Scavenger receptor class B type I localizes to a late endosomal compartment

Malika Ahras, Thet Naing, and Ruth McPherson[1]

Division of Cardiology, University of Ottawa Heart Institute, Ottawa, Canada K1Y 4W7

Abstract **Scavenger receptor class B type I (SR-BI) has an established role in mediating the selective uptake of cholesterol from HDL in hepatocytes, steroidogenic cells, and other tissues. SR-BI is present on the plasma membrane but also localizes to stable intracellular compartments of unknown function. Using indirect immunofluorescence and subcellular fractionation, we have investigated the subcellular distribution of SR-BI. We report that red fluorescent protein-tagged mouse SR-BI (RFP-mSR-BI) colocalizes with the late endosomal and lysosomal markers, Rab7, LBPA, and Rab9. In addition, endogenous SR-BI is also found on lysosomes and colocalizes with LAMP-2 in primary hepatocytes. Furthermore, we demonstrate that the trafficking of SR-BI through these compartments is Rab7 dependent. Interestingly, filipin staining indicates accumulation of lysosomal cholesterol in SR-BI-deficient ($^{-/-}$) as compared with wild-type hepatocytes. In addition to its role as a plasma membrane receptor, SR-BI may function in cholesterol trafficking from late endosomes/lysosomes.—**Ahras, M., T. Naing, and R. McPherson. **Scavenger receptor class B type I localizes to a late endosomal compartment.** ***J. Lipid Res.*** **2008.** 49: **1569–1576.**

Supplementary key words cholesterol • selective uptake • late endosomes • lysosomes • intracellular trafficking

Scavenger receptor class B type I (SR-BI) belongs to the class B scavenger receptor family of cell surface glycoproteins (1). The receptor is expressed ubiquitously, with the highest levels of expression in steroidogenic, intestinal, and hepatic cells (2, 3). SR-BI is a receptor for HDL, and several lines of evidence indicate an anti-atherogenic role for this protein (4–6) in the promotion of the terminal stages of macrophage reverse cholesterol transport (7). SR-BI mediates the net transfer of cholesteryl ester from HDL into cells without degradation of the HDL protein, a process known as selective uptake (4, 8, 9). SR-BI localizes to the basolateral and canalicular membranes in polarized cells such as primary hepatocytes and polarized Madin-Darby canine kidney cells (10–13).

Although SR-BI is an established cell surface lipoprotein receptor, intracellular localization of SR-BI has also been observed. In mouse hepatocyte couplets, SR-BI was found in juxta-nuclear compartments (13, 14). In adipocytes, anti-SR-BI antibody staining revealed a punctate intracellular distribution (15). These data suggest that in addition to its well-described role in selective uptake at the plasma membrane, a pool of SR-BI that resides in intracellular structures may have an independent role in intracellular lipid movement. The functional significance of SR-BI trafficking is not known; however, we and others have reported that endocytosis and recycling of SR-BI occur in but are not required for efficient selective uptake (16, 17).

Using indirect immunofluorescence and subcellular fractionation, we have investigated the subcellular distribution of both red fluorescent protein-tagged mouse SR-BI (RFP-mSR-BI) and endogenous SR-BI in primary hepatocytes. We demonstrate that SR-BI localizes in part to late endosomes/lysosomes and that the trafficking of SR-BI through these compartments is Rab7 dependent.

MATERIALS AND METHODS

Cell lines and reagents

HeLa cells were obtained from American Type Culture Collection (Manassas, VA). Wild-type human fibroblasts (AG10803) and human Niemann-Pick type C (NPC-1) fibroblasts (GM03123) were obtained from Coriell Cell Repositories (Camden, NJ). Cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Primary hepatocytes were isolated from retired breeders of the C57BL6 (wild-type) mouse strain purchased from Charles River (Wilmington, MA) and SR-BI$^{-/-}$ mice (18) as previously described (16). Other reagents and suppliers included: filipin (Sigma Mississauga, ON, Canada), human plasma HDL (Calbiochem, San Diego, CA), anti-LAMP-2 and anti-EEA1 Abs (Transduction Laboratories, Mississauga, ON, Canada), anti-SR-BI Ab (NB400-101) (Novus Biologicals, Little-

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[1] To whom correspondence should be addressed.
e-mail: rmcpherson@ottawaheart.ca

ton, CO), anti-Rab7 Ab (Sigma), and Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). LBPA was a gift from Pr J. Gruenberg (Geneva, Switzerland).

Constructs

The mSR-BI was digested with *Kpn*I and *Xma*I and cloned into the pERFP-C1 vector (a gift from R. Tsien). Adenoviral (Ad)-RFP-mSR-BI was from Vector Biolabs (Philadelphia, PA). GFP-Rab5^{WT}, Rab7^{WT}, and Rab7^{Q79L} were a gift of C. Bucci (Università di Lecce, Italy). GFP-Rab7^{T22N} was from M. Zerial (Dresden, Germany). YFP-Rab4 and GFP-Rab11 were from T. Jeffries (Cancer Research UK). GFP Rab9 and GFP-Rab9^{S21N} were from S. Pfeffer (Stanford University, CA).

