal/lysosomal, immunolabeled with anti-lgp120) (a couple of hepatocytes in Fig. 7B and C). These results are in agreement with work published by Malaba et al., and our own studies in isolated rat-liver endosomal fractions.

Hepatocytes were treated with reagents that interfere with cholesterol, filipin and cyclodextrin, and therefore modify the structure and the function of caveolae. Figure 8 shows the effect of cyclodextrin on the internalization of RBP, ASF, and LDL. Internalization of RBP was significantly inhibited by cyclodextrin (88%) (Fig. 8A) (filipin only inhibited the internalization of RBP by 26%, data not shown). On the other hand, treatment of hepatocytes with CD hardly affected the internalization of ASF (Fig. 8B) or LDL (Fig. 8C). Figure 8D, E, and F show representative images of the internalization of RBP-FITC, ASF-FITC, and DiI-LDL, respectively, in hepatocytes after CD treatment.

Finally, we also compared and quantified the entry of 125I-CT and 125I-ASF by the hepatocytes after K+ depletion, a treatment known to markedly reduce the rate of endocytosis.

### Table 1. Morphometric Analysis of Clathrin and Caveolin Labeling in Liver Parenchymal Cells

<table>
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NOTE. Clathrin and caveolin distribution in hepatocyte membranes of rat liver was analyzed using ultrathin cryosections immunolabeled with anti-caveolin or anti-clathrin X22 antibodies and examined and photographed using a JEOL JEM 1010 electron microscope. A total of 384 and 230 gold particles associated with membranes were counted after clathrin and caveolin labeling, respectively. A total of 57 (clathrin) and 72 (caveolin) micrographs labeled cryosections (at × 50,000 magnification) were quantified. Gold particles associated with hepatocytes were counted; labeling at the plasma membrane (PM) was distinguished from labeling associated with intracellular membranes (ICM). Linear membranes in the cytoplasm of unknown organelle type were scored as intracellular membranes. On close examination of the plasma membranes, gold particles associated with plasma membranes were distinguished from those associated with caveolae (PM caveolae) or clathrin-coated pits (CCP) attached to the plasma membranes and the caveolae or clathrin-coated vesicles that were apparently detached (free) (vesicles).

Abbreviations: PM, plasma membrane; CCP, clathrin-coated pits; CC vesicles, clathrin-coated vesicles; ICM, intracellular membrane; PM, caveolae, plasma membrane caveolae.
FIG. 5. Comparison of ultrathin sections and rapid-freeze, deep-etching replicas of the dorsal (A, C) and ventral (B, D) hepatocyte plasma membrane. (A) A detail of the dorsal surface of an isolated hepatocyte with a fully formed caveola can be observed (arrow); (B) a flat region of the ventral membrane with some clathrin-coated pits and vesicles (arrowheads); (C) the dorsal membrane with several caveolae (arrows); (D) freeze-dry replicas of this ventral surface show the enrichment of clathrin-lattices (arrowheads). Bar = 100 nm.

FIG. 6. Combination of deep-etching and immuno-gold techniques with a polyclonal anti-caveolin (15 nm) in dorsal membranes (A) and (B) and a double labeling with a rabbit anti-caveolin (15 nm) and a mouse anti-plgR (10 nm) in ventral hepatocyte plasma membranes (C, D). Detail of the ventral surface with a clathrin-coated pit labeled with anti-plgR (D). Arrowheads indicate the labeling of the anti-plgR in clathrin-coated pits; arrows indicate the labeling with anti-caveolin. Bar = 100 nm.
of ligands that enter by clathrin-coated pits. Internalization of $^{125}$I-ASF was significantly reduced (39%) compared with the entry of $^{125}$I-CT that was almost unaffected (9%) (Fig. 9A). However, endocytosis of $^{125}$I-CT in hepatocytes treated with cyclodextrin was reduced by 45% (Fig. 9B).

**DISCUSSION**

The presence of caveolae in the hepatocyte plasma membrane was directly shown by rapid-freeze, deep-etching electron microscopy. Moreover, combined deep-etching and immunogold techniques revealed the presence of caveolin in prototypic caveolae of the dorsal membrane of primary cultured hepatocytes. Interestingly, immunofluorescence analysis of isolated nonparenchymal cells also revealed that Kupffer and stellate cells contained caveolin but endothelial cells lacked it. In this study, we achieved a morphologic and functional characterization of caveolae in hepatocytes.

Hepatocytes are extremely active in endocytic processes, and its major intracellular trafficking pathways have been examined in detail. The "default" pathway for the delivery of the content of endosomes to lysosomes, the transcytotic route involved in apical secretion but also in the transport of apical plasma membrane proteins to the bile canalicular plasma membrane (e.g., GPIs) and the recycling pathway, exemplified by the transferrin receptor, have been investigated. These endocytic pathways begin in clathrin-coated pits at the sinusoidal plasma membrane. However, different ports of entry have now been shown to be operative in different cells: clathrin-independent and through caveolae.

We have described a subcellular fraction from rat liver highly enriched in caveolin and morphologically identical to the caveolar-membrane fraction isolated from endothelial cells. A comprehensive characterization of this caveolin-enriched plasma membrane fraction (CEF) showed that besides caveolin it contained other molecules present in the caveolae of endothelial, smooth muscle, adipocytes, or fibroblast cells (e.g., PKC, Ras, Raf-1, Mek or SR-BI). Interestingly, although several publications have shown that the caveolin detected in liver may originate from caveolae of hepatocytes, no clear-cut morphologic or immunocytochemical evidence has been presented.

