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The Low Density Lipoprotein Receptor-related Protein Contributes to Selective Uptake of High Density Lipoprotein Cholesteryl Esters by SW872 Liposarcoma Cells and Primary Human Adipocytes*

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The concept that selective transfer of high density lipoprotein (HDL)-derived cholesteryl esters (CE) does not require lipoprotein internalization has been challenged recently by evidence that implicates HDL recycling during the selective uptake process. This has prompted us to examine the role of the low density lipoprotein receptor-related protein (LRP) in selective uptake. LRP is an endocytic receptor for lipoprotein lipase (LpL) and apolipoprotein E (apoE) ligands that are able to mediate selective uptake. We report that molecules that interfere with ligand binding to LRP, such as the receptor-associated protein (RAP), suramin, α_2-macroglobulin, or lactoferrin, inhibit HDL-CE selective uptake by human primary adipocytes and SW872 liposarcoma cells by 35–50%. This partial inhibition of selective uptake from total HDL was not due to preferential inhibition of the HDL_2 or HDL_3 subfractions. Selective uptake by the scavenger receptor BI was not inhibited by RAP, excluding its involvement. Furthermore, in SW872 cells in which LRP was reduced to 14% of control levels by stable antisense expression, selective uptake was attenuated by at least 33%, confirming a role for LRP in this process. RAP, α_2-macroglobulin, lactoferrin, and suramin (individually or in paired combinations) also attenuated selective uptake of HDL-CE by primary human adipocytes by about 40%. On the other hand, human skin fibroblasts express LRP abundantly but lack the capacity for selective uptake, demonstrating that other molecules are required. In SW872 cells, exogenous apoE or LpL can facilitate selective uptake but only the apoE-enhanced uptake can be inhibited by RAP, implicating apoE as a likely co-mediator. We discuss the possible mechanisms by which the endocytic receptor, LRP, can mediate selective uptake.

Cells acquire cholesterol from lipoproteins either by receptor-mediated endocytosis of the entire particle or by the "selective uptake" of particular lipids (notably cholesteryl esters, CE)[1] that the cell accrues independently and in excess of the apolipoproteins (see Refs. 1 and 2 and for reviews see Refs. 3 and 4). Selective uptake is the principal means by which HDL, the major donor in this pathway (5), delivers cholesterol to ovary, testes, and adrenal cells for steroidogenesis (6) and to adipocytes for storage (7, 8). The tenet of selective uptake (delivery without degradation) may conserve HDL and consolidate its anti-atherogenic role (for review see Ref. 9).

The mechanism of selective uptake is poorly understood. Evidently, HDL first binds to the cell surface. Then, in a crucial but unclear step, CE is passively and reversibly transferred into a plasma membrane pool (10, 11), possibly cholesterol-rich domains such as caveolae (12). Subsequently, the CE is irreversibly internalized and hydrolyzed (13). Selective uptake may involve multiple pathways. This is underscored by the observation that several diverse molecules can selectively internalize CE.

Chief among these is the scavenger receptor BI (SR-BI), a physiologically relevant, high affinity HDL receptor (14–16) expressed in many tissues active in selective uptake (15, 17). Based on thermodynamic and kinetic data, Rodrigueza *et al.* (18) proposed that SR-BI forms a lipophilic channel through which CE and other lipids traverse from the bound lipoprotein to the plasma membrane. As yet, there is no physical evidence for such a model. In addition to SR-BI, selective uptake is mediated by lipoprotein lipase (LpL) (19, 20), hepatic lipase (21), and apoE in some cells (22) but not others (6). These molecules are proposed to enhance CE transfer by tethering lipoproteins to the cell surface, thereby increasing their effective concentration at sites of selective uptake. Tethering may also explain the contribution of heparin sulfate proteoglycans (20) as well as CETP (8) to selective CE delivery. Additionally, HDL remodeling by CETP may produce a lipoprotein that is a better substrate for selective uptake (23). The assertion that distinct pathways underlie selective uptake is further supported by evidence for HDL recycling during selective CE transfer in *ob/ob* hepatocytes (24). This finding implies that CE transfer occurs at an intracellular location, as yet undefined. Additionally, this implicates an endocytic step in a process that was originally proposed to be non-endocytic (25). If true, the low density lipoprotein receptor-related protein (LRP) (for reviews see Refs. 26 and 27) is a likely contributor given that it binds apoE (28, 29), LpL (30, 31), and hepatic lipase (32), and it collaborates with heparin sulfate proteoglycans to me-

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[1] The abbreviations used are: CE, cholesteryl ester; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; HDL, high density lipoprotein; HSF, human skin fibroblasts; LDL, low density lipoprotein; Lf, lactoferrin; LPDS, lipoprotein-deficient serum; LRP, LDL receptor-related protein; LpL, lipoprotein lipase; α_2M, α_2-macroglobulin; PBS, phosphate-buffered saline; RAP, receptor-associated protein; SR-BI, scavenger receptor BI; HDL, high density lipoprotein; HSF, human skin fibroblasts; CHO, Chinese hamster ovary; CETP, cholesteryl ester transfer protein.

diate the internalization of apoE and LpL (33, 34). For these reasons we tested the involvement of LRP in selective uptake. LRP is already implicated in a broad range of biological processes because it binds numerous, diverse ligands. In addition to those already mentioned, LRP binds and internalizes (26, 27) α_2-macroglobulin (α_2M), lactoferrin (Lf), plasminogen activators, complexes of plasminogen activators and their inhibitors, tissue factor-pathway inhibitor, thrombospondin, amyloid precursor protein, $\alpha\beta$-peptide, and the 39-kDa RAP that copurifies with the receptor and inhibits binding of all known ligands to the LRP (26, 27).

In this paper we present evidence that the endocytic receptor, LRP, contributes to at least 35% of HDL cholesteryl ester selective uptake by SW872 adipocytes and primary human adipocytes.

EXPERIMENTAL PROCEDURES

Materials—All common reagents were analytical grade purchased from Fisher, Sigma, or BDH (Poole, UK).

