HDL Uptake by Scavenger Receptor SR-BII

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Abstract.

Scavenger Receptor BI (SR-BI) mediates selective uptake of HDL lipids. It is unclear whether this process occurs at the cell membrane or via endocytosis. Our group previously identified an alternative mRNA splicing variant of SR-BI, named SR-BII, with an entirely different, yet highly conserved cytoplasmic carboxy-terminus. In this study we aimed to compare HDL uptake by both isoforms. Whereas SR-BI was mainly (~70%) localized on the surface of transfected CHO cells, as determined by biotinylation, HDL binding at 4°C, and studies of GFP-tagged SR-BI/II fusion proteins, the majority of SR-BII (~80-90%) was expressed intracellularly. The cellular distribution of SR-BI was not affected by deletion of the C-terminus, which suggests that the distinct C-terminus of SR-BII is responsible for its intracellular expression. Pulse-chase experiments showed that SR-BII rapidly internalized HDL protein, whereas in case of SR-BI most HDL protein remained surface bound. Like its ligand, SR-BII was more rapidly endocytosed compared to SR-BI. Despite more rapid HDL uptake by SR-BII than SR-BI, selective cholesteryl ether uptake was significantly lower. Relative to their levels of expression at the cell surface, however, both isoforms mediated selective uptake with similar efficiency. HDL protein that was internalized by SR-BII largely co-localized with transferrin in the endosomal recycling compartment (ERC). Within the ERC of SR-BII cells there was extensive co-localization of internalized HDL lipid and protein. These results do not support a model that selective lipid uptake by SR-BI requires receptor/ligand recycling within the cell. We conclude that SR-BII may influence cellular cholesterol trafficking and homeostasis in a manner that is distinct from SR-BI.
Introduction.

Scavenger Receptor BI (SR-BI), a major HDL receptor (1-3), plays an important role in reverse cholesterol transport, a major pathway for the clearance of excess cholesterol from the body. In this process, peripheral cholesterol is packaged into HDL from which it is subsequently removed in the liver and excreted into bile. SR-BI mediates uptake and biliary secretion of HDL cholesterol by the liver (4,5). Therefore, and perhaps also for other reasons, SR-BI is of great importance in the prevention of atherosclerosis (6). SR-BI is most highly expressed in liver and steroidogenic tissues (4) where it mediates selective uptake of cholesterol from HDL, i.e. without concomitant uptake and degradation of HDL apoproteins. The detailed mechanism of this process has not been elucidated yet, including whether it occurs at the cell membrane (7) or during endocytosis and retro-endocytosis of HDL (8). SR-BI is known to influence membrane structure (9,10) and membrane cholesterol distribution (11,12), which all may contribute to SR-BI-facilitated selective lipid uptake.

SR-BI is a ~82 kDa protein with two short cytoplasmic termini, two transmembrane domains, and a large, heavily glycosylated extracellular loop (2). In non-polarized cultured cells, only the extracellular loop of SR-BI is essential for selective uptake (13-15); both the N- and C-terminal cytoplasmic tails can be deleted or exchanged with the corresponding region of the other class B receptor CD36 (14,15). Co-operation of other proteins with SR-BI seems not to be essential for this process, since mere liposomes containing purified SR-BI can selectively absorb HDL-cholesterol (16). However, in polarized hepatocytes, the interaction of the C-terminus of SR-BI with a protein called CLAMP, or PDZK1, is required for cell surface expression of the receptor (17,18). A
domain consisting of the three C-terminal amino-acids in murine SR-BI, Arg-Lys-Leu, is required for binding PDZK1. Lack of the terminal leucine residue results in decreased surface expression of SR-BI in the mouse hepatocyte, and consequently to impaired HDL clearance (18).

Previously, our group discovered an alternative mRNA splicing variant of SR-BI, called SR-BII, which differs from SR-BI by an entirely different C-terminal cytoplasmic tail (19,20). In liver, adrenals, adipose- and testicular tissue, SR-BII mRNA levels are comparable to the SR-BI isoform, though protein levels are lower (19). Nevertheless, in the liver, SR-BII accounts for some 10-15% of SR-BI/II protein, and in rats treated with 17\(\beta\)-estradiol, SR-BII in liver is substantially upregulated (21). SR-BII protein was also detected in human retinal epithelial cells (22) and in rat Leydig cells (23). Another indicator for a possible biological function of SR-BII is the high degree of conservation of the C-terminus between various species (19).

