High-density-lipoprotein subfraction 3 interaction with glycosylphosphatidylinositol-anchored proteins

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To elucidate further the binding of high-density-lipoprotein subfraction 3 (HDL_3) to cells, the involvement of glycosylphosphatidylinositol-anchored proteins (GPI-proteins) was studied. Treatment of cultured cells, such as fibroblasts or SK-MES-1 cells, with a phosphatidylinositol-specific phospholipase C (PI-PLC) significantly decreases specific HDL_3 binding. Moreover, PI-PLC treatment of cultured cells or cellular plasma membrane fractions results in releasing proteins. These proteins have a soluble form and can also bind HDL_3, as revealed by ligand blotting experiments with HDL_3. In order to obtain enriched GPI-proteins, we used a detergent-free purification method to prepare a caveolar membrane fraction. In the caveolar fraction, we obtained, by ligand blotting experiments, the enrichment of two HDL_3-binding proteins with molecular masses of 120 and 80 kDa. These proteins were also revealed in a plasma membrane preparation with two other proteins, with molecular masses of 150 and 104 kDa, and were sensitive to PI-PLC treatment. Electron microscopy also showed the binding of Au-labelled HDL_3 inside the caveolar membrane invaginations. In SK-MES-1 cells, HDL_3 are internalized into a particular structure, resulting in the accumulation and concentration of such specific membrane domains. To sum up, a demonstration has been made of the implication of GPI-proteins as well as caveolae in the binding of HDL_3 to cells.

INTRODUCTION

The risk of premature cardiovascular disease has long been known to be inversely correlated with the plasma concentration of high-density lipoproteins (HDL). These relative vasoprotective effects have still not been elucidated. Several functions of HDL could be implicated in this process. The most important mechanism by which HDL could exert their protective role is certainly the removal of excess cholesterol from peripheral cells and transport to the liver, a process commonly called reverse cholesterol transport. The initial step in this mechanism is the binding of HDL to the plasma-membrane bilayer. Specific HDL binding to mammalian cells has been proved by numerous investigators. Several candidate proteins have been partially purified on the plasma membranes of cells, including fibroblasts, hepatocytes, adipocytes and macrophages [1–5]. Molecular masses of 80–210 kDa have been reported. Among these, an HDL-binding protein has been cloned [6]. Unexpectedly, its cDNA sequence predicts no obvious membrane-spanning sequence. Acton et al. [7] identified the class B scavenger receptor SR-B1 as a HDL receptor, but this receptor does not promote cholesterol efflux and mediates selective cholesteryl esters uptake.

Recently, Fielding and Fielding [8] have identified specialized plasma-membrane domains called caveolae, as a major intermediate for the efflux of free cholesterol from cultured fibroblasts to native plasma and in particular to HDL. Caveolae were originally identified by electron microscopy as flask-shaped membrane invaginations (50–100 nm in diameter) on the surface of epithelial and endothelial cells [9]. Caveolae are clathrin-free cell-surface organelles shown to be implicated in transmembrane transport. For the entirety of the caveolae, the level of membranous cholesterol is of prime importance. In fact, treatment of cells with cholesterol-binding drugs, such as filipin or digitonin [10], makes the caveolae flat and completely disorganizes them. It is also in the caveolar compartment that most of the glycosylphosphatidylinositol-anchored proteins (GPI-proteins) are concentrated. The functions of many GPI-linked proteins are not known; however, in several cells, signalling and activation of cellular processes may occur via the GPI anchor.

We have studied the possible involvement of proteins anchored to the plasma membrane through a GPI anchor in HDL association to cells. The implication of a GPI-anchored protein can be shown by treating cellular membrane extracts and cultured cells with a phosphatidylinositol-specific phospholipase C (PI-PLC). Then, HDL subfraction 3 (HDL_3) binding is studied, and proteins liberated in the medium by PI-PLC are analysed.

By electron microscopy, we have identified caveolae at the cell surface and clusters of Au-labelled HDL_3 inside the caveolar invaginations of SK-MES-1 cells. A detergent-free method was used for purifying caveolae from tissue-culture cells [11]. In the caveolar membrane fraction obtained, we studied the presence of HDL_3-binding proteins using Western-blotting experiments. As a result, it can be suggested that proteins that bind HDL could be anchored to cell membranes by GPI and could be concentrated in the caveolae.

We have also tried to find out if the implication of a GPI-anchored protein in HDL binding has any importance in the first step of reverse cholesterol transport, i.e. cholesterol efflux. For

Abbreviations used: apo A-I, apolipoprotein A-I; DAF, decay-accelerating factor; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine; GPI, glycosylphosphatidylinositol; $HDL_{(3)}$, high-density lipoprotein (subfraction 3); LDL, low-density lipoprotein; PI-PLC, phosphatidylinositol-specific phospholipase C.

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these experiments, two cell types were used, fibroblasts, where caveolae and cholesterol efflux were largely investigated, and SK-MES-1 cells, which are epithelial-type cells, where we demonstrated that caveolae were largely present and where cholesterol efflux occurs.

EXPERIMENTAL

Materials

Fibroblasts were obtained from a skin biopsy from normal human volunteers, and the human SK-MES-1 cell line was purchased from the American Type Culture Collection (HTB 58). [1α,2α(n)-^{3}H]cholesterol and ^{125}I were from NEN (Les Ulis, France). Nitrocellulose membranes (0.45 μm pores) and the enhanced chemiluminescence detection kit were from Amersham International (Les Ulis, France). Multiscreen vacuum filtration system and 96-well filtration plates (0.22 μm pores) were from Millipore (Molsheim, France). PI-PLC from *Bacillus cereus* (EC 3.1.4.10), chlorpromazine and BSA free fatty acid were from Sigma (L'Isle d'Abeau, France). 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (ET-18-OCH_3) was from TEBU (L-108; Le Perray en yvelines, France). Percoll was from Pharmacia. Optiprep was from Life Technologies (Cergy Pontoise, France). Rabbit polyclonal anti-caveolin and mouse monoclonal anti-clathrin IgGs were from Transduction Laboratories (Intecchim, Montlucon, France). Mouse monoclonal anti-[decay-accelerating factor (DAF)] IgGs were from Biocytex (Marseille, France). Colloidal gold probe ($HAuCl_4$) was from Electron Microscopy Sciences (Fort Washington, PA, U.S.A.). Peroxidase-labelled anti-mouse and anti-rabbit antibodies were from Sanofi-Pasteur (Maines la Coquette, France).

