High-Density Lipoprotein 3 Receptor-Dependent Endocytosis Pathway in a Human Hepatoma Cell Line ($HepG_2$)

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ABSTRACT: The internalization of HDL_3 into $HepG_2$ cells at 37 °C was precisely measured, taking advantage of the previously observed rapid dissociation of HDL_3 from its two binding sites [Barbaras, R., et al. (1994) *Biochemistry 33*, 2335−2340]. We observed a high level of HDL_3 internalization (100 ng/mg of cell protein, corresponding to 45.5% of the total HDL_3 associated to the cells at 37 °C) reaching a plateau at 15 min. Apolipoprotein A-I (the main HDL_3 apolipoprotein) associated with dimyristoylphosphatidylcholine (DMPC) complexes was also internalized by $HepG_2$ cells, at levels comparable to those obtained with HDL_3 lipid-free apolipoprotein A-I, which can bind only to the HDL_3 high-affinity binding site, and displayed a weak internalization (5 ng internalized/mg of cell protein compared to 250 ng/mg for apolipoprotein A-I complexed with DMPC). Clathrin-coated vesicle purification following HDL_3 or LDL internalization at 37 °C showed radioactivity associated with these vesicles, and further content analysis evidenced the presence of radiolabeled apoA-I and apoB, respectively. Treatment of the cells either by saccharose hypertonic shock or by potassium depletion, in order to block clathrin-coated vesicle formation, completely inhibited HDL_3 internalization, as also observed with LDL. Altogether, these observations clearly demonstrate that HDL_3 internalization into $HepG_2$ cells occurs through an endocytosis pathway involving an interaction between apolipoprotein A-I and a cell surface protein, leading to the formation of clathrin-coated vesicles.

Cholesterol homeostasis in humans is dependent on the balance between two main pathways: first, cholesterol synthesis and its transport from the liver to peripheral cells, which involve the cellular clearance of low-density lipoprotein (LDL),[1] and second, cholesterol removal from peripheral tissues to the liver for reutilization or excretion as bile sterols, a process called "reverse cholesterol transport", which mainly concerns high-density lipoprotein (HDL) (Eisenberg, 1984). Such a role is consistent with the negative correlation observed between HDL serum concentrations and the incidence of cardiovascular diseases (Miller & Miller, 1975). The LDL clearance has been widely studied for the last 20 years and is well elucidated after the pioneering work from Brown and Goldstein (Goldstein et al., 1985). Briefly, LDL bind to a specific apolipoprotein B and E (apoB/E) receptor and are then internalized by a clathrin-coated vesicle pathway followed by lysosomal degradation and cholesterol delivery inside the cells. The LDL apoB/E receptor is considered as an established model for the study of the endocytosis mechanisms. In contrast with LDL, the interaction of HDL with liver cells is still a matter of debate, and appears more complex. This discrepancy between LDL and HDL may partly be explained by the much greater heterogeneity of HDL particles compared to LDL, in terms of size and apolipoprotein or lipid compositions. Thus, studies of HDL cellular interactions require first the characterization of the fate of different HDL molecular partners. Nevertheless, some information concerning HDL−hepatocyte interaction is available today. HDL can directly interact with liver cells (Mackinnon et al., 1986; Arbeeny et al., 1987), presumably *via* a specific binding protein (Kambouris et al., 1988; Barbaras et al., 1994), recognizing apolipoprotein A-I. This interaction may lead to the uptake of HDL components, such as cholesteryl esters and phospholipids or apoA-I, by a still unelucidated pathway. Further, the internalization of HDL may lead to retroendocytosis (Kambouris et al., 1990) or to lysosomal degradation (Miller et al., 1977), depending on which cell type and which experimental protocol are used.

In the case of hepatocytes, many factors might contribute to the discrepancy of the results obtained by different authors. First, hepatocytes secrete lipases (Laposata et al., 1987; Busch et al., 1990), lipid transfer proteins, and apolipoproteins E, B, A-I (McCall et al., 1988), etc., and the longer HDL stay in the presence of these factors and the more likely they are to modify HDL. Second, there have been reports that such proteins, like the cholesteryl ester transfer protein (CETP), can modulate the uptake of HDL cholesteryl esters by hepatoma cells (Granot et al., 1987). Conversely, the uptake of apolipoprotein A-I (apoA-I), the main HDL apolipoprotein, is probably less influenced by those cellular proteins. Moreover, the short time required to study this uptake should minimize the potential influence of cellular factors. Thus, it appears simpler to follow the fate of HDL protein than that of the lipid moiety. However, such studies require highly labeled HDL to be able to detect an usually weak signal.

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[1] Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; apo, apolipoprotein; SDS, sodium dodecyl sulfate; DMPC, L-α-dimyristoylphosphatidylcholine; TCA, trichloroacetic acid.

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In a previous study, we have used such highly radiolabeled HDL_3 (a subclass of HDL without apoE) and apoA-I, and demonstrated the presence of two HDL binding sites on intact human hepatoma cells ($HepG_2$) (Barbaras et al., 1994). Furthermore, lipid-free apoA-I specifically bound only to the high-affinity binding sites. The purpose of our present work was to define the role of apoA-I in the internalization of HDL_3 into $HepG_2$ cells and to determine the relative contributions of the two HDL_3 binding sites in this internalization process. Moreover, in this paper, we show for the first time that this internalization occurs through clathrin-coated vesicles. This observation strongly supports the importance of the HDL_3 binding sites in the internalization process.