The presence of an entirely different C-terminus in SR-BII is intriguing in light of the reported critical importance of the corresponding domain in the cellular distribution of SR-BI. In the present study we investigated SR-BII expression in more detail, focusing on its cellular distribution and interaction with HDL. Our data show that SR-BII is mainly expressed intracellularly in non-polarized (CHO) cells, with reduced selective uptake capacity. In contrast to SR-BI, SR-BII appeared to mediate the rapid internalization of HDL particles and their accumulation in the transferrin-positive endosomal recycling compartment (ERC). Our results suggest that selective lipid uptake by SR-BI does not require receptor/ligand recycling. SR-BII may influence cellular cholesterol trafficking and homeostasis in a manner distinct from SR-BI.
**Materials and Methods.**

**Cells**

CHO-A7 cells (donated by Dr. M. Krieger, M.I.T.) were grown in 250 ml tissue culture flasks in Ham’s F12 medium (Gibco, Carlsbad, CA) containing 5% heat-inactivated fetal calf serum ("FCS", Gibco). MDCK and COS cells (both American Type Culture Collection, Manassas, VA) were grown in DMEM (Gibco) containing 10% FCS. Both media were supplemented with 2 mM L-Glutamine, 0.1 mM non-essential amino acids, 50 U/L penicillin-G and 50 µg/L streptomycin (all Gibco). Cells were sub-cultured until 70% confluency was reached, using a 1:5 split ratio. Stably transfected CHO cells expressing murine SR-BI or SR-BII were generated as described elsewhere (19) and were cultured in medium containing 0.25 g/l genitin (Gibco). Clones were screened for expression of the scavenger receptors by immunoblotting with a rabbit polyclonal antibody recognizing the common extracellular domain ("red-1"; receptor extracellular domain 1) (19); one of each clone was selected based on similar expression levels ("CHO-SRBI" and "CHO-SRBII"). COS cells were transiently transfected with pCMV5-based expression vectors (24) (1 µg DNA per ml) using the lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) and the cells were used experimentally 16 hours after transfection. For biochemical assays, CHO and COS cells were grown in 12 well clusters (Corning Corp., Corning, NY). For microscopy, CHO and COS cells were grown on glass cover slips, MDCK cells on Transwell Clear® filter membranes (Corning).
Isolation and labeling of lipoproteins

Human HDL (d 1.063 to 1.21 g/ml) fractions were isolated from fresh human plasma by density gradient ultracentrifugation as previously described (25). Human HDL₃ (d = 1.13 - 1.18 g/ml) was obtained from total HDL by density gradient fractionation. All isolated fractions were dialyzed against 150 mM NaCl-2.5 mM EDTA, sterile filtered, and stored under N₂ gas at 4°C.

Lipoproteins were iodinated in presence of ¹²⁵I (Amersham Pharmacia Biotech, Piscataway, NJ) by the iodine monochloride method (26). HDL-associated cholesteryl ester was traced with non-hydrolysable [1, 2(n)-³H] cholesteryl oleoyl ether (Amersham Pharmacia Biotech) according to the method of Gwynne and Mahaffee (27), with the following modifications: [1, 2(n)-³H] cholesteryl oleoyl ether was dried in a 12x75 mm glass borosilicate tube (20 µCi per mg HDL protein) after which HDL and partially purified cholesteryl ester transfer protein were added. Following 16 hours incubation at 37°C, HDL was re-isolated by ultracentrifugation at a density of 1.21 g/ml. The specific activity of the [³H] HDL ranged from 10-30 dpm/mg protein. Labeled lipoproteins were verified by SDS-PAGE and gradient gel electrophoresis.

HDL₃ lipid was labeled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) as described elsewhere (1) and protein with Alexa 488 (Molecular Probes) according to manufacturer’s instructions, and were stored at 4°C under N₂ gas.

HDL association and selective uptake

Cell association assays were performed as previously described (19), in radio-labeled lipoprotein-containing medium with 0.5% Bovine Serum Albumin (“BSA”) instead of
serum. Cells were seeded on 12-well cell culture clusters at an initial density of $1 \times 10^5$ cells/cm$^2$. When cells reached confluency, they were either incubated at 37º C for indicated times or at 4ºC for 2 h with double-labeled HDL$_3$, in the latter case with HEPES-buffer instead of bi-carbonate. Medium was removed and cells were washed four times with cold buffer (50 mM Tris-HCl, 150 mM NaCl (pH 7.4)) containing BSA (2 mg/ml) followed by two washes in the same buffer without BSA. Cells were then solubilized in 0.1 N NaOH for 60 min at room temperature, and proteins and radioactivity were measured in the lysate. $^{125}$I represented HDL protein association according to the protein tracer, $[^3H]$ that according to the cholesteryl-ether tracer. The trichloracetic acid-soluble degraded material in cell media was assayed as described (19). Values are expressed as apparent HDL protein uptake assuming the uptake of intact holoparticles. This was done to compare association of both tracers on the same basis. Selective uptake is defined as $[^3H] - ([^{125}I]$ cell associated + $[^{125}I]$ degraded) and represents the uptake of cholesteryl ester that cannot be accounted for by the internalization of intact particles. SR-B1/II- specific values were calculated as the difference between CHO-SR-B1/II and CHO-A7 values.

