Human scavenger receptor class B type II (SR-BII) and cellular cholesterol efflux

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Although studies in recombinant cells indicate that scavenger receptor class B, type I (SR-BI) can promote cholesterol efflux, investigations in transgenic mice overexpressing or deficient in SR-BI endorse its physiological function as selectively sequestering cholesteryl esters from high-density lipoproteins (HDLs). Less clear is the role of SR-BII, a splice variant of the *SR-B* gene that differs only in the C-terminal cytoplasmic domain. Here, we identify several putative signalling motifs in the C-terminus of human SR-BII, which are absent from SR-BI, and hypothesize that these motifs interact with signalling molecules to mobilize stored cholesteryl esters and/or promote the efflux of intracellular free cholesterol. 'Pull-down' assays using a panel of tagged SH3 (Src homology 3) domains showed that cytoplasmic SR-BII, but not cytoplasmic SR-BI, bound the SH3 domain of phospholipase C- γ 1; this interaction was not, however, detected under more physiological conditions. Specific anti-peptide antisera identified SR-BII in human monocyte/macrophage THP-1 cells and, in re-

combinant cells, revealed receptor localization to caveolae, a plasma membrane microdomain that concentrates signal-transducer molecules and acts as a conduit for cholesterol flux between cells and lipoproteins. Consistent with its caveolar localization, expression of human SR-BII in recombinant Chinese hamster ovary cells (CHO-SR-BII) was associated with increased HDL-mediated cholesterol efflux. Nevertheless, when CHO-SR-BII cells were pre-loaded with cholesteryl [³H]oleate and incubated with HDL, cholesteryl ester stores were not reduced compared with control cells. We conclude that although human SR-BII is expressed by macrophages, contains cytoplasmic signalling motifs and localizes to caveolae, its ability to stimulate cholesterol efflux does not reflect enhanced hydrolysis of stored cholesteryl esters.

Key words: caveolae, cholesteryl ester, intracellular signalling, lipid raft, scavenger receptor class B type I, Src homology 3 (SH3).

INTRODUCTION

Mouse scavenger receptor class B, type I (SR-BI) was the first physiological HDL (high-density lipoprotein) receptor to be identified [1]. It is highly expressed in tissues with an elevated cholesterol demand, such as the liver (for bile-acid synthesis) and the adrenal gland, ovaries and testes (for steroidogenesis), where it functions to bind mature HDL particles and selectively remove cholesteryl esters [2,3]. In addition, SR-BI promotes HDL-mediated cellular cholesterol efflux [4] and, as the receptor is expressed at low levels in many cells, including macrophages [4,5], it may also contribute to cellular cholesterol homeostasis by facilitating cholesterol egression.

Alternative splicing of the *SR-B* gene transcript generates two isoforms (types I and II) with identical extracellular regions, but distinct C-terminal cytoplasmic tails [6]. Significant proportions of SR-BII mRNA are expressed in the mouse; the receptor binds HDL with high affinity, and mediates both selective lipid uptake and cellular cholesterol efflux, albeit less efficiently than mouse SR-BI [7]. Both isoforms localize to caveolae [7,8], cholesterol-rich plasma membrane microdomains that are implicated in cellular cholesterol homeostasis and in cell signalling [9,10]. In contrast, there is little information on human SR-BII. In the human monocytic (THP-1) and hepatoblastoma (HepG2) cell lines, SR-BII represents approx. 30% and 20% of total SR-BI/II mRNA respectively [7], but SR-BII protein has not been detected in THP-1 cells or unstimulated HepG2 cells, and its physiological significance has been questioned [5,11].

Atherosclerotic lesions are characterized by cholesteryl ester-engorged macrophages within the arterial intima. Cholesteryl ester mobilization involves HDL binding to an unknown cell-surface receptor, activation of signalling pathways and hydrolysis to free cholesterol, which is translocated to the plasma membrane for efflux [12,13]. In the present study, we describe signalling motifs in C-terminal cytoplasmic human SR-BII, which have the potential to interact with SH (Src homology) 2 and 3 domains [14,15]; these are absent from SR-BI. We hypothesize that the main physiological function of human SR-BII is to bind HDL and transduce signals, via intracellular protein–protein interactions, that promote cholesteryl ester hydrolysis and free cholesterol translocation to the plasma membrane for efflux. We have therefore undertaken studies: (i) to determine whether THP-1 macrophages express SR-BII; (ii) to verify caveolar localization of the human receptor; (iii) to identify signalling molecules that might bind cytoplasmic SR-BII; and (iv) to investigate whether human SR-BII promotes cholesteryl ester mobilization and/or free cholesterol efflux.