Protein Purification and Labeling—ApoA-I was expressed and purified using the IMPACT T7 system (New England Biolabs, Beverly MA). In this system, proteins are expressed in fusion with a self-cleavable affinity tag (intein fused to a chitin binding domain) that allows a single step purification of the fusion protein on a chitin column and elution of the target protein after dithiothreitol-induced self-cleavage of the intein. The apoA-I sequence corresponding to the mature protein (without the pre- and pro-sequences) was amplified by PCR using the full-length apoA-I cDNA as template. The forward and reverse primers included *Nde*I and *Sap*I restriction sites, respectively. The PCR product and pTYB1 were double-digested with *Nde*I and *Sap*I and then ligated together. This plasmid was designated pTYB1(AI). This cloning strategy produces apoA-I without any additional amino acids after cleavage from the intein-chitin binding domain. pTYB1(AI) was amplified in DH5α. To express the protein, 100 ng of pTYB1(AI) was electroporated into ER2566 cells that were grown for 1 h at 37 °C in LB medium and then grown at 37 °C in 1 liter of LB, 100 μg/ml ampicillin, to a density of 0.65 at A_{600}. Solid isopropyl-β-D-thiogalactopyranoside (ICN, Aurora, OH) was added to 250 μM, and the protein was expressed for 16 h at 17 °C. The bacteria were harvested by centrifugation at 6,000 × *g* for 15 min at 4 °C. The cell pellet was suspended in 74 ml of ice-cold 20 mM Hepes, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.3% Triton X-100, 20 μg/ml lysozyme (Roche Molecular Biochemicals), and 1 tablet Complete protease inhibitors (Roche Molecular Biochemicals), and the cells were then clarified by sonication on ice. Cellular debris was removed by centrifugation at 20,000 × *g* for 30 min at 4 °C, and the apoA-I was purified on a 6-ml chitin column according to instructions from New England Biolabs. The apoA-I-containing fractions were concentrated by centrifugation using a Centriprep-10 (Millipore, Bedford, MA). By repeated (5 times) dilution and concentration of the apoprotein, the buffer was replaced with 200 mM sodium phosphate, pH 7.4. Typically, about 2 mg of apoA-I was purified from 1 liter, and the proteins appeared pure after SDS-PAGE and Coomassie staining. ApoA-I (200 μg) was labeled with 200 μCi of $Na^{125}I$ (Amersham Biosciences) using IODO-GEN tubes (Pierce) by the indirect method (35). The ^{125}I-apoA-I was dialyzed twice in 2 liters of 5 mM ammonium bicarbonate, 1 mM EDTA, 3 mM sodium azide and then frozen at −80 °C and freeze-dried for 16 h. The dried ^{125}I-apoA-I was dissolved in 500 μl of 100 mM Na_2HPO_4, 6 M guanidine HCl, incubated for 1 h at 37 °C, and then dialyzed 4 times, each time for 4 h against 2 liters of nitrogen-sparged PBS, pH 7.4, with 2 g of Chelex (Bio-Rad). The ^{125}I-apoA-I was used to label HDL (see below). ApoE was purified from human triacylglycerol-rich lipoproteins as described (36). Native α_2M was purified from human plasma by zinc-chelate chromatography (37). The α_2M was activated with methylamine and labeled with ^{125}I as described (38). RAP-glutathione *S*-transferase was expressed in DH5α and purified (39). The RAP was cleaved from the fusion protein and purified as described (38).

HDL Isolation and Radiolabeling—Plasma was collected from healthy normolipemic donors, and HDL or HDL_2 and HDL_3 subfractions were purified by density gradient ultracentrifugation (40). To prepare lipoprotein-deficient serum (LPDS), blood was allowed to clot at room temperature for 30 min and centrifuged at 2,000 × *g* for 15 min at room temperature. The serum was adjusted to a final density of 1.215 g/ml with KBr and centrifuged at 150,000 × *g* for 16 h at 4 °C. The bottom fraction, excluding all lipoproteins, was harvested and dialyzed 3 times in 2 liters of nitrogen-sparged PBS, pH 7.4, with 2 g of Chelex. The dialyzed LPDS was heated at 60 °C for 10 min to inactivate lecithin-cholesterol acyltransferase activity and then stored at −20 °C. Prior to use, the LPDS was centrifuged at 15,000 × *g* for 5 min to remove any precipitate. The HDL was labeled with [^{3}H]CE or [^{3}H]cholesteryl ether according to Rodrigueza *et al*. (18) with the following amendments. LPDS (1.5 ml, typically about 120 mg of protein), 5 mg of HDL, 0.01% glutathione, and Complete protease inhibitors were added to a 2-ml polypropylene tube. To this, 100 μCi of [^{3}H]CE or [^{3}H]cholesteryl ether (Amersham Biosciences) in 50 μl of ethanol was injected rapidly. The tube was sealed under nitrogen and mixed slowly by rotation for 36 h at 37 °C. The HDL was isolated by density gradient ultracentrifugation (40) and dialyzed 3 times in 2 liters of nitrogen-sparged PBS, pH 7.4, with 2 g of Chelex. Typically, the specific activity was 15,000–20,000 cpm/μg HDL. To prepare HDL-labeled with ^{125}I-apoA-I, 2.5 mg of HDL that had been labeled with [^{3}H]CE (Amersham Biosciences) was incubated with 200 μg of ^{125}I-apoA-I and mixed slowly by rotation for 6 h at 37 °C. The specific activity of HDL-labeled with ^{125}I-apoA-I was typically 40,000–50,000 cpm/μg HDL, and greater than 99% of the radioactivity was trichloroacetic acid-precipitable.

Cell Culture—All cells were cultured at 37 °C in a humidified 5% CO_2 incubator. SW872 liposarcoma cells were cultured in Dulbecco's modified Eagle's medium/F-12 (3:1), 5% fetal bovine serum (Life Technologies, Inc.), with 2 mM L-glutamine (Life Technologies, Inc.) and 50 μg/ml gentamicin (Schering-Plough, Quebec, Canada). Human skin fibroblasts (HSF) were from the Coriell Institute of Medical Research, repository number GM00038B, and were cultured as described (38). SR-BI-expressing CHO cells and control ldl-A7 CHO cells were cultured as described (15). Primary, subcutaneous human adipocytes were obtained from healthy subjects undergoing reduction mammoplasty for cosmetic purposes, and preadipocytes were isolated, seeded at about 50% confluence into 24-well plates, and differentiated as described (41). Human subcutaneous adipocytes were also obtained commercially from Zen-Bio (Research Triangle Park, NC).

LRP-null SW872 cells were prepared by stable expression of antisense RNA. A 120-bp fragment of human LRP (nucleotides 371–490, which includes the ATG start site) was amplified by PCR using full-length human LRP cDNA as template. The forward and reverse primers (5-gcgcgTCTAGAAAAGGAGGAAAAGGGGCGCG-3 and 5-gcaaaACGCGTCAGGAGCAACGGCGG-3, respectively) contained *Xba*I or *Mlu*I restriction sites (underlined) and spacer nucleotides. The PCR product was digested with *Xba*I and *Mlu*I and ligated (in reverse orientation) into pCMV5 that had been similarly digested. The resultant vector was called pCMV5(αsLRP). Stable transfection into SW872 cells was achieved by co-transfection of 20 μg of pCMV5(αsLRP) and 0.4 μg of pSV2-neo by calcium phosphate precipitation (42). Stable transformants were selected with 1000 μM G418 and maintained with 800 μM G418 (Life Technologies, Inc.). LRP-null clones were confirmed by Western blotting.