The squirting technique for exposing the protoplasmic surface of the plasma membrane reveals the topography of large areas of plasma membrane and allows direct identification of those structural elements that are morphologically distinct, such as clathrin sheets, coated vesicles, caveolae or actin-microfilaments. A striking finding in hepatocytes was the presence of multiple caveolae clustered in restricted areas of the dorsal membrane, whereas this face of hepatocyte was poor in clathrin complexes, compared with their ventral face. The presence of numerous clathrin complexes on the protoplasmic surface of the ventral plasma membrane of hepatocytes has been reported elsewhere. These authors suggested that the first stages of cell adhesion to the substratum are mediated by specialized regions of the plasma membrane, rich in receptors, with clathrin-coated structures, that strongly interact with the extracellular matrix components (e.g., collagen). On the other hand, the presence of scattered caveolin containing structures (other than caveolae) in the ventral membrane of hepatocytes might be related with its function of linking integrins with signal transduction. Indeed, a recent article showed that caveolin-1 functions as a membrane adaptor to link the $\alpha$-subunit of integrin to the tyrosine kinase Fyn. Once activated, Fyn binds to Src, which on phosphorylation recruits Grb and eventually regulates the Ras-ERK cascade. The assembly of signaling molecules surrounding the integrin family of adhesion receptors remains poorly understood. Caveolin binds cholesterol and several signaling molecules potentially linked to integrin function, e.g., the Src family of kinases, although caveolin has not been directly implicated in integrin-dependent cell adhesion.

Finally, although hepatocytes are quiescent cells, it was shown that there is basal activity of Raf-1 and Mek, restricted to the early/sorting endocytic compartment, and EGF triggers a recruitment of caveolin from the plasma membrane to the early endocytic compartment. Thus, the machinery for signal transduction pre-organized in the caveolae at the cell surface could be recruited into the early/sorting endosomes, where phosphorylation of Raf-1, Mek, and eventually MAPK takes place. Binding of EGF could induce the dissociation of the caveolin/Ras complex and allow access of Ras to GTP, thus activating the MAPK cascade.
Is There a Caveolar-Dependent Endocytic Pathway in Hepatocytes? Evidence for a caveolar-dependent endocytic pathway has been reported in various systems.\textsuperscript{65-70} In hepatocytes, at least the following possibilities should be considered: (1) the uptake of retinol-binding protein for subsequent transport to stellate cells, (2) “fluid phase” transcytosis (e.g., albumin)\textsuperscript{71} or the specific targeting of GPI-anchored proteins from the basolateral to the apical plasma membrane, and (3) receptor-mediated endocytosis of HDL by the scavenger receptor SR-BI specifically detected in caveolae\textsuperscript{72} or in caveolin-enriched rat liver membrane fractions.\textsuperscript{59} Furthermore, because the hepatocyte is the only site of catabolism of free cholesterol to bile acids, which are secreted to the bile canaliculi along with free cholesterol and phospholipids, one may question the possible involvement of the caveolae in cholesterol transport.

Internalization assays were performed using 2 ligands that internalize via caveolar-mediated processes such as retinol-binding protein and cholera toxin subunit B. Treatment of cells with drugs that bind or alter cholesterol-rich rafts such as caveolae significantly inhibit the internalization of these ligands, indicating that, in hepatocytes, their internalization is dependent on cholesterol-rich rafts. Thus, in this study, using the squirting technique for exposing the proplasmic surface of plasma membranes and by biochemical means, we show that hepatocytes from rat liver have prototypic caveolae and that these cell surface microdomains are functional.

Finally, caveolin was also detected in nonparenchymal rat liver Kupffer and stellate cells. Its precise cellular or subcellular distribution, compared with hepatocytes, may be different and is currently under investigation.

**Fig. 8.** Effect of cyclodextrin on the internalization and distribution of RBP-FITC, ASF-FITC, and Dil-LDL in hepatocytes. Histograms represent the quantification of internalized ligands: (A), RBP-FITC, (B), ASF-FITC, (C), Dil-LDL (see Materials and Methods for details), and the corresponding panels on the right are representative fields visualized by confocal microscopy at the same experimental conditions: (D), RBP; (E), ASF; (F), LDL. Control, untreated; CD, cyclodextrin.

**Fig. 9.** Effects of cyclodextrin and K\textsuperscript{+} depletion on \textsuperscript{125}I-CT and \textsuperscript{125}I-ASF internalization in isolated hepatocytes. Cells were cultured for 12 hours in 10% FCS and then incubated for 1 hour in serum-free without (control) and with cyclodextrin (CD) for 15 minutes. \textsuperscript{125}I-CT or \textsuperscript{125}I-ASF were incubated at 0°C for 60 minutes and then internalized for 30 minutes at 37°C (A). In parallel experiments, hepatocytes were K\textsuperscript{+}-depleted and incubated with \textsuperscript{125}I-CT and \textsuperscript{125}I-ASF (B). Controls in (B) were incubated with 10 mmol/L KCl.
Acknowledgment: The authors are grateful to Esther Titos (Liver Unit, Hospital Clinic, Barcelona) and to Dr. Sofia Perez del Pulgar (Immunology Unit, Fundació Clinic, Barcelona) for the advice in the preparation of Kupffer cells and the gift of stellate cells respectively. They also thank the technical staff, Anna Bosch and Marta Taules, from Serveis Cientifitècnics de la Universitat de Barcelona, for skillful assistance in confocal and electron microscopy.

REFERENCES

7. Anna Bosch and Marta Taules, from Serveis Científico-technics stellate cells respectively. They also thank the technical staff, Anna Bosch and Marta Taules, from Serveis Cientificotècnics de la Universitat de Barcelona, for skillful assistance in confocal and electron microscopy.


52. Enrich C, Jackle S, Havel RJ. The polymeric immunoglobulin receptor is the major calmodulin-binding protein in an endosome fraction from rat liver enriched in recycling receptors. HEPATOLOGY 1996;24:226-232.