Cell cultures

Cells were grown routinely in 75 cm² flasks in 15 ml of culture medium. Fibroblasts were grown in Dulbecco's modified Eagle's medium, and the human lung carcinoma SK-MES-1 cells were grown in minimum essential medium; each medium contained 10 % (v/v) fetal-calf serum, 3 % (w/v) glutamine, 0.2 % (w/v) $NaHCO_3$, 1000 units/ml penicillin and 1000 μg/ml streptomycin, supplemented for the SK-MES-1 cells with non-essential amino acids and 1 mM sodium pyruvate. Cells were grown to confluence in 6-well tissue-culture plates before using for HDL_3-binding or cholesterol-efflux experiments and in 100 or 150 mm diameter plates for plasma membrane or caveolar preparations.

For PI-PLC treatment, cells were incubated with 1 unit/ml PI-PLC for 1 h at 37 °C. Then, the incubation medium was lyophilized and analysed by SDS/PAGE. Cells were washed once with serum-free medium and used for HDL_3-binding or cholesterol-efflux studies.

For chlorpromazine treatment, cells were incubated with 50 μM chlorpromazine for 30 min at 37 °C. Then cells were washed once with serum-free medium and used for HDL_3 binding.

For bicarbonate treatment, cells were incubated with 500 mM NH_4HCO_3, pH 11, for 5 min at room temperature [12]. Then the incubation medium was lyophilized and analysed by SDS/PAGE.

Lipoprotein isolation and labelling

Low density lipoprotein (LDL) and HDL_3 were isolated from human plasma by ultracentrifugation at densities of 1.030–1.063 and 1.12–1.21 g/ml respectively [13]. Protein content was measured by Petersen's procedure [14]. Lipoprotein-deficient serum was obtained from the bottom after ultracentrifugation of fetal-calf serum at a density of 1.21 g/ml.

HDL_3 and LDL apolipoproteins were radiolabelled with ^{125}I as described by Bilheimer et al. [15]. The specific radioactivity usually obtained was 300–600 c.p.m./ng of protein.

LDL–$Au_{16\,nm}$ and HDL–$Au_{16\,nm}$ complexes were conjugated according to the citric acid method of Handley et al. [16]. Lipoprotein–Au complexes were revealed by negative staining to ensure the structural integrity of the ligands. About three to six HDL and eight to twelve LDL bind to each centrally positioned Au particle.

Membrane preparation

Fibroblasts and SK-MES-1 cells were grown to confluence in 100-mm-diameter plates. Cell membranes were prepared according to Basu et al. [17] with a few modifications. Briefly, washed monolayers were dislodged from dishes with a nylon policeman into 50 mM Tris/HCl (pH 7.4)/50 mM NaCl/300 mM saccharose containing a mixture of anti-proteases (1 mM benzamidine, 1 mM iodoacetamide, 1 mM phenanthroline, 1 μM pepstatine and 1 mM PMSF). Cells from dishes were combined, pelleted by centrifugation at 200 ***g*** for 5 min, resuspended in the same buffer, homogenized with two 5 s pulses at the 80 % level and one 5 s pulse at 100 % level using a Bioblock 375 W ultrasonic homogenizer, and then centrifuged at 800 ***g*** for 10 min at 4 °C. The supernatant was centrifuged at 100000 ***g*** for 60 min at 4 °C, and the pellet was resuspended in the Tris buffer used above. The membranes were either analysed immediately for HDL-binding and ligand blotting activity or stored frozen.

For the PI-PLC treatment, 2 mg of membrane proteins were incubated with 5 units of PI-PLC in 900 μl of phosphate buffer for 1 h at 37 °C. Then samples were centrifuged at 100000 ***g*** for 60 min at 4 °C. For the detection of HDL_3-binding activity, membrane pellets were solubilized in phosphate buffer and used as described below. For SDS/PAGE studies, supernatants were collected, dialysed and lyophilized, and then used as membrane pellet in ligand blotting as described later.

Purification of caveolar membranes from cultured cells

Caveolar membranes were prepared according to the method of Smart et al. [11]. Briefly, plasma-membrane fractions of fibroblasts and SK-MES-1 cells were prepared from 20 150-mm-diameter dishes of confluent tissue-culture cells. Cells were homogenized with a Teflon homogenizer, and the postnuclear supernatant was collected. After gradient Percoll fractionation [30 % (v/v) Percoll centrifuged at 84000 ***g*** for 30 min in a Beckman Ti 60 rotor], a plasma-membrane fraction ($\approx$ 5.7 cm from the bottom of the centrifuge tube) was obtained and was subjected to sonication. The caveolar membranes were separated from the remainder of the plasma membrane using two OptiPrep density gradients. First, the sample reached a final OptiPrep concentration of 23 % (v/v). Then, a linear 20–10 % (v/v) OptiPrep gradient was poured on the top of the sample and centrifuged at 52000 ***g*** for 90 min in a swinging-bucket rotor. The top 5 ml of the gradient was collected, adjusted to a final OptiPrep concentration of 23 % (v/v) and overlaid with 5 % (v/v) OptiPrep. After centrifugation at 52000 ***g*** for 90 min at 4 °C, a distinct opaque band was present in the 5 % (v/v) OptiPrep overlay. This band was collected and given the name 'caveolar membranes'. In a standard preparation 79 and 229 μg of caveolar protein was obtained from 20 fibroblast and SKMES-1 dishes respectively. Controls of preparations were performed after SDS/PAGE electrophoresis, electroblotting and detection, as described later with specific antibodies against caveolin, a specific

marker of caveolae [11], against DAF, a GPI-anchored protein [18] present in SK-MES-1 and fibroblasts, and against clathrin, a specific marker of coated pits on plasmic membrane [19].

Binding of ^{125}I-labelled HDL_3 to isolated membrane preparations

For the binding of human ^{125}I-labelled HDL_3 to plasma membranes, we used a 96-well filtration plate with a nitrocellulose membrane of 0.22 μm pore size sealed at the bottom of each well. Each well was saturated overnight at room temperature with Tris buffer (10 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA) containing 0.2 % (w/v) BSA. In the standard test, HDL_3 binding was determined by incubating increasing concentrations of labelled lipoprotein with 15 μg of the membranes in Tris buffer containing 0.01 % (w/v) BSA in a final volume of 0.25 ml. Incubation was carried out at 37 °C for 2 h. Then, vacuum was applied and each well was washed eight times in 0.25 ml of Tris buffer containing 0.2 % (w/v) BSA. The 96-well filtration plate was dried for 30 min at 37 °C before punching the filtration plate. The radioactivity of each well was then determined on a Beckman gamma scintillation counter.

