The Di-Leucine Motif Contributes to Class A Scavenger Receptor-Mediated Internalization of Acetylated Lipoproteins

Yaoyu Chen, Xiaohua Wang, Jingjing Ben, Shen Yue, Hui Bai, Xiaoxiang Guan, Xiaoming Bai, Li Jiang, Yong Ji, Leming Fan, Qi Chen

Objective—The di-leucine motif exists in the intracellular domains of certain cell surface receptors, participating in the receptor-mediated endocytosis. The present study was aimed at determining the role of the di-leucine motif in class A scavenger receptor (SR-A)-mediated ligand endocytosis.

Methods and Results—cDNA coding for a mutant (SR-A mutant N3132LM) with deletion of the di-leucine structure was transfected into Chinese hamster ovary (CHO) cells. Compared with wild-type SR-A–expressing cells, the cells expressing the SR-A mutant N3132LM showed a significant decrease in uptake but almost no change in binding of the SR-A ligand acetylated low-density lipoprotein (AcLDL). Western blot analysis revealed coimmunoprecipitation of SR-A mutant and clathrin from the lysates of the mutant but not wild-type CHO cells, suggesting that AcLDL-bound SR-A mutant N3132LM is associated with the clathrin-coated pit of cellular membrane. Removal of the first 27 amino acid residues from the SR-A N-terminus further reduced AcLDL uptake by the cells with the di-leucine motif mutation.

Conclusions—The di-leucine motif of SR-A intracellular domain contributes to the SR-A–mediated cellular internalization of AcLDL. Di-leucine pair exists in the cytoplasmic domain of class A scavenger receptor. The cells expressing di-leucine mutants showed decreased uptake and unchanged binding of AcLDL. The di-leucine pair was not associated to coated pits. It suggests that di-leucine motif acts as a signal sequence to mediate SR-A into cell. (Arterioscler Thromb Vasc Biol. 2006;26:1317-1322.)

Key Words: atherosclerosis ■ class A scavenger receptor ■ di-leucine motif ■ internalization ■ clathrin-coated pit

Atherosclerosis is a leading cause of coronary arterial disease characterized by cholesterol deposition and inflammatory infiltration with macrophage and lymphocytes in response to “invading” pathogenic lipoproteins in the arterial wall.1–4 The first macroscopically recognizable lesion of atherosclerosis or “fatty streak” arises from intimal accumulation of macrophage-derived foam cells with cholesterylester-rich lipid droplets.5 The formation of macrophage-derived foam cells involves increased expression of a family of integral membrane glycoproteins, namely scavenger receptors, that mediate binding and uptake of native and modified lipoproteins by macrophages.6 In terms of lipid transport, the class A scavenger receptor (SR-A) and CD36 account for 75% to 90% of degradation of low-density lipoprotein (LDL) modified by acetylation or oxidation.7 Acetylated low-density lipoprotein (AcLDL), an artificially modified LDL, serves as a ligand of SR-A widely used for in vitro determination of SR-A activities.8 Thus, analysis of SR-A–mediated AcLDL uptake and accumulation may help unearth the potential molecular mechanism underlying lipid-laden foam cell formation.

Structurally, SR-A is a trimeric integral membrane glycoprotein with 2 isoforms generated through alternative splicing of a single gene.9,10 The carboxyl-terminal extracellular region of SR-A type I or the longer isoform contains a positively charged groove that allows SR-A to bind to a variety of polyanionic ligands including modified lipoproteins, bacterial products, and extracellular matrix proteins. The amino-terminal cytoplasmic tail of SR-A is composed of 50 amino acids in human, bovine, and rabbit that is responsible for the internalization of the ligands.10,11 SR-A–mediated internalization of modified LDL triggers a phagocytotic cascade containing the following steps: (1) cell surface ligand binding to SR-A; (2) concentration of the ligand-receptor complexes in coated pits; and (3) internalization into endosomes. The ligand dissociates from the receptor and is transported from endosomes to lysosomes, where it is metabolized, while the receptor recycles back to the cell surface.12

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Y. Chen and X. Wang contributed equally to this study.
Correspondence to Qi Chen, Nanjing Medical University, Nanjing, People’s Republic of China. E-mail qichen@njmu.edu.cn
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1317
This endocytic process is highly efficient, and regulated by certain receptor-mediated mechanisms. Unlike other lipoprotein receptors such as native LDL receptor, SR-A does not appear to cycle continuously through the metabolic pathway in the absence of ligands. This property reflects its unique regulatory mechanism behind uptake of a broad spectrum of ligands.