EXPERIMENTAL

Materials

A 22-amino-acid sequence within cytoplasmic human SR-BII (residues 493–514; EDTVSQPLAAGPDRPPSPYTP) was conjugated to KLH (keyhole-limpet haemocyanin) and used to produce anti-peptide antisera (anti-SR-BII) in rabbits. Anti-CLA-1

Abbreviations used: ABCA1, ATP-binding cassette transporter A1; apo, apolipoprotein; CHO, Chinese hamster ovary; CLM, caveolin-rich light membrane; ECL, enhanced chemiluminescence; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; GST, glutathione S-transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PLC, phospholipase C; SH, Src homology; SR-BI/II, scavenger receptor class B, type I/II.

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(Transduction Laboratories, Lexington, KY, U.S.A.) and anti-RED-1 (Novus Biologicals, Littleton, CO, U.S.A.), which recognize different parts of the extracellular domain common to human SR-BI/II, were used for immunoblotting and flow cytometry respectively. The GST (glutathione S-transferase)-tagged SH3 domain-containing proteins were generously given by Dr I. Gout (Ludwig Institute, London, U.K.). Low-density lipoproteins (LDLs) (1.019–1.063 g/ml) and HDL₃ particles (1.125–1.21 g/ml) were isolated from fresh human plasma by sequential ultracentrifugation [16]. 'HDL plasma' was prepared by chemically precipitating apoB (apolipoprotein B)-containing lipoproteins [17], whereas human apoAI was purified from plasma as described previously [18].

Cell culture

HepG2, THP-1 and Chinese hamster ovary (CHO)-K1 cells (European Collection of Cell Cultures, Porton Down, Salisbury, Wilts., U.K.) were grown in DMEM, RPMI-1640 and Ham's F12 medium respectively at 37 °C in 5 % CO₂. Each medium was supplemented with 10 % (v/v) FBS (fetal bovine serum), 2 mM glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin. Suspension cultures of THP-1 cells were differentiated to adherent mature macrophages over 7 days by adding PMA (250 nM) to the medium every 2 days [19].

Pull-down binding assays with cytoplasmic human SR-BI and -BII

The 3' end of C-terminal cytoplasmic human SR-BII was amplified (35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 62 °C, and elongation for 1 min at 72 °C) from a THP-1 cDNA template using specific forward (5'-GAT CTC CTG AGG ACA CCG TGA GC) and reverse (5'-GAT CCG ATC CGA GGC TCA GGC TGT GG) primers containing *Bsu*36I and *Bam*HI restriction sites (underlined) respectively. After cloning the sequence into the pGEM-TTM vector, the plasmid was cut with *Pst*I and *Bsu*36I. Finally, a pair of annealed oligonucleotides encoding the first 7 amino acids of the cytoplasmic tail, and a *Hind*III restriction site (underlined) for subsequent manipulation, was ligated into the vector using the relevant sticky ends (forward oligonucleotide: 5'-GAA GCT TCA AAT CCG GAG CCA AGG TCC; reverse oligonucleotide: 5'-TCA GGA CCT TGG CTC CGG ATT TGA AGC TTC TGC A).

Similarly, the full-length human SR-BI sequence was amplified from the THP-1 cDNA, using specific primers (forward: 5'-GAT CAA GCT TCC TGT GTC GTC TCT GTC G; reverse: 5'-GAT CCG ATC CGA GGC TCA GGC TGT GG) and the AdvantageTM-GC cDNA PCR kit (Clontech, Basingstoke, Hants., U.K.), and ligated into pGEM-TTM. The plasmid was then cut with *Sac*II and *Acc*III to remove the 5' end of the SR-BI sequence and replaced with a pair of annealed oligonucleotides to generate the complete C-terminal cytoplasmic SR-BI tail sequence (forward oligonucleotide: 5'-GGA AGC TTC AAA T; reverse oligonucleotide: 5'-CCG GAT TTG AAG CTT CCG C).