Selective Uptake Assay—Cells were plated in 24-well tissue culture plates at about 25% confluence and grown for 2 days as described above. After 2 days the cells were about 90% confluent. Prior to the selective uptake assay, the cells were washed once at 37 °C in 1 ml of ligand buffer (HBSS, 25 mM Hepes, 5 mg/ml BSA, pH 7.45) and then incubated in this buffer at 37 °C for 30 min. This buffer was replaced with 300 μl of ligand buffer containing [^{3}H]CE-labeled HDL or ^{125}I-apoA-I-labeled HDL (at the concentrations indicated in the figure legends), and the cells were incubated at 37 °C for up to 8 h. In some experiments, unlabeled HDL, RAP, suramin, α_2M, Lf, or antibodies were included with the labeled HDL. At the end of the incubation the extracellular buffer was removed, and the cells were washed 5 times in HBSS, 25 mM Hepes, pH 7.45, at 10 °C. After 8 h of incubation, the cells were shown to be greater than 99% viable according to the trypan blue exclusion assay. After the final wash the medium was removed, and the cells were solubilized with 500 μl of 200 mM NaOH for 2 h at room temperature with gentle mixing. The protein content of 40 μl from each well was measured using the bicinchoninic acid protein reagent (Pierce) according to the manufacturer's instructions with BSA for standard comparison. The ^{3}H or ^{125}I radioactivity of 440 μl of each cell lysate was measured by liquid scintillation counting using Ecolite (ICN, Costa Mesa, CA) or by gamma counting, respectively. Proteolytically degraded and resecreted fragments of ^{125}I-apoA-I in the ligand-containing medium were measured according to Goldstein *et al*. (43). The cell association of [^{3}H]CE or ^{125}I-apoA-I is plotted in units of the amount of these labels contained in 1 ng (protein content) of HDL. Plotted thusly, an equivalent cell association of [^{3}H]CE and ^{125}I-apoA-I indicates holoparticle uptake, and any additional [^{3}H]CE cell association is attributed to selective uptake. Kinetic parameters for selective uptake and constants for inhibition of selective uptake were determined by non-linear

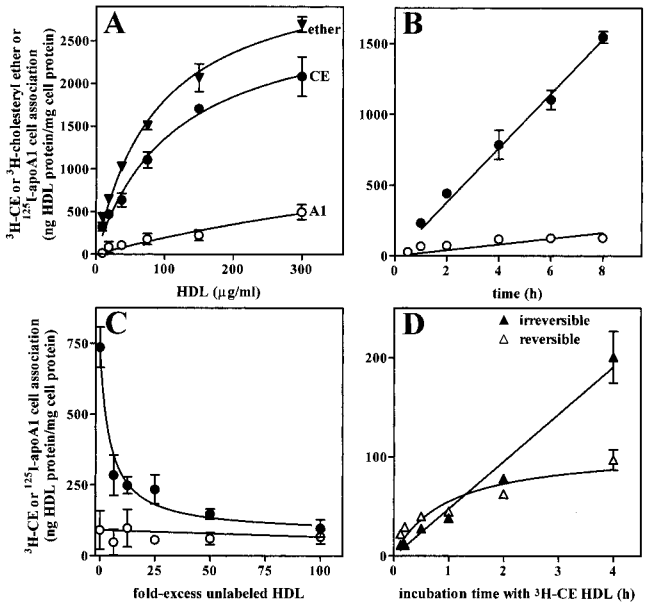

FIG. 1. **Characterization of selective uptake by SW872 cells.** Cells were incubated at 37 °C for 8 h with up to 300 μg/ml [^{3}H]CE-labeled, [^{3}H]cholesteryl ether-labeled, or ^{125}I-apoA-I-labeled HDL (*A*) or with 100 μg/ml labeled HDL for various times up to 8 h (*B*) or for 8 h with 25 μg/ml labeled HDL in the presence of the indicated excess of unlabeled HDL (*C*). After washing the cells, the cell-associated radioactivity and cellular protein were measured, and the incorporation of [^{3}H]CE (●), [^{3}H]cholesteryl ether (▼), or cell-associated and degraded ^{125}I-apoA-I (○) was plotted in units of the amount of these labels contained in 1 ng (protein content) of HDL and normalized for cellular protein. *D,* cells were incubated with 10 μg/ml [^{3}H]CE-labeled HDL to permit selective uptake for up to 4 h. After each time point the cells were washed twice and incubated for 2 h with medium containing 1 mg/ml unlabeled HDL. After this period the radioactivity in the medium or the cells was deemed to be reversibly (△) or irreversibly (▲) incorporated, respectively. Each point is the mean of four measurements, and the S.E., which lies within the symbols in some cases, is shown.

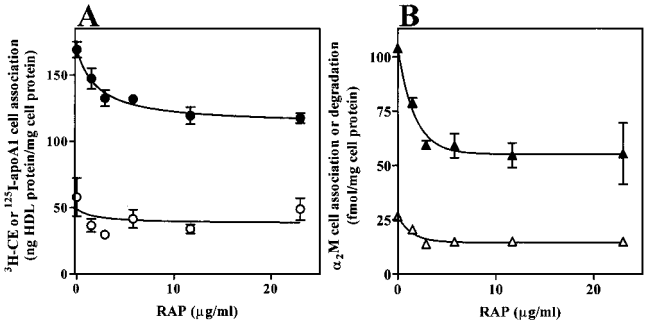

FIG. 2. **Inhibition of selective uptake with RAP.** SW872 cells were incubated at 37 °C for 8 h with 8 μg/ml [^{3}H]CE-labeled or ^{125}I-apoA-I-labeled HDL in the presence of the indicated concentrations of RAP (*A*). The cellular incorporation of [^{3}H]CE (●) or cell-associated and degraded ^{125}I-apoA-I (○) was measured and plotted as described in Fig. 1. To confirm the inhibitory effect of RAP on LRP, ^{125}I-α_2M at 1 μg/ml was incubated at 37 °C for 8 h in the presence of the indicated concentrations of RAP (*B*). After this time, the ligand medium was precipitated with ice-cold trichloroacetic acid, and the soluble material was used as a measure of the degraded protein (▲). After removal of the ligand medium, the cells were then washed and from the cell-associated radioactivity and protein content, the cell-associated ^{125}I-α_2M (△) was calculated. Background cell association and degradation (not plotted) were measured in the presence of a 50-fold excess of unlabeled α_2M, and this was generally about 50% of values in the absence of unlabeled α_2M. Each point is the mean of four measurements, and the S.E. is shown.

regression using the Prism computer program (GraphPad Software).

Demonstration of Reversible and Irreversible Phases of Selective Uptake—SW872 cells were incubated with 50 μg/ml [^{3}H]CE or ^{125}I-apoA-I-labeled HDL for various times up to 4 h. Following this period the cells were rapidly washed 5 times in HBSS, 25 mM Hepes, pH 7.45, at 10 °C to remove the labeled HDL from the extracellular medium. The cells were then incubated with 400 μg/ml unlabeled HDL in 300 μl of HBSS, 25 mM Hepes, pH 7.45, 5 mg/ml BSA. After 2 h, the ^{3}H and ^{125}I radioactivity in the medium or the cells was counted.

Cell surface binding and degradation of ^{125}I-α_2M was measured as described (38). Specific ^{125}I-α_2M binding (total minus nonspecific) was used to generate parameters of K_d and B_{max} after non-linear regression analysis.

Western Blot of LRP—Total cellular protein (5 μg) from SW872 cells or LRP antisense-expressing cells was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (44). The LRP was detected (44) using a polyclonal rabbit antisera (from Dr. G. Bu) followed by chemiluminescent detection (Roche Molecular Biochemicals) of a secondary antibody conjugated to horseradish peroxidase. We have confirmed that the band detected co-migrates with LRP that has been purified on a GST-RAP column.