EXPERIMENTAL PROCEDURES

Materials. Dimyristoylphosphatidylcholine (DMPC), bovine serum albumin (BSA), 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and Tricine were obtained from Sigma (La Verpilliere-France). ^{125}I-Na was from Amersham (France). All other reagents used were of analytical grade.

Cells. The human hepatoblastoma-derived cell line $HepG_2$ was obtained from the American Type Culture Collection (Rockville, MD). Cells were plated at $(1-2) \times 10^5$ cells per well in 48-multiwell plates (Costar) and grown in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 and 95% air incubator. The cells were subcultured every 7−8 days.

Lipoprotein, Apolipoprotein, and Proteoliposome Preparations. High-density lipoproteins (HDL_3) were isolated from plasma of normolipidemic healthy human donors. Na_2-EDTA, sodium azide, and phenylmethanesulfonyl fluoride were added to a final concentration of 1 mM, 0.05% (w/v), and 1 mM, respectively. The lipoproteins were obtained by sequential flotation ultracentrifugation (VLDL, $d = 1.006$ g/mL; LDL, $1.006 < d > 1.063$ g/mL). HDL_2 were first isolated (110000*g* for 40 h) at $d = 1.125$ g/mL followed by HDL_3 (110000*g* for 40 h) at $d = 1.21$ g/mL. Using a specific radioimmunoassay, no apoE was detected in HDL_3. HDL_3 were kept at 4 °C in the dark and under nitrogen and were extensively dialyzed against phosphate-buffered saline (PBS: 0.137 M NaCl, 4.3 mM sodium phosphate, 1.5 mM potassium phosphate, and 2.7 mM KCl, pH 7.4), before use.

Low-density lipoproteins (LDL) (1.006−1.063 g/mL) were depleted of any possible apoA-I, by affinity chromatography using anti-apoA-I antibodies immobilized on a CNBr-activated Sepharose 4B gel. LDL were incubated overnight at 4 °C in PBS. Nonretained LDL were then eluted with PBS and were checked for the presence of apoA-I by Western blot.

ApoA-I was isolated from HDL_3 by ion-exchange chromatography as outlined before (Mezdour et al., 1987). The apoA-I preparations were checked on an HPLC apparatus using a reverse-phase C18 column and an acetonitrile gradient. Furthermore, Western blot analyses (Towbin et al., 1979) were carried out using different antibodies directed against human apoB, apoA-II, and apo-C's for apoA-I analysis. In this case, the apolipoprotein homogeneity was more than 99% (as measured by densitometry).

Complexes containing apoA-I and DMPC were prepared by the cholate dialysis procedure as described by Chen and Albers (1982), at a phosphatidylcholine:protein molar ratio of 150:1. The Stokes radius of the complexes was estimated to be 100 ± 10 Å (Chen & Albers, 1982).

Labeling of apoA-I, LDL, and HDL_3 with $Na^{125}I$ was performed by the *N*-bromosuccinimide method according to Sinn et al. (1988). Specific radioactivities ranged from 3000 to 5000 cpm/ng of protein. In the case of ^{125}I-apoA-I−DMPC complexes, apoA-I was labeled before insertion into the lipid complexes. The same preparation of ^{125}I-apoA-I was used either in a free form or complexed to DMPC in further binding experiments (Barbaras et al., 1994). The specific radioactivities ranged from 10 000 to 20 000 cpm/ng. More than 97% of the radioactivity was associated with protein.

Binding Assays. Binding of labeled lipoproteins or proteoliposomes to $HepG_2$ cells was performed at 4 °C as previously described (Steinmetz et al., 1990). Briefly, cell monolayers were incubated in PBS with labeled or unlabeled ligands, and then washed twice with ice-cold PBS (maximum washing time was 15 s). To determine the cell-associated radioactivity, 500 μL of 0.1 N NaOH was added to the washed monolayer. The NaOH digest was then used for protein determination and radioactivity measurement. Nonspecific binding was determined in the presence of a 100-fold excess (compared to the K_d value) of the corresponding unlabeled ligand; the values of nonspecific binding varied from 30 to 40% of total binding.

Internalization Assays. Internalization of labeled lipoproteins and proteoliposomes was performed at 37 °C as previously described (Garcia et al., 1996). Cells were incubated in PBS with labeled or unlabeled ligands, and then washed twice with ice-cold PBS at 4 °C. Depletion of radioactive ligands present on the cell surface was performed at 4 °C for 3 × 30 min, with a 100-fold excess of corresponding nonlabeled ligand or, in some experiments, with PBS alone. In some experiments, depletion of radioactive ligands from the cell surface was realized using treatment with trypsin 0.05% (w/v) according to Biesbroeck et al. (1983). Radioactivity measurements were performed as above. Radioactivity measured at 37 °C before depletion is referred to as "binding+internalization". For each experiment, the depletion efficiency was determined by measuring the cell-associated radioactivity remaining after binding at 4 °C followed by depletion, which represented less than 10% of the initial cell surface binding value. Nonspecific internalization was determined in the presence of a 100-fold excess of the corresponding unlabeled ligands and varied from 40 to 45% of the total internalization. Labeled HDL_3 degradation was assessed, after internalization, by measure of the TCA-nonprecipitable material present in the medium as previously described (Brown et al., 1975).

