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High Density Lipoprotein Endocytosis by Scavenger Receptor SR-BII Is Clathrin-dependent and Requires a Carboxyl-terminal Dileucine Motif*

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The high density lipoprotein (HDL) receptor Scavenger Receptor BII (SR-BII) is encoded by an alternatively spliced mRNA from the *SR-BI* gene and is expressed in various tissues. SR-BII protein differs from SR-BI only in the carboxyl-terminal cytoplasmic tail, which, as we showed previously, must contain a signal that confers predominant intracellular expression and rapid endocytosis of HDL. We have shown that SR-BII mediates HDL endocytosis through a clathrin-dependent, caveolae-independent pathway. Two candidate amino acid motifs were identified in the tail that could mediate association with clathrin-containing endocytic vesicles: a putative dileucine motif at position 492–493 and an overlapping tyrosine-based *YXXZ* motif starting at position 489. Although substitution of tyrosine at position 489 with alanine or histidine did not affect endocytosis, substitution L492A resulted in increased surface binding of HDL and reduced HDL particle endocytosis. Substitution L493A had a less dramatic effect. No other regions in the carboxyl-terminal tail appeared to contain motifs required for HDL endocytosis. Substitutions of leucine at position 492 with the hydrophobic amino acids valine or phenylalanine also reduced HDL endocytosis, stressing the importance of leucine at this position. Introducing the SR-BII YTPLL motif into the carboxyl-terminal cytoplasmic tail of SR-BI converted SR-BI into an endocytic receptor resembling SR-BII. These results demonstrated that SR-BII differs from SR-BI in subcellular localization and trafficking and suggest that the two isoforms differ in the manner in which they target ligands intracellularly.

Scavenger Receptor BI (SR-BI),[2] a member of the CD36 receptor family, is considered to be the major receptor for high density lipoproteins (HDL) and mediates selective lipid uptake from lipoproteins in a variety of tissues (1–4). During the selective uptake process, only lipids are taken up from lipoproteins; apolipoproteins are not degraded (5). There is uncertainty as to whether the selective uptake process occurs at the cell surface or during uptake and retro-endocytosis of the ligand. In polarized cells, such as hepatocytes and transfected Madin-Darby canine kidney cells, evidence points toward SR-BI-dependent retro-endocytosis of at least part of the internalized HDL (6–8), although it is still unclear whether, or to what extent, endocytosis is required for selective uptake. In non-polarized cells, such as CHO, COS, and human embryonic kidney cells, selective uptake appears to occur at the cell membrane (9–12) and, as shown recently, does not require endocytosis (13). Furthermore, selective uptake could be observed in artificial membranes containing only purified SR-BI (14).

The *SR-BI* gene gives rise to at least two mRNA splice variants (15, 16). The SR-BII variant is the main isoform in terms of mRNA levels in some tissues, exceeding SR-BI mRNA levels by up to 48-fold in brain cells (17), and protein levels reach 10–15% of SR-BI in the liver (16). SR-BII immunoreactivity has also been detected in significant amounts in other tissues, such as testes (18), retinal pigment epithelial cells (19), and rabbit spleen, where protein levels of SR-BII are relatively high when compared with SR-BI (20). Interestingly, the SR-BII isoform differs from SR-BI only in its entirely different, yet highly conserved, cytoplasmic carboxyl terminus. We recently showed that the carboxyl terminus of SR-BII must contain a signal that mediates predominantly intracellular expression in CHO cells and polarized Madin-Darby canine kidney cells (21). However, through endocytosis, SR-BII mediates the accumulation of significant amounts of ligand intracellularly, largely within the transferrin-positive endosomal recycling compartment (21). Selective uptake of cholesteryl ether from endocytosed HDL was rather inefficient, however, and virtually all of the selective uptake mediated by SR-BII could be accounted for by the surface pool of the receptor (21). The physiological significance of retro-endocytosis of lipoproteins via the SR-BII pathway remains unclear.

In this study, we aimed to identify the mechanism by which SR-BII mediates uptake of lipoproteins as well as the signal in the carboxyl terminus that is responsible for its endocytic activity. Alignment of predicted and published SR-BII protein sequences identified two candidate motifs: a dileucine motif, which was conserved among all species, and an overlapping tyrosine-based motif, which was less conserved. It was found that SR-BII endocytosis occurs in a clathrin-dependent, caveolae-independent fashion. The conserved dileucine motif in the carboxyl terminus of SR-BII appeared essential for endocytosis, whereas the tyrosine residue was not required. No other motifs affecting endocytosis were found in the carboxyl terminus of SR-BII. Interestingly, introduction of the dileucine motif into the carboxyl terminus of SR-BI resulted in endocytosis of SR-BI.