Molecular biology techniques were essentially as described by Sambrook *et al.* (45).

RESULTS

Characterization of Selective Uptake in SW872 Cells—We have demonstrated that SW872 liposarcoma cells grown to 80% confluence express the LRP as well as other functionally important adipocyte genes including adipsin, LpL, CETP, CD-36, and caveolin-1 and -2 and accumulate lipid (not shown), making this cell line a potentially useful human adipocytic model to study selective uptake. We first established that SW872 cells could acquire HDL-CE by selective uptake, with the characteristics previously reported for other cells. When SW872 cells were incubated with [^{3}H]cholesteryl oleate-labeled HDL or ^{125}I-apoA-I-labeled HDL, CE cell association exceeded (by about 5-fold) the cell association and degradation of apoA-I, characteristic of selective uptake (Fig. 1*A*). This selective accumulation of CE was not due to the internalization of radiolabeled degradation products of CE because selective uptake of cholesteryl ether, a non-hydrolyzable analogue of CE, was also observed (Fig. 1*A*). The CE cell association reached a plateau at around 200 μg/ml HDL, confirming that selective uptake was saturable (6, 46). Non-linear regression analysis, assuming a single class of transporters, generated a maximal transport rate (T_{max}) of 269 (±14) ng of HDL protein/mg of cell protein/h, and the half-maximal transport (K_m) was at 88 (±12) μg/ml HDL. The time dependence of selective uptake (Fig. 1*B*) showed that the process rapidly attained and maintained steady state over 8 h. To show specificity, 25 μg/ml labeled HDL was incubated with up to a 100-fold excess of unlabeled HDL (Fig. 1*C*). The cellular incorporation of [^{3}H]CE was inversely related to the concentration of unlabeled HDL, indicating competition and specificity. In this experiment, which was repeated with more data points, the selective uptake kinetics measured from the competition curve (T_{max} = 355 ng HDL protein/mg cell protein/h and K_m = 90 μg/ml HDL) reasonably matched the kinetics measured using labeled HDL (Fig. 1*A*). Selective uptake characteristically includes a reversible and irreversible phase (10), and we have replicated this observation in SW872 cells (Fig. 1*D*). The reversible phase is proposed to include CE that has entered the plasma membrane, a compartment with a limited capacity to store CE (hence the plateau in the curve). This CE is accessible to extraction by extracellular unlabeled HDL. The plasma membrane CE is subsequently internalized and becomes inaccessible to extraction by extracellular unlabeled HDL, and this CE accumulates within the cell. Overall, these data show that SW872 cells selectively internalize [^{3}H]cholesteryl oleate from HDL.

RAP Inhibits Selective Uptake by SW872 Cells—We assessed the involvement of LRP in selective uptake by inhibition with up to 23 μg/ml RAP. We found that RAP inhibited [^{3}H]CE cell association by about 42%, whereas ^{125}I-apoA-I cell association and degradation were not changed significantly (Fig. 2*A*) indi-

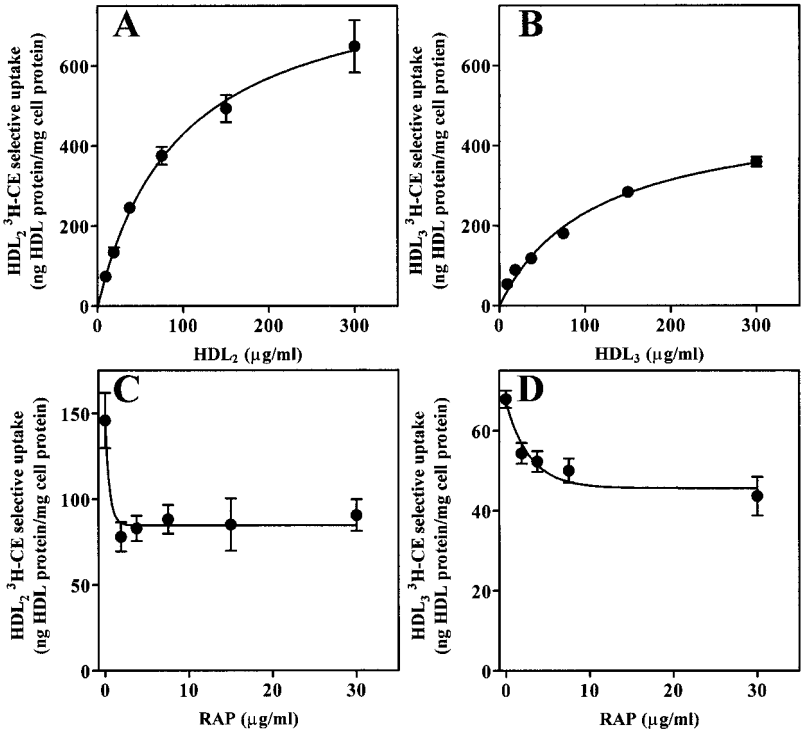

FIG. 3. **Inhibition of HDL_2- and HDL_3-selective uptake with RAP.** SW872 cells were incubated at 37 °C for 8 h with up to 300 μg/ml [^{3}H]CE-labeled or ^{125}I-apoA-I-labeled HDL_2 (*A*) or HDL_3 (*B*). The [^{3}H]CE-selective uptake was measured as described in Fig. 1 and plotted after subtraction of the ^{125}I-apoA-I cell association and degradation. To measure RAP inhibition, 20 μg/ml labeled HDL_2 or HDL_3 was incubated in the presence of the indicated concentrations of RAP (*C* and *D*), and the [^{3}H]CE selective uptake was plotted. Each point is the mean of four measurements, and the S.E. is shown.

cating that selective uptake, rather than holoparticle uptake, was being inhibited. The inhibition was not increased further by increasing RAP to 50 μg/ml. Hence, in SW872 cells there are distinct RAP-inhibitable and RAP-independent pathways contributing to selective uptake. As a positive control, the RAP was shown to inhibit effectively association and degradation of ^{125}I-α_2M by SW872 cells at 37 °C (Fig. 2*B*). The background degradation of ^{125}I-α_2M, measured in the presence of a 50-fold excess of unlabeled α_2M or 500 μg/ml suramin, was similar in SW872 cells and in HSF (not shown), but in SW872 cells the background degradation was about 50% of the total degradation because of the low LRP-dependent degradation. From these curves, half-maximal inhibition is attained when RAP is 1.7 and 1.9 μg/ml for [^{3}H]CE and ^{125}I-α_2M, respectively. The similarity of these values suggests a common site of action. This is particularly true for allosteric inhibitors such as RAP (47) whereby the effectiveness of inhibition depends only on the binding of the inhibitor to the receptor and is independent of the ligand being inhibited. In this regard, half-maximal inhibition would occur when half the receptors are occupied and the RAP inhibitory constant of selective uptake translates to a dissociation constant (K_d) of about 43 nM which is higher than, but not inconsistent with, the reported K_d of 4–20 nM for RAP binding to the LRP (48).