**Biotinylation of SR-B1 and SR-BII**

Biotinylation was carried out as described elsewhere (28), with slight modifications. CHO-cells were incubated in serum-free Ham’s F12 + 0.5% BSA for 2 h prior to the experiment. After washing cells 5 times with cold PBS containing 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$ (“PBS-CM”), cells were incubated on ice twice for 20 min each with 0.5 mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL), in PBS-CM. Unbound biotin was inactivated and removed by a 5 min wash with Tris-buffered saline followed by two
washes with PBS-CM. Cells were solubilized with cold MES-buffered saline with 1% Triton X-100 and 1.75% n-octyl-Beta-D-glucopyranoside ("MBSTOG") supplemented with Complete-Mini® protease inhibitors (Roche, Indianapolis, IN). Protein concentrations of cell lysates were estimated with a bicinchoninic acid assay (Pierce), whereupon 5 µg protein lysate were incubated with the pellet of 50 µl immobilized, BSA-blocked streptavidin beads (Sigma, St. Louis, MO) for 16 h, at 4°C. The supernatant (representing intracellular proteins) and the pellet (representing surface proteins), the latter 2x washed with 0.5 ml MBSTOG, were analyzed by SDS-PAGE and immunoblotting using the red-1 antibody.

In order to study potential redistribution of receptor in response to exposure to HDL, cells were incubated overnight with medium containing 0.5% BSA instead of serum, followed by 5 h in the absence or presence of 50 µg/ml unlabeled human HDL₃. Biotinylation was performed immediately after this incubation.

**Uptake of HDL and transferrin**

Cells were grown on glass cover slips until 50-70% confluency and were incubated in serum-free medium containing 0.5% BSA for 1 h at 37°C before addition of diI- or Alexa-labeled HDL₃ (10µg/ml, unless indicated otherwise). Cells were either continuously labeled with ligand at 37°C, or for 2 h at 4°C followed by cold washes with PBS-CM and a subsequent 37°C chase with complete medium. After the incubations, cells were washed 5 times with cold PBS-CM and fixed in 1% paraformaldehyde in PBS. Cells were then mounted on glass slides with Vectashield mounting medium (Vector Labs, Burlingame, CA) and analyzed with an Olympus BX51 fluorescence microscope or a Leica TCS confocal laser scanning fluorescence microscope.
To visualize the endosomal recycling compartment, cells were incubated for 1 h with Alexa-568-labeled human transferrin (Probes), at a final concentration of 50 µg/ml.

**Internalization of SR-BI and SR-BII**

COS cells grown on glass cover slips till 50% confluency were transfected with the pCMV-5 based vector encoding the appropriate scavenger receptor and were incubated overnight in complete medium. Before the experiment, cells were incubated in serum-free medium with 0.5% BSA for 1 h at 37°C and put on ice. Cells were then incubated with red-1 antibody (1:200) in PBS + 0.5% BSA for 30 min, at 4°C. Cells were washed with PBS-CM, incubated with complete medium for various times at 37°C, washed with cold PBS-CM to prevent further endocytosis, fixed in cold 4% paraformaldehyde in PBS and permeabilized with cold 0.1% Triton X-100 in PBS for 10 min. Thereafter, secondary antibody, Cyanine Cy2 (green)-conjugated anti-rabbit IgG (1:200; Jackson Immunoresearch Laboratories, West Grove, PA) was added for 30 min. Cells were mounted on glass slides in Vectashield mounting medium and were observed under a confocal laser scanning microscope.
**Construction of GFP fusion proteins**

pCMV-5-based expression vectors encoding SR-BI or SR-BII (19) were PCR amplified with the forward oligo 5’-CAT AGA TCT ATG GGC GGC AGC TCC AGG GCG-3’ and the reverse oligos 5’-TAT AGG GCC CTA TAG CTT GGC TTC TTG-3’ and 5’-GCC TGA GGG CCC TCA GGC CAT GGC ACT GGT GGG-3’, respectively. The resulting products where digested with BglII and ApaI and introduced into similarly digested pEGFP-C1 (Clontech, Palo Alto, CA). The resulting recombinant expression vector encodes EGFP-SRBI/II fusion proteins with the EGFP signal at the N-terminus of the receptor. The DNA sequence of the coding region was verified and protein expression and function was studied in transfected COS cells. All GFP-fusion proteins were found to yield SR-BI/II immunoreactive proteins of predicted size, and COS cells expressing these constructs showed HDL binding and selective uptake comparable to COS cells expressing non-GFP SR-BI/II (not shown).