EXPERIMENTAL PROCEDURES

Cell Lines—CHO-A7-based clones expressing similar amounts (21) of either mouse SR-BI or SR-BII protein ("CHO-SRBI" and "CHO-SR-BII") were grown in Ham's F12 medium supplemented with 5% heat-

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[2] The abbreviations used are: SR-Bx, Scavenger Receptor class B, type I or II; HDL, high density lipoproteins; CHO, Chinese hamster ovary.

inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin-G, 100 mg/liter streptomycin, and 0.25 g/liter Geneticin (all from Invitrogen). CHO-A7 cells used for transient transfections were maintained in the same medium but without Geneticin.

Isolation and Labeling of Ligands—Human HDL_3 (d = 1.13 − 1.18 g/ml) was obtained from human plasma (donated by volunteers following a protocol approved by the University of Kentucky Internal Review Board) by KBr density gradient fractionation as described before (22), dialyzed against 150 mM NaCl, 0.01% EDTA, sterile-filtered, and stored under nitrogen gas at 4 °C. HDL_3 apolipoproteins were labeled by iodination in presence of ^{125}I (Amersham Biosciences) by the iodine monochloride method (23). HDL-associated cholesteryl ester was traced with non-hydrolyzable [1,2(n)-^3H]cholesteryl oleoyl ether (Amersham Biosciences) according to the method of Gwynne and Mahaffee (24), with the following modifications: [1,2(n)-^3H]cholesteryl oleoyl ether was dried in a 12 × 75-mm glass borosilicate tube (20 μCi/mg of HDL protein), after which HDL and partially purified cholesteryl ester transfer protein were added. Following a 16-h incubation at 37 °C, HDL was refloated by ultracentrifugation at a density of 1.21 g/ml. The size and apolipoprotein integrity of labeled lipoproteins were verified by SDS-PAGE and non-denaturing gradient gel electrophoresis. The specific activity typically ranged between 50–100 and 3–10 dpm/ng of protein for ^{125}I and ^3H, respectively. For microscopy studies, fatty acid-free albumin or the protein fraction of HDL_3 was labeled with Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

HDL Cell Association and Selective Uptake—Cell association assays were performed in radiolabeled lipoprotein-containing medium with 0.5% bovine serum albumin instead of serum as described previously (16). CHO-A7 cells were seeded on 12-well culture clusters at an initial density of 1×10^5 cells/cm^2 and were grown until ~50% confluency was reached. Plasmids were introduced with FuGENE 6 (Roche Diagnostics; 0.5 μg DNA and 1.5 μl FuGENE/well) and were grown for an additional 48 h. Thereafter, the cells were either incubated with double-labeled HDL_3 at 37 °C for indicated times or incubated with double-labeled HDL_3 at 4 °C for 2 h, in the latter case with HEPES-buffered medium. Medium was removed, and cells were washed four times with ice-cold buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing albumin (2 mg/ml) followed by two washes in the same buffer without albumin. Cells were then solubilized in 0.1 N NaOH for 60 min at room temperature, and protein content and radioactivity were measured in this lysate; ^{125}I represented HDL protein association according to the protein tracer, and ^3H represented cholesteryl ester accumulation, calculated from cell-associated cholesteryl-ether tracer. The non-iodide, trichloroacetic acid-soluble ^{125}I in cell media corresponding to degraded ^{125}I-apolipoprotein was assayed as described (16). 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. Receptor-specific values were calculated as the difference between CHO-SR-B*x* and CHO-A7 values. Selective uptake efficiency was defined as the amount of selective uptake normalized for total (*i.e.* surface bound and internalized) cell association of ^{125}I-HDL.

Inhibition of Endocytosis—The formation of clathrin-coated pits was disrupted by different treatments. Potassium depletion was performed according to the method of Larkin *et al.* (25), and the hypertonic shock treatment, in 40% sucrose, was performed according to the method of Heuser and Anderson (26). Caveolae-dependent endocytosis was inhibited by preincubating cells for 2 h with serum-free medium containing 200 μM genistein (Sigma) (27).