RAP Inhibits HDL_2- and HDL_3-selective Uptake—In Fig. 2*A*, RAP partially inhibited [^{3}H]CE-selective uptake from total HDL. To determine whether the inhibition was incomplete because RAP exclusively inhibited either HDL_2 or HDL_3, we measured the ability of RAP to inhibit [^{3}H]CE-selective uptake from these HDL subfractions (Fig. 3). We found that SW872 cells selectively internalized [^{3}H]CE from HDL_2 with a T_{max} of 106 (±8) ng of HDL protein/mg of cell protein/h and a K_m of 97 (±18) μg/ml HDL (Fig. 3*A*). In contrast, the T_{max} for HDL_3-selective uptake was 61 (±4) ng of HDL protein/mg of cell protein/h with a K_m of 111 (±14) μg/ml HDL (Fig. 3*B*). RAP inhibited HDL_2- and HDL_3-selective uptake to a maximal extent of about 38 and 36%, respectively (Fig. 3, *C* and *D*). Half-maximal inhibition is attained when RAP is 0.35 and 2 μg/ml for HDL_2 and HDL_3, respectively, showing that HDL_2 is more sensitive to RAP inhibition. This result was confirmed by two separate experiments. Because the partial inhibition of HDL-selective uptake is not due to the preferential inhibition of either HDL subfraction, further experiments were performed using total HDL.

RAP Inhibition of Selective Uptake Is Specific and Not Mediated through SR-BI—The SW872 cells express less than 10% of the levels of SR-BI expressed by normal CHO or HepG2 cells, as measured by Western blotting (not shown). Nevertheless, we determined whether the inhibition of selective uptake by RAP was mediated through SR-BI. First, we characterized the ability of SR-BI-expressing CHO cells and the SR-BI-deficient, ldl-A7 CHO cells to accumulate [^{3}H]CE selectively. SR-BI-expressing CHO cells were at least 10-fold more effective at mediating selective uptake compared with SW872 cells or the ldl-A7 cells (Fig. 4*A*). The [^{3}H]CE-selective uptake by ldl-A7 cells could be described adequately by a single class of transporters with a T_{max} of 308 (±15) ng of HDL protein/mg of cell protein/h. The SR-BI-expressing cells included an additional class of high capacity transporters with a T_{max} of 4408 (±536) ng of HDL protein/mg of cell protein/h. SR-BI was responsible for at least 96% of the selective uptake at 9.4 μg/ml HDL, but at this concentration [^{3}H]CE selective uptake was resistant to RAP inhibition (Fig. 4*B*). This indicates that RAP does not inhibit selective uptake by inhibition of SR-BI. Furthermore, it is evident that RAP does not inhibit selective uptake through a nonspecific interaction with HDL, which then prevents HDL from interacting with the cell. [^{3}H]CE-selective uptake by ldl-A7 cells was also resistant to RAP inhibition (not shown).

Suramin Inhibits Selective Uptake to the Same Extent as RAP—Another potent inhibitor of ligand binding to the LRP is the polysulfated drug suramin (49). We found that 1 mg/ml suramin or 20 μg/ml RAP inhibited selective uptake by SW872 cells by almost the same extent, 49 and 46%, respectively (Fig. 5). Half-maximal inhibition was achieved with about 38 μg/ml suramin. By 250 μg/ml suramin, the inhibition of [^{3}H]CE-selective uptake had leveled to its nadir, and this same concentration of suramin was required to fully inhibit α_2M or RAP binding to LRP (38). To test whether pretreatment of the cells with suramin could inhibit selective uptake, SW872 cells were

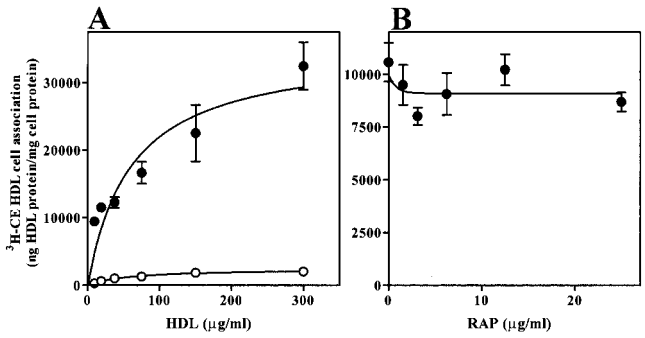

FIG. 4. **The effect of RAP on SR-BI-mediated selective uptake.** *A,* the cellular incorporation of [^{3}H]CE was measured in SR-BI-expressing CHO cells (●) and in the background ldl-A7 CHO cells (○) as described in Fig. 1, after incubation of these cells with [^{3}H]CE-labeled or ^{125}I-apoA-I-labeled HDL. The ^{125}I-apoA-I cell association and degradation is not shown but was about 20% of the value of [^{3}H]CE cell association. [^{3}H]CE-labeled HDL at 9.4 μg/ml was incubated with SR-BI-expressing CHO cells at 37 °C for 8 h the presence of the indicated concentrations of RAP (*B*) and the cell-associated incorporation of [^{3}H]CE was measured and plotted as described in Fig. 1. Each point is the mean of four measurements, and the S.E. is shown.

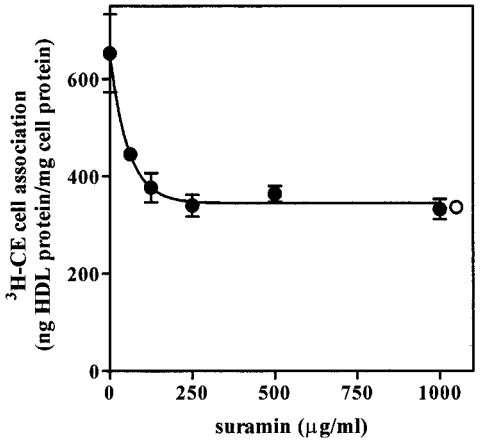

FIG. 5. **Inhibition of selective uptake with suramin.** SW872 cells were incubated at 37 °C for 8 h with 25 μg/ml [^{3}H]CE-labeled HDL in the presence of the indicated concentrations of suramin, and the cell association of [^{3}H]CE (●) was measured and plotted as described in Fig. 1. In a parallel experiment, 25 μg/ml [^{3}H]CE-labeled HDL was incubated as described above with up to 25 μg/ml RAP, and a single point at this maximal concentration is included on the graph for comparison (○). Each point is the mean of four measurements, and the S.E., which lies within the symbols in some cases, is shown.

incubated with 500 μg/ml suramin in 1 ml of ligand buffer for 1 h at 4 °C. The suramin was removed by washing the cells twice in ligand buffer, and the cells were incubated with 50 μg/ml [^{3}H]CE-labeled HDL at 37 °C for 30 min. The cellular accumulation of [^{3}H]CE was unchanged by suramin pretreatment (not shown) indicating that suramin did not affect selective uptake by irreversibly denaturing a cell surface site.