Both cytoplasmic tail sequences were manipulated into the Pin-PointTM vector using the *Bam*HI and *Hind*III restriction sites in the reverse primers and polylinker sequences respectively. Biotinylated fusion proteins were expressed in JM109 *Escherichia coli* cells and isolated by avidin resin chromatography. The tail sequences were also transferred into pGEX-4T (Amersham Biosciences, Little Chalfont, Bucks., U.K.) to generate GST-tagged fusion proteins. These were expressed in the BL21 *E. coli* bacterial strain and purified using the GST Purification Module (Amersham Biosciences).

Affinity matrices were prepared by immobilizing biotinylated cytoplasmic SR-BI/II to TetraLinkTM tetrameric avidin resin

(Promega) and GST-tagged SH3 domain-containing fusion proteins to glutathione-agarose beads [20]. The individual matrices were incubated with target proteins in PBS on a rotating wheel at 4 °C for 2 h. After washing the beads 5 times, bound protein was eluted by boiling in SDS/PAGE sample buffer containing 2-mercaptoethanol, and analysed by immunoblotting using either strept-avidin-horseradish peroxidase and ECL (enhanced chemiluminescence) or anti-GST (Amersham Biosciences) for detection.

Generating recombinant CHO-SR-BII clones

Full-length SR-BII cDNA was cloned into the pCDNA3 expression vector (Invitrogen, Groningen, The Netherlands) and used to transfect CHO-K1 cells. Stably transfected cells were selected by culturing in G418 (500 µg/ml), and cell-surface expression was monitored by flow cytometry using anti-RED-1 (1:1000 dilution) and a goat anti-rabbit secondary antibody conjugated to FITC (1:800 dilution) for detection. FITC fluorescence was measured in fixed single-cell suspensions using 488 nm for excitation with emissions collected between 520 and 530 nm (FACS Calibur; Becton Dickinson, Bedford, U.K.). The selected cells were then cloned by limiting dilution, analysing the expanded clones by Western blotting with anti-CLA-1 to assess relative expression levels of SR-BII protein. For accurate comparisons, CHO-SR-BII clones were surface-labelled with 0.5 mg/ml EZ-Link Sulpho-NHS-LC-BiotinTM (Perbio Science Ltd., Cheshire, U.K.), lysed and the biotinylated membrane proteins were isolated using pre-washed streptavidin beads. Samples were separated by SDS/PAGE on Novex gels [8 % or 4–20 % (w/v) polyacrylamide; Invitrogen] and relative levels of SR-BII in the surface membrane were quantified using anti-CLA-1 for Western blotting and scanning densitometry. Caveolin-rich light membranes (CLMs) from CHO-SR-BII cells were prepared using a detergent-free method, and were analysed as described previously [21].

Free cholesterol efflux studies

Recombinant CHO-SR-BII cells and CHO-K1 control cells were seeded overnight in 12-well plates (175 000 cells/well) and then incubated for 24 h with 1 ml of medium containing 5 % (v/v) FBS and 1 µCi of [³H]cholesterol. Cell monolayers were washed twice and equilibrated for 16 h with 1 ml of serum-free medium, containing 1 % (w/v) fatty-acid-free BSA. Cholesterol efflux was stimulated by adding 1 ml of serum-free medium containing 200 µg of HDL₃ protein. Background efflux was determined by incubating cells with serum-free medium alone. Efflux was stopped by removing the medium and dissolving the cells in 0.1 M NaOH (1 ml). Radioactive [³H]cholesterol in cells and the medium was measured by liquid scintillation counting to calculate the percentage efflux.