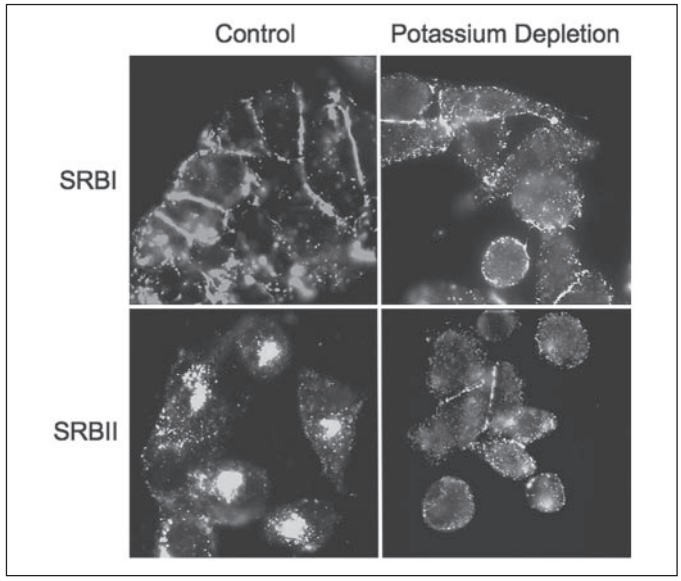

FIGURE 1. **Effect of disruption of clathrin-coated pit formation on HDL endocytosis.** CHO-SRBI and -SRBII cells were incubated in normal or potassium-depleted medium according to a previously published procedure (25) and subsequently incubated with Alexa Fluor-labeled HDL_3 for 30 min, at 37 °C, fixed in paraformaldehyde, and visualized by fluorescence microscopy. Non-transfected CHO-A7 cells did not bind Alexa Fluor-HDL_3 (Fig. 5) (Ref. 21 and data not shown).

Generation of Point Mutations in SR-Bx—Point mutations were introduced in the carboxyl terminus of full-length murine SR-BI or SR-BII cDNA that had been subcloned into pCMV5 or pcDNA5 FRT/TO (Invitrogen). Amino acid substitutions were introduced with the PCR-based QuikChange kit from Stratagene, using synthetic DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) carrying the desired mutation. Mutants were verified by DNA sequence analysis, and the predicted protein size (~82 kDa) was verified by immunoblotting using the Red-1 antibody (16) following transient transfection into CHO-A7 cells.

Microscopy—CHO-A7 cells were grown on glass coverslips, transfected with FuGENE 6, and maintained for an additional 48 h. The medium was changed on the morning of the experiment. Fluorescently labeled ligand was added directly to the medium, at a final concentration of 10 μg/ml, followed by incubations at 37 °C, 5% CO_2 for the indicated amount of time. Thereafter, cells were washed seven times with cold phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline (at room temperature), and subsequently mounted on glass slides with VECTASHIELD mounting medium containing nuclear stain 4′,6-diamidino-2-phenylindole (Vector Laboratories). Slides were observed under an Olympus BX51 fluorescence microscope.

Statistical Analysis—Statistics were calculated with Graphpad's Prizm software, using one-way analyses of variance with post-hoc Bonferroni's test. Statistical significant differences, with p values lower than 0.05, are indicated with *asterisks* in Figs. 2 and 6.

RESULTS

SR-BII-mediated HDL Endocytosis Occurs via a Clathrin-dependent Pathway—Previous studies showed that in CHO-SRBI cells, most cell-associated HDL remains on the cell surface (21). SR-BII, in contrast, mediated extensive endocytosis of HDL, through a transferrin-positive pathway (21). To elucidate the endocytic mechanism underlying HDL