α_2M inhibits Selective Uptake to the Same Extent as RAP—Neither suramin nor RAP are specific inhibitors of LRP. Suramin intervenes in a broad range of functions (50), and RAP binds to other members of the LDL receptor family (51, 52), to the sortilin/neurotensin receptor-3 (53), and to a low affinity, high capacity site on human skin fibroblasts (38). On the other hand, cell surface binding studies indicated that α_2M specifically binds LRP. Although a distinct α_2M signaling receptor on macrophages has been postulated (54), this putative receptor apparently does not bind RAP. We therefore used α_2M as a specific inhibitor of LRP and found that it could inhibit selective uptake by about 35%, which was not statistically different from the extent of RAP inhibition measured in a parallel experiment (Fig. 6). We have also attempted to block LRP binding with polyclonal antibodies directed against this receptor. Unfortunately, the antibodies available were unable to block the cell association of ^{125}I-α_2M and were therefore ineffective inhibitors of LRP.

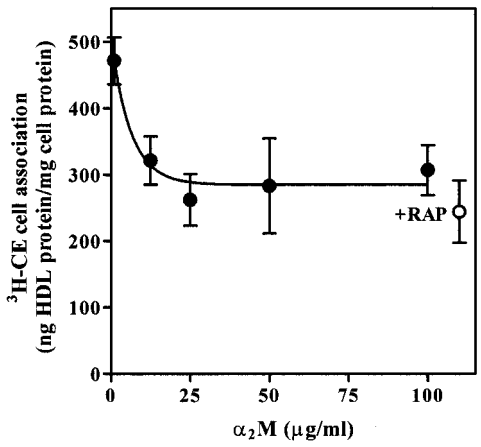

FIG. 6. **Inhibition of selective uptake with α_2M.** SW872 cells were incubated at 37 °C for 8 h with 20 μg/ml [^{3}H]CE-labeled HDL in the presence of the indicated concentrations of unlabeled α_2M, and the cell association of [^{3}H]CE (●) was measured and plotted as described in Fig. 1. In a parallel experiment, 20 μg/ml [^{3}H]CE-labeled HDL was incubated as described above with up to 25 μg/ml RAP, and a single point at this maximal concentration is included on the graph for comparison (○). Each point is the mean of four measurements, and the S.E. is shown.

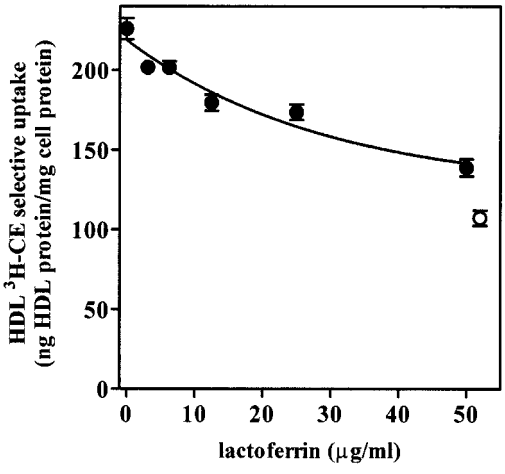

FIG. 7. **Inhibition of selective uptake with bovine Lf.** SW872 cells were incubated at 37 °C for 8 h with 25 μg/ml [^{3}H]CE or ^{125}I-apoA-I-labeled HDL in the presence of the indicated concentrations of unlabeled bovine Lf, and the [^{3}H]CE-selective uptake has been measured as described in Fig. 1 and plotted after subtraction of the ^{125}I-apoA-I cell association and degradation (●). In a parallel experiment, 25 μg/ml [^{3}H]CE or ^{125}I-apoA-I-labeled HDL was incubated as described above with 25 μg/ml RAP, and the [^{3}H]CE-selective uptake is included on the graph for comparison (○). Each point is the mean of four measurements, and the S.E. is shown.

Lactoferrin Inhibits HDL [^{3}H]CE-selective Uptake by SW872 Cells—Lf is another LRP ligand that is also a potent inhibitor of apoE and LpL binding to LRP (31). We found that bovine Lf inhibited HDL [^{3}H]CE-selective uptake and that the half-maximal inhibition was at about 20 μg/ml Lf (Fig. 7). Furthermore, non-linear regression analysis indicated that the maximal inhibition would occur at about 124 (±19) ng of HDL protein/mg of cell protein that is not significantly different from the maximal level of RAP inhibition of 107 ng of HDL protein/mg of cell protein, measured in parallel.

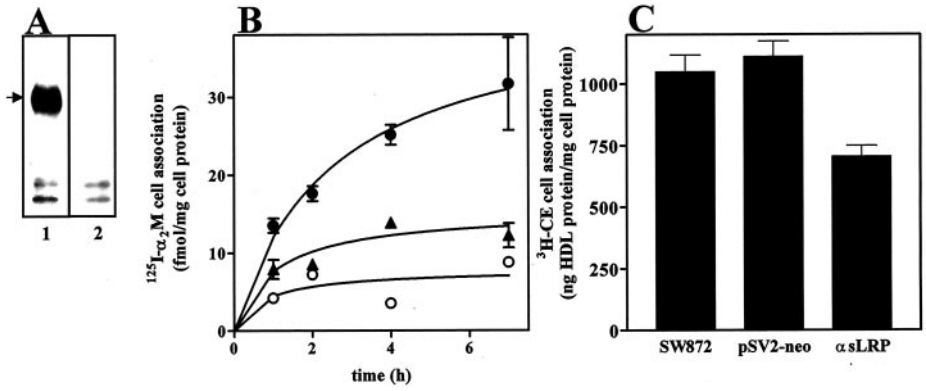

FIG. 8. **Reduced cellular LRP attenuates selective uptake.** *A,* Western blot of LRP (*arrow*) detected with polyclonal rabbit antisera against LRP in control SW872 cells (*lane 1*) but undetectable in cells stably expressing LRP antisense mRNA (*lane 2*). *B,* ^{125}I-α_2M at 1 μg/ml was incubated with SW872 cells (●) or LRP antisense-expressing cells (▲) for 7 h at 37 °C, and then the cells were washed, and the cell association of ^{125}I-α_2M was measured as described in Fig. 2. Background binding (which was similar in both cell types) was measured in the presence of 250 μg/ml suramin (○). *C,* the cell association of [^{3}H]CE was measured as described in Fig. 1 by incubating cells at 37 °C for 7 h with 25 μg/ml [^{3}H]CE-labeled HDL. The cells were either wild-type SW872 cells or SW872 cells that were stably transfected only with the vector carrying the selection marker (*pSV2-neo*) or with SW872 cells that were stably transfected with pSV2-neo and the antisense LRP vector (*αsLRP*). Background binding of ^{125}I-α_2M was a single measurement at each concentration, but all other values plotted are the mean of four measurements, and the S.E. is shown.