Cholesteryl ester mobilization studies

For each experimental condition, triplicate wells of recombinant CHO-SR-BII cells and CHO-K1 cells were seeded overnight in 12-well plates and then serum-starved for 24 h in serum-free medium containing 1 % (w/v) BSA. Next, the cells were labelled by incubation for 36 h with 1 ml of serum-free medium containing 40 µM [³H]oleic acid (0.5 µCi/ml), complexed with BSA [22] and 100 µg of LDL protein. The medium was removed and, following an overnight equilibration in serum-free medium containing 1 % (w/v) BSA, the cells were incubated with or without 200 µg of HDL₃ protein. After 4, 8 and 24 h, cellular lipids were extracted using hexane/propan-2-ol (3:2, v/v) 'spiked' with 100 000 d.p.m. [¹⁴C]cholesterol to control recoveries. Free

Figure 1 Localization of human SR-BII to CLMs and co-immunoprecipitation with caveolin-1

Recombinant CHO-K1 cells expressing either human SR-BI or -BII were sonicated in buffer containing 500 mM sodium carbonate, and the homogenates were fractionated on a 5–35% (w/v) discontinuous sucrose density gradient. **(A)** Fractions (12 × 1 ml) were collected and analysed by reduced SDS/PAGE and silver staining (4–20% acrylamide gels; molecular-mass markers are shown to the right). **(B)** Replicate gels were immunoblotted with antibodies against caveolin-1, clathrin heavy chain and SR-BII. **(C)** CLM (250 μ l; fractions 4–6) were immunoprecipitated with polyclonal anti-caveolin Ig. Equivalent volumes of the starting CLM, post-adsorption supernatant (S/N), and adsorbed proteins eluted from the beads (eluate) were analysed by Western blotting using SR-BII antiserum or anti-caveolin monoclonal antibody.

and esterified cholesterol were separated by TLC [18], and their radioactivities were measured by liquid scintillation counting, while the residual cell monolayer was dissolved in 0.1 M NaOH (1 ml) for protein quantification. The intracellular cholesteryl [3 H]oleate content was calculated as d.p.m. of 3 H/mg of cell protein, and expressed as a percentage of that present at zero time. This value was then subtracted from 100% to give the percentage of cholesteryl ester mobilized for each of the two cell types studied: CHO-K1 control cells and CHO-SR-BII recombinant cells.

Statistical analysis

Data in text and figures are expressed as means $\pm$ S.E.M. Statistical differences between means were determined by Student's unpaired *t* test, and considered significant if *P* < 0.05.

RESULTS

Co-immunoprecipitation of SR-BII with caveolin

CLM were prepared from recombinant CHO-SR-BII cells using the detergent-free carbonate method and discontinuous density-gradient ultracentrifugation (Figure 1A). Immunoblotting for caveolin-1 confirmed that CLM had localized at the 5/35% (w/v) sucrose interface in fractions 4–6 (Figure 1B). CLM comprised about 5% of total cellular protein, whereas the remaining protein was found in the bottom fractions, along with clathrin heavy chain,

Figure 2 Potential signalling motifs in C-terminal cytoplasmic SR-BII

Several sequences were identified within cytoplasmic SR-BII that have the potential to bind intracellular signalling molecules: six proline-rich motifs (PxxP) (shaded box), which could recognize SH3 domains, and a candidate SH2 binding site, containing a tyrosine residue and a hydrophobic amino acid at position +3 (dotted line).

the main structural protein of clathrin-coated pits [23] (Figure 1A). Human SR-BII was localized in the caveolin-1-containing CLM fractions from CHO-SR-BII cells (Figure 1B), as shown by immunoblotting with our specific anti-peptide antisera directed against its unique C-terminal cytoplasmic tail. When the caveolin-containing vesicles were separated from other lipid rafts using polyclonal anti-caveolin-1 [21,24], the majority of SR-BII co-immunoprecipitated with caveolin-1 (Figure 1C). This finding confirms that SR-BII localizes to true caveolae, a plasma membrane microdomain involved in cholesterol homeostasis and signal transduction.

Signalling potential of cytoplasmic SR-BII

The type I and II splice variants of the *SR-B* gene differ in their C-terminal cytoplasmic tails. We hypothesized that HDL binding to the extracellular domain of SR-BII promotes signal transduction via intracellular protein–protein interactions involving this C-terminal region. In support, we identified six PXXP (Pro-Xaa-Xaa-Pro) motifs in the 44-amino-acid SR-BII tail sequence that might serve as ligands for SH3-domain-containing proteins [14,15,25] (Figure 2), whereas intriguingly only two are present in mouse SR-BII [7]. Furthermore, the (phospho)tyrosine residue in the YTPL sequence is a potential SH2 domain recognition site, since there is a hydrophobic amino acid at position +3 [14,26]; conceivably, HDL binding might activate phosphorylation of this cytoplasmic tyrosine.