Subcellular fractionation

Subcellular fractionation of SR-BI was performed as described previously (19). Briefly, HeLa cells were transfected with RFP-mSR-BI using Lipofectamine 2000. Transfected cells were scraped and washed in PBS then homogenized in homogenization buffer (0.25 M sucrose, 0.5 mM EDTA). Cells were fractionated by passing through a cell-cracker (H and Y Enterprises, Redwood City, CA), and the postnuclear supernatant (PNS) was prepared. The PNS was loaded on a continuous 20% to 58% sucrose gradient and centrifuged in an SW40 rotor at 87,000 *g* overnight. Fractions were collected, and proteins were precipitated in acetone and resolved by SDS-PAGE.

Indirect immunofluorescence and microscopy

Cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions. In other experiments, cells were infected with a 100 multiplicity of infection adRFP-mSR-BI in complete medium overnight. Cells were then washed, fixed with 3% paraformaldehyde, permeabilized in 0.2% triton X-100 or 0.05% saponin, and stained with the appropriate primary and secondary Abs. For filipin staining, cells were fixed as above and incubated in 0.05 mg/ml filipin for 2 h. Cells were washed with PBS, then H_2O, and finally mounted in Mowiol (Dako, Mississauga, ON, Canada). Images were acquired using a confocal laser scanning microscope (Olympus 1 × 80, Olympus, Center Valley, PA), FV1000 software, and equipped with a 100×/1.4 plan-APOCHROMAT oil immersion objective. To quantify the intensity of filipin in wild-type (WT) and SR-BI$^{-/-}$ primary hepatocytes, images were taken with an Olympus 1 × 70 inverted confocal microscope operated with Tillvison software 4.0, and the mean intensity of filipin for individual cells was measured using ImageJ software (National Institutes of Health; Bethesda, MD).

For live-cell microscopy, transfected Hela cells were grown on glass-bottomed MaTeK dishes. Cells were analyzed live in full media supplemented with 30 mM HEPES-NaOH, pH 7.4, at 37°C and 10% CO_2, which was maintained by an environmental control chamber enclosing the microscope. Images were acquired on the same Olympus 1 × 80 confocal microscope as mentioned above. Frames were captured as indicated in figure legends.

HDL labeling

HDL was labeled using a Cy5 mono-reactive dye pack (Amersham Biosciences, Oakville, ON, Canada) according to the manufacturer's instructions. To label with HDL-Cy5, transfected Hela cells were incubated with 30 μg/ml HDL-Cy5 at 4C° for 15 min. Cells were then rinsed and transferred to 37C° media, and HDL-Cy5 was chased for various times.

RESULTS

Intracellular SR-BI is mainly localized to late endosomes/lysosomes

To investigate the intracellular localization of SR-BI, we colabeled mouse primary hepatocytes with anti-SR-BI and anti-LAMP-2 Abs. We find that endogenous SR-BI displays a punctate perinuclear localization, which colocalizes with the late endosomal/lysosomal marker, LAMP-2 (**Fig. 1A**).

To further study the intracellular localization of SR-BI, we expressed RFP-mSR-BI in HeLa cells and studied its colocalization with a number of specific intracellular markers. HeLa cells were chosen for these studies because both primary hepatocytes and HepG2 cells are difficult to transfect. Using the late endosomal markers GFP-Rab7 and LBPA, we demonstrate that RFP-mSR-BI displays a strong colocalization with Rab7 (Fig. 1B) and LBPA (Fig. 1C). In addition, live imaging of cells expressing RFP-mSR-BI and GFP-Rab7 show SR-BI to be associated with Rab7-positive structures, and this association is stable. Moreover, SR-BI was found to maintain its lysosomal localization even after an 8 h cycloheximide chase, indicating that this is a stable pool of SR-BI (data not shown). These data suggest that SR-BI is not degraded but rather is a resident protein on late endosomal organelles. Furthermore, we found that RFP-mSR-BI also displays a strong colocalization with YFP-Rab9 (Fig. 1D), indicating that SR-BI traffics through Rab9-positive endosomes.

To confirm the immunofluorescence data, we performed subcellular fractionation to establish the distribution of SR-BI. The PNS from HeLa cells transfected with RFP-mSR-BI was separated on a continuous sucrose gradient and the collected fractions were loaded on a SDS-PAGE gel. The membrane was blotted with anti-EEA1, anti-SR-BI and anti-Rab7 Abs. SR-BI was found mainly in the Rab7-positive fraction and not with EEA1-enriched fractions (**Fig. 2A, B**). Similarly, indirect immunofluorescence analysis showed that RFP-mSR-BI displays a small colocalization with the early endosomal marker, GFP-Rab5, as well as with the sorting/recycling endosome markers YFP-Rab4 and Rab11 (data not shown). Together, these findings confirm that the intracellular SR-BI is mainly present on late endosomes/lysosomes.