In general, receptor-mediated endocytosis requires specific internalization motifs within the cytoplasmic domain. The NXY motif in the cytoplasmic domain of LDL receptor is required for efficient ligand internalization and biological signaling. In insulin-like growth factor-I receptor, a similar motif is identified too. Our previous study has shown that the N-terminal cytoplasmic domain of SR-A is necessary to the receptor-mediated internalization of AcLDL. Furthermore, the pA3 to 27 amino acid sequence, phosphorylation sites, and VXFD in SR-A cytoplasmic domain has been identified as the potential signal motifs for internalization and adhesion of SR-A. The multiple signal motifs in SR-A are likely adapted to the intrinsic multifunctional properties of SR-A. Thus, it is of interest to explore the signal motif of SR-A for its functional regulation.

Di-leucine sequence represents one of the signal motifs important for endocytosis of plasma membrane proteins at coated pit. For instance, the di-leucine motif plays a key role in the internalization mediated by insulin receptor. In IL-6 receptor, glucose transporter-4 and CD4 the di-leucine motif acts in cooperation with an upstream serine. The motif function is not strictly dependent on its position in the cytoplasmic domain because it may function at the carboxyl terminus or as an internal sequence. All of the cytoplasmic domains of human, bovine and rabbit SR-A share a di-leucine motif at amino acid residues 31 and 32. However, the exact function of this pair of leucine residues remains unknown. To investigate the role of di-leucine motif in SR-A-mediated uptake of lipids, in this study, various SR-A mutants were constructed and expressed in Chinese hamster ovary (CHO) cells that lack the endogenous SR-A. Our data demonstrated for the first time to our knowledge that the SR-A di-leucine motif is critical for internalization of lipids by the receptor-expressing cells.

Methods

Human SR-A/EGFP (enhanced green fluorescence protein) was kindly provided by Dr Harald Heider at Institute of Biochemistry, University of Basel, Switzerland. The plasmid encoding the correct fusion protein (referred to as EGFP/SR-A) was mutagenized using the Stratagene Quickchange™ site-directed mutagenesis kit according to the instructions supplied by the manufacturer. Plasmids containing the correct mutation were identified with automated sequencing. CHO cells were transiently transfected with different constructed plasmids. Expression of SR-A and SR-A mutants in transfected cells was detected by Western blot analysis with anti-SR-A antibodies. C, Surface proteins of transfected CHO cells were biotinylated, lysed, and immunoprecipitated with anti-SR-A antibodies.

Results

Expression of SR-A and SR-A Mutant in Transfected CHO Cells

To track the dynamic changes of targeted proteins inside the cells, plasmid with cDNA insert coding for SR-A with or without mutation in the di-leucine motif fused to the C-terminal end of EGFP was constructed and used for transfection of CHO cells. The forced expression of SR-A or its mutant in the transfected CHO cells was verified by Western blot analysis with anti-SR-A antibodies (Figure 1) and by fluorescence microscopy (Figure 1, please see http://atvb.ahajournals.org). Quantification of SR-A protein expression by fluorescence-activated-cell sorter (FACS) measurement showed similar levels of expression of SR-A and its mutant N3132LM between SR-A and SR-A mutant cDNA-transfected cells (Figure 1A). This result was confirmed by Western blot analysis (Figure 1B). Biotinylation analysis of transfected cells revealed that both SR-A and SR-A mutant expressed on cellular plasma membrane. There was no significant difference in cell-surface expression between wild-type and mutant SR-A when expression levels of the plasmid were normalized with ImageJ software. No biotinylated proteins were detected in EGFP vector-transfected cells (Figure 1C).

Replacement of the Di-Leucine Motif Alters DiI-AcLDL Uptake by CHO Cells Expressing SR-A