**Site-directed mutagenesis**

Deletion of the C-terminus of murine SR-BI in the pEGFP-C1 expression vector was achieved by introducing a stop codon at position of Q463, employing the PCR-based Quickchange kit (Stratagene, La Jolla, CA) and primer pair 5’- GTG CCC ATC ATC TGC TAA CTG CGC AGC CAG GAG-3’ and 5’- CTC CTG GCT GCG CAG TTA GCA GAT GAT GGG CAC-3’. Immunoblotting of transfected COS-cell extracts yielded an immunoreactive protein with the predicted size (not shown).
Results.

Expression of SR-BII

Stably transfected CHO-A7 clones were screened by immunoblotting for expression of murine SR-BI or SR-BII using the red-1 antibody which detects the common extracellular domain of the receptors (19). Two lines expressing similar amounts of either SR-BI (CHO-SRBI) or SR-BII (CHO-SRBII) were selected. Surface expression of both isoforms was studied by a biotinylation approach. In CHO-SRBI cells labeled at 4°C with non-absorbable biotin, approximately ~70% of total scavenger receptor could be precipitated with immobilized streptavidin, indicating that this isoform was mainly expressed at the cell surface (Figure 1A). In the case of CHO-SRBII cells, only ~10-20% of SR-BII protein could be precipitated, indicating that this isoform is much less expressed on the cell surface, with 80-90% being expressed intracellularly. The surface expression of both receptors was unchanged by 5 h exposure of cells to HDL in the medium, as opposed to cells not exposed to HDL (Figure 1B), indicating that the presence of the ligand did not induce redistribution of receptors.

The distinct cellular distribution of SR-BI and SR-BII was confirmed by fluorescence microscopy of CHO cells expressing GFP-fusion constructs of either SR-BI or SR-BII. These fusion proteins were functionally active, as shown by their capacity to mediate selective uptake with an efficiency similar to non-GFP isoforms in transiently transfected COS cells (not shown). As shown in Figure 2A, SR-BI is highly expressed on the cell surface whereas SR-BII is expressed predominantly intracellularly with little enrichment of the cell surface being evident (Figure 2B). Interestingly, GFP-SRBI lacking the normal cytoplasmic C-terminus showed a similar cellular distribution (Figure 2C) to full-length
GFP-SRBI, suggesting that the presence of the SR-BII C-terminal cytoplasmic tail, and not the absence of the SR-BI C-terminus, is responsible for the more intra-cellular distribution of SR-BII. Figure 2D shows cells expressing only GFP protein in a diffuse intracellular manner. The marked difference in intracellular localization of SR-BI and SR-BII was also observed in stably transfected polarized MDCK cells. GFP-SRBI is predominantly expressed on the cell surface of these cells (Figure 2E), mostly on the basal membrane (Figure 2F). In contrast, GFP-SRBI is localized mainly intracellularly (Figure 2G and 2H).

**HDL binding and selective uptake**

The predominantly intracellular expression of SR-BII was in line with the observation that receptor-specific binding of HDL at 4°C was significantly lower for CHO-SRBII than CHO-SRBI cells (Figure 3A), whereas receptor levels in the two cell types were similar (data not shown). Receptor-specific cell association of HDL and selective cholesteryl-ester uptake at 37°C were also measured. Unlike HDL binding at 4°C, cell association at 37°C was similar for SR-BI- and SR-BII- expressing cells (Figure 3B). Both receptors mediated selective uptake, but this was significantly lower for SR-BII (Figure 3C). For both receptors, HDL protein degradation was low, indicating little delivery of ligand to lysosomes via typical endocytic pathways (Figure 3D). If the efficiency of selective uptake is expressed as selective uptake relative to cell associated HDL at 37°C, the efficiency of SR-BII was approximately 2-fold lower than SR-BI. However, when efficiency of selective uptake is calculated relative to the number of receptors at the cell surface, calculated from HDL binding at 4°C, the efficiencies of uptake for SR-BI and SR-BII are similar. The relatively greater difference between HDL
cell association at 37°C and 4°C in case of SR-BII, compared to SR-BI, suggests that, at 37°C, SR-BII may accumulate HDL in an intracellular compartment to a greater extent than SR-BI. Cell association and selective uptake, as a function of time, are shown in Figure 4A and B. Cell association for both SR-BI and SR-BII reached a plateau after 1-2 h (Figure 4A), whereas the selective uptake in cells continued over time (Figure 4B). These results are consistent with the previously described selective uptake process (29).