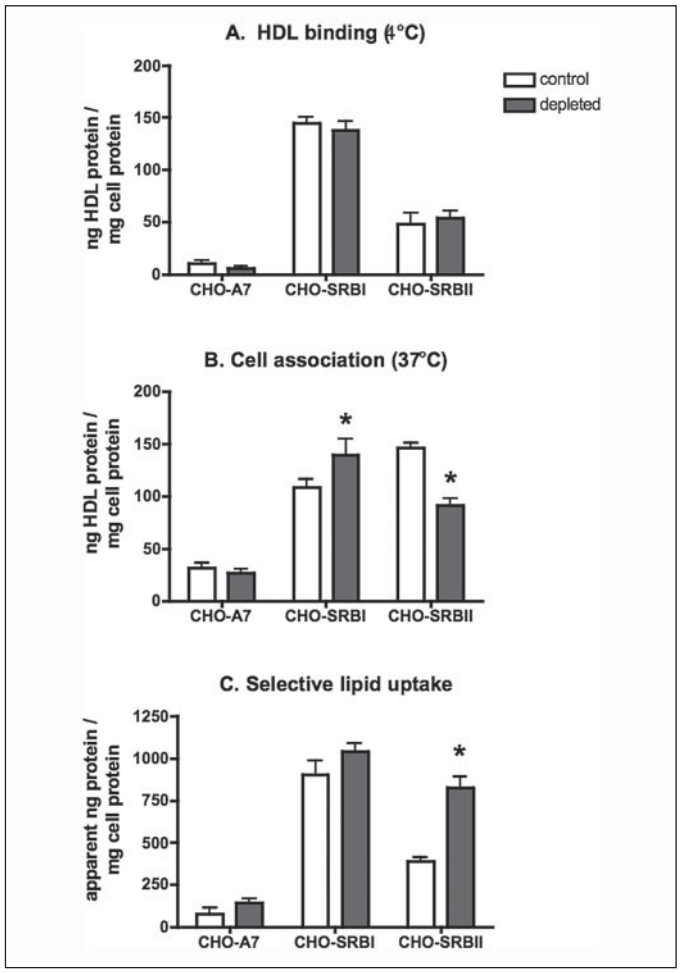

FIGURE 2. **Effect of disruption of clathrin-coated pit formation on HDL cell association and selective uptake.** CHO-SRBI, CHO-SRBII, and control CHO-A7 cells were treated with serum-free control medium (*open bars*) or potassium-depleted medium (*dark gray bars*) and then incubated with HDL_3 that was labeled at the protein moiety with ^{125}I at 4 °C (*A*) or at 37 °C (*B*) or with HDL_3 additionally labeled with [^{3}H]cholesteryl oleoyl ether at 37 °C (*C*). *Error bars* indicate S.D. with $n = 3$; the figure is representative of four independent experiments, all with similar outcomes. *Asterisks* indicate statistically significant differences in CHO-SRBx cells, control *versus* potassium-depleted medium.

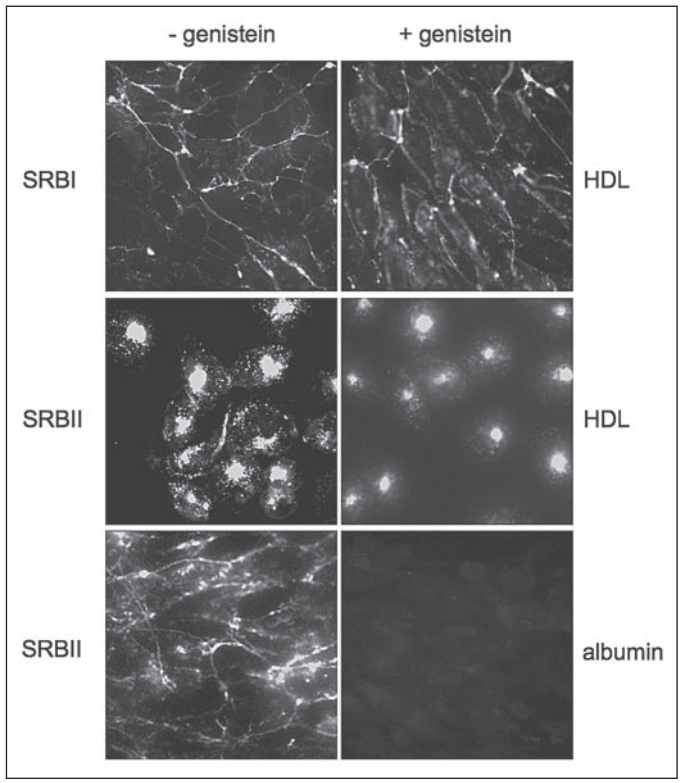

FIGURE 3. **Effect of genistein, an inhibitor of caveolae-dependent endocytosis, on HDL uptake.** CHO cells expressing SR-BI or SR-BII were preincubated in serum-free medium for 2 h, either with or without 200 μM genistein. Thereafter, Alexa Fluor 488-labeled HDL_3 (10 μg/ml) or Alexa Fluor 488-labeled albumin (10 μg/ml) was added, and cells were further incubated for 30 min, fixed, and mounted on a cover slide for fluorescence microscopy.

uptake by SR-BII, we first studied uptake of HDL, fluorescently labeled at the protein moiety, in CHO-SRBII cells after a short potassium depletion treatment to block clathrin-coated pit formation (25). As shown in Fig. 1, potassium depletion led to a marked decrease of HDL endocytosis by CHO-SRBII cells. Cells grown in potassium-containing medium, however, showed intracellular localization of the ligand, as we have reported before (21). In the case of CHO-SRBI cells, the bulk of cell-associated HDL was found at the cell surface, both in control cells and in treated cells. Incubation in high sucrose buffer, also known to disrupt clathrin-coated pit formation (26), had similar results (not shown). For both receptor isoforms, potassium depletion did not alter 4 °C binding of ^{125}I-HDL, indicating that the steady-state amount of surface receptors was unaltered by the treatment (Fig. 2*A*). At 37 °C, however, cell association (the sum of surface-bound and internalized ligand) in potassium-depleted CHO-SRBII cells was significantly decreased when compared with untreated cells (Fig. 2*B*). This is in line with an inhibitory effect of potassium depletion on intracellular accumulation of HDL (Fig. 1). In the case of SR-BI, potassium depletion resulted in a slight but significant increase in 37 °C cell association. No effect of potassium depletion on HDL-cell association was seen in receptor-deficient control cells. Surprisingly, although potassium depletion had little effect on selective uptake by SR-BI, selective uptake by SR-BII was actually significantly increased following the treatment (Fig. 2*C*), despite unaltered surface receptor numbers in the steady-state (Fig. 2*A*). The explanation for this is unclear. Perhaps the displacement of SR-BII out of clathrin-coated pits to other areas of the cell surface enhances selective uptake efficiency. Nevertheless, it can be concluded that blocking SR-BII-dependent HDL endocytosis does not impair selective uptake. Genistein, an inhibitor of caveolae-dependent uptake (28), had no effect on HDL uptake by either CHO-SRBI or CHO-SRBII cells (Fig. 3), whereas the compound potently inhibited uptake of Alexa Fluor-labeled albumin, a marker for caveolae-dependent endocytosis (29) (Fig. 3).