Incubation with DiI-AcLDL (5 μg/mL) at 37°C for 5 hours made the transfected cells fluorescently visible under a laser.
fluorescent scanning confocal microscope. The N3132LM mutant-expressed cells showed both green and red fluorescences in plasma membrane region, which is the same pattern as wild-type SR-A–expressed cells both in the receptor protein expression (green fluorescence) and in the receptor uptake of ligand (red fluorescence) (Figure I). However, quantitative analysis by FACS revealed a significant difference in the uptake of DiI-AcLDL between SR-A- and N3132LM mutant-expressed cells. The overall uptake of DiI-AcLDL by N3132LM mutant-expressed cells was reduced by 37.98%, even though the same amount of DiI-AcLDL (5 μg/mL) was used for test (Figure 2A). Concerning the possible influence of transfection efficiency on the receptor expression and activity in cells, the intensity of red fluorescence was divided by the relative intensity of green fluorescence to reflect the uptake and binding of DiI-AcLDL by transfected CHO cells. It was demonstrated that the cellular uptake of DiI-AcLDL reduced by 38.13% in N3132LM mutant-transfected cells, as compared with that of wild-type SR-A. The difference was statistically significant (Figure 2B). To determine whether the impaired effect of DiI-AcLDL uptake was the consequence of reduced AcLDL binding, a measurement for DiI-AcLDL binding with transfected cells was performed. No significant difference in binding of DiI-AcLDL with cells was found between wild-type and mutant SR-A transfected cells (Figure 2C). Both uptake and binding of DiI-AcLDL by SR-A–transfected cells were dramatically inhibited in the presence of excess amounts of unlabeled AcLDL (Figure 2) (SR-A/AcLDL).

**Di-Leucine Motif-Mediated Endocytosis Is Not Associated With Clathrin-Coated Pit in CHO Cells**

The clathrin-coated pit is known to serve as an important membrane structure in mediating cellular endocytosis. SR-A locates in clathrin-coated pits to bind with its ligands, in which the cytoplasmic domain of SR-A plays an important role. To explore the role of di-leucine motif in localization of SR-A in clathrin-coated pit, the lysates of transfected cells were immunoprecipitated by an antibody against clathrin. Western blot analysis showed that the immunoprecipitate of SR-A–transfected cell contained clathrin as well as SR-A. Similar Western blot pattern was seen in the immunoprecipitate of N3132LM-transfected cell. However, in EGFP-transfected cell only clathrin was detected from the immunoprecipitates (Figure 3).

**Impact of Multiple Structural Changes in the SR-A Cytoplasmic Domain on DiI-AcLDL Uptake by CHO Cells**

The cytoplasmic domain of SR-A contains several unique amino acid sequences, such as the VXFD and phosphorylated sites, which mediate SR-A–mediated phagocytosis. Most of these motifs localize in the first half of cytoplasmic domain. To investigate the potential relationship of the di-leucine motif with those motifs, SR-A1 to 27 and SR-A1 to 27...
with the mutation of leucine to alanine (NΔ1 to 27LM) were constructed and transfected to CHO cells (Table). The expression of these truncated receptors was confirmed by Western blot analysis (data not shown). The cellular uptake of DiI-AcLDL decreased markedly in the cells expressing SR-A with either mutation of the di-leucine sequence or truncation of 1 to 27 amino acid sequence or both in the cytoplasmic domain as measured by FACS. Compared with that in the cells transfected by wild-type SR-A, the cellular uptake of DiI-AcLDL via these truncated SR-A were 61.87%, 27.93%, and 22.79%, respectively (Figure 4).

Discussion

It is known that SR-A–mediated internalization of lipids into cell plays an important role in the formation of foam cells, a pathological characteristic of atherosclerosis.6,7 Among the 6 structural components of SR-A, the cytoplasmic domain of the receptor is responsible for highly efficient internalization of the receptor-ligand complex and cell surface expression of the receptor.12,16,17,22 Our previous results have demonstrated that deletion of SR-A cytoplasmic domain has no effect on its surface expression and binding to its lipoprotein ligands. However, the SR-A–expressing cells experience a defect in internalization of the bound ligands into cell, suggesting that within the cytoplasmic domain there may be a signal motif responsible for endocytosis of the receptor-ligand complex into SR-expressing cells.15 The VXFD, FDARS, and KLKSFK sequences were reported to constitute the possible signal motifs to mediate the internalization of SR-A into cell.12,16,17,22 These motifs are evolutionarily conserved and exist in SR-A of different mammalian species. In addition, phosphorylation of Ser48 was reported to act as a key mediator for internalization.23 However, the influence of the heterogeneity in SR-A cytoplasmic domain on mediating uptake of ligand into cell would not be neglected. For example, a significant difference in the structure of cytoplasmic domain exists between human and murine SR-A. The murine SR-A has 5 more amino acid residues in its cytoplasmic domain than does human SR-A, and their homology is 76% (blast in NCBI). Whether the difference in the cytoplasmic domain of SR-A contributes to the difference in the sensitivity of foam cell formation remains to be elucidated. The present study shows that the di-leucine motif of the SR-A cytoplasmic domain at amino acid residues 31 and 32 may contribute to DiI-AcLDL internalization by SR-A-expressed CHO cells, suggesting that this motif is needed for lipid-laden foam cell formation.