Non-specific binding of ^{125}I-labelled HDL_3 to the membranes was determined on parallel sample runs that also contained a 50-fold excess of unlabelled lipoproteins. The difference between the ^{125}I-labelled HDL_3 bound to the membranes in the absence and in the presence of an excess of unlabelled HDL_3 is considered as the amount of ^{125}I-labelled HDL_3 bound specifically to the membranes. Results are the mean of triplicate assays.

Binding of ^{125}I-labelled HDL_3 or ^{125}I-labelled LDL to cultured cells

To determine specific binding of ^{125}I-labelled HDL_3 to cells, the 6-well plates were washed with serum-free culture medium for 1 h at 37 °C. Cells were then incubated for 2 h at 4 °C with serum-free medium containing increasing concentrations of ^{125}I-labelled HDL_3 with or without a 50-fold excess of unlabelled HDL_3. Dishes were washed twice with ice-cold 2 % (w/v) albumin/Tris buffer (10 mM Tris/HCl, pH 7.4, containing 150 μM NaCl) and washed twice again with cold Tris buffer. Cells were then digested in 0.1 M NaOH, and the ^{125}I radioactivity was determined with a Beckman gamma scintillation counter. An aliquot of each sample was assayed for protein content [14]. Receptor-bound ^{125}I-labelled HDL_3 is defined as high-affinity binding calculated by subtracting the binding of ^{125}I-labelled HDL_3 in the absence and presence of an excess of unlabelled HDL_3. Results are the mean of triplicate assays.

For ^{125}I-labelled LDL binding, cells were preincubated for 24 h at 37 °C in medium containing 10 % (v/v) lipoprotein-deficient serum to overexpress LDL-receptor, then LDL binding was determined at 4 °C as indicated for HDL binding.

Ligand-blot and immunoblot analysis

Membrane proteins were isolated under non-reducing conditions by SDS/PAGE using 4–15 % gradient gels as described by Laemmli [20]. Membrane pellets were solubilized in SDS gel sample buffer [1 % (w/v) SDS, 100 mM Tris/HCl, pH 6.8, 1 % (v/v) Bromophenol Blue and 10 % (v/v) glycerol], heated to 95 °C for 2 min, and applied to separate lanes. The proteins were blotted on to nitrocellulose membranes by electrotransfer at 10 mA/cm² of gel for 2 h in 0.15 M glycine/20 mM Tris/HCl/20 % (v/v) methanol. The nitrocellulose sheets were first incubated for 2 h at room temperature in blocking buffer [10 mM phosphate buffer, pH 7.4, 150 mM NaCl and 10 % (w/v) non-fat dried milk]. The buffer was then replaced with buffer containing 5 % (w/v) non-fat dried milk and 30 μg of HDL_3/ml, and the blots were shacked at room temperature for a further 2 h incubation. After washing, the sheets were incubated overnight and agitated at the same time at 4 °C with 0.5 μg/ml peroxidase-labelled anti-[apolipoprotein A-I (apo A-I)] antibodies [21] diluted in phosphate buffer containing 5 % (w/v) non-fat dried milk. Finally, the blots were rinsed in the same manner as previously described. The presence of apolipoproteins was highlighted by enhanced chemiluminescence, followed by autoradiography for an exposure time of 10–60 s. The apparent molecular mass of each protein was determined in comparison with molecular-mass standards (Bio-Rad). For immunoblot analysis the nitrocellulose sheets were first incubated for 2 h with the specific antibody (0.25 μg/ml), then, after washing, with the peroxidase-labelled rabbit anti-mouse or goat anti-rabbit antibodies (0.20 μg/ml) overnight at 4 °C. The presence of the proteins was highlighted by enhanced chemiluminescence as for ligand blotting.

Measurement of cellular cholesterol efflux

Cellular cholesterol efflux was determined following the procedure previously described by de la Llera Moya et al. [22]. Briefly, the cells were maintained in culture medium containing 10 % (v/v) fetal-calf serum. Cells were plated on 6-well tissue-culture plates using 2 ml/well. At 2 days after plating, the cells were labelled during 48 h of incubation with 1 μCi/well [^{3}H]cholesterol. To attain equilibration of the label between the various cellular cholesterol pools, cells were rinsed and incubated for 24 h in serum-free culture medium containing 0.5 % (w/v) BSA. To determine cholesterol efflux, the cells were washed with PBS and incubated at 37 °C with HDL diluted in serum-free medium. At the end of the efflux period, the medium was removed and centrifuged. The cell monolayer was washed three times with PBS and harvested with 1 ml of 0.1 M NaOH. Finally, radioactivity was measured in both medium and cells, making it possible to determine the total radioactivity content in each well. Fractional cholesterol efflux, expressed as a percentage, was calculated as the amount of label recovered in the medium divided by the total label in each well.

Electron microscopy

Subconfluent fibroblasts and SK-MES-1 cells grown on porous filters coated with rat tail collagen prepared by a modification of the method of Bornstein [23] were incubated with LDL–$Au_{16\,nm}$ or HDL–$Au_{16\,nm}$ for 15, 45 and 90 min at 37 °C. Cells on filters were fixed with 2.5 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer, and postfixed for 60 min with 1 % (w/v) aqueous uranyl acetate containing 50 mg/ml sucrose. Cells were dehydrated in alcohol and embedded in Epon. Sectioning was performed perpendicularly to the plane of the filters and counter-staining was carried out with uranyl acetate and lead citrate. Sections were examined at 80 kV through a Philips EM 420 microscope.

RESULTS

To demonstrate if GPI-anchored proteins could participate in HDL_3 binding and in the HDL_3-mediated cholesterol efflux in fibroblasts and SK-MES-1 cells, we exploited two preserved structural features of GPI-anchored proteins, namely (i) their presence solely on the exoplasmic leaflet of the lipid bilayer and (ii) the susceptibility of the GPI membrane anchor to cleavage by PI-PLC. In fact, studies have shown that bacterial PI-PLC can cleave GPI anchors and release proteins in a soluble form from intact cells. This cleavage is the most common criterion used in GPI-anchor identification [18,24].