The signalling potential of cytoplasmic SR-BII was investigated by 'pull-down' assays. Initially, we screened a panel of GST-tagged SH3 domains for binding interactions with biotinylated cytoplasmic SR-BII. These assays repeatedly highlighted an interaction between the SH3 domain of phospholipase C- γ 1 (PLC- γ 1) and the cytoplasmic tail of SR-BII (Figure 3A). This interaction was confirmed as type II-isoform-specific by using human SR-BI and -BII affinity matrices in reverse pull-down assays (Figure 3B). To determine its physiological significance, further experiments were performed under more demanding conditions. First, pull-down assays were repeated using the cytoplasmic SR-BII affinity matrix and human cell lysates. Despite readily demonstrating full-length PLC- γ 1 in the lysates by immunoblotting, we failed to detect any interaction with cytoplasmic SR-BII (Figure 4A). Secondly, CHO-SR-BII cells were pre-incubated with HDL to activate the receptor, and then the cell lysate was incubated with a

Figure 3 'Pull-down' assays suggest a specific interaction between cytoplasmic SR-BII and the SH3 domain of PLC-γ1

(A) GST-tagged SH3 domains (2 μ g) from several signalling proteins were immobilized on glutathione-agarose beads (50 μ l) and incubated with biotinylated cytoplasmic SR-BII (2 μ g in 200 μ l of PBS). The beads were washed, and any bound cytoplasmic SR-BII was eluted in SDS/PAGE sample buffer for detection by Western blotting with streptavidin-horseradish-peroxidase. (B) To determine whether this interaction was isoform specific, the 'pull-down' assay was reversed. Affinity matrices of biotinylated cytoplasmic SR-BI or II (2 μ g) bound to avidin beads (50 μ l) were incubated with GST-tagged PLC-γ1 SH3 domain (2 μ g in 200 μ l PBS); any interacting protein was detected using anti-GST antibody.

Figure 4 Failure to verify potential cytoplasmic SR-BII signalling under more physiological conditions

(A) Affinity matrices of GST-tagged cytoplasmic SR-BI/II or GST tag alone (25 μ g) on glutathione-agarose beads (50 μ l) were incubated with 1 ml of THP-1 cell lysate (1 mg/ml). Any interacting proteins were analysed by Western blotting with an anti-PLC-γ1 antibody; 25 μ g of cell lysate was used as a positive control. (B) Dishes of recombinant CHO-SR-BII were maintained in growth media (controls) or incubated for 5 and 30 min in serum-free media with and without 5% (v/v) 'HDL plasma'. Cell lysates were immunoprecipitated with anti-CLA-1 antibody and analysed by Western blotting, probing with anti-peptide SR-BII antiserum and anti-phosphotyrosine (anti-pY).

GST-PLC-γ1 affinity matrix; a binding interaction was still not detected (results not shown). Thirdly, we investigated recruitment of PLC-γ1 to CHO-SR-BII caveolae upon HDL stimulation, but failed to find an increase in PLC-γ1 content when CLM were immunoprecipitated with caveolin-1 (results not shown). This implied that ligand binding did not recruit PLC-γ1 into caveolae microdomains to interact with cytoplasmic SR-BII. We also analysed the immunoprecipitates by SDS/PAGE and silver staining,

but noted no obvious differences in protein composition between caveolae from stimulated or unstimulated cells. Finally, we monitored tyrosine phosphorylation in cytoplasmic SR-BII in response to HDL stimulation. Although the receptor was successfully immunoprecipitated from CHO-SR-BII cell lysates, Western blotting with anti-phosphotyrosine [27] consistently failed to reveal any phosphorylation (Figure 4B).