Inhibition of Receptor-Mediated Endocytosis. Cells were washed twice with DMEM medium at 37 °C and were incubated for 30 min in DMEM at the same temperature as previously described (Hansen et al., 1993). Cells were further washed twice with DMEM supplemented with 0.45 M saccharose and were incubated in this medium for the indicated times. Internalization was then measured at 37 °C with 20 μg/mL of labeled HDL_3 (or labeled LDL) with or without a 100-fold excess of unlabeled HDL_3 (or LDL) for 10 min. Depletion of HDL_3 bound to the cell surface was performed at 4 °C as described above. In the case of LDL, surface depletion was carried out using heparin incubations

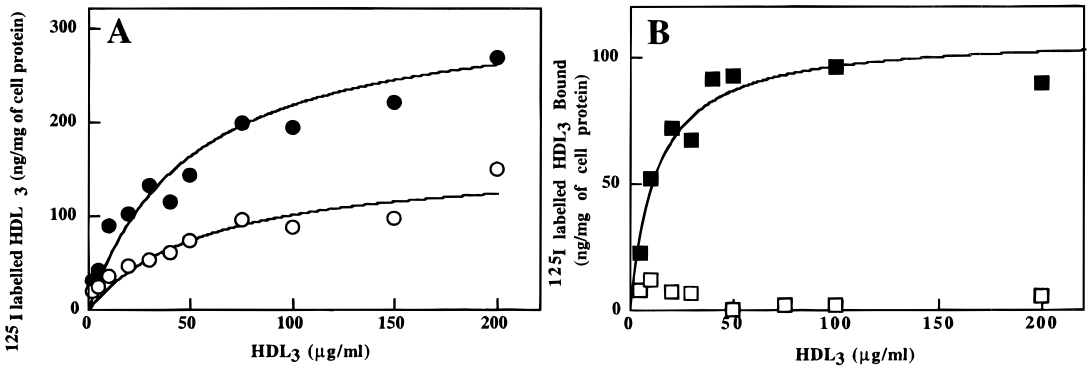

FIGURE 1: Binding isotherm and internalization of ^{125}I-labeled HDL_3 to $HepG_2$ cells. Specific binding (■) and internalization (○) of ^{125}I-labeled HDL_3 were measured on duplicate wells after 90 min incubation at 4 °C (B) or 37 °C (A). Nonspecific binding and internalization were assessed as described under Experimental Procedures. Control experiments were the radioactivity measured at 37 °C before depletion (●), corresponding to the sum of binding and internalization, and the radioactivity measured after binding at 4 °C, and then depletion (□). The mean values from duplicate wells are reported (±10% from the mean) and are representative of three independent experiments performed on three different series of cells.

(2 mg/mL in PBS) at 4 °C. Reverse experiments were performed after a 30 min preincubation of the cell with DMEM medium supplemented with 0.45 M saccharose followed by a wash-out incubation in DMEM for the indicated periods of time. In some experiments, endocytosis was inhibited using potassium depletion as previously described (Hansen et al., 1993).

Kinetic Assays. The kinetics of binding and internalization were performed at 4 and 37 °C, respectively. The concentration of the labeled ligand was constant during the entire experiment. Nonspecific binding/internalization was determined with a 100-fold excess of the corresponding nonradioactive ligand. All the washing and radioactivity measurements were performed as for the binding assays. The pseudo-first-order method was used for k_{ob} (k_{ob} is the observed association rate constant) determination: considering L as the concentration of the labeled ligand and B_e as the equilibrium binding value, a plot of $\ln[B_e/(B_e - B)]$ versus time will have a slope of k_{ob} (Bennett, 1978; Williams & Lefkowitz, 1978).

Clathrin-Coated Vesicle Purification. Clathrin-coated vesicles were purified as already published by Pearse (1982) except that we used MES buffer (pH = 6.5) instead of HEPES buffer. The purity of the preparations was assessed by electron microscopy (negatively stained in 2% uranyl acetate on carbon grids, and examined in a JEOL 200 CTX microscope) and by typical clathrin detection on SDS−PAGE. Furthermore, potential contaminations by other subcellular compartments were detected by measurement of specific enzymatic markers such as alkaline phosphatase or 5′-nucleotidase for plasmic membranes, galactosyltransferase for Golgi, lactate dehydrogenase for cytosol, NADH−dehydrogenase for total endoplasmic reticulum, *N*-acetyl-β-D-glucosaminidase for lysosomes, and monoamine oxidase for mitochondria (Tercé et al., 1988). Possible contamination by caveolae vesicles was assessed by Western blotting using anti-caveolin antibody (Transduction Laboratories). Protein concentrations in purified clathrin-coated vesicle preparations were measured using a μBCA kit (Pierce) with serum albumin as a standard. They represented 100 μg of protein in a final volume of 1 mL, starting from 9×10^7 cells.

Electrophoresis. After clathrin-coated vesicle purification, samples suspended in a SDS buffer were run on Tricine/SDS/polyacrylamide (0.1 M/0.1%/4−20%, w/v) gradient gel electrophoresis as previously described (Schägger & von Jagow, 1987). Under these conditions, both labeled apoB and labeled apoA-I were visualized using a Phosphorimager apparatus (Molecular Dynamics) after 1 week exposure.

Analytical Procedures. The protein concentration of lipoproteins and proteoliposomes was determined by the method of Bradford (1976), using the Bio-Rad protein assay dye and bovine serum albumin (BSA) as a standard. The distributions of free and esterified cholesterol in the lipid extracts from clathrin-coated vesicles were determined by gas−liquid chromatography as described by Vieu et al. (1996).