SR-BI trafficking through late endosomes/lysosomes is Rab7 dependent

To gain further insight into the trafficking of SR-BI through late endosomes/lysosomes and to determine whether this trafficking is dependent on Rab7, we coexpressed RFP-mSR-BI with the dominant active (DA) GFP-Rab7^{Q79L}, which constitutively binds GTP, leading to the formation of enlarged late endosomes (20). This resulted in almost all of SR-BI localizing on the enlarged late endosomes (**Fig. 3A**), suggesting that the expression of the DA Rab7^{Q79L} traps SR-BI in Rab7-positive endosomes. We then overexpressed RFP-mSR-BI with the dominant negative (DN) Rab7^{T22N} (20) and examined the distribution of SR-BI in these cells. Interestingly, in cells expressing GFP-Rab7^{T22N}, SR-BI was dispersed throughout the cytoplasm,

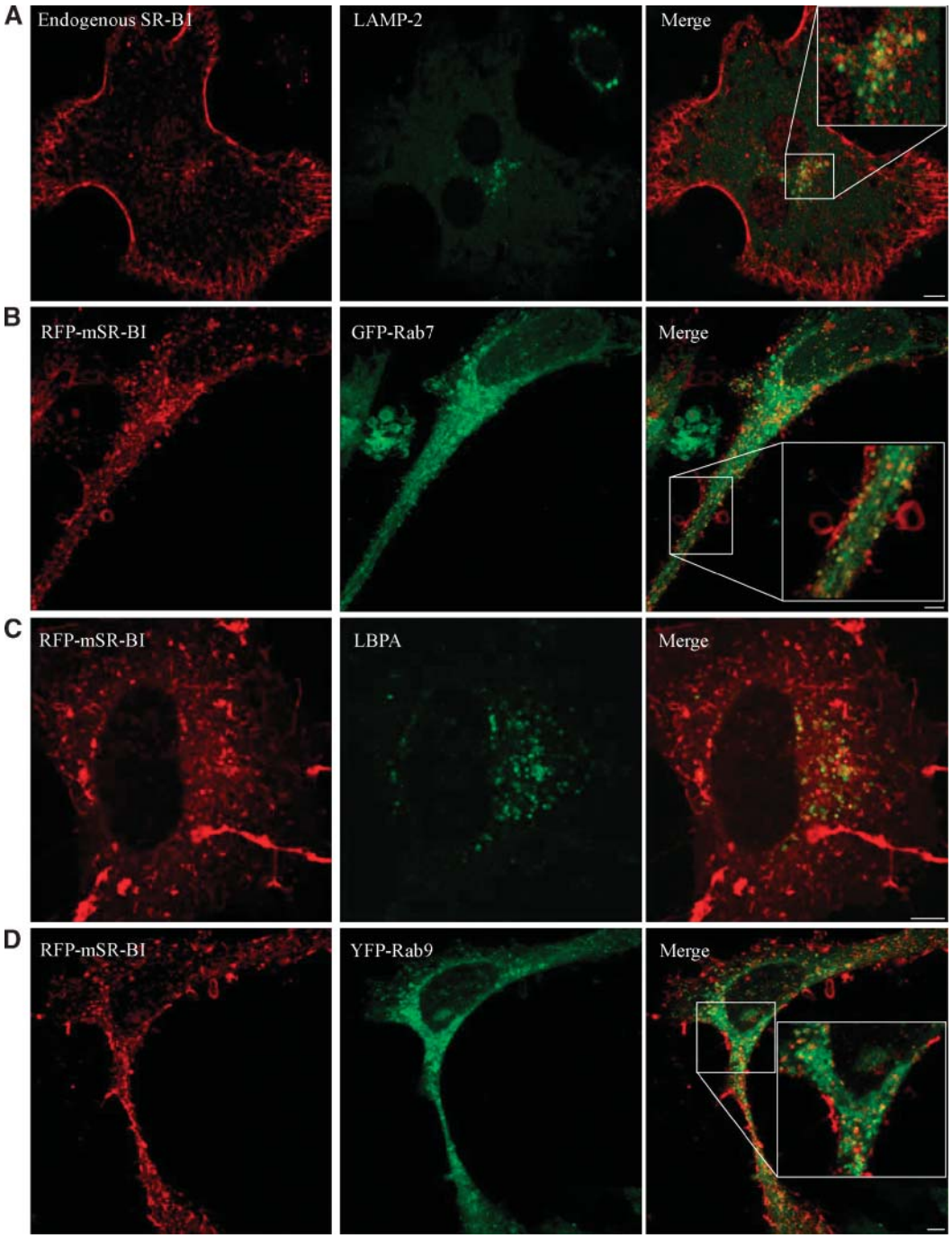

Fig. 1. Intracellular scavenger receptor class B type I (SR-BI) is mainly localized to late endosomes/lysosomes. A: Primary hepatocytes were colabeled with anti-SR-BI and LAMP-2 Abs, followed by secondary anti-rabbit 488 and anti-mouse 647. HeLa cells were cotransfected with red fluorescent protein-tagged mouse SR-BI (RFP-mSR-BI) and GFP-Rab7 (B), RFP-mSR-BI alone (C), or RFP-mSR-BI and YFP-Rab9 (D). Cells were processed directly for imaging or labeled with anti-LBPA Ab, followed by a secondary anti-mouse 647, respectively. Bar = 5 μm. LBPA, lysobisphosphatidic acid.