SR-BII and free cholesterol efflux

CHO-K1 cells were stably transfected with an expression vector encoding full-length human SR-BII cDNA and expanded under selection pressure. A flow cytometry screen with anti-RED-1 of the cells showed a clear, albeit modest, right shift in the fluorescence peak of recombinant CHO-SR-BII cells compared with CHO-K1 controls (Figure 5A). To determine whether human SR-BII promotes HDL-mediated cholesterol efflux, as reported for mouse SR-BII [7], we labelled cellular free cholesterol plasma membrane pools with [3 H]cholesterol, and incubated them with HDL or lipid-free apoAI for 4 h to stimulate efflux. As expected, HDL stimulated significantly the efflux of more radiolabelled cholesterol from CHO-SR-BII cells than from CHO-K1 controls ($10.0 \pm 0.4\%$ cholesterol efflux promoted/4 h compared with $6.9 \pm 0.3\%$; means $\pm$ S.E.M; $P < 0.001$) (Figure 5B). Importantly, lipid-free apoAI released less than one-fifth of the cholesterol from CHO-SR-BII cells compared with HDL ($1.85 \pm 0.1\%$; $P < 0.001$), implying that apoAI must be lipoprotein-bound to interact efficiently with SR-BII. Nevertheless, lipid-free apoAI stimulated significantly ($P < 0.001$) more cholesterol efflux than serum-free media, presumably via ATP-binding cassette transporter A1 (ABCA1) activity [28].

We also compared recombinant CHO-SR-BII clones expressing SR-BII at low, medium and high levels with CHO-K1 cells, screening the relative amounts of receptor protein in the surface membrane by Western blotting with anti-CLA-1 (Figure 5C). Cells expressing even low levels of SR-BII showed significantly greater efflux capacity than CHO-K1 controls ($12.3 \pm 0.5\%$ cholesterol efflux promoted/4 h compared with $10.9 \pm 0.3\%$; $P < 0.05$), while those expressing medium and high amounts of SR-BII had markedly enhanced increases ($30.5 \pm 0.3\%$ and $42.2 \pm 0.6\%$, respectively; both $P < 0.001$) (Figure 5C). These data suggest that human SR-BII is biologically active in our recombinant cells and is capable of promoting HDL-dependent cholesterol efflux.

SR-BII and cholesterol ester mobilization

Although SR-BII mRNA transcripts are present in both THP-1 and HepG2 human cell lines, protein expression is reported only in hormone-induced HepG2 cells [11]. To detect SR-BII protein in THP-1 monocytes and macrophages, as well as HepG2 cells, we analysed biotinylated cell-surface proteins by Western blotting using our isoform-specific anti-peptide antiserum. This revealed SR-BII in unstimulated HepG2 cells, as well as lower levels in THP-1 monocytes and macrophages (Figure 6A). The mobility of SR-BII was slightly lower by SDS/PAGE in CHO-SR-BII cells compared with the human cell lines, but this may reflect cell-type differences in glycosylation, as noted for other lipoprotein receptors [9]. An extra low-molecular-mass band was also seen in HepG2 and THP-1 cells; whether this represented a cross-reacting protein or SR-BII with different post-translational modifications was not investigated. The finding that THP-1 macrophages express SR-BII supports the hypothesis that this receptor might function to mobilize stored cholesterol esters, thus promoting regression of macrophage foam cells within atherosclerotic lesions.

Figure 5 Promotion of human SR-BII by free cholesterol efflux

(A) Stably transfected CHO-SR-BII cells and CHO-K1 controls were incubated with anti-RED-1 primary antibody (non-immune serum as a negative control), followed by a FITC-conjugated secondary antibody. FITC fluorescence of 5000 intact gated cells or 'events' was detected using flow cytometry and is displayed as a histogram against a one-decade log¹⁰ fluorescence scale. The experiment was performed in triplicate, but only one representative histogram for each cell type is shown. (B) Recombinant CHO-SR-BII cells (shaded bars) and CHO-K1 controls (white bars) were pre-labelled with [³H]cholesterol and then incubated with 5% (v/v) 'HDL plasma', 10 µg/ml lipid-free apoA1 or serum-free medium for 4 h. Efflux of [³H]cholesterol in triplicate wells from two separate experiments was expressed as a percentage of total [³H]cholesterol in the media and cells (means ± S.E.M.). (C) Cloned CHO-SR-BII cell lines expressing low, medium and high levels of receptor, as judged by Western blotting of isolated membranes (inset), and control CHO-K1 cells were pre-labelled with [³H]cholesterol and then incubated with HDL₃ (200 µg of protein/ml) for 4 h. Efflux of [³H]cholesterol in quadruplicate wells was expressed as a percentage of total [³H]cholesterol in the media and cells (means ± S.E.M.), with background values subtracted. The CHO-SR-BII clones promoted the efflux of significantly more cholesterol than the control cells (**P* < 0.05 and ****P* < 0.001; Student's *t* test).