RESULTS

To assess the internalization of HDL_3 in $HepG_2$ cells, we took advantage of the rapid dissociation of ^{125}I-labeled HDL_3 from their binding sites on $HepG_2$ cells, with or without an excess of unlabeled HDL_3 (Barbaras et al., 1994). Using this observation, we were able to dissociate at 4 °C the ^{125}I-labeled HDL_3 bound to the cell surface after preincubation with $HepG_2$ cells at 37 °C, and thus to precisely measure the HDL_3 internalization (Figure 1A). As shown in Figure 1B, control experiments were realized in the same conditions but at 4 °C. At this temperature, we observed, as in our previous studies, an almost complete dissociation (>95%) of the labeled HDL_3 binding from the cell surface (in contrast to what was observed with human fibroblasts; data not shown). All further internalization studies have included this type of control experiment. In order to check whether two cell washes at 4 °C would allow complete dissociation of membrane-bound HDL, particularly from sites that would be uncovered during a prior incubation at 37 °C, we have tested increased washing procedures (up to 5 times) without any change in the binding parameters and in the amount of cell-internalized HDL_3. Under these conditions, we never exceeded 2 min for the total washes, because longer washes would induce dissociation of HDL_3 from its specific binding sites, as evidenced by our previous studies on the dissociation kinetics (Barbaras et al., 1994). As shown in Figure 1, the internalization of ^{125}I-labeled HDL_3 into $HepG_2$ cells represents about 50% of the total radioactivity measured at 37 °C. No HDL_3 degradation (less than 1%) was detected as TCA-nonprecipitable material, neither in the medium nor in the cells. Furthermore, we have performed "chase" experi-

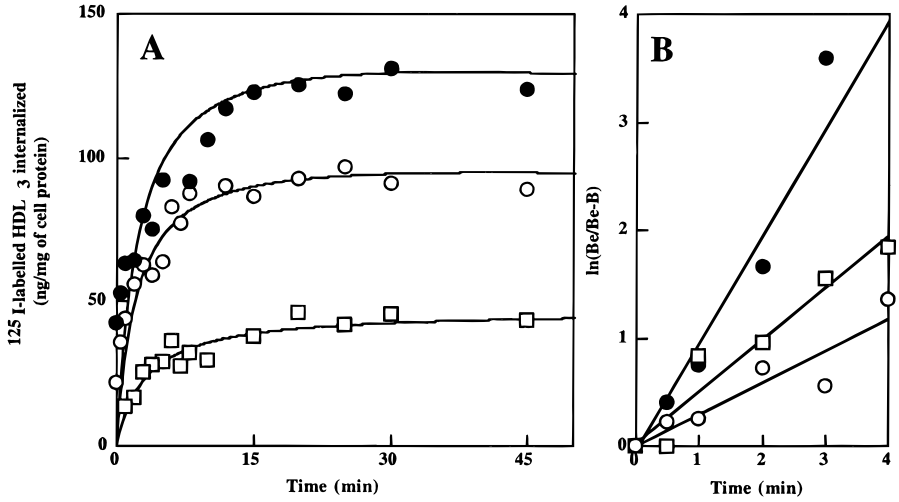

FIGURE 2: Kinetics of binding and internalization of ^{125}I-labeled HDL_3 onto $HepG_2$ cells. A concentration of 20 μg/mL was used for binding association (□) and internalization experiments (○). A pseudo-first-order equation was used to calculate the observed rate constant (k_{ob}) of the association and internalization. A plot of ln [$B_e/(B_e - B)$] versus time (B) enables us to calculate the observed rate constant as described under Experimental Procedures (B). Radioactivity measured during kinetic experiments at 37 °C without depletion (●) represents the sum of binding and internalization. Nonspecific binding and internalization averaged 40% of the total binding. The curves are representative of two experiments performed on two independent series of cells.

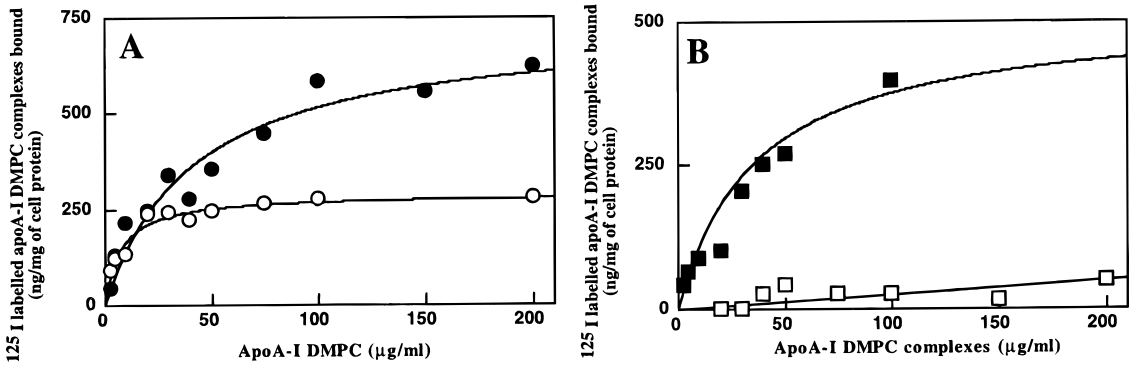

FIGURE 3: Binding isotherm and internalization of ^{125}I-labeled apoA-I−DMPC complexes onto $HepG_2$ cells. Specific binding (■) and internalization (○) of ^{125}I-labeled apoA-I−DMPC complexes were measured on duplicate wells after 90 min of incubation at 4 °C (B) or 37 °C (A). Nonspecific binding and internalization were assessed as described under Experimental Procedures. Control experiments were the radioactivity measured at 37 °C before depletion (●), corresponding to the sum of binding and internalization, and the radioactivity measured after binding at 4 °C, and then depletion (□). The mean values from duplicate wells are reported (±10% from the mean) and are representative of three independent experiments performed on three different series of cells.