Tyr-489 Is Not Required for Endocytosis of HDL by SR-BII—Previously published data showed that deletion of the SR-BI carboxyl terminus did not affect surface expression of SR-BI in CHO cells (21). We therefore hypothesized that the strongly conserved carboxyl terminus of SR-BII (16) (Fig. 4) must contain a motif that mediates receptor endocytosis. Alignment of hitherto known SR-BII cDNA sequences as well as SR-BII sequences predicted based on published SR-BI data, shown in Fig. 4, revealed a putative *YXXZ* (*Z* representing a hydrophobic amino acid) motif (30) at amino acid positions 489–492 with a partially overlapping, completely conserved dileucine motif (Leu-492–Leu-493). It should be noted, however, that, in contrast to the dileucine repeat, the tyrosine residue is not entirely conserved; in the rabbit, this residue is replaced with a histidine (20).

Mutagenesis of the carboxyl terminus of SR-BII was carried out to define amino acid residues necessary for SR-BII-mediated HDL endocytosis. Constructs encoding wild-type SR-BI or SR-BII or mutant isoforms were transiently transfected into CHO-A7 cells grown on glass coverslips. Endocytosis was determined microscopically after incuba-

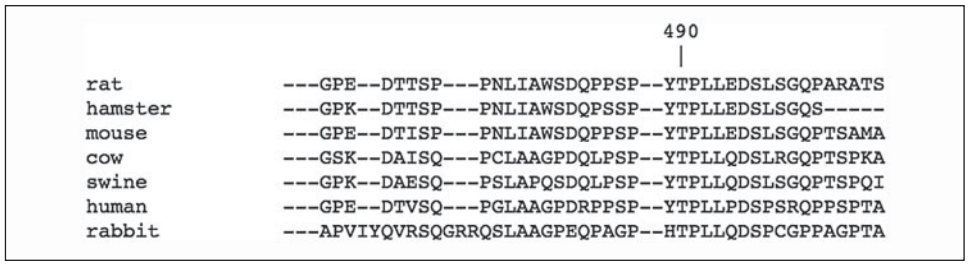

FIGURE 4. **Alignment of known amino acid sequences of the SR-BII carboxyl terminus.** Shown are carboxyl termini from human (Ref. 37), mouse (Refs. 15 and 16) and rabbit (Ref. 20) as well as from predicted sequences from cow, hamster (incomplete), swine, and rat (GenBank™ accession numbers AF019384, U11453, NM213967, and D89655, respectively.

TABLE 1

Mutational analysis of the carboxyl terminus of SR-BI and SR-BII

The putative endocytosis signal in SR-BII is underlined, point-mutations are in bold. Endocytosis was determined microscopically as shown in Fig. 5.

Amino acid sequence	Mutation	Endocytosis?
SR-BII		
GPEDTISPPNLIAWSDQPPSPYTPLLEDSLSGQPTSAMA	SR-BII	Yes
GPEDTISPPNLIAWSDQPPSP**A**TPLLEDSLSGQPTSAMA	SR-BII Y489A	Yes
GPEDTISPPNLIAWSDQPPSP**H**TPLLEDSLSGQPTSAMA	SR-BII Y489H	Yes
GPEDTISPPNLIAWSDQPPSPY**A**PLLEDSLSGQPTSAMA	SR-BII T490A	Yes
GPEDTISPPNLIAWSDQPPSPYT**A**LLEDSLSGQPTSAMA	SR-BII P491A	Yes
GPEDTISPPNLIAWSDQPPSPYTP**A**LEDSLSGQPTSAMA	SR-BII L492A	No
GPEDTISPPNLIAWSDQPPSPYTP**V**LEDSLSGQPTSAMA	SR-BII L492V	No
GPEDTISPPNLIAWSDQPPSPYTP**F**LEDSLSGQPTSAMA	SR-BII L492F	No
GPEDTISPPNLIAWSDQPPSPYTPL**A**EDSLSGQPTSAMA	SR-BII L493A	Yes
GPEDTISPPNLIAWSDQPPSP	SR-BII Y489stop	No
G----------YTPLLEDSLSGQPTSAMA	SR-BII delP469P488	Yes
GPEDTISPPNLIAWSDQPPSPYTPLL	SR-BII E494stop	Yes
SR-BI		
EKCFLFWSGSKKGSQDKEAIQAYSESLMSPAAKGTVLQEAKL	SR-BI	No
EKCFLFWSGSKKGSQDKEAIQAYTPLLMSPAAKGTVLQEAKL	SR-BI Y490TPLL	Yes

tion with Alexa Fluor 568-labeled HDL_3 (10 μg/ml HDL protein, 40–60 min, 37 °C). Table 1 shows the analyzed mutations and summarizes the effect of the mutations on HDL endocytosis by SR-BII.