**Intracellular distribution of HDL-lipids and -proteins**

To assess possible HDL particle uptake, CHO cells were first incubated for 2 h at 4°C with HDL3 labeled with alexa-488 in the protein moiety and then washed and incubated at 37°C in medium for 1 h. Confocal laser-scanning microscopy showed surface binding of HDL at 4°C to both SR-BI- (Figure 5A) and SR-BII- (Figure 5B) expressing cells. Following incubation at 37°C, the cell-associated HDL remained largely localized at the cell surface in the case of SR-BI (Figure 5C). In contrast, significant amounts of surface-bound HDL3 were internalized by CHO-SRBII cells (Figure 5D). No significant cell association of alexa-labeled HDL was observed in CHO-A7 cells that do not express SR-BI or SR-BII (data not shown).

When cells were continuously exposed to alexa-labeled HDL3 at 37°C, for up to 120 minutes, CHO-SRBI cells accumulated limited amounts of HDL protein intracellularly, with the majority of the ligand after 30 min (Figure 5E) and 120 min (Figure 5G) being associated with the cell surface. In case of CHO-SRBII cells, significant amounts of ligand were rapidly taken up, such that the amount of surface bound ligand was small compared to intracellular ligand (Figure 5F, H).
To study HDL-particle uptake in more detail, CHO cells were incubated with a mixture of diI- and alexa 488- labeled HDL$_3$. After a one hour continuous pulse at 37°C, CHO-SRBI cells appeared to accumulate some HDL protein within the cell, yet most HDL protein remained on or in proximity to the cell membrane (Figure 6A). There appeared to be some heterogeneity in terms of protein distribution, however, with some cells showing more intracellular accumulation of HDL protein than others. Though most diI label was clearly enriched in the plasma membrane and colocalized with alexa, in the case of CHO-SRBI cells, a significant portion of diI appeared dissociated from the protein fraction and was dispersed throughout the cell (Figure 6C, E). This is consistent with selective lipid uptake mediated by SR-BI (1). In CHO-SRBII cells, in contrast, most of the HDL proteins and lipids were accumulated in a peri-nuclear region, with strong colocalization of both HDL components (Figure 6B, D, F). Though colocalization suggests that the protein and lipid fractions of HDL remain associated, at this resolution it cannot be excluded that at least partial dissociation had occurred. CHO-A7 cells that do not express either scavenger receptor isoform did not accumulate detectable amounts of ligand (not shown). Similar results on the uptake of these tracers by SR-BI and SR-BII were observed in transiently transfected COS cells (data not shown).

The cellular distribution of SRBI/II and acquired HDL was also studied in CHO cells expressing GFP-SRBI or GFP-SRBII fusion constructs following a 1 h exposure of cells to alexa-568 (red) labeled HDL at 37°C. As seen in Figure 7, SR-BI (Figure 7A) and SR-BII (Figure 7B) showed extensive co-localization with HDL proteins, with SR-BI being mainly expressed on the cell-surface and SR-BII within the cell.
These observations support the biochemical data, in that SR-BII mediates intra-cellular accumulation of HDL into a compartment in which selective uptake does not occur or occurs only slowly. In the case of SR-BI, however, lipids seem to dissociate more readily from the HDL particle, in a process that seems to occur mainly at or in proximity of the cell membrane.

**SR-BII accumulates HDL protein into a transferrin-positive endosomal recycling compartment**

To characterize the intracellular compartment into which SR-BII accumulates ligand, CHO cells were simultaneously incubated for 1 h with alexa-488 (green) labeled HDL and alexa-568 (red) labeled transferrin that is known to recycle to the plasma membrane from the endosomal recycling compartment (ERC). In case of SR-BI, little co-localization of HDL and transferrin could be observed (Figure 8A, C and E). Strikingly, as shown in Figure 8B, D and F, virtually all HDL acquired by CHO-SRBII cells co-localized with transferrin. These results indicate the internalization of HDL by SR-BII into the transferring-containing ERC occurred at a relatively rapid rate compared to SR-BI.