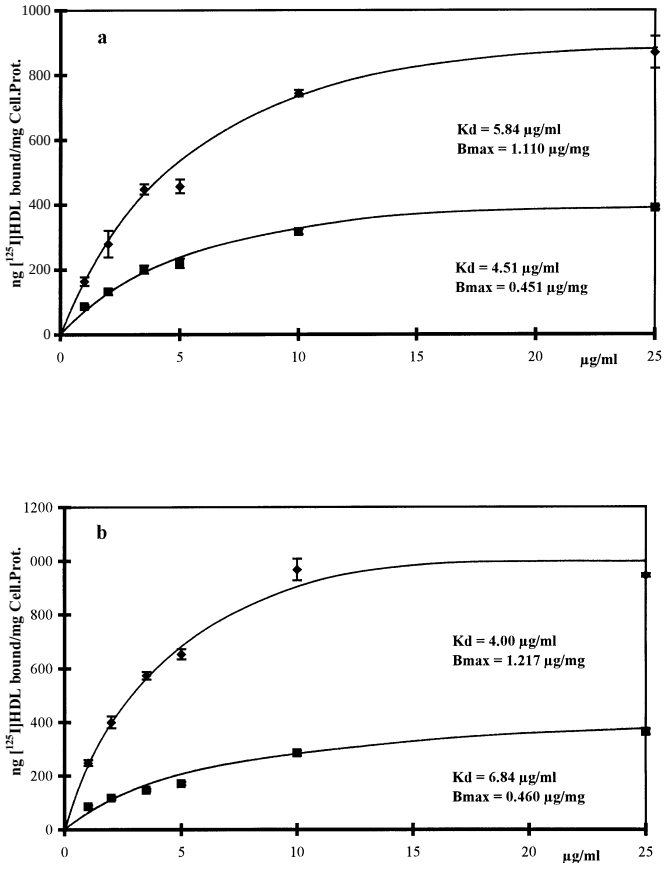

Figure 1 HDL_3 binding to membrane extracts is sensitive to PI-PLC

Radiolabelled HDL_3 was incubated with cell membranes for 2 h at 37 °C, and incubation mixtures (250 μl) were then processed as described in the Experimental section. Control incubations were processed without PI-PLC in phosphate buffer containing phospholipase inhibitor ET-18-OCH_3. (**a**) PI-PLC treatment (■) and control (◆) binding of ^{125}I-labelled HDL_3 on fibroblast membranes. (**b**) PI-PLC treatment (■) and control (◆) binding of ^{125}I-labelled HDL_3 on SK-MES-1 cell membranes. Binding-data points in graphs represent means and ranges of triplicate determinations. These values were used to make a Scatchard analysis. From the Scatchard plot, K_d (μg of ligand protein/ml) and B_{max} (μg of ligand-protein bound/mg of membrane protein) values were obtained. Cell.Prot., cell protein.

HDL_3 binding to membrane extracts and cultured cells is sensitive to PI-PLC

We first quantified binding activity in a preparation of cellular plasma membranes with and without PI-PLC treatment.

The binding curves shown in Figure 1 were obtained when isolated plasma membranes at a fixed concentration (15 μg/ml) were incubated with increasing concentrations of human ^{125}I-labelled HDL_3. The high-affinity component of two plasma-membrane cell types (fibroblasts and SK-MES-1) showed evidence of saturation at about 25 μg of ^{125}I-labelled HDL_3 protein/ml. A Scatchard analysis of these high-affinity and saturable binding curves gave a linear plot, suggesting the presence of a single class of specific binding sites for HDL_3 in the membranes. From the Scatchard plot, an equilibrium dissociation constant (K_d) of 5.84 μg of HDL_3 protein/ml and a maximum binding capacity (B_{max}) of 1.11 μg of HDL_3 protein/mg of fibroblast membrane protein were obtained. For SK-MES-1 cells, we obtained comparable values: K_d 4.00 μg/ml and B_{max} 1.217 μg of HDL_3 protein/mg SK-MES-1 membrane protein.

Table 1 Effect of PI-PLC and chlorpromazine on HDL and LDL binding to fibroblasts and SK-MES-1 cells

Cells were preincubated for 1 h without or with 1 unit/ml PI-PLC or for 30 min with 50 μM chlorpromazine at 37 °C. Then binding experiments were performed at 4 °C with 50 μg/ml ^{125}I-labelled HDL or 15 μg/ml ^{125}I-labelled LDL as indicated in the Experimental section. Results are expressed as percentages of binding compare with control cells preincubated in the medium (means ± S.D. of triplicate experiments).

	HDL	LDL
Fibroblasts	100 ± 7	100 ± 2
Fibroblasts + PI-PLC	75 ± 2	90 ± 2
Fibroblasts + chlorpromazine	98.2 ± 15	48.6 ± 5
SK-MES-1	100 ± 4	100 ± 4
SK-MES-1 + PI-PLC	78 ± 4	95 ± 5
SK-MES-1 + chlorpromazine	122 ± 8	70 ± 7

On Figures 1(a) and 1(b) we can see that PI-PLC treatment of fibroblasts and SK-MES-1 membranes drastically decreased HDL_3 binding: B_{max} decreased by 59 % for fibroblasts and by 62 % for SK-MES-1 cells, without any significant modification of the K_d values.

The binding curves of increasing amounts of ^{125}I-labelled HDL_3 on whole cells (fibroblasts and SK-MES-1 cells) at 4 °C gave a specific and saturable fixation (results not shown).

The PI-PLC treatment of SK-MES-1 or fibroblast cells results in a 22–25 % decrease in HDL_3 binding (Table 1). This decrease in HDL_3 binding takes place at the specific binding level, because non-specific binding (in the presence of unlabelled HDL_3) does not differ according to whether cells are PI-PLC treated or not. As was predictable, PI-PLC had no effect on LDL binding. Chlorpromazine, a specific drug that interacts with clathrin-coated-pits formation [19], decreased LDL but not HDL binding on the two cell types. These results clearly indicate a specific action of PI-PLC on HDL-binding sites on these cells.

HDL_3 binds to membrane proteins

The membranous cellular fractions of fibroblasts and SK-MES-1 cells were subjected to ligand blotting analysis. The sizes of the individual bands that bind HDL_3 were revealed. Figure 2(a) shows that, for fibroblasts, HDL_3 binds to four major proteins of 150, 120, 104 and 80 kDa. In agreement with an earlier report [25], there is a doublet of a strong 120 kDa and a weaker 104 kDa protein bands. There is also a 150 kDa protein that could correspond to the predicted molecular mass of the primary HDL-binding-protein gene product described by McKnight et al. [6].

With the SK-MES-1 cell extracts, the same patterns were obtained (Figure 2b), but the 120 and 80 kDa proteins were more intense than in fibroblasts.