Di-leucine motif was originally identified as an important element for the sorting of intracellular proteins between the trans-Golgi and lysosome compartments.18 It was proven as a mediator in the process of ligand entry into cells in insulin receptor, growth hormone receptor, and scavenger receptor BII.19,24,25

Using the approach of somatic mutation, we have constructed the di-leucine mutant of human SR-A N3132LM and transfected it into CHO cells that do not express endogenous SR-A.20,21 The di-leucine–mutated SR-A was successfully expressed in the transfected cells, as demonstrated by fluorescence-based morphological observation on intact cells and Western blot analysis with cell lysates. The expression of SR-A on the cell surface was not impaired by replacement of

### The Amino Acid Composition of SR-A Mutants

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>MEQWDHFHNQEDTOSCESVKFDARSMTALLPPNPKNPSLQEOKLKSF</td>
</tr>
<tr>
<td>N3132LM</td>
<td>MEQWDHFHNQEDTOSCESVKFDARSMTAAPNPKNPSLQEOKLKSF</td>
</tr>
<tr>
<td>NΔ1–27</td>
<td>MTALLPPNPKNPSLQEOKLKSF</td>
</tr>
<tr>
<td>NΔ1–27LM</td>
<td>MTAAPNPKNPSLQEOKLKSF</td>
</tr>
</tbody>
</table>

The amino acid sequence of SR-A N-terminal cytoplasmic tail is listed. The substituted amino acids are bold marked.
the di-leucine motif. However, uptake of DiI-AcLDL by cells expressing the SR-A mutant N3132LM was 61.87% of that exhibited by wild-type SR-A-transfected cells. The lower uptake of DiI-AcLDL by mutant N3132LM-transfected cells does not appear to be accounted for by differences in transfection efficiency of the different plasmids, because in our study all transfected cells were measured for β-galactosidase activity, which was used as an internal transfection control. In addition, the ratio of cellular DiI-AcLDL (red fluorescence intensity in cell) to SR-A proteins (relative intensity of green fluorescence in cell) may be a better indicator of the ligand-binding and internalization function of SR-A. One of the possible explanations for lower ligand uptake in N3132LM mutant cells is that mutation of the SR-A di-leucine motif impairs transport of DiI-AcLDL from the cell surface into the cytoplasm. This view is clearly supported by the result obtained through confocal microscopy of the fluorescent ligand binding and uptake, showing the ligand retention on the mutant cell surface. Furthermore, ligand binding assay demonstrated that no obvious difference exists between the binding of DiI-AcLDL with the wild-type and mutant SR-A transfected cells. Thus, the di-leucine motif in SR-A plays an important role in mediation of ligand internalization into the cells.

Clathrin-coated pit is a key plasma membrane structure for the receptor-mediated internalization. Two categories of receptors exist in the cells, and they differ in biological function because of their dependence on clathrin-coated pits. Class I receptors, like LDL receptor, are spontaneously segregated in clathrin-coated pit and are continuously internalized and recycled even in the absence of its ligand.25 Class II receptors, like insulin receptor, firstly bind with ligands to gain the access to clathrin-coated pits which subsequently initiate their internalization.23 The internalization of ligands by SR-A is thought to act as classical coated pit–dependent endocytosis.17 After binding with its ligand, the receptor–ligand complexes form and they are rapidly associated with coated pit structure.27–30 The cluster of receptors at coat-pits is a stimulus-dependent process that is activated by ligand binding.23 Di-leucine motif is required for protein interaction with heterotetrameric clathrin adaptor protein (AP) complexes. Several distinct heterotetrameric AP complexes have been identified to exhibit specific protein trafficking functions.32 Among them, the AP-2 complex mediates the internalization of proteins from the plasma membrane through clathrin-coated vesicles. To investigate a positive relation of the di-leucine structure to clathrin-coated pits, the antibody-based analysis was performed in the study. The Western blot analysis showed that both SR-A and clathrin were in the cell lysates immunoprecipitated by the anti-clathrin antibody. The mutant N3132LM-expressed cells showed same Western blot pattern as that of wild-type SR-A. These results confirmed that SR-A–mediated internalization of DiI-AcLDL into cell was via coated pit. As to the di-leucine pair, its role seemed not obvious in binding of SR-A with clathrin because no obvious change in protein mass was detected in mutant N3132LM-expressed cells. Furthermore, both wild-type SR-A and SR-A mutant N3132LM were found to colocalize with clathrin in cells under microscope when transfected cells were pre-incubated with AcLDL (data not shown).