First, it was found that the endocytic signal in the carboxyl terminus of SR-BII is indeed contained within the YTPLL stretch since the remainder of the carboxyl terminus could be deleted (deletion of residues between Pro-469 and Pro-488, not shown; and mutant E494stop, Fig. 5*C*) without an apparent effect on the SR-BII phenotype (Table 1). To address the question of whether Tyr-489 is required for HDL endocytosis by SR-BII, this residue was replaced by histidine or alanine, and the ability of the mutant receptors to mediate endocytosis of Alexa Fluor 568-labeled HDL_3 at 37 °C was studied in transiently transfected CHO-A7 cells. As shown in Fig. 5*D*, mutant Y489A still mediated HDL endocytosis; the same was observed for mutant Y489H (not shown). Thus, tyrosine 489 appears not to be required for endocytosis of HDL.

The Dileucine Motif Leu-492–Leu-493 Is Required for Intracellular Accumulation of HDL—Dileucine motifs are known to mediate endocytosis into endosomal compartments (31) and usually correspond to a (DE)*XXX*L(LI) or D*XX*LL consensus. Despite the fact that SR-BII lacks the acidic residue(s) preceding Leu-492, we nevertheless tested the hypothesis that the strongly conserved dileucine repeat Leu-492–Leu-493 is required for SR-BII endocytosis. Interestingly, substitution L492A resulted in a virtual abolishment of intracellular accumulation of Alexa Fluor-labeled HDL in CHO cells (Fig. 5*E*). Substitution L493A had a less striking effect, with only partial loss of endocytic activity (Fig. 5*F*), whereas Thr-490 and Pro-491 appeared not critical for the SR-BII phenotype (not shown; Table 1). Further, we tested whether leucine, one of the most hydrophobic amino acids, is required at position 492 or whether it can be replaced by another hydrophobic amino acid such as the structurally similar hydrophobic amino acid valine or by phenylalanine, which carries a "bulky" side chain. Interestingly, as seen in Fig. 5*G*, CHO cells expressing the L492V mutant accumulated HDL mainly on the cell surface, not dissimilar from wild-type SR-BI but in strong contrast with wild-type SR-BII. The same was observed for L492F (not shown). Thus, leucine 492 is specifically required for SR-BII endocytosis and might be part of an unorthodox dileucine motif.

Introduction of the SR-BII Endocytic Motif into SR-BI Results in HDL Endocytosis—The carboxyl terminus of SR-BI contains a tyrosine residue at position 490 (Table 1), which is highly conserved, and a leucine residue at position 494. To test whether introduction of the YTPLL motif into SR-BI would be sufficient to mediate endocytosis, the amino acids in between Tyr-490 and Leu-494 were substituted with the amino acid sequence TPL (Table 1). Strikingly, when introduced into CHO cells, this SR-BI mutant mediated endocytosis of HDL similarly to SR-BII (Fig. 5*H*). These results show that the YTPLL residue is sufficient to mediate endocytosis. Furthermore, since the YTP residues are dispensable for endocytosis by SR-BII, these results strongly suggest that the dileucine motif is the mediator of endocytosis.

Effect of Mutations in the Carboxyl Terminus of SR-BII on Selective Uptake Efficiency—To determine whether mutations in the endocytic motif of SR-BII that block HDL endocytosis also affect selective uptake efficiency, wild-type SR-BI and SR-BII as well as various mutant forms of these receptors were transfected into CHO-A7 cells. Non-transfected and transfected cells were then incubated with $^{125}I/^{3}H$-labeled HDL_3, and receptor-mediated selective uptake efficiency was determined by measuring the ratio of selective uptake per unit of cell association. In agreement with published results (21), SR-BII mediated selective uptake from HDL with reduced efficiency when compared with SR-BI (Fig. 6), most likely due to the fact that HDL endocytosed by SR-BII did not significantly contribute to selective uptake. All mutants that abolished HDL endocytosis showed statistically significant increases in selective uptake efficiency when compared with SR-BII, reaching similar efficiencies as observed with wild-type SR-BI. Interestingly, two SR-BII mutants that maintained endocytic capacity, *i.e.* the Y489A mutant and the mutant lacking the residues following the endocytic motif (E494stop), showed increased selective uptake efficiency (Fig. 6) when compared