in contrast to the Rab7^{WT}, where SR-BI displayed a more perinuclear localization (Fig. 3B). Similarly, LAMP-2 displayed a dispersed localization upon the expression of GFP-Rab7^{T22N}, as shown previously (Fig. 3C) (20). In addition, in these cells, SR-BI appears to colocalize with the dispersed LAMP-2 (Fig. 3C), suggesting that SR-BI may fail to exit late endosomes/lysosomes in the presence of the DN Rab7 mutant. In contrast, expression of the DN Rab9 S^{21N} did not alter the intracellular localization of SR-BI (data not shown). Together these data suggest that the trafficking of SR-BI through the late endosomal/lysosomal system is Rab7 dependent.

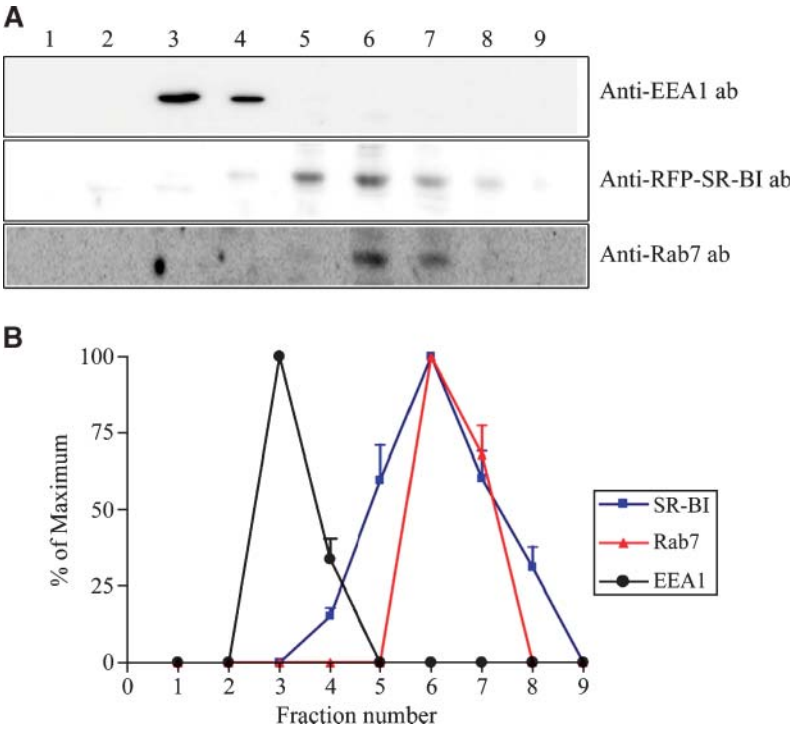

Fig. 2. SR-BI is found in the late endosomal-rich fractions on sucrose gradient. A: HeLa cells were transfected with RFP-mSR-BI. Postnuclear supernatant was loaded on a 20% to 58% sucrose gradient, followed by centrifugation. Fractions were collected and subjected to a Western blot analysis using anti-EEA1 (top panel), anti-SR-BI (middle panel), or anti-Rab7 (bottom panel) Abs. B: Signals were quantified by densitometry using ImageJ software. Data represent the mean of three independent experiments. Error bars are the SEM.

The late endosomal/lysosmal localization of SR-BI does not affect HDL internalization

Although it is established that SR-BI can effectively mediate selective uptake without the concomitant internalization of the HDL particle, there is evidence that a fraction of HDL is internalized together with SR-BI (13, 14, 16). The functional significance of HDL internalization is not understood. To establish whether HDL internalization is SR-BI dependent, we incubated HDL-Cy5 with RFP-mSR-BI-expressing cells at 0 C° for 15 min, and followed HDL binding at 0 min, followed by its internalization after a 1 hr chase at 37C°. At 0 min, HDL bound to the plasma membrane in cells expressing RFP-mSR-BI (**Fig. 4A**). After a 1 h chase at 37C°, HDL was almost completely internalized (**Fig. 5A**). In contrast, cells that did not express RFP-mSR-BI did not bind or internalize HDL. These data confirm other findings that in cells that do not express cholesteryl ester transfer protein (CETP) (21, 22), binding and internalization of HDL are solely dependent on SR-BI (4, 13, 16, 23).