PI-PLC treatment releases HDL_3-binding proteins from membrane extracts and cultured cells

We tested the liberation of HDL_3-binding proteins by PI-PLC treatment of membranes. Membranes were incubated for 1 h with purified PI-PLC. After centrifugation at 100000 ***g*** for 60 min at 4 °C, the supernatant containing the released proteins and the membrane pellets were collected separately and studied by ligand blotting. HDL_3 revealed proteins with molecular masses of 150,

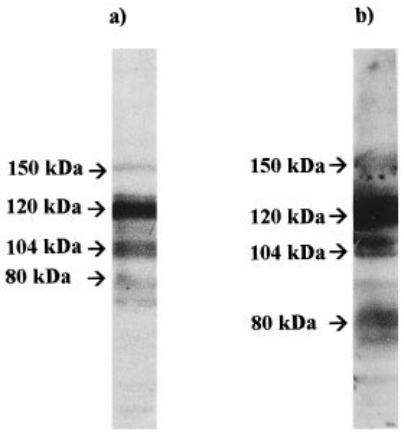

Figure 2 Detection of HDL-binding proteins by ligand blotting

Membranes from fibroblasts (**a**) and SK-MES-1 cells (**b**) were prepared, and 300 μg of protein was loaded on to each lane of an SDS/4–15% polyacrylamide gel and processed for ligand blotting. Strips of nitrocellulose were cut and incubated with HDL_3 (30 μg/ml), and then, after washing, with peroxidase-labelled anti-(apo A-I) antibodies. The positions of molecular-mass markers are shown on the left of each panel.

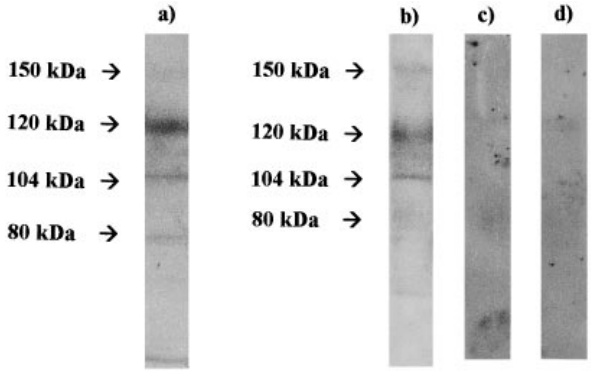

Figure 3 Detection by ligand blotting of HDL-binding proteins released by PI-PLC treatment of cellular membranes

Membranes from fibroblasts (**a**) and SK-MES-1 cells (**b**) were prepared, and 2 mg of protein was incubated with PI-PLC (5 units) for 1 h at 37 °C. After centrifugation at 100000 ***g*** for 60 min at 4 °C, supernatant was collected, lyophilized, loaded on to each lane of an SDS/4–15% polyacrylamide gel and processed for ligand blotting. Supernatants obtained for the control incubations on membranes from SK-MES-1 cells with phosphate buffer (**c**) or with phosphate buffer containing phospholipase inhibitor ET-18-OCH_3 (**d**) were also studied by ligand blotting. Nitrocellulose strips were incubated with HDL_3 (30 μg/ml), then, after washing, with peroxidase-labelled anti-(apo A-I) antibodies. The positions of molecular-mass markers are shown on the left of each panel.

120, 104 and 80 kDa for fibroblast supernatant (Figure 3a) and 150, 120, 104 kDa for SK-MES-1 supernatant (Figure 3b), which are released by PI-PLC. PI-PLC treatment leads to a decrease in signal intensity in the membrane pellets, particularly for the 120 kDa protein band (results not shown).

In the control incubation with a phosphate buffer on SK-MES-1 membranes (Figure 3c), a weak signal for the 120 kDa protein can be noted. The release of these proteins, without PI-PLC treatment, could be attributed to endogenous phospholipase activity, which is confirmed by incubating SK-MES-1 membranes with the ET-18-OCH_3 phospholipase inhibitor [26], which

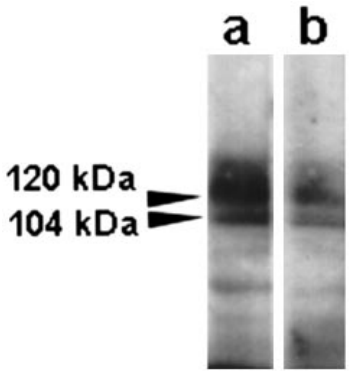

Figure 4 Detection by ligand blotting of HDL-binding proteins released by PI-PLC treatment of cultured cells

SK-MES-1 cells were incubated with PI-PLC (1 unit/ml) (**a**) or with phosphate buffer (**b**), for 1 h at 37 °C. Incubation media were lyophilized, loaded on to each lane of an SDS/4–15% polyacrylamide gel and processed for ligand blotting. Nitrocellulose strips were incubated with HDL_3 (30 μg/ml), then, after washing, with peroxidase-labelled anti-(apo A-I) antibodies. The positions of molecular-mass markers are shown on the left of each panel.

prevents the release of HDL_3-binding proteins in the supernatant (Figure 3d).

It was also interesting to find out if proteins released from cells by PI-PLC could be recovered from the cultured cell medium. Figure 4(a) shows proteins released in the culture medium after PI-PLC treatment of cultured SK-MES-1 cells. Those proteins with molecular masses of 120 and 104 kDa, cleaved from their anchorage at the outer leaflet of the cytoplasmic membranes by PI-PLC treatment, bind HDL_3, as shown by ligand blotting. In the control incubation with a phosphate buffer, a weaker signal was noted for these proteins (Figure 4b).

After NH_4HCO_3 incubation of cells for 5 min [12], the proteins revealed by HDL_3 in the culture medium were not more significant than with the control incubation (results not shown). This excludes the attachment of HDL-binding proteins by protein–protein interaction with an integral membrane protein.

Caveolae from fibroblasts and SK-MES-1 cells can be revealed by electron microscopy

When grown on filter supports, fibroblasts and SK-MES-1 cells form tight monolayers; they exhibit well characterized morphologies and show some typical membrane domains on their surfaces.

Electron microscopic studies have revealed that the surface of human fibroblasts displays numerous clathrin-coated pits associated with abundant smaller flask-shaped membrane invaginations, also known as caveolae.

In contrast, our ultrastructural observations revealed that caveolae were the predominant subdomains observed on the surface of human lung carcinoma cells SK-MES-1 (Figure 5a).

HDL–$Au_{16\,nm}$ is processed by the caveolar pathway

To determine if HDL_3 could distinguish between accessible and inaccessible cell-membrane structures that could be concentrated in the caveolae, cells were incubated at either 4 °C or 37 °C with HDL–Au conjugates.