Table 1: Internalization Parameters of HDL_3, ApoA-I−DMPC Complexes, and Free ApoA-I[a]

	HDL_3	apoA-I−DMPC	free apo-A-I
internalization max (ng/mg of cell protein)	100	250	5
total association at 37 °C (ng/mg of cell protein)	220	500	30
% of internalization	45.5	50	16.7

[a] Data are obtained from Figures 1, 3, and 4 and are representative from three independent experiments performed on three different series of cells.

ments at 37 °C, following a prior internalization step (as defined in our procedure), in order to follow the fate of cell-associated HDL. Under these conditions, only 20−25% of the radioactivity associated with cells could be released to the medium after 60−180 min chase incubations. These data demonstrate that at least 75% of cell-associated HDL cannot be displaced by further incubations (at 4 or 37 °C), and thus represent HDL_3 internalized in $HepG_2$ cells.

To further confirm that nondissociable cell-associated radioactivity represented HDL_3 internalization and not merely irreversible cell surface binding, we have performed trypsin treatment of the cells preincubated with labeled HDL_3 at 37 °C, as already published by Biesbroeck et al. (1983). Under these conditions, a similar proportion of trypsin-resistant cell-internalized HDL_3 was measured as compared to the data from dissociation experiments (45% in both cases; data not shown). Kinetics of ^{125}I-labeled HDL_3 internalization were performed (Figure 2). These kinetics were very fast, reaching a plateau of internalization at about 10 min. Furthermore, the observed kinetic parameters (k_{ob}, Figure 2B) determined for labeled HDL_3 binding and internalization were not significantly different, which suggests that the binding could be the rate-limiting step.

To characterize the implication of the main HDL_3 apolipoprotein (apoA-I) in the internalization process, we repeated the internalization experiments with ^{125}I-labeled apoA-I−DMPC (dimyristoylphosphatidylcholine) complexes (Figure 3). We have previously reported that these ^{125}I-labeled apoA-I complexes are able to bind to the two families of binding sites on $HepG_2$ cells, as HDL_3 do. As shown in Figure 3, the cells internalized a high amount of apoA-I−DMPC complexes, comparable to the values observed with HDL_3 (Figures 1 and 3 and Table 1).

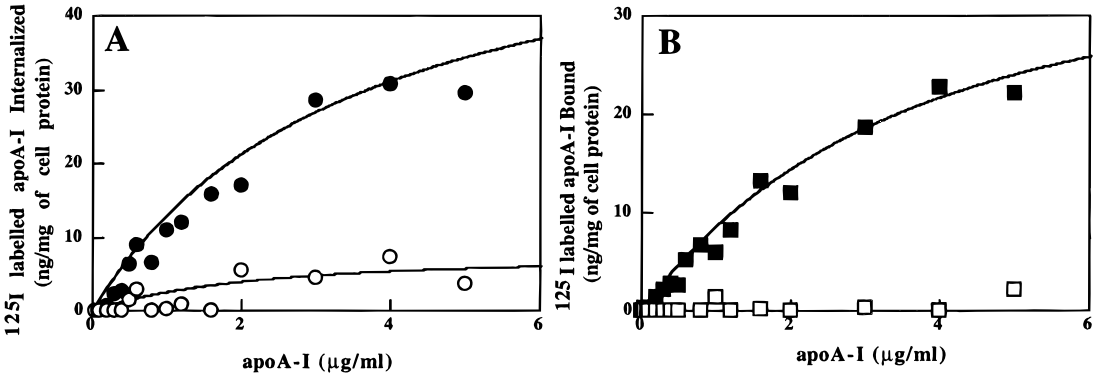

FIGURE 4: Binding isotherm and internalization of ^{125}I-labeled apoA-I onto $HepG_2$ cells. Specific binding (■) and internalization (○) of ^{125}I-labeled apoA-I were measured on duplicate wells after 90 min of incubation at 4 °C (B) or 37 °C (A). Nonspecific binding and internalization were assessed as described under Experimental Procedures. Control experiments were the radioactivity measured at 37 °C before depletion (●), corresponding to the sum of binding and internalization, and the radioactivity measured after binding and then depletion at 4 °C (□). The mean values from duplicate wells are reported (±10% from the mean) and are representative of three independent experiments performed on three different series of cells.

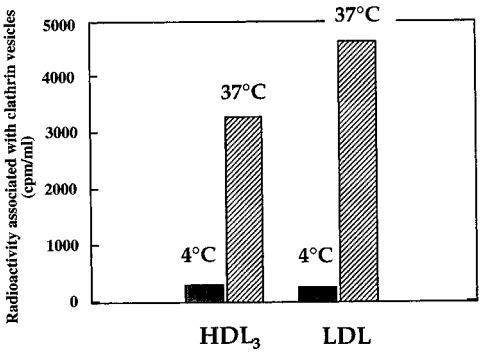

FIGURE 5: Measurement of the radioactivity associated with purified clathrin-coated vesicles after internalization of ^{125}I-labeled HDL_3 or ^{125}I-labeled LDL. Cells were incubated 20 min with 20 μg/mL ^{125}I-labeled HDL_3 (A) or ^{125}I-labeled LDL (B) at 37 or 4 °C. Clathrin vesicles were then purified and tested for purity as described under Experimental Procedures. Protein concentrations in the purified preparation represented 100 μg of protein in a final volume of 1 mL, starting from 9×10^7 cells. The data are representative of five experiments performed on five independent series of cells.