To investigate whether HDL endocytosis is dependent on the late endosomal/lysosmal pool of SR-BI, we performed a pulse chase experiment with HDL-Cy5 in cells expressing RFP-mSR-BI alone or with GFP-Rab7^{WT}, GFP-Rab7^{Q79L}, or GFP-Rab7^{T22N}. Cells were incubated with HDL-Cy5 for 15 min at 0 C°. Bound HDL was chased for 0, 5, 30, and 60 min at 37 C°. We found that expression of the GFP-Rab7Q^{79L}, which trapped SR-BI in late endosomes, did not stimulate the internalization of HDL (Fig. 4B), as compared with that observed in SR-BI- or SR-BI and GFP-Rab7^{WT}-expressing cells (Fig. 4B). Similarly, GFP-Rab7^{T22N} did not inhibit the SR-BI-dependent internalization of HDL (Fig. 4B), but rather the internalization of HDL occurred at a similar rate in all three conditions. Thus, the endocytosis of HDL is not linked to the late endososmal/lysosomal pool of SR-BI, but appears solely dependent on plasma membrane-localized SR-BI.

Potential role of SR-BI in cholesterol trafficking from lysosomal compartments

Next, we aimed to investigate the functional properties of lysosomal SR-BI, using primary hepatocytes isolated from SR-BI$^{-/-}$ and WT mice and labeled with filipin. We observed an increase in filipin staining in the perinuclear area in primary hepatocytes from SR-BI$^{-/-}$ mice compared with that of WT mice (Fig. 5A). Moreover, the accumulated cholesterol in those perinuclear compartments colocalized with the lysosomal marker, LAMP-2 (Fig. 5B). The quantification of filipin fluorescence revealed an approximately 30% increase in lysosomal cholesterol in the SR-BI$^{-/-}$ primary mouse hepatocytes compared with that of WT (Fig. 5C). These findings suggest that SR-BI may be involved in the trafficking of cholesterol out of lysosomes.

We then determined whether SR-BI can compensate for NPC-1 deficiency. NPC-1 has been shown to be involved in the trafficking of cholesterol from late endosomes/lysosomes, and cells lacking this protein accumulate cholesterol in these compartments (24, 25). It has been reported that some proteins, such as the LDL receptor-related protein-1 adaptor protein, GULP, follow the same pathway as NPC-1 in the removal of cholesterol from lysosomal compartments, and the expression of this protein corrects for the NPC-1 phenotype (26). We infected NPC-1 cells with RFP-mSR-BI and stained them with filipin. In NPC-1 cells expressing RFP-mSR-BI, cholesterol accumulated in lysosomal structures to an extent similar to that seen in cells not expressing RFP-mSR-BI (data not shown).