As the above cholesterol efflux studies confirm that our recombinant CHO-SR-BII cells express functional receptors, we used these cells to investigate whether SR-BII mobilizes intracellular cholesteryl ester. The cholesteryl ester pools in low- and high-expressing CHO-SR-BII clones were labelled with [³H]oleate, and then reductions in radioactive cholesteryl ester were followed during 4, 8 and 24 h of HDL₃ stimulation (Figure 6B). The low CHO-SR-BII clone showed substantial reductions in cholesteryl [³H]oleate (about 40% and 50% at 4 h and 8 h respectively), but these were not significantly different from control CHO-K1 cells (*P* > 0.05). Surprisingly, HDL₃ mobilized much less cholesteryl [³H]oleate from the high CHO-SR-BII clone than from the low CHO-SR-BII clone or CHO-K1 cells (for example, at 8 h,

Figure 6 SR-BII protein is expressed by THP-1 macrophages, but the receptor does not stimulate HDL-mediated mobilization of intracellular cholesteryl ester in recombinant CHO cells

(A) Biotinylated membrane proteins were isolated from THP-1 monocytes/macrophages and HepG2 cell lysates (1 mg/ml) using streptavidin beads, and receptor expression was analysed by ECL-Western blotting using isoform-specific anti-peptide SR-BII antiserum; CHO-SR-BII lysate served as a positive control. (B) CHO-K1 control cells and recombinant CHO-SR-BII clones expressing low and high levels of SR-BII were labelled with [³H]oleate and stimulated with HDL₃ (200 µg of protein/ml) for up to 24 h. The percentage of cholesteryl [³H]oleate mobilized (means ± S.E.M.) was calculated for each cell line and plotted against time. Similar results to those shown were obtained in two additional experiments.

18 ± 2% compared with 51 ± 4% and 45 ± 3% respectively; *P* < 0.001). These data suggest that SR-BII does not play a role in mobilizing cholesteryl ester stores.

DISCUSSION

Peripheral cells, including those in arterial tissue, are unable to catabolize cholesterol and promote the efflux of any excess to circulating HDL acceptors [28]. This process is critical to cellular cholesterol homeostasis and, if inefficient, esterified cholesterol accumulates in lipid-laden macrophages within the arterial intima. Here, we investigated the role of the HDL receptor, SR-BII, in cholesterol metabolism and whether this involves signal transduction. We found SR-BII to localize to caveolae microdomains, to be present in THP-1 macrophages and to potentially bind signalling molecules via cytoplasmic SH3 motifs. However, although we showed that SR-BII promoted a 2-fold-increased efflux of free cholesterol compared with the major splice variant, SR-BI, we failed to find evidence that SR-BII mobilizes intracellular cholesteryl ester stores.

When CLMs from CHO-K1 cells expressing human SR-BII were immunoprecipitated with anti-caveolin-1, the receptor was also isolated. This caveolar localization was predicted. Although anti-caveolin fractionation was not used, both mouse SR-BII and SR-BI localize to these cholesterol-enriched microdomains [29], which implies that the targeting signal resides in their homologous regions and not in their distinct C-terminal cytoplasmic tails. Indeed, fatty acylation of SR-BI and II is the likely trigger [30], most probably via palmitoylation of Cys⁴⁶², which is common to both [7]. Caveolae act as conduits for cellular cholesterol flux [10] and also concentrate signalling proteins to promote signal

transduction from transmembrane receptors via second messengers [9]. In mouse, caveolar SR-BI selectively removes cholesteryl esters from HDL [8,31,37] and promotes HDL-mediated cholesterol egress [4]. Mouse SR-BI is also implicated as a signal transducer, interacting with a PDZ domain-containing protein named PDZK1 [32], and binding HDL to stimulate nitric oxide synthase (NOS3) activity [33]. By contrast, the role of SR-BII is ill-defined, although our finding that human SR-BII localizes to caveolae highlights its potential for signal transduction and for mediating cholesterol flux.