In addition to the di-leucine motif other signal motifs in SR-A cytoplasmic domain have been identified recently.12,16,17,23 In human beings these motifs seem to participate in SR-A–mediated uptake of DiI-AcLDL. First, if the sequence containing these motifs is artificially truncated in SR-A, the uptake of ligand by cells may decrease dramatically.15 Second, truncation of the SR-A N-terminal 1 to 27 caused greater loss in DiI-AcLDL uptake by cells, as compared with that of mutant of di-leucine (72% to 38%). The N-terminal 27 amino acids sequence hides at least VXXFD and Ser phosphorylation site.12,16 Third, truncation of the sequence of 1 to 27 amino acid plus mutation of di-leucine affected DiI-AcLDL uptake by cells more than that of either kind of truncated SR-A alone, although the difference between SR-AΔ1 to 27 and SR-AΔ1 to 27LM was not statistically significant (≈5%). Thus, it is hypothesized that each signal motif in SR-A may contribute its own role to the ligand uptake by cells. The uptake of ligand involves several complicated steps and interactions. Different signal motifs can be adapted to multiple functions of native SR-A associated with the high efficient ligand internalization. This phenomenon may also occur in other types of receptors. For example, 3 independent internalization motifs have been identified in insulin receptor.25 Whether more signal motifs exist in SR-A is still an issue warranted for further exploration.

In summary, we have shown that the di-leucine structure in human SR-A cytoplasmic domain contributes to AcLDL internalization into cells. Manipulation of this motif provides a novel mechanism to regulate SR-mediated internalization of modified lipids and foam cell formation during atherogenesis.

Acknowledgments

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Methods

Preparation of lipoproteins

Human LDL (d = 1.019-1.063 g/ml) was isolated from EDTA-treated plasma by density gradient ultracentrifugation. LDL was labeled with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindo-carbocyanine perchlorate (DiI) to specific activities of 20-40 ng/mg LDL. The acetylation of LDL was performed as described previously. The protein concentrations of LDL were measured using the method of BCA protein assay (BCA Protein Assay Kit, PIERCE, USA).

Construction of SR-A mutants

Human SR-A/EGFP (enhanced green fluorescence protein) was kindly provided by Dr. Harald Heider at Institute of Biochemistry, University of Basel, Switzerland. The plasmid encoding the correct fusion protein (referred to as EGFP/SR-A) was mutagenized using the Stratagene Quickchange™ site-directed mutagenesis kit according to the instructions supplied by the manufacturer. The method is based on the incorporation of a mutation using Pfu DNA polymerase and complementary mutagenic oligonucleotide primers. The native receptor mutant construct (50 ng) was incubated with 125 ng of each mutagenic oligonucleotide primer (32–38 bases in length) and Pfu DNA polymerase (2.5 units). The temperature cycling parameters followed the recommendations suggested by the manufacturer. The reaction mixture was incubated with DpnI (20 units) to digest the parental DNA template and then used to transform DH5α competent cells. DNA from selected bacterial colonies was isolated.

The sense and antisense mutagenesis primers were designed by replacement of the Leu-31 codon TTG with the alanine codon GCG. (Sense: 5'-GCTCGCTCAATGACAGCTGCCGCTTCTCCG AATCC-3′; antisense: 5'-CGAGCGGAGTTACTGTCGACGCCGAAGGAGGCTTAGG-3′; altered
nucleotides are shown in bold letters). The primers for the replacement of the Leu-32 codon CTT to the alanine codon GCT are as follows: sense: 5’-GCTCGCTCAATGACAGCTTTGGCTCCTCGGAATCC-3’, antisense: 5’-CGAGCGAGTTACTGTCGAAACCGAAGGGCTTGG-3’, altered nucleotides are shown in bold letters. The primers for the double mutation, the Leu-31 codon TTG to the alanine codon GCG and the Leu-32 codon CTT to the alanine codon GCT, are as follows: sense: 5’-GCTCGCTCAATGACAGCTGCGGCTCCTCCGAATCC-3’, antisense: 5’-CGAGCGAGTTACTGTCGACGCCGGAGGGCTTGG-3’ (the altered nucleotides are shown in bold letters). The mutant was named as N3132LM. DNA of the SR-AΔ1-27 was amplified by PCR. The PCR products were cleaved with the restriction enzymes BamHI and XhoI, and then subcloned to the mammalian expression vector pEGFP-c with T4 ligase (New England Biolabs). The vectors were amplified in DH5α competent cells. Plasmids containing the correct mutation were identified with automated sequencing.