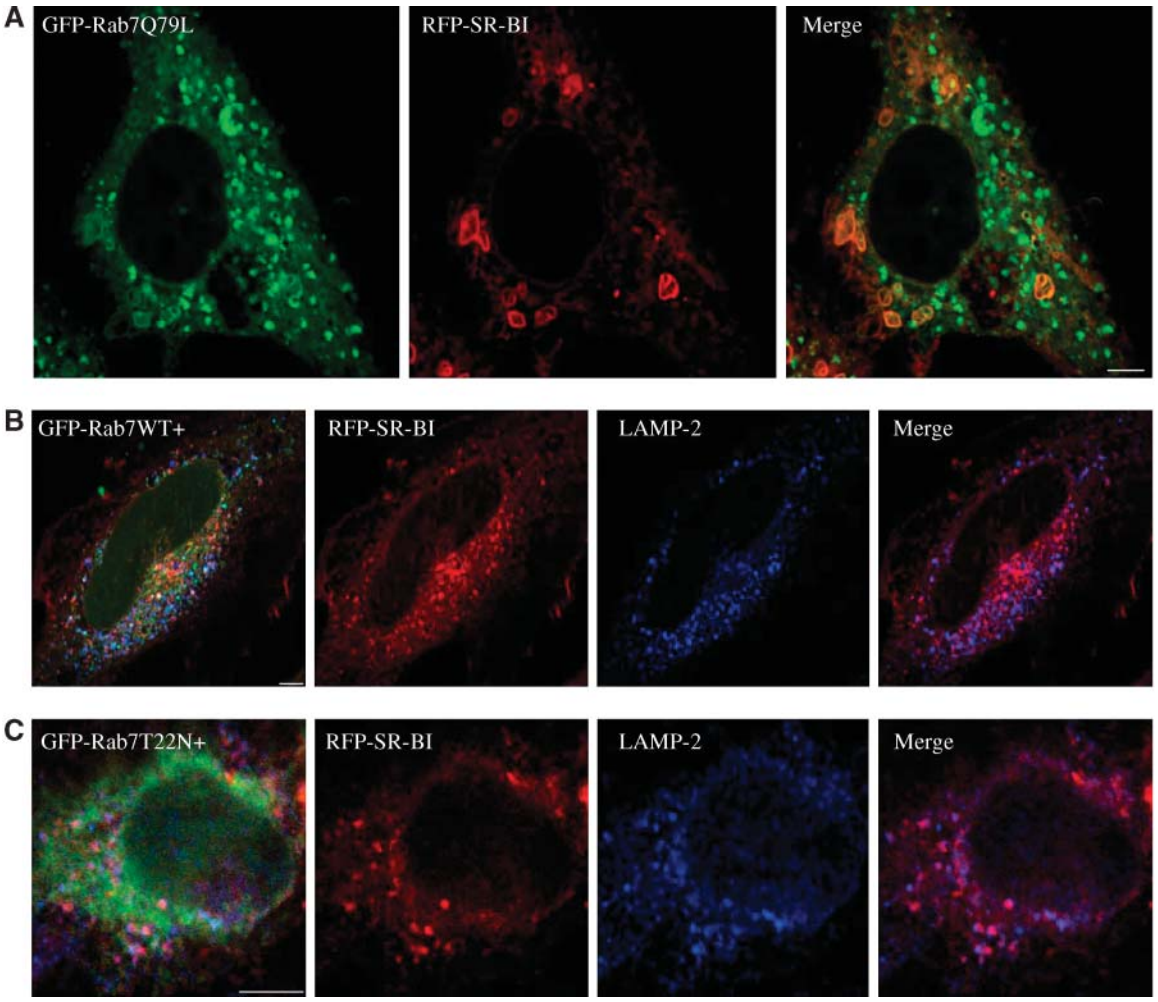

Fig. 3. SR-BI localization on late endosomes is stimulated by the expression of the dominant active Rab7^{Q79L} and disrupted upon the expression of the dominant negative Rab7 T22N. Hela cells were cotransfected with RFP-mSR-BI and either GFP-Rab7^{Q79L} (A), GFP-Rab7^{WT} (B), or GFP-Rab7^{T22N} (C). Cells were fixed and processed for imaging. Bar = 5 μm.

DISCUSSION

Although the majority of SR-BI is situated on the plasma membrane, we have demonstrated that a stable pool of SR-BI is localized to the late endosome. Using indirect immunofluorescence and subcellular fractionation techniques, we find that RFP-mSR-BI displays significant colocalization with the late endosomal/lysosomal markers LBPA, Rab7, and Rab9 and minor colocalization with the early endosomal marker Rab5. However, we could not detect SR-BI on Rab4- or Rab11-positive endosomes, indicating that although SR-BI may traffic through these compartments, it does not form a stable structure on sorting or recycling endosomes. These imaging studies were carried out using HeLa cells because both primary hepatocytes and HepG2 cells are difficult to transfect.

Importantly, we also demonstrate that endogenous SR-BI colocalizes with LAMP-2 in primary mouse hepatocytes, indicating that in addition to its plasma membrane localization, SR-BI is situated on lysosomal structures. Rab7 mediates early-to-late endosome and late endosome-to-lysosome transport. Moreover, we demonstrate that overexpression of the DA Rab7^{Q79L} traps SR-BI on enlarged late endosomes, whereas the expression of the DN Rab7^{T22N} results in the redistribution of SR-BI from a perinuclear compartment to a more dispersed intracellular region. Thus, endogenous and expressed SR-BI localize in part to a late endosomal/lysosomal compartment, and trafficking of SR-BI through this compartment is Rab7 dependent. Rab9 is involved in transport from LE to the trans Golgi network (TGN). We also find that RFP-SR-BI displays a strong colocalization with YFP-Rab9 (Fig. 1D), indicating that SR-BI traffics through Rab9 positive endosomes. Thus, in addition to its known function in selective uptake at the plasma membrane, SR-BI may be involved in the trafficking of cholesterol through these structures.

Interestingly, filipin staining reveals accumulation of lysosomal cholesterol in primary hepatocytes from SR-BI $^{-/-}$ as compared with WT mice. This is consistent with the hypothesis that SR-BI may have a role in the further trafficking of cholesterol from the LE to other organelles, including ER, Golgi and mitochondria, which are found in close association with LE. The steroidogenic acute regulatory protein (StAR) is an absolute requirement in