Reduced Cellular LRP Attenuates Selective Uptake—To confirm that LRP mediates selective uptake, we reduced cellular LRP by stable antisense expression and determined its effect on selective uptake. In the antisense-expressing cells, cellular LRP was reduced to undetectable levels according to Western blotting (Fig. 8*A*), and the cellular association of α_2M at 37 °C confirmed that LRP was reduced to at least 14% of control levels (Fig. 8*B*). The LRP antisense-expressing cells also had a reduced ability (33%) to accumulate selectively [^{3}H]CE compared with control SW872 cells (Fig. 8*C*) or to cells that had been stably transfected with the vector carrying the selection marker (pSV2-neo). These results were confirmed by repeating the experiment twice with different antisense-expressing cells (not shown). Overall, these results confirm that LRP contributes to selective uptake.

Inhibition of Selective Uptake by Primary Human Adipocytes with Combinations of RAP, α_2M, Lf, and Suramin—To confirm that LRP-dependent selective uptake is physiologically important and not merely observed in the SW872 liposarcoma cells, we measured the ability of RAP, α_2M, Lf, and suramin (individually or in paired combinations) to inhibit selective uptake in primary human adipocytes. Primary human adipocytes were obtained from healthy subjects undergoing reduction mammoplasty for cosmetic purposes and preadipocytes were isolated, seeded into 24-well plates, and differentiated (41). HDL-CE-selective uptake was measured in the presence of the various inhibitors (Fig. 9). Compared with control values, selective uptake was inhibited by around 40% by each of these inhibitors. The use of two inhibitors simultaneously did not yield further decreases, indicating that the inhibitors are blocking the same LRP-dependent transport mechanism. This experiment was repeated with primary, human abdominal adipocytes obtained from Zen-Bio with essentially the same results.

LRP Is Contributory but Not Sufficient for Selective Uptake—A significant portion, but not all, of adipocyte-selective uptake is dependent on LRP. Thus, we determined whether LRP alone was sufficient to mediate the LRP-dependent fraction of selective uptake. Human skin fibroblasts express about 20-fold more cell surface LRP compared with SW872 cells (Fig. 10*A*). If about one-third of the selective [^{3}H]CE accumulation by SW872 cells can be attributed to the LRP alone, then HSF should mediate selective uptake at least 6.6-fold (0.33 × 20) more than the total selective uptake by SW872 cells. Yet we found that HSFs were essentially unable to mediate selective uptake compared with SW872 cells (Fig. 10*B*) indicating that

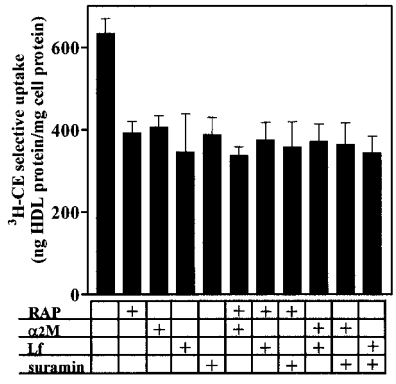

FIG. 9. **Inhibition of selective uptake by primary human adipocytes with combinations of RAP, α_2M, Lf, and suramin.** Primary adipocytes were incubated with 25 μg/ml [^{3}H]CE-labeled or ^{125}I-apoA-I-labeled HDL in HBSS, 25 mM Hepes, 5 mg/ml BSA, pH 7.45, for 8 h. The [^{3}H]CE-labeled HDL was also incubated with one or combinations of two of the following inhibitors (as indicated): 30 μg/ml RAP, 100 μg/ml α_2M, 100 μg/ml Lf, or 250 μg/ml suramin. The [^{3}H]CE-selective uptake was measured as described in Fig. 1 and plotted after subtraction of the ^{125}I-apoA-I cell association and degradation. Each value plotted is the mean of four measurements, and the S.E. is shown. This experiment was repeated with primary subcutaneous adipocytes obtained from Zen-Bio with essentially the same results.

LRP is not sufficient for the LRP-dependent pathway of selective uptake and that at least one other co-mediator, provided by SW872 cells but deficient in HSF, is necessary.

We have attempted to demonstrate a direct interaction between LRP and the HDL used in our studies, with negative results. First, binding of HDL to the SW872 cell surface at 4 °C was not inhibited by RAP (not shown). Second, we failed to detect binding between HDL and purified LRP by surface plasmon resonance although binding of RAP to LRP was confirmed (not shown). When [^{3}H]CE and ^{125}I-apoA-I-labeled HDL (at 25 μg/ml) were incubated for 8 h with SW872 cells at 8 °C, selective uptake was observed confirming that selective uptake occurs at this temperature (18), but under these conditions the selective uptake of [^{3}H]CE from HDL was not inhibited by RAP (not shown).

ApoE, but Not LpL, Is a Candidate Co-mediator of LRP-dependent Selective Uptake—The ability of Lf to inhibit selective uptake (Fig. 7) implicates apoE or LpL as co-mediators of LRP-dependent selective uptake because both these ligands are known to mediate selective uptake, and the binding of these

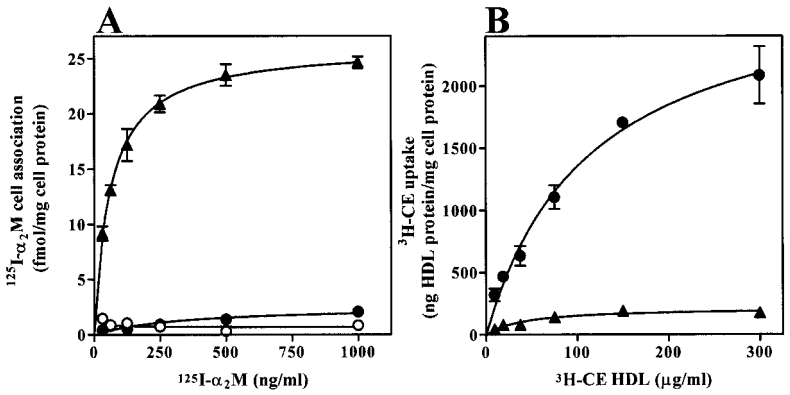

FIG. 10. **LRP is not sufficient for selective uptake.** *A,* to measure cell surface LRP levels, cells were incubated at 4 °C for 2 h with up to 1 μg/ml ^{125}I-α_2M, and after washing the cells, the cell surface binding was quantified in HSF (▲) and SW872 cells (●). Background binding (which was similar in both cell types) was measured in the presence of 250 μg/ml suramin (○). *B,* SW872 cells (●) or HSF (▲) was incubated at 37 °C for 8 h with up to 300 μg/ml [^{3}H]CE-labeled or ^{125}I-apoA-I-labeled HDL, and the cellular incorporation of [^{3}H]CE was measured and plotted as described in Fig. 1. The values for SW872 cells have already been plotted in Fig. 1*A,* and for clarity the cell association of ^{125}I-apoA-I has not been plotted. The incorporation of ^{125}I-apoA-I in HSF (not plotted) was the same as the incorporation of [^{3}H]CE. Background binding of ^{125}I-α_2M was a single measurement at each concentration, but all other values plotted are the mean of four measurements, and the S.E. is shown.

ligands to LRP is inhibited by Lf (31). Furthermore, SW872 adipocytes express about 600 ng of LpL/mg of cell protein and 30 ng of apoE/mg of cell protein according to semi-quantitative Western blotting (not shown). Addition of exogenous LpL or apoE accentuated selective uptake by SW872 cells (Fig. 11). In the absence or presence of LpL, RAP reduced HDL-CE selective uptake by about the same degree (by 125 and 110 ng of HDL protein/mg of cell protein, respectively) indicating that the LpL-stimulated selective uptake was not inhibited by RAP (Fig. 11*A*). In contrast, RAP inhibited the apoE-stimulated selective uptake in addition to the level of inhibition observed in the absence of apoE (Fig. 11*B*). This implicates apoE as a likely co-mediator of LRP-dependent selective uptake.