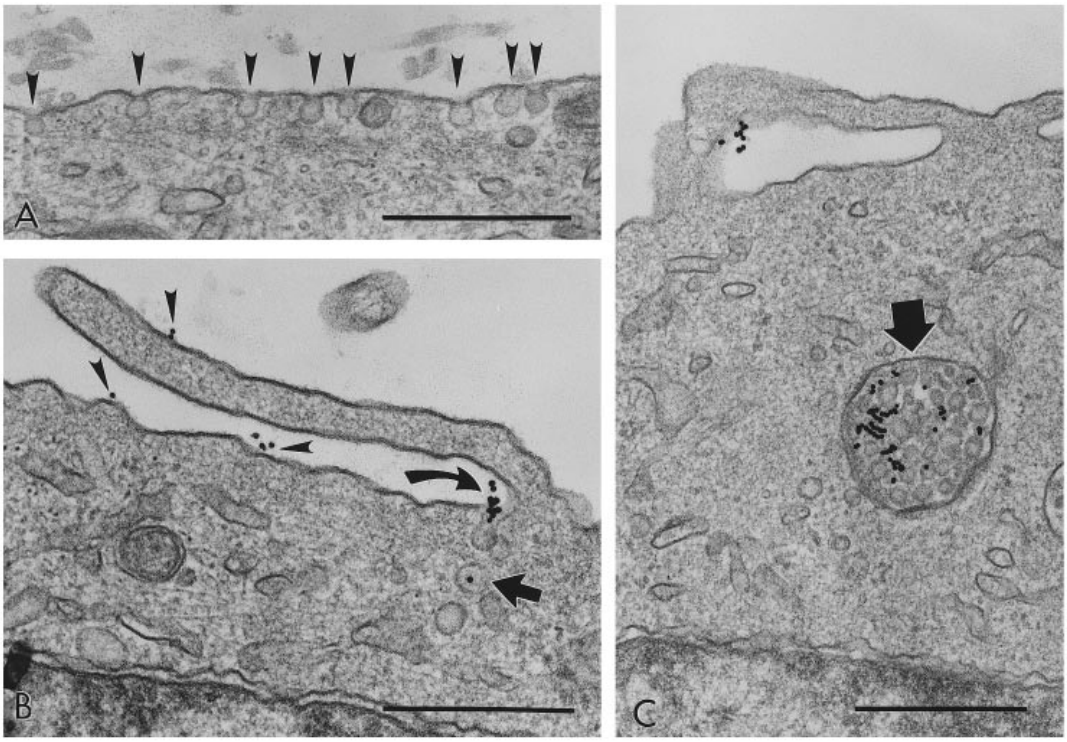

Figure 5 Electron-microscopic revelation of caveolae-mediated endocytosis of HDL–Au in SK-MES-1 cells

(**A**) Cell-surface invaginations are 55–65 nm in diameter and do not show any defined coat. They are seen as single pits (arrowheads) with the typical morphology of caveolae. (**B**, **C**) SK-MES-1 cells were incubated with HDL–$Au_{16\ nm}$ for 15–45 min at 37 °C. (**B**) Au particles were seen on the cell surface (arrowheads) and were also observed at the edges of a caveola (curved arrow). An Au particle is also evident in an intracellular non-coated vesicle (straight arrow). (**C**) Au particles were observed in a spherical endosomal structure containing empty vesicular inclusions (arrow). The bars represent 0.5 μm.

Following processing for electron microscopy, cells incubated at 4 °C for 5–90 min were devoid of any Au-complex staining pattern.

HDL–Au tracer was not observed before the 15 min time point of continuous incubation at 37 °C. At this time, thin sections of SK-MES-1 cells revealed clusters of HDL–$Au_{16\ nm}$ complexes, as well as some single Au particles on the cell surface (Figure 5B). Clusters of Au particles were also found at the edges of caveolae and in intracellular non-coated vesicles probably derived from the pinching off of caveolae (Figure 5B). After continuous internalization of the ligand for 45 min at 37 °C, Au particles were observed in typical spherical structures containing numerous empty vesicular inclusions (Figure 5C) and appeared to be segregated outside internal profiles. The latter structures had a remarkably constant diameter of 45–60 nm. With longer incubations at 37 °C, accumulation in lysosomes was not observed.

In fibroblasts, HDL–Au incubations did not result in detectable cell-surface binding and uptake at 37 °C, which might be explained by cell interactions that are either non-permanent or too transient. Our results provide clear evidence that caveolar functions can be regulated quite differently according to cell type.

To investigate in greater detail the differences that have been noted for caveolae in the two cell lines, fibroblasts and SK-MES-1 were incubated with LDL–$Au_{16\ nm}$ for a period of 45 min or longer at 37 °C.

At 45 min and 90 min, LDL–Au particles were found, as predicted, within multivesicular bodies or late endosomes and were concentrated within compartments of the human fibroblasts with a typical lysosome-like morphology (results not shown). No localization of the marker could be observed in caveolae. These results accord well with receptor-mediated endocytosis using clathrin-coated pits as the vehicle for internalization and generating endosomes that deliver LDL–Au to lysosomes.

Extensive localization of LDL–Au complexes was observed at the cell surface of SK-MES-1 (Figure 6A). Surprisingly, an accumulation of complexes was found in areas of the plasma membrane exhibiting tubulo-vesicular invaginations (Figures 6A and 6B). At later stages of internalization, LDL–Au was detected in endosomal structures without any signs of lysosome accumulation, as is shown in Figure 6(C).

Caveolae can be purified from cultured cells

We used a detergent-free method described by Smart et al. [11] to purify caveolar membrane from cultured cells of fibroblasts and SK-MES-1 cells.

A post-nuclear fraction was prepared from 20 dishes of 150 mm diameter of each type of confluent tissue-culture cells; that represents 6.4 mg of total protein for the fibroblasts and 7.3 mg for SK-MES-1 cells. After Percoll gradient fractionation, we obtained 1.75 mg for the plasma-membrane fraction of fibroblasts and 4.37 mg for the SK-MES-1 cells. And finally, after two OptiPrep gradients, we obtained in the caveolar membrane fraction 79 μg of the original 1.750 mg of protein for the fibroblast plasma membrane and 229 μg of the original 4.370 mg of protein for the SK-MES-1 plasma membrane. We set up an immunoblot