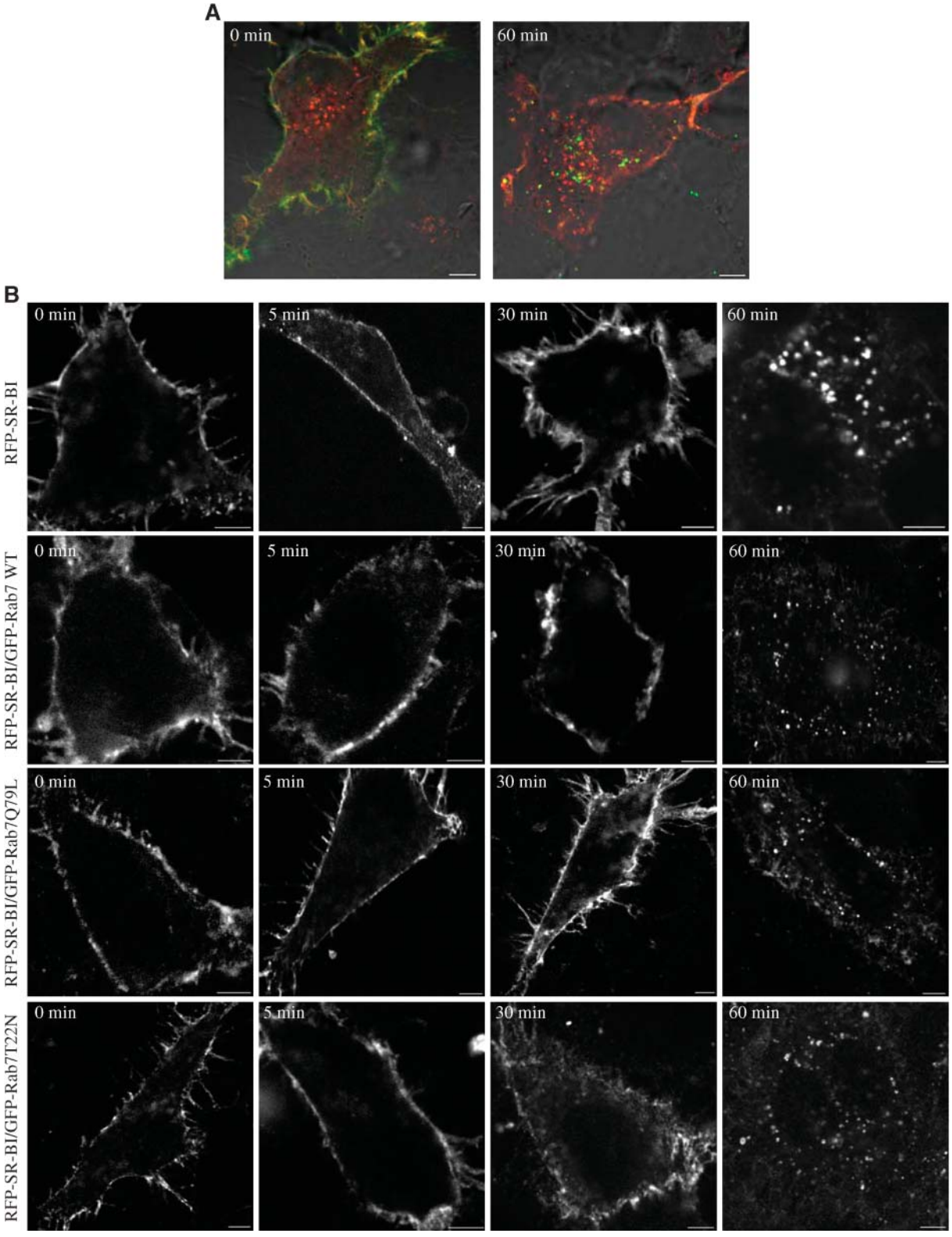

Fig. 4. The late endosomal/lysosmal localization of SR-BI does not affect HDL internalization. A: Hela cells were transfected with RFP-mSR-BI. Twenty-four hours post transfection, cells were incubated with 30 μg/ml HDL-Cy5 for 15 min on ice. Cells were washed, and HDL-Cy5 was chased for 60 min before visualization. B: HeLa cells were transfected with RFP-mSR-BI and either GFP-Rab7^{WT}, GFP-Rab7^{Q79L}, or GFP-Rab7^{T22N}. Twenty-four hours post transfection, cells were incubated with 30 μg/ml HDL-Cy5 for 15 min on ice. Cells were then washed, and HDL-Cy5 was chased for 0, 5, 30, and 60 min at 37C°. HDL-Cy5 trafficking was monitored in transfected cells. For clear visualization, only the HDL-Cy5 channel is presented in this figure. Bar = 5 μm.