Overall, these data demonstrate that LRP and another co-mediator (most likely apoE) on SW872 cells and primary human adipocytes are partially responsible for the selective accumulation of [^{3}H]CE from HDL.

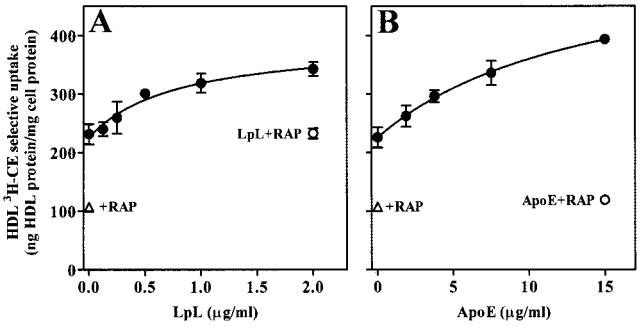

FIG. 11. **LpL or apoE-stimulated selective uptake in the absence or presence of RAP.** SW872 cells were incubated at 37 °C for 8 h with 25 μg/ml [^{3}H]CE or ^{125}I-apoA-I-labeled HDL in the presence of the indicated concentrations of unlabeled bovine LpL (*A*) or human apoE (*B*), and the [^{3}H]CE selective uptake (●) was measured as described in Fig. 1 and plotted after subtraction of the ^{125}I-apoA-I cell association and degradation. The [^{3}H]CE-selective uptake was also measured in the presence of 25 μg/ml RAP either in the absence of LpL or apoE (Δ) or in the presence of either 2 μg/ml LpL or 15 μg/ml apoE (○). Each point is the mean of four measurements, and the S.E. is shown.

DISCUSSION

Selective uptake has been defined as a process distinct from the classical receptor-mediated pathway leading to holoparticle internalization. Consequently, the possible role of endocytic receptors in selective uptake has been neglected. The implication of HDL recycling in selective uptake (24) has prompted us to examine the role played by the endocytic receptor, LRP. The LRP was studied because a number of ligands that it binds, such as apoE (28, 29), LpL (30, 31), and HL (32), also mediate selective uptake (19–22). In this paper we have examined the involvement of LRP in CE-selective uptake from HDL by primary human adipocytes and human SW872 liposarcoma cells. We have shown that antagonists of LRP ligand binding inhibit selective uptake by 35–50%. These antagonists include RAP, suramin, α_2M, and Lf. The effect of RAP at least is not mediated through SR-BI. Furthermore, reduction in cellular LRP to 14% of control levels, by stable antisense expression in SW872 cells, leads to a 33% reduction in selective uptake. The ability of RAP, α_2M, Lf, and suramin to inhibit selective uptake by primary human adipocytes by about 40% suggests that LRP may contribute physiologically to HDL-CE-selective uptake in adipocytes. In addition, the use of paired combinations of inhibitors did not yield further decreases indicating that the inhibitors are blocking the same LRP-dependent transport mechanism. Nevertheless, LRP by itself is not sufficient to mediate selective uptake, and we show that apoE (but not LpL) is a likely co-mediator given that selective uptake mediated by exogenous apoE can be inhibited by RAP. The possible involvement of LRP in LpL and hepatic lipase-mediated selective uptake by hepatic cells was examined (55, 56) with negative results, confirming our data with LpL.

How could an endocytic receptor mediate selective rather than particle uptake? During the preparation of this manuscript, Swarnakar *et al*. (57) demonstrated that LDL-CE (but not HDL-CE)-selective uptake by apoE-expressing adrenocortical cells is dependent on apoE and can be inhibited by RAP and α_2M. Based on these observations, the authors proposed that LRP contributes to LDL-selective uptake by tethering the lipoprotein. We do not believe that tethering explains the LRP-dependent selective uptake of HDL-CE because RAP was unable to inhibit selective uptake at 8 °C, a temperature that permits selective uptake (our data and Ref. 18) but does not permit membrane trafficking- suggesting that LRP trafficking is required. We favor three other models. (*a*) HDL may acquire apoE from the cell surface enabling LRP to bind and internalize the HDL to an intracellular compartment where transfer of CE is enhanced, as is implied by the recycling model of HDL-selective uptake (24). The CE-depleted HDL may then return to the cell surface (possibly still attached to the LRP) and be resecreted into the media. We know that apoE-enriched HDL will bind and then be internalized by LRP (58), and LRP recycling has been demonstrated (59). Indeed, the LRP has

been proposed to contribute to the recycling through the Golgi of apoE-containing very low density lipoprotein (60). Nevertheless, it remains to be demonstrated that LRP can recycle HDL. (*b*) Alternatively, cell-surface apoE may bind transiently to HDL and microsolubilize some of the HDL lipids (particularly CE) when it dissociates. This is analogous to the model of apolipoprotein-mediated lipid microsolubilization proposed as a mechanism of cholesterol efflux (61). As it applies to LRP-dependent selective uptake, the CE-lipidated apoE would then bind LRP and be internalized independently of the HDL apolipoproteins. (*c*) According to a third model, molecules such as RAP may accumulate intracellularly and interfere with the ability of LRP to recruit mediators of selective uptake (such as apoE) to the cell surface. All of these models may include the participation of proteoglycans. Indeed, the interaction of apoE (33), LpL (34), and hepatic lipase (32) with LRP is facilitated by proteoglycans, and proteoglycans contribute to selective uptake (20). The study with apoE-expressing adrenocortical cells (57) showed that LDL-CE-selective uptake required chondroitin sulfate proteoglycans in addition to apoE and LRP. In that study and in contrast to the present study, HDL-CE-selective uptake was not inhibited by RAP, suggesting that apoE and LRP may not be sufficient to mediate HDL-CE-selective uptake. HDL-CE-selective uptake may require another a class of proteoglycan that was not expressed by the adrenocortical cells. We are currently investigating the mechanism of LRP-dependent selective uptake and the possible contribution of proteoglycans.

Selective uptake of HDL-CE is a significant pathway for cholesterol delivery to human adipocytes that have a very low capacity for cholesterol synthesis. SR-BI, the well defined HDL receptor mediating selective uptake in adrenal cells and liver, is expressed at very low levels in adipocytes and human liposarcoma cells. We demonstrated previously (8) that CETP facilitates selective uptake in human adipocytes. The present study demonstrates that LRP also contributes to selective uptake in adipocytes and identifies a novel role for this receptor beyond that of ligand uptake and degradation. Whether these different mediators of selective uptake involve mechanistically distinct processes remains to be determined.

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