**Internalization of SR-BII**

Since SR-BII appeared to accumulate substantial amounts of HDL within the ERC, we assessed whether the receptor undergoes endocytosis. COS cells were transiently transfected with either SR-BI or SR-BII. Surface receptors were labeled at 4°C with the red-1 antibody that recognizes the common extracellular domain. Cells were then chased at 37°C with complete medium. Figure 9 shows that after 45 minutes, significant amounts of SR-BII antibody were found within the cell, whereas SR-BI remained mainly on the
cell-surface. These observations indicate a relatively rapid rate of internalization of SR-
BII compared to SR-BI and are consistent with the observed rapid SR-BII-mediated
endocytosis of HDL.
Discussion.

In this study we show that the C-terminal cytoplasmic tail of SR-BII, which is entirely different from that of SR-BI (20), contains a signal that leads to reduced surface expression compared to SR-BI in both polarized and non-polarized cells. Furthermore, SR-BII, but not SR-BI, undergoes rapid endocytosis and mediates intracellular accumulation of HDL in the endosomal recycling compartment (ERC). Despite the fact that SR-BII mediates cell-association of HDL at 37°C in similar amounts compared to SR-BI, much of it intracellularly, the selective uptake capacity of SR-BII is less than SR-BI. Indeed, intracellular ligand shows considerable co-localization of HDL lipids and proteins, suggesting that selective uptake from the ERC is not efficient.

Our finding that SR-BII is expressed mainly intracellularly in polarized MDCK cells is not unexpected, since a PDZ-domain in the C-terminus of SR-BI is essential for surface expression in polarized cells (18), through interaction with the “scaffolding” protein PDZk1 (17). Our finding, however, that SR-BII is mainly expressed intracellularly in CHO cells is surprising, since previous reports suggest that the C-terminus of SR-BI is not required for HDL cell-association in non-polarized cells (14,15,17,18). Thus, the C-terminus of SR-BII contains a signal that confines this isoform to the cell interior. This points to a possible interaction of the C-terminal tail of SR-BII with another cellular protein that serves to regulate its trafficking and intracellular distribution.

Previously, it was reported that SR-BII is capable of mediating selective uptake of HDL lipids, albeit at a lower efficiency (13,19). Here we used CHO clones that were carefully selected for expression of equal levels of murine SR-BI or SR-BII protein and found that
selective uptake by SR-BII was indeed less efficient, despite the fact that SR-BII expressing cells showed similar amounts of HDL protein association at 37°C compared to SR-BI expressing cells. The relatively high level of HDL protein association to SR-BII could not be accounted for by surface bound material, since biotinylation studies and HDL binding at 4°C clearly showed that less SR-BII was expressed on the cell surface than SR-BI. We therefore hypothesized that SR-BII mediates the internalization and accumulation of HDL in an intracellular pool from which selective uptake occurs only inefficiently. Our results indeed showed a marked difference in behaviour of SR-BI and SR-BII with respect to the rate at which they accumulate intracellular HDL particles. SR-BII was found to rapidly internalize HDL particles as judged by the internalization of HDL protein together with HDL lipid (Figure 6). In contrast, SR-BI mediated internalization at a markedly slower rate.

Interestingly, selective uptake by SR-BI and SR-BII was shown to correlate closely with the surface expression of SR-BI and SR-BII rather than the rate at which the two receptors accumulate HDL intracellularly. This strongly suggests that the majority of selective uptake occurs at the cell membrane rather than intracellularly during SR-BI/II-mediated recycling of HDL. This finding provides support for a current model of the mechanism of SR-BI mediated selective lipid uptake. This model suggests that selective uptake occurs exclusively at the cell surface (29-33), in a two-step mechanism involving ligand binding followed by lipid transfer from HDL at the cell-surface (14), perhaps through the formation of a hydrophobic channel through which cholesteryl esters subsequently enter the cell (7).
The majority of HDL protein that was internalized in CHO cells by SR-BII appeared in the endosomal recycling compartment (ERC). HDL protein uptake into this compartment was accompanied by the uptake of HDL lipid, indicating whole particle endocytosis into the ERC. Following exposure of cells to HDL, the amount of internalized protein reached a steady state level within about 2 h (Figure 4A), indicating that HDL recycles back to the cell surface, with little intracellular degradation of HDL apolipoproteins. Transferrin is a well-known protein that recycles through the ERC. Transferrin releases its iron load in early endosomes, and recycles back to the cell surface, together with its receptor (34). Interestingly, it was recently suggested that HDL proteins are at least partially recycled through the ERC in hepatocytes (35). Later, these findings were related to recycling of SR-BI through this compartment (8). Silver et al. then proposed an intriguing concept of SR-BI-dependent selective sorting of HDL lipids in hepatocytes, in which basolaterally expressed SR-BI would internalize HDL into a selective-sorting compartment that partially overlaps with the ERC (8). Apolipoproteins would be re-secreted basolaterally and cholesterol apically, thus providing an attractive model for SR-BI-dependent biliary cholesterol secretion. This model seems plausible in light of the well documented retroendocytosis of HDL particles in polarized cells (36-38). A potential shortfall of the study by Silver et al. was, however, that the authors could not discriminate between the movement of SR-BI and SR-BII in hepatocytes, since they used an antibody that recognizes the common extracellular domain of both receptors (8). Nevertheless, the authors showed partial co-localization of SR-BI and HDL in the ERC of stably transfected CHO-SRBI cells, an observation that we failed to repeat (data not shown). A recent study also shows SR-BI-dependent intracellular accumulation of HDL protein in a
pulse-chase experiment employing SR-BI transfected HeLa cells, although the relative amount of internalized material was rather small, given the long chase period used (4 h) (39). In our study, virtually all HDL proteins taken-up by SR-BII co-localize with transferrin, whereas co-localization in case of SR-BI was limited and occurred only after prolonged incubation. We were also unable to directly demonstrate endocytosis of the SR-BI receptor itself (Figure 9A, C). In contrast, SR-BII was endocytosed quite rapidly (Figure 9B, D). The cellular distribution of SR-BII was not altered by the presence or absence of HDL, indicating that receptor recycling was constitutive (Figure 1B).