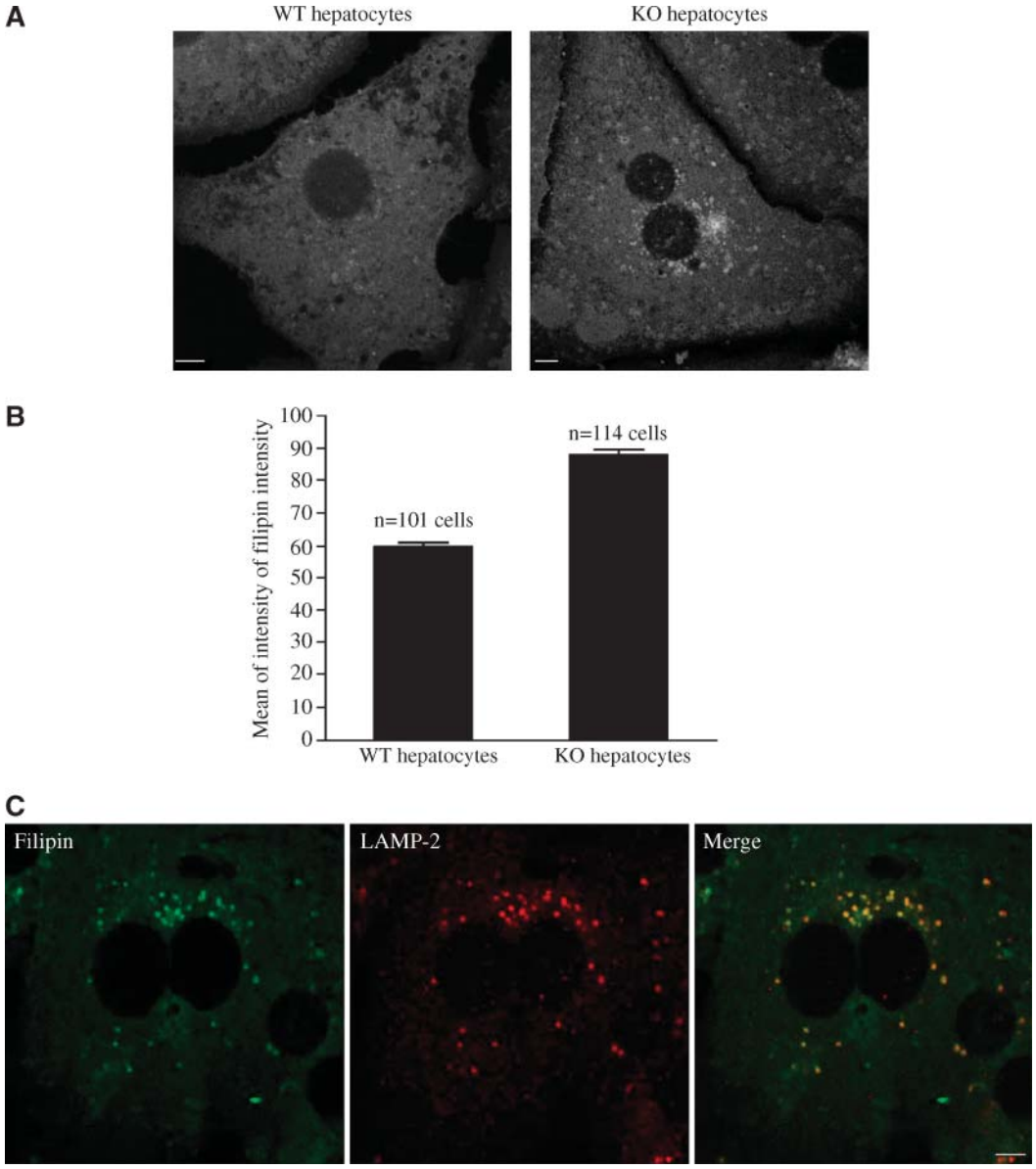

Fig. 5. SR-BI may function in cholesterol trafficking from lysosomal compartments. Wild-type (WT) and SR-BI$^{-/-}$ mouse primary hepatocytes were stained with filipin, and cells were processed for imaging. A: SR-BI $^{-/-}$ mouse primary hepatocytes were labeled with filipin and anti-LAMP-2 Ab. B: Cells were individually selected and the mean of intensity of the filipin channel was measured using ImageJ. Columns represent average of the mean of intensity of the filipin channel. Mean (SEM), where n = 101 cells for WT hepatocytes and 114 cells for SR-BI$^{-/-}$ hepatocytes. C: Images of filipin-stained primary hepatocytes from WT and SR-BI$^{-/-}$ mice. Bar = 5 μm.

the rate-limiting step in steroidogenesis, the transfer of cholesterol into the mitochondria (27). Notably, SR-BI mRNA and protein are expressed at high levels in the adrenal glands of StAR$^{-/-}$ mice and a subset of SR-BI is localized to LE, in close association with other cholesterol trafficking proteins including the START domain protein MLN64, and NPC1. However in these studies, overexpression of SR-BI in NPC1 deficient cells background failed to rescue the lysosomal cholesterol accumulation phenotype, suggesting that NPC1 and SR-BI may regulate cholesterol removal from lysosomes by independent pathways.

These data confirm previous reports that in cells that do not express CETP, binding and internalization of HDL requireSR-BI. Of note, the pool of SR-BI which is localized to the late endosome, is not involved in HDL binding or internalization. Expression of the constitutively active GFP-Rab7Q^{79L}, which trapped SR-BI in late endosomes, did not stimulate the internalization of HDL, as compared with that observed in SR-BI- or SR-BI and GFP-Rab7^{WT}-expressing cells. Similarly, expression of the DN GFP-Rab7^{T22N} did not inhibit the SR-BI-dependent internalization of HDL. Thus, the endocytosis of HDL is not linked to the late endososmal/lysosomal pool of

SR-BI but appears solely dependent on plasma membrane-localized SR-BI.

CONCLUSIONS

These studies show that in addition to its plasma membrane location, SR-BI forms a stable structure on late endosomes/lysosomes and that this localization is dependent on the protein Rab7. This raises the possibility that SR-BI may be involved in the trafficking of cholesterol from lysosomal compartments to other organelles, possibly endoplasmic reticulum, Golgi, or plasma membrane. It is also possible that in hepatocytes, lysosomal SR-BI is involved in the transfer of cholesterol from those organelles to the bile canaliculi (BC). Indeed, experiments in a hepatocyte cell line, WIF-B, showed that SR-BI and cholesterol follow the trafficking pathway (28). In addition, immunolabelling of lysosomal SNAREs, syntaxin 7 in the same cell type, revealed lysosomal structures in close vicinity to the BC (29), suggesting that cholesterol may traffic from lysosomes to BC and that this trafficking could be dependent on SR-BI. More experiments are under way to investigate this possibility.

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