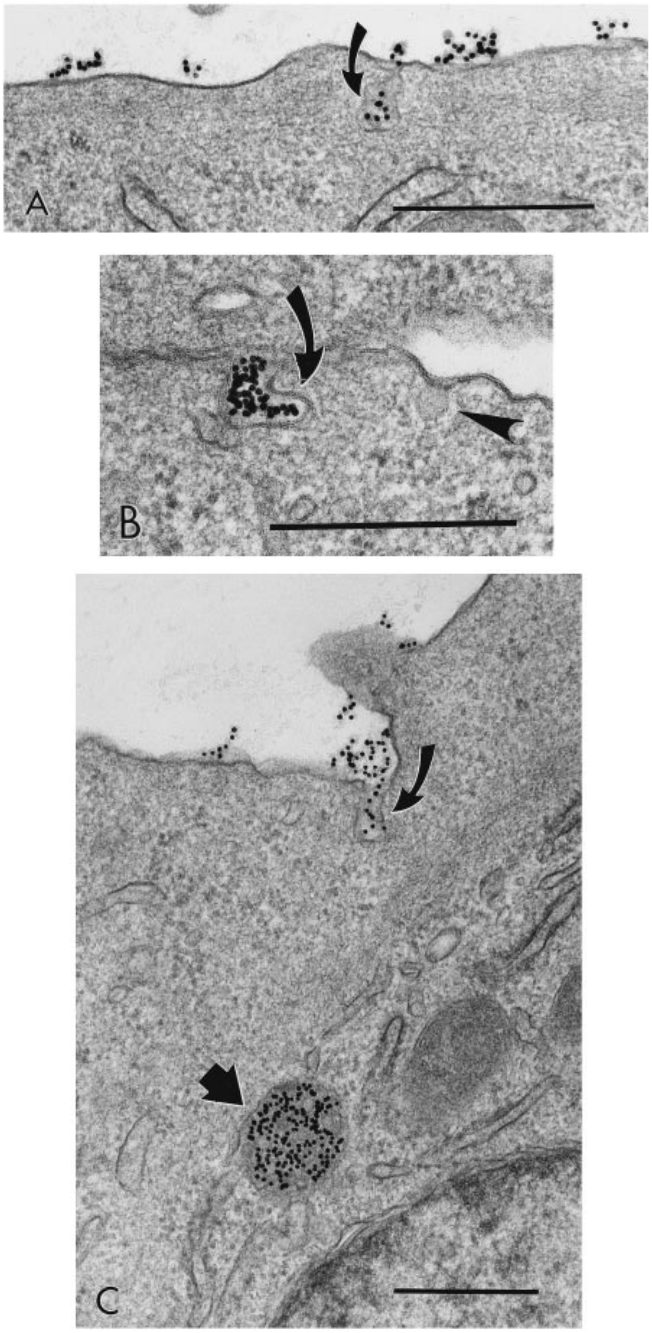

Figure 6 Electron-microscopic localization of LDL–Au complexes in SK-MES-1 sections

(**A**, **B**) After 15 min incubation with cells, extensive localization of LDL–$Au_{16\ nm}$ was observed at the membrane surface of the human lung carcinoma cells and in areas of the plasma membrane exhibiting tubulo-vesicular invaginations (curved arrows) as compared with the characteristic flask-shape invagination of caveolae (**B**, arrowhead). (**C**) Accumulation of LDL–$Au_{16\ nm}$ was evident in membrane invaginations of the cell surface of an SK-MES-1 cell incubated for 90 min at 37 °C (curved arrow). Endocytosed Au tracer was detected in a spherical endosome-like structure. The bars represent 0.5 μm.

assay on 350 μg of post-nuclear fraction and 25 μg of the caveolar fraction of SK-MES-1 cells with anti-caveolin, anti-DAF and anti-clathrin antibodies. In Figure 7, we can see that caveolin was detected in post-nuclear and in caveolar fractions as intense

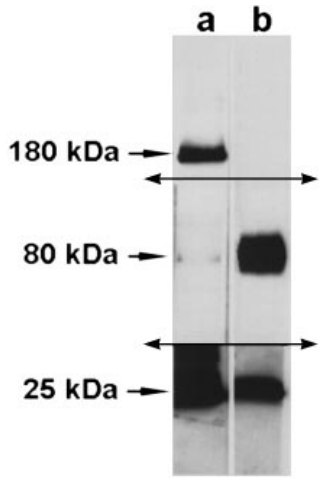

Figure 7 Detection of the caveolin and DAF on the caveolar preparation from SK-MES-1 cells

Post-nuclear extracts (350 μg of protein) (**a**) and the caveolar fraction (25 μg of protein) (**b**) were loaded on to each lane of an SDS/4–15% polyacrylamide gel and processed for immunoblotting. Nitrocellulose strips were cut in three parts, separated on the Figure by double-headed arrows. The lower part was incubated with anti-caveolin, the intermediate with anti-DAF and the upper with anti-clathrin. Then each piece of sheet was incubated with peroxidase-labelled anti-species antibodies (0.5 μg/ml), and positive bands were identified by autoradiography after using the enhanced-chemiluminescence Western-blotting system. The positions of the molecular-mass markers are shown on the left.

reactive bands (25 kDa). DAF, a GPI-anchored protein, was more concentrated in caveolae (80 kDa), and clathrin was only detected in post-nuclear fractions (180 kDa). We obtained the same pattern for the fibroblasts (results not shown). The presence of a high concentration of caveolin and the absence of clathrin in the fraction obtained from plasma membrane is a good indicator of the enrichment of this fraction in caveolar membrane domains [11].

HDL_3 binds to proteins from caveolae extracts

Figure 8 shows the results obtained by ligand-blot analysis with HDL_3 on the caveolar membranes (40 μg) from fibroblasts (Figure 8a) and from SK-MES-1 cells (Figure 8b). In the caveolar fraction compared with SK-MES-1 membrane fraction (300 μg) (Figure 2b), the same four proteins were obtained with molecular masses of 150, 120, 104 and 80 kDa. In spite of such different amounts of total protein in the two lanes, the intensity of the 120 kDa protein is stronger in the caveolar sample. With the caveolar SK-MES-1 cell fraction, there is not only enrichment in the 120 kDa protein, but also a more intensified signal for the 80 kDa protein.

HDL_3-induced cholesterol efflux is sensitive to PI-PLC

It therefore seemed from these results that GPI-anchored proteins could be implicated, at least partly, in HDL_3 binding on plasma membranes and caveolae. The next step was to investigate the physiological implication of such proteins on cholesterol efflux from cultured cells.

Cholesterol efflux was evaluated as a transfer of [^{3}H]cholesterol from prelabelled cells to human HDL_3. Labelled cells were incubated for varying lengths of time in the presence of HDL_3 (50 μg/ml). When the amount of [^{3}H]cholesterol released from

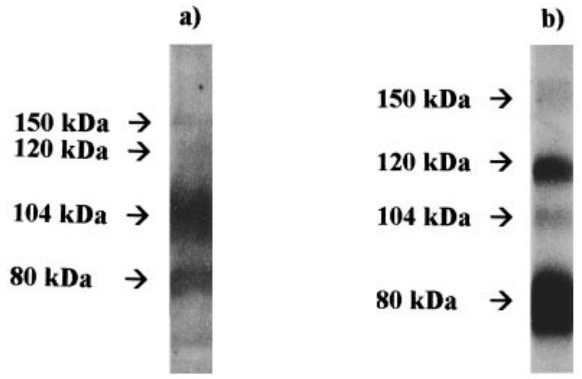

Figure 8 Detection by ligand blotting of HDL-binding proteins on the caveolar fraction

Caveolar fractions from fibroblasts (**a**) and SK-MES-1 cells (**b**) were loaded on to each lane of an SDS/4–15% polyacrylamide gel and processed for ligand blotting. Nitrocellulose strips were incubated with HDL_3 (30 μg/ml), then, after washing, with peroxidase-labelled anti-(apo A-I) antibodies. The positions of the molecular-mass markers are shown on the left.