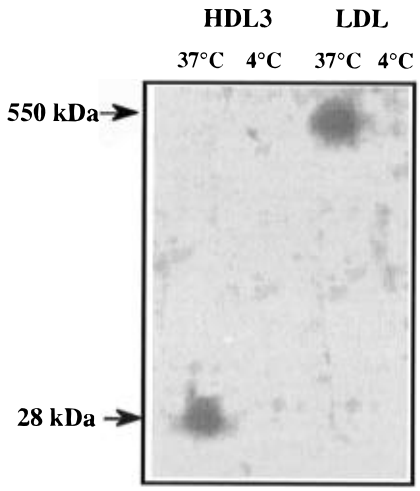

FIGURE 6: SDS−PAGE of purified clathrin-coated vesicles after internalization of ^{125}I-labeled HDL_3 or ^{125}I-labeled LDL. Cells were treated exactly as for Figure 5. After clathrin-coated vesicles purification, half of the preparation was spotted on a 4−20% SDS−PAGE under reducing conditions and radioactivity detected as described under Experimental Procedures.

To determine the relative contributions of the high-affinity binding sites in the HDL_3 internalization process, we repeated the same kind of experiments using ^{125}I-labeled apoA-I non-associated with lipids (namely, ^{125}I-labeled free apoA-I). This ^{125}I-labeled free apoA-I can bind only to the high-affinity binding sites (Barbaras et al., 1994). As expected, we found a much weaker amount of ^{125}I-labeled free apoA-I internalized compared to the HDL_3 internalization (Figure 4A and Table 1).

To test whether the internalization process of HDL_3 may occur through the clathrin-coated vesicle pathway, we first measured the radiolabeled apoA-I recovered in clathrin-coated vesicles, following labeled HDL_3 internalization at 37 °C. Similar experiments were performed with radiolabeled LDL, as a control. Thus, we have purified clathrin-coated vesicles after incubations of $HepG_2$ cells with ^{125}I-labeled HDL_3 or ^{125}I-labeled LDL, at 4 or 37 °C. As shown in Figure 5, we found radioactivity associated with the clathrin-coated vesicles only at 37 °C, either with ^{125}I-labeled HDL_3 or with ^{125}I-labeled LDL incubations, but not at 4 °C. The purity of the preparations of clathrin-coated vesicles was assessed from different arguments. Electron microscopy qualitative analysis showed the absence of contamination with other subcellular compartments. Furthermore, quantitative measurements of specific markers for these subcellular components, expressed as a percentage of the specific activity for these markers compared to initial cell homogenates, have confirmed the lack of contamination: NADH dehydrogenase and 5′-nucleotidase represented less than 0.1%, while galactosyltransferase, lactate dehydrogenase, *N*-acetyl-β-D-glucosaminidase, and monoamine oxidase were undetectable. It is noteworthy that caveolin, a typical protein marker of caveolae, was not detectable in the preparations as evidenced by Western blot analysis (data not shown).

We have further tested the clathrin-coated vesicle content using SDS−PAGE followed by detection with the phosphorimager. We found a single band corresponding to radiolabeled apoA-I and apoB, following 37 °C incubations with HDL_3 and LDL, respectively (Figure 6). The amount of apoA-I recovered in the purified clathrin-coated vesicles from 9 - 10^7 cells was 1 ng, and that of apoB, 1.5 ng, following HDL or LDL incubations at 37 °C, respectively, as estimated from the apolipoprotein specific radioactivities. The distribution of free and esterified cholesterol was also assessed, by gas−liquid chromatography, in the purified vesicles. The esterified cholesterol versus free cholesterol molar ratio was