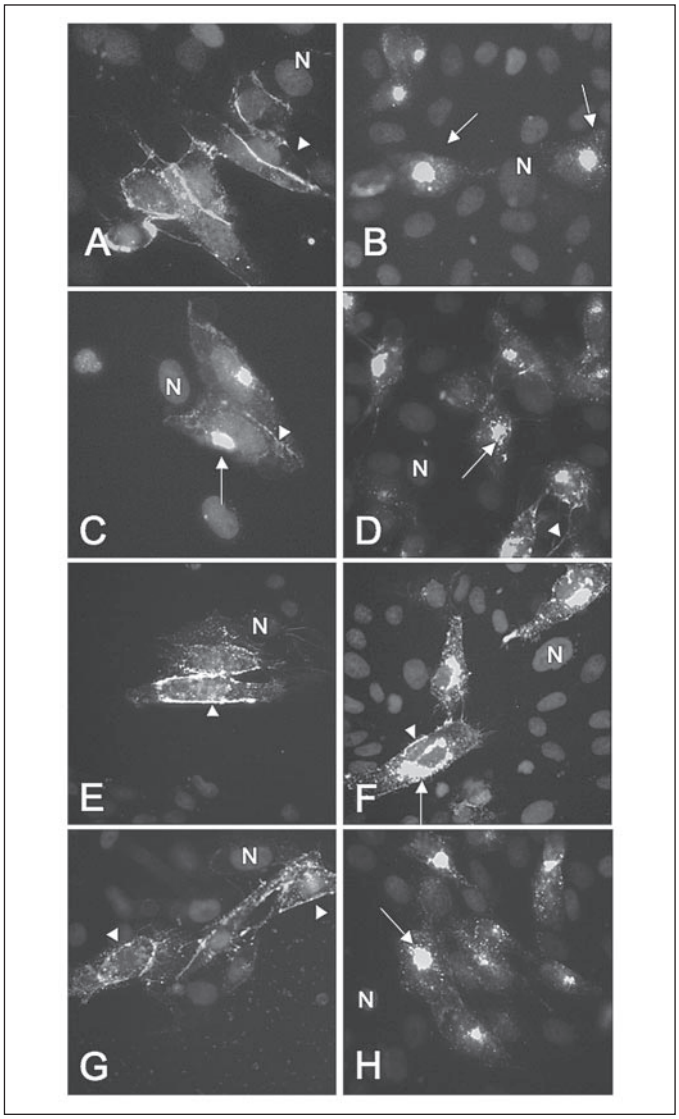

FIGURE 5. **Effect of point mutations in the carboxyl terminus of SR-BI and SR-BII on HDL uptake.** CHO cells were transfected with SR-B*x*-encoding plasmids expressing wild-type SR-BI or SR-BII or with mutant forms of the receptors. 24 h later, the cells were incubated for 40–60 min with Alexa Fluor 568-labeled HDL_3, fixed, and mounted for fluorescence microscopy. Note that for all of the transiently transfected cell populations, Alexa Fluor 568 HDL is not found associated with cells not expressing SR-B*x*. Nuclei (*N*) were stained with 4′,6-diamidino-2-phenylindole. *Arrows* point to intracellular ligand, whereas ligand on the cell surface is indicated with *arrowheads*. *A* = SR-BI; *B* = SR-BII; *C* = E494stop; *D* = Y489A; *E* = L492A; *F* = L493A; *G* = L492V; *H* = SR-BI YTPLL.

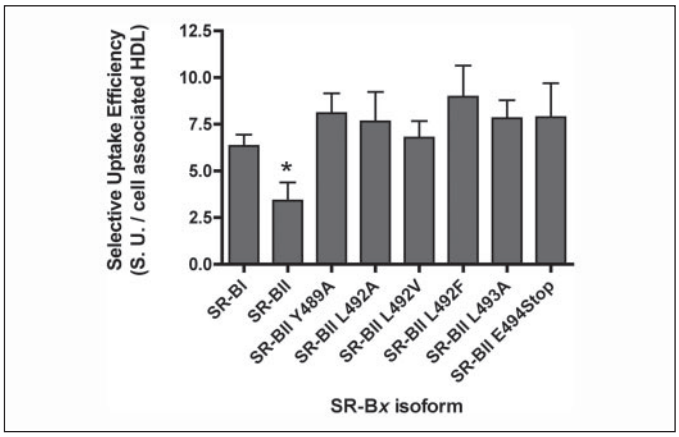

FIGURE 6. **Selective uptake efficiency (*i.e.* selective uptake divided by cell association) by CHO cells expressing SR-BI, SR-BII, and indicated mutants.** Shown are averages ± S.D. ($n = 6$) obtained by combining values from two independent experiments. The efficiency obtained with SR-BII differed significantly from all other isoforms ($p < 0.05$; Dunnett's multiple comparison test). *Asterisks* indicate statistically significant differences between SR-BII and all other receptors.

with wild-type SR-BII. These results suggest that these mutants deliver HDL to different cellular compartments than SR-BII and that this allows for selective uptake to occur.