The physiological relevance of extensive and rapid HDL internalization by SR-BII into the ERC is not yet clear. Protein levels of SR-BII are generally low, compared to SR-BI. In the liver, it is estimated to represent 10-15% of total SR-Bx protein mass (19), even though it has been reported that SR-BII may be strongly upregulated under certain conditions (21). SR-BII internalization of HDL may play an important role in previously reported cellular trafficking of HDL (8,35). Internalization of HDL and its retroendocytosis has been demonstrated in macrophages and CaCo-2 cells (36,40-43). Such HDL recycling has been reported to be important in the process of cholesterol efflux from macrophages (41) and also in the pathway by which apolipoprotein E is recycled in hepatocytes (44). It is possible that SR-BII plays a key role in mediating HDL retroendocytosis. In the liver, as discussed, SR-BI appears to play a role in biliary cholesterol secretion. Evidence has been presented that this involves HDL internalization by SR-BI and intracellular selective sorting of HDL lipid from HDL protein. In the light of our findings that SR-BII internalizes HDL at a much faster rate SR-BI, it is possible that SR-BII plays an important or major role in this pathway. Thus, SR-BII allows for
endocytic uptake of HDL lipid and protein, in a manner that is distinct from cell surface selective uptake and that allows for lipid delivery into a different subcellular compartment in which cholesterol may be processed differently.

In conclusion, we have demonstrated that the C-terminus of SR-BII, an alternative splicing-variant of the important HDL receptor SR-BI, contains a signal that leads to marked alterations in the cellular distribution and cellular trafficking of SR-BII compared to SR-BI. Surface expression of SR-BII and selective uptake capacity are reduced compared to SR-BI. The lack of correlation between the rates of SR-BI and SR-BII mediated HDL internalization and the rates of selective lipid uptake by these receptors provides strong evidence that selective lipid uptake by SR-BI occurs at the cell surface and does not require HDL internalization and recycling. In contrast to SR-BI, SR-BII mediates the rapid uptake of significant amounts of HDL into the ERC, by a pathway that is distinct from selective lipid uptake at the cell surface.
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**Abbreviations.**

BSA = Bovine Serum Albumin  
ERC = Endosomal Recycling Compartment  
FCS = Fetal Calf Serum  
GFP = Green Fluorescent Protein  
MBSTOG = MES-buffered saline with 1% Triton X-100 and 1.75% n-octyl-Beta-D-glucopyranoside  
SR-BI = Scavenger Receptor Class B Type I
References.

Figure Legends.

Figure 1. Surface expression of SR-BI or SR-BII in CHO cells.
A. CHO-SRBI and CHO-SRBII cells were biotinylated at 4°C and lysed, and 5 µg extract was subsequently incubated with immobilized streptavidin. Precipitated protein represents surface protein, the corresponding supernatant represents intracellular protein as detected by immunoblotting after SDS-PAGE of equivalent cell amounts of each fraction. B. Surface expression of SR-BI or SR-BII in CHO cells with (-HDL) or without (+HDL) 5h incubation with HDL₃ (50 µg / ml). Cells were biotinylated at 4°C after the 5h incubation, and streptavidin precipitable material from 5µg cell protein was analyzed by immunoblotting.