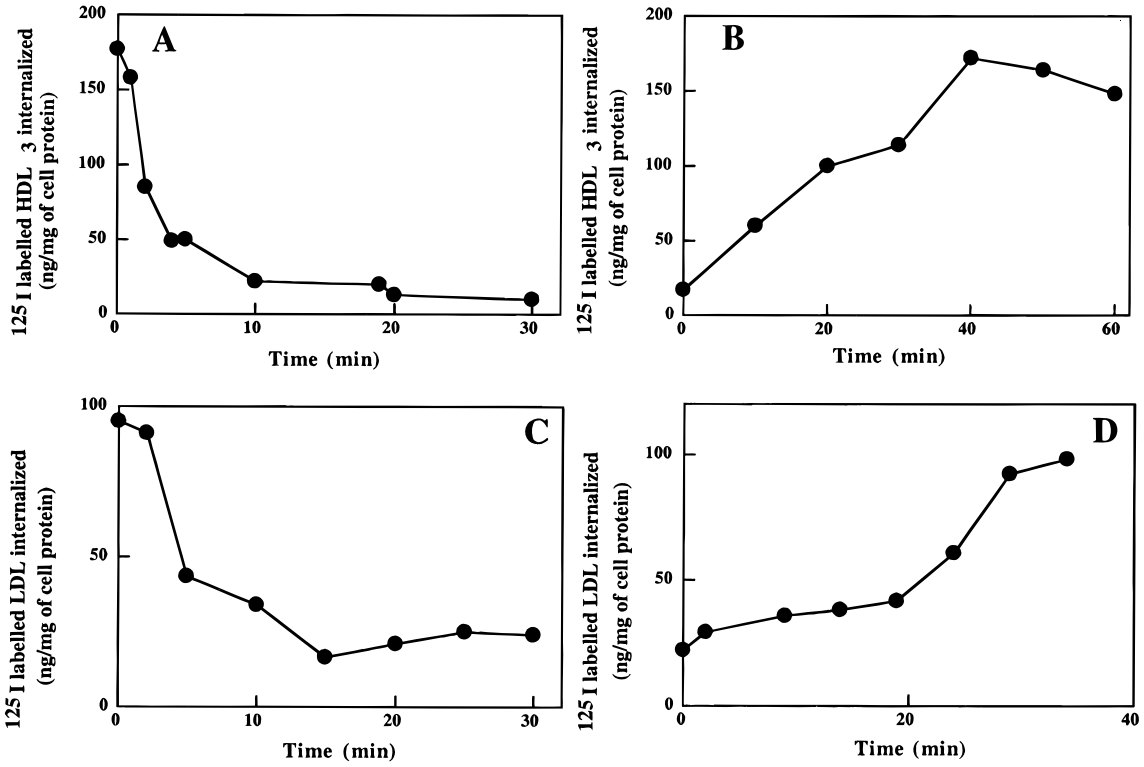

FIGURE 7: Inhibition of the clathrin-coated vesicle internalization pathway onto $HepG_2$ cells. Specific internalization of HDL_3 or LDL (C) was measured for 10 min after the indicated different periods of time of hypertonic shock as described under Experimental Procedures. The cells were then transferred to DMEM buffer (B and D), and again specific HDL_3 or LDL endocytosis was measured. The mean values from duplicate wells are reported (±10% from the mean) and are representative of two independent experiments performed on two different series of cells.

0.32−0.33 following 4 °C incubations and became 0.52−0.55 after 37 °C incubations. For comparison, this ratio was 0.09 in purified plasma membranes.

Finally, to quantify the amount of HDL_3 internalized through the clathrin-coated vesicle pathway, we inhibited this process by different methods: potassium depletion of the cells (data not shown) or slight hypertonic shock (Figure 7A,B). Similar experiments were performed in parallel with LDL (Figure 7C,D). When the clathrin-coated vesicle pathway was impaired, we found a rapid and complete inhibition of the HDL_3 internalization, comparable with that observed for the LDL internalization. In both conditions, we were able to restore the clathrin-coated vesicle pathway by reversing the experimental conditions (Figure 7B,D), indicating that the cells were not altered during these experiments. To confirm the absence of major cell alterations, we measured the release of the cytosolic enzyme lactate dehydrogenase (LDH) under clathrin-coated pathway inhibition conditions, and found less than 1% of activity in the culture medium.

DISCUSSION

In the present study, we have followed the intracellular fate of HDL_3 (−apolipoprotein), following incubations at 37 °C and removal of the membrane-bound HDL. For that purpose, we took advantage of our previously published observations that the complete dissociation of HDL_3 from the cell surface does occur within 30 min, by simple addition, to the cells, of fresh incubation medium complemented or not with an excess of unlabeled HDL_3 (Barbaras et al., 1994). Two main conclusions can be pointed out at this time. (i) HDL_3 are internalized at a high level in $HepG_2$ cells. Indeed, the level of HDL_3 internalized represents about 45.5% of the total HDL_3 associated with the cells at 37 °C. A similar proportion of cell-internalized HDL_3 was measured after protease treatment of the cells, allowing us to exclude the possibility of an irreversible $HepG_2$ cell surface binding of HDL_3 at 37 °C, in contrast to the data reported for human fibroblasts (Oram et al., 1993). (ii) This internalization is very fast with a plateau at about 15 min, suggesting a very active endocytosis pathway.

HDL are generally described as the main lipoprotein particles involved in cholesterol transport from peripheral tissues to the liver. In the liver, this cholesterol will be reutilized or excreted as bile acids or sterols. However, the intracellular fate of HDL cholesterol in hepatocytes is still unknown. One reasonable hypothesis is that the movement of cholesterol from extracellular HDL to bile canaliculus would follow a transcytosis pathway. Indeed, as for many epithelial cells, hepatocytes present a cell polarity which suggests the existence of such transcytosis pathways. The absence of HDL_3 degradation in $HepG_2$ cells might support this transcytosis hypothesis, instead of a strict endocytosis pathway which finally leads to lysosomal degradation as in the case of LDL endocytosis (Rodman et al., 1990).

A retroendocytosis pathway of HDL_3 after internalization into hepatocytes was suggested by others (Kambouris et al., 1990). In our case, the 25% release of HDL_3 to the medium as TCA-precipitable material may represent either a retroendocytosis pathway or eventually a further dissociation of HDL_3 binding. Although we cannot definitely exclude either hypothesis, our conclusion at this stage is that most of the cell-associated HDL (75%) measured in our protocol represents internalization of the lipoprotein, and any retroen-

docytosis would be very limited (a plateau of 25% after 3 h).

We have shown that the interaction between HDL_3 and $HepG_2$ cells was mainly protein-dependent and apolipoprotein A-I was a likely ligand candidate, through both the HDL_3 high-affinity and the HDL low-affinity binding sites (Barbaras et al., 1994). The following questions were to determine whether apolipoprotein A-I was also involved in the HDL_3 internalization process and by which mechanism, and what were the relative contributions of the two HDL_3 binding sites in this pathway.