Table 2 Time-dependent decrease in specific HDL_3-mediated cholesterol efflux of fibroblasts and SK-MES-1 cells, after PI-PLC treatment of cells

^{3}H-labelled fibroblast and SK-MES-1 cells, with or without PI-PLC treatment, were incubated for various lengths of time in the presence of HDL_3 (50 μg/ml), as described in the Experimental section. The results were expressed as the decrease in specific efflux obtained after 30 min, 2 h and 4 h with HDL_3. Cholesterol efflux after a 4 h incubation was low but very reproducible [2.83 ± 0.09 (S.D.)% in triplicate experiments for fibroblasts and 4.22 ± 0.34% for SK-MES-1 cells].

Cell type	Time	Decrease of efflux after PI-PLC treatment (%)
Fibroblast	30 min	−7
	2 h	−11
	4 h	−9
SK-MES-1	30 min	−2
	2 h	−18
	4 h	−28

SK-MES-1 cells was compared with that obtained in serum-free medium, a time-dependent increase in the specific HDL_3-mediated efflux became apparent. The percentage of specific efflux obtained after 4 h with HDL_3 was 4.22 ± 0.34 (S.D.)%. For fibroblasts, there was also a time-dependent increase, and the value at 4 h was 2.83 ± 0.09%. PI-PLC treatment of SK-MES-1 cells (1 h at 37 °C) induced a decrease in the HDL_3-mediated cholesterol efflux of about 20% after 2 h and about 30% after a 4 h incubation time with the HDL_3-containing medium (Table 2). It is important to note that inhibition of cellular cholesterol efflux by PI-PLC treatment is higher when the incubation time with HDL_3 increases.

DISCUSSION

To clarify the possible involvement in HDL-binding and HDL-mediated cholesterol efflux of a membrane protein and its anchorage at the cell surface, two cell types were chosen, namely fibroblasts, which have been widely used and reported in publications, and SK-MES-1 (epithelial cell-like) cells, which, as has already been shown, can bind HDL_3, can be loaded by cholesterol and can produce measurable efflux (results not shown).

First, specific and saturable sites were shown to be involved in the interaction of HDL_3 on the surface of whole cells and on membrane extracts. Next, an equilibrium dissociation constant (K_d) and a maximum binding capacity (B_{max}) were characterized. We then showed that HDL binding to the cell membranes was sensitive to PI-PLC treatment, suggesting that it is mediated by a GPI-anchored structure. This latter finding is strengthened by the fact that PI-PLC treatment releases two proteins with molecular masses of 120 and 104 kDa from membrane extracts and also from whole cells. These proteins, cleaved from their anchorage at the outer leaflet of the cytoplasmic membranes, still bind HDL_3, as is shown by ligand blotting.

In spite of the evident release of proteins by PI-PLC from SK-MES-1 cells and cultured fibroblasts, the diminution of HDL_3 binding on cells is much weaker than that observed for membranes. This could be attributable to a considerable potential renewal of proteins released at the cell surface, and a rapid mobilization of new HDL-binding proteins from intracellular compartments to plasma membranes. We could also hypothesize a mechanism such as for the insulin receptor [27], by which GPI-anchored proteins are released from their anchor after ligand binding, then bind via their residual inositol phosphate to membranous proteins or inositol receptors and still remain efficient for ligand binding.

Proteins anchored by GPI have been reported to be clustered at the cell surface [28], and in most cases cross-linking of the protein is a prerequisite for their signalling function within a caveolar domain [29]. To test for such an HDL_3-binding effect, fibroblasts and SK-MES-1 cells were selected for their high concentration of caveolae at the surface, as observed by electron-microscopy experiments. For SK-MES-1, the involvement of caveolae in HDL_3 binding and internalization was clearly identified morphologically. The HDL_3–Au preferentially accumulated within caveolae was internalized in spherical endosomal structures with numerous empty vesicular inclusions. It is interesting that, for cultured fibroblasts, we could not detect any cell-surface binding and uptake of HDL_3 or any internalization structures deriving from the pinching off of caveolae. Taken as a whole, our data accords with the demonstration that caveolae are not always static invaginations on the plasma membrane and are capable of being internalized in a regulated manner under special experimental conditions [30]. Our results provide new evidence for various kinetics and functions of caveolar domains concerning tissue-specific variability between cell types. In this connection, it is interesting to note that LDL–$Au_{16\,nm}$ also takes various pathways in endocytosis.

At present, we have no evidence that caveolae play a role in the HDL_3-mediated cholesterol efflux under physiological conditions. Nevertheless, for the two cell types, the physiological implication of GPI-anchored proteins is clearly demonstrated by the decrease in HDL_3-mediated cholesterol efflux after PI-PLC treatment of cultured cells. This result has to be linked (i) to the demonstration that GPI-anchored proteins are involved in the release of intracellular signals such as diacylglycerol after HDL_3 stimulation [31], (ii) to the evident concentration of trimeric G-proteins in caveolae [32] and the suggestion that caveolae may represent signal-transduction centres [33], and (iii) to the proposal that cholesterol efflux and activation of PKC are connected [34,35]. Recently, Fielding and Fielding [8] have suggested that caveolae could be a localized reference for the efflux of free cholesterol from cultured fibroblasts to native plasma and in particular to HDL. The rapid protease-dependent selective efflux of free cholesterol that was identified

[8] could explain that HDL–Au tracer was not observed before 15 min during our continuous incubations at 37 °C. This is particularly interesting in view of earlier studies showing the importance of cholesterol and caveolin in helping to uncluster GPI-anchored proteins [36,37].

To sum up, our results indicate that releasing GPI-anchored proteins results not only in a decrease in HDL binding but also in a decrease in cholesterol efflux. The mechanism by which caveolae are involved in HDL_3 cellular routing and processing has still to be investigated. The identification of GPI-anchored proteins is being carried out in our laboratory, and in the near future the question to be elucidated is whether the association of HDL with GPI-anchored proteins is simply required to increase HDL binding in the cholesterol-abundant area known as caveolae, or if the HDL-binding pathway via GPI-anchored proteins in the caveolae is at the beginning of a specific efflux mechanism.

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