DISCUSSION

In the present study, we show that SR-BII, a protein encoded by alternatively spliced SR-BI mRNA, undergoes endocytosis by a clathrin-dependent, caveolae-independent mechanism. Endocytosis appeared to be mediated by a unique signal in the cytoplasmic carboxyl terminus, which was identified as a conserved dileucine-motif.

When compared with previously described dileucine motifs, the putative dileucine motif of SR-BII is somewhat unusual. Although it carries a serine residue after the dileucine repeat, the motif lacks the preceding acidic amino acids conforming to the consensus (DE)*XXX*-L(LI) or D*XX*LL (31). These dileucine motifs mediate rapid endocytosis and generally target the receptor to late endosomes or lysosomes (31). One example of a protein with such a signal is lysosomal integral membrane protein-II (LIMP-II), which belongs to the same scavenger receptor family as CD36, SR-BI, and SR-BII. However, SR-BII does not appear to direct HDL to lysosomes since apolipoprotein degradation is negligible (21). Perhaps the lack of the acidic residues prior to the dileucine repeat, which might decrease lysosomal targeting efficiency (31, 32), prevents lysosomal targeting of SR-BII. Interestingly, introduction of the SR-BII dileucine motif into the carboxyl terminus of SR-BI confers the SR-BII-like endocytic phenotype to an otherwise unaltered SR-BII, providing additional evidence that this motif alone is sufficient to target receptors for internalization.

We previously showed that SR-BII-mediated selective lipid uptake from HDL is less efficient than selective uptake by SR-BI and argued that endocytosed HDL does not contribute significantly to the selective uptake process (21). Our present data indicate that SR-BII mutants that are deficient in endocytosis have enhanced selective uptake activity. In addition, blocking SR-BII-mediated HDL endocytosis by potassium depletion actually enhanced selective uptake. We interpret these results to suggest that localization of the receptor to the cell surface is optimal for selective uptake. SR-BI-dependent endocytosis of HDL has been reported by various groups (6, 7, 8, 13), but increasing evidence points against a role for HDL endocytosis in selective uptake. As recently shown by Nieland *et al.* (13), for example, blocking of SR-BI-dependent HDL endocytosis appeared not to inhibit selective uptake, and blocking of selective uptake by specific inhibitors did not affect endocytosis of HDL. Furthermore, selective uptake can be mediated by purified SR-BI inserted into artificial membranes (13), and electron microscopic studies showed that little HDL is taken up by cells that naturally exhibit strong SR-BI-dependent selective uptake activities (10, 33). Thus, whatever the functional significance of HDL endocytosis by SR-BI or SR-BII, it does not seem to be related to selective lipid uptake from the lipoprotein. HDL endocytosis by SR-BII appears unique in this respect, however, since mutations in the carboxyl terminus of SR-BII that did not abolish endocytosis did not show the reduced selective uptake efficiency characteristic for SR-BII. This is somewhat unexpected and could indicate that endocytosis of HDL does not necessarily preclude selective uptake. Possibly, the endocytic route taken by wild-type SR-BII is different from mutant SR-BII and targets HDL to compartments where selective uptake is not efficient. Nevertheless, both wild-type SR-BII (21)

and SR-BII mutants that retained endocytic activity (data not shown) do not degrade significant amounts of HDL apolipoproteins.

Although SR-BII protein levels in most tissues are relatively low when compared with SR-BI, it cannot be excluded that some of the severe occlusive atherosclerosis displayed by SR-BI(II)/ApoE double knock-out mice (34) can be attributed to lack of SR-BII. It is not likely that SR-BII plays a role in bulk HDL metabolism, but the isoform might nevertheless indirectly be involved in cholesterol homeostasis. SR-BII recycles HDL in a transferrin-positive peri-centriolar compartment (21) close to the nucleus. HDL carries many biologically active hydrophobic compounds that are ligands for nuclear receptors, including oxysterols, fat-soluble vitamins, estrogens, and bile salts, compounds that normally have to diffuse across the plasma membrane and through the cytoplasm to reach the nucleus. Perhaps efficient internalization and peri-nuclear recycling of HDL by SR-BII enhances diffusion of such compounds into the nucleus and thus potentiates the cellular response; this is a possibility we are currently investigating. Additionally, SR-BII-mediated retro-endocytosis of HDL, a potent acceptor of free cholesterol, might affect the endocytic recycling compartment itself by altering its cholesterol content, thus potentially affecting endocytic pathways of other receptors. Recycling of glycosylphosphatidylinositol-anchored receptors, for example, strongly depends on endosomal cholesterol content (35, 36).

Taken together, we have identified an endocytic motif in the carboxyl terminus of SR-BII that mediates clathrin-dependent endocytosis of the receptor and have provided additional evidence against involvement of HDL endocytosis in selective uptake.

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