Figure 2. Cellular distribution of GFP-SR-BI/II fusion constructs.
Fusion proteins consisting of GFP at the N-terminus and either full-length SR-BI (A), SR-BII (B), SR-BI with deleted C-terminus (C), or GFP alone (D) were expressed in transiently transfected CHO cells. Cells were observed under a fluorescent microscope. MDCK cells, grown on permeable membranes and expressing GFP-SRBI (E and F) or GFP-SRBII (G and H) were observed by confocal laser-scanning microscopy, and the merged image of 6 planes was projected in the XYZ plane (E and G) or XZY plane (F and H), the latter allowing to distinguish the apical from the basolateral side.

Figure 3. HDL binding and selective uptake.
Cell association of [³H]/¹²⁵I double-labeled HDL₃ (5, 10, 20, 40 or 80 µg/ml) with CHO-SRBI cells (closed symbols) or CHO-SRBII cells (open symbols) at 4°C for 4 h (A) or at 37°C for 2 h (B). Receptor-specific selective uptake values from HDL₃ (5, 10, 20, 40 or
80 µg/ml) after 2 h incubation with [3H]/[125$I double-labeled HDL$_3$ are shown in C and are expressed in terms of apparent HDL protein. For both receptor isoforms, degradation of HDL proteins was equally low (D). Points represent the average of three values and are representative of two independent experiments.

**Figure 4. Time course of HDL cell association and selective uptake**

A time-course of 37°C cell-association of 10 µg/ml [3H]/[125$I double-labeled HDL$_3$ with CHO-SRBI cells (closed symbols) or CHO-SRBII cells (open symbols) is shown in A, as is receptor-specific selective uptake from these ligands (B). Points represent the average of three values and are representative of two independent experiments.

**Figure 5. Internalization of HDL protein.**

Pulse-chase of alexa-488 (green) labeled HDL$_3$ (10 µg/ml) in CHO cells expressing SR-BI (A and C) or SR-BII (B and D). A and B show confocal images after the 2 h 4°C pulse, C and D after a subsequent 1 h 37°C incubation. E and G show HDL protein after a 30 min and 120 min continuous pulse at 37°C in CHO-SRBI cells, F and H in CHO SR-BII cells.

**Figure 6. Uptake of HDL lipid and protein.**

CHO cells expressing SR-BI (A, C, E) or SR-BII (B, D, F) were incubated with a mixture of 10µg/ml each of alexa-488 HDL$_3$ (green; A and B) and diI-HDL$_3$ (red; C and D) for 1 h at 37°C. Co-localization of HDL lipid and protein is evidenced by yellow staining (E and F).
Figure 7. Co-localization of SR-BI/BII and HDL protein.

CHO cells expressing GFP-SRBI (A) or GFP-SRBII (B) were incubated at 37°C with alexa-568 (red) labeled HDL$_3$ (10 µg/ml) for 1 h. Co-localization of receptor and ligand is shown as yellow.

Figure 8. Uptake of HDL protein and transferrin.

CHO-SRBI (A, C, E) or CHO-SRBII (B, D, F) cells were incubated at 37°C for 1 h with alexa-488 labeled HDL$_3$ (green; 10 µg/ml) and alexa-568 labeled transferrin (red; 20 µg/ml). As seen in E, after one hour, little HDL was found in the endosomal recycling compartment in case of SR-BI. In case of SR-BII, however, extensive co-localization was observed (yellow signal in F).

Figure 9. Endocytosis of SR-BI and SR-BII.

COS cells were transiently transfected with either SR-BI (A, C) or SR-BII (B, D), then incubated with red-1 antiserum at 4°C for 1 h. Cells were then incubated with complete medium for 45 minutes at 37°C (C, D) or remained at 4°C (A, B). After extensive washing cells were fixed, permeabilized and incubated with fluorescent secondary antibody. Each image contains at least one cell that was not stained with the antibody, showing specificity of the antiserum. Furthermore, untransfected cells showed no fluorescent signal when incubated with red-1 and secondary antibody or secondary antibody alone (not shown).
A

SR-BI

Surface  Intracellular

SR-BII

Surface  Intracellular

B

SR-BI

-HDL  +HDL

SR-BII

-HDL  +HDL

Eckhardt et al. “HDL Uptake by Scavenger Receptor SR-BII” Figure 1
Eckhardt et al. “HDL Uptake by Scavenger Receptor SR-BII”
SR-BI

A

SR-BII

B

C

D

Eckhardt et al. “HDL Uptake by Scavenger Receptor SR-BII” Figure 9
HDL uptake by scavenger receptor SR-BII
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