We initially investigated SR-BII signalling potential in 'pull-down' assays. As we had identified six putative SH3 motifs within C-terminal human SR-BII (Figure 2), four more than in mouse SR-BII [7], we screened a panel of immobilized SH3 domain proteins for their ability to capture purified cytoplasmic SR-BII, using SR-BI as a negative control. Preliminary results revealed PLC- γ 1 as a binding partner, an attractive candidate for SR-BII signalling since this modular enzyme associates with caveolae and activates protein kinase C [34,35], a feature of translocation and efflux of intracellular cholesterol mediated by HDL [12,36]. However, binding between PLC- γ 1 and SR-BII could not be confirmed in cell lysates; nor could we show recruitment of PLC- γ 1 to CHO-SR-BII caveolae upon HDL stimulation. Thus, although a proline-rich motif(s) in cytoplasmic SR-BII interacts with the SH3 domain of PLC- γ 1 in an isolated system, this partnership seems unlikely in a cellular environment even when the receptor is activated by HDL binding. The potential tyrosine-containing SH2 binding site within cytoplasmic SR-BII was also rejected as a signal transducer for HDL binding; anti-phosphotyrosine Western blotting of immunoprecipitates from HDL-stimulated CHO-SR-BII cells repeatedly failed to detect tyrosine phosphorylation in activated SR-BII.

Next, we investigated whether human SR-BII functions to promote HDL-stimulated free cholesterol efflux, as reported for the type I isoform and also mouse SR-BII [4,7], and/or to mobilize cholesteryl ester stores. By labelling cholesterol in three lines of recombinant CHO-SR-BII cells, we showed that the efflux of free cholesterol to HDL increased as levels of SR-BII protein rose, implying a link between cellular SR-BII content and HDL-dependent cholesterol efflux. Before studying cholesteryl ester mobilization in CHO-SR-BII cells, we tried to detect SR-BII in human macrophages, the precursors of cholesteryl ester-rich 'foam cells' which characterize atherosclerotic lesions. Although Matveev et al. [5] briefly stated that SR-BII protein was not detected in human THP-1 monocytes or macrophages, our Western blotting combination of isolated cell-surface proteins and specific anti-SR-BII clearly demonstrated SR-BII expression. This sensitive technique also revealed SR-BII protein in unstimulated HepG2 cells, contradicting an earlier report [11]. To assess whether SR-BII mobilizes cholesteryl ester, recombinant CHO-SR-BII cells expressing low and high levels of the receptor were pre-incubated with [3 H]oleate and LDL to produce intracellular stores of cholesteryl [3 H]oleate. However, stimulation with HDL for up to 24 h failed to reduce the level of radiolabelled cholesteryl oleate below that of control CHO-K1 cells. Indeed, when expression of SR-BII was high, mobilization of intracellular cholesteryl ester was inhibited.

In summary, we have shown that human SR-BII is expressed by macrophages, localizes to caveolae and contains cytoplasmic signalling motifs. However, our findings do not support a direct role for SR-BII in mobilizing intracellular cholesteryl ester deposits, although they do suggest an important role in cholesterol efflux and cellular cholesterol homeostasis. Whether the signalling motifs present in cytoplasmic human SR-BII have functional significance remains to be elucidated. However, it is noteworthy

that several lipoprotein receptors are increasingly recognized to function as signal transducer molecules, rather than as passive endocytic agents to capture circulating lipids [21,33,38–40]. Furthermore, although the target of SR-BII signalling is not cholesteryl ester hydrolase, there are several other potential targets that could enhance cholesterol efflux. These include inhibition of acyl-CoA:cholesterol acyltransferase, stimulation of cholesterol synthesis, and an acceleration of intracellular trafficking of cholesterol to the plasma membrane, perhaps by promoting the movement of ABCA1 from endocytic vesicles to the cell surface [41]. As yet, these mechanisms are speculative, but will clearly be of interest to examine in future studies.

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