To answer the first point, we have followed the internalization of apolipoprotein A-I−DMPC complexes, which recognize the same HDL_3 high- and low-affinity binding sites (Barbaras et al., 1994). ApoA-I−DMPC complexes were also internalized by $HepG_2$ cells, at levels comparable to those obtained with HDL_3. This is the first evidence of a direct implication of a HDL_3 apolipoprotein in the endocytosis process. The mechanism leading to HDL_3 internalization following apolipoprotein binding needs to be elucidated. However, one can hypothesize the existence of a "classical" clathrin-coated vesicle pathway for the HDL_3 internalization.

Recently, using electron microscopy, Herold et al. (1994) described the association of apoA-I complexes with coated pits and their presence into endosomes in a human enterocyte cell line (CaCo-2 cells). A simple approach to test the possible involvement of clathrin-coated vesicles in HDL_3 uptake was first to purify clathrin-coated vesicles and then to test for the presence of HDL_3 components inside these vesicles. The presence of radioactive apoA-I associated with clathrin-coated vesicles purified from cells, only after incubation with HDL_3 at 37 °C, strongly supports the existence of HDL_3 internalization through the clathrin-coated vesicle pathway into HepG2 cells. Then, we attempted to quantify the contribution of clathrin-coated vesicles in HDL_3 uptake. For that purpose, we induced inhibition of the formation of clathrin-coated vesicles and then measured the HDL_3 internalization. Three techniques are usually used for clathrin-coated vesicle pathway inhibition: pH shock, K^+ depletion of the cells, or slight hypertonic shock. The acidification of the cytosol affects clathrin-coated membrane domains in a way that interferes with the budding of clathrin-coated vesicles from the plasma membrane as well as from the trans Golgi network (TGN). Potassium depletion and incubation in hypertonic medium prevent clathrin-coated vesicles and adaptors from interacting (Hansen et al., 1993). Although these techniques might be harsh, it was already demonstrated that these treatments, during a short period of time, do not affect other internalization processes such as caveolae, noncoated vesicle endocytosis or bulk phase budding (Daukas & Zigmond, 1985; Oka & Weigel, 1988; Raposo et al., 1989; Cupers et al., 1994). We preferentially used first the hypertonic shock technique and then the potassium depletion methods. All these experiments were realized with LDL as controls, since LDL are well-known to be internalized through the clathrin-coated vesicle pathway. We show, for the first time, the complete lack of HDL_3 internalization after hypertonic shock or potassium depletion (not shown). It is noteworthy that the cells were able to reinternalize HDL_3 (or LDL) after reconstitution of a normal medium, indicating that they were not irreversibly affected by the treatments. These observations clearly show that HDL_3 internalization into $HepG_2$ cells occurs through an endocytosis pathway involving the formation of clathrin-coated vesicles. This strongly suggests that the binding of HDL_3 to receptor sites is the first obligatory event for the HDL_3 endocytosis. The identity of a HDL_3 receptor on hepatic cells is still controversial. However, it was recently reported that the previously described scavenger receptor SR-B1 binds HDL and promotes internalization of HDL lipids (Acton et al., 1996). Although this SR-BI has not been described as of yet in human cells, we performed competition experiments using PS/PI liposomes, as already published by Rigotti et al. (1995). Indeed, these liposomes are reported to be specific ligands for the SR-BI receptor. In our experiments with $HepG_2$ cells, radiolabeled HDL_3 were not competed by PS/PI liposomes, strongly suggesting that the binding observed does not concern the SR-BI receptor (data not shown).

Concerning a possible transfer of HDL-esterified cholesterol (CE) during cellular internalization, we did not use radiolabeled CE, as our experiments were focused on the fate of radiolabeled apoA-I. A preliminary approach was to determine the relative proportions of free and esterified cholesterol in the purified vesicles. A substantial enrichment in esterified cholesterol was noted following incubations with the lipoproteins at 37 °C. However, at the present stage, we cannot conclude that this only reflects the contribution of lipoprotein-derived cholesteryl esters, and alternative approaches will be required to elucidate this phenomenon. Nevertheless, we can exclude an increase of the cholesteryl ester values by contamination from endoplasmic reticulum of the clathrin-coated vesicle preparations, since NADH dehydrogenase, an ER enzymatic marker, was almost undetectable in our preparations.

Concerning the contribution of the HDL_3 high-affinity binding site into the internalization process, we have used apolipoprotein A-I with no associated lipid (namely, free apoA-I) which is able to bind only to the HDL_3 high-affinity binding site (Barbaras et al., 1994). Internalization experiments with lipid-free apoA-I evidenced a very low amount of intracellular free apoA-I. Indeed, only 16.7% of the total free apoA-I associated at 37 °C was found internalized compared to 50% of apoA-I−DMPC complexes, as generally observed. Furthermore, the absolute amount of apoA-I internalized through the high-affinity binding site represented only 5 ng/mg of cell protein compared to 250 ng/mg of cell protein of apoA-I internalized through the low-affinity binding site. Our conclusion at this time is that the high-affinity site cannot trigger all the HDL_3 endocytosis measured in the $HepG_2$ cells. Nevertheless, we cannot exclude the possibility that this HDL_3 high-affinity binding site may regulate the general internalization process, by triggering the low-affinity binding sites and consequently regulating their expression or function.

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