Cell culture and transfection

Wild type CHO-K1 cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA, Cat. No. CCl-61) and maintained in Dulbecco's modified Eagle's medium (DMEM/F12) with high glucose plus 10 % fetal calf serum at 37°C in 5 % CO₂. Transient transfections were performed with the Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Briefly, 10^5 cells were plated onto two 6-well plates 24 h before addition of the precipitate containing 5 μg of SR-A or SR-A mutant expressing plasmid and 1 μg of β-galactosidase expression plasmid (pSV-β-gal, Promega), which was used as an internal control for assessment of transfection efficiency. The expression vector pEGFP-c was used as a negative control. After incubation for 24 h, parts of cells were lysed in reporter lysis buffer and
β-Galactosidase activity was assayed spectrophotometrically according to the manufacturer's instructions. Other cells were scraped off the culture dishes and analyzed by flow cytometry. The intensity of green fluorescence was normalized to β-galactosidase activity (the relative intensity of green fluorescence) as an internal transfection control. Each treatment was repeated three times.

**Preparation of cell lysates**

Cell lysates were prepared with the lysis buffer (25 mM MES, 150 mM NaCl, 60 mM octylglucopyranoside, 1 % Triton X-100, pH 6.4) for 30 min in ice. Protein concentrations were determined using the method of BCA protein assay.

**Western blot analysis**

Cell lysates were separated with 8 % SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride membranes (Amersham, BS, UK). SR-A protein bands were stained with 2F8 antibody (3 µg/ml, Serotec, Raleigh, NC, UK), recognizing an extracellular epitope of SR-A, and detected by adding a horseradish peroxidase-labeled rabbit antibody against goat IgG (1:2000 dilutions; Boster biotechnology, China). The membrane was visualized by use of enhanced chemiluminescence Western blotting kit (ECL, Amersham, BS, UK).

**Biotinylation**

Transfected and untransfected cells were grown for 24 h in six-well plates, and washed twice with 4 ml ice cold PBS (pH 8.0). After incubation with 0.5 mg biotin-sulfo-N-hydroxysuccinimide at room temperature for 30 min with gentle agitation, cells were then washed twice with 4 ml ice-cold PBS and solubilized in lysis buffer. Immunoprecipitation was performed with anti-SR-A antibodies. Blots were incubated in blocking buffer composed of 3 % BSA in PBS for 1 h at room temperature before they were incubated for another 2 h in horseradish peroxidase streptavidin
conjugate (2 µg/ml, Pierce, USA). After washing four times with PBS plus 0.1 % Tween 20 (PBST), biotinylated SR-A bands were detected using the enhanced chemiluminescence system (Amersham, UK). Thereafter, the membranes were reprobed with anti-SR-A antibodies for detection of total cellular SR-A.

**Morphological observation**

CHO cells expressing SR-A or SR-A mutant were seeded on a glass coverslip in a 6-well plate. After incubation for 24 h, the cells were washed twice with fresh medium without FCS. The cells were then incubated in a medium containing 10 % lipoprotein deficient serum (LPDS) and 5 µg/ml DiI-AcLDL at 37°C for 5 h. After washing three times, with PBS containing Ca\(^{2+}\) and Mg\(^{2+}\) at room temperature, the cells were observed at the excitation/emission wave lengths of EGFP and rhodamine filter sets under a laser scanning confocal microscope (Zeiss, Germany).

**Measurements of DiI-AcLDL binding and uptake by SR-A**

CHO cells were seeded and transfected as described above. After washing twice with fresh medium without FCS, the cells were incubated in a medium containing 10 % LPDS and 5 µg/ml DiI-AcLDL at 37°C for 2 h. For binding assays, cells were incubated for 2 h at 4°C with 5 µg/ml DiI-AcLDL. Unlabeled AcLDL in excess amounts (300 µg/ml) were added together with the fluorescent lipoproteins for competition assays.\(^3\) In the end of incubation, the transfected cells were scraped off the culture dishes with a rubber policeman and washed three times with PBS. The amounts of bound and internalized DiI-AcLDL by transfected cells were calculated by flow cytometry using the ROI (region of interest) function (FACS, Becton Dickinson, USA). The green fluorescent cells positive to SR-A with comparable intensity were gated for analysis of red fluorescent intensity. A single selected green fluorescence-positive cell was defined as one ROI.
The appropriate excitation filter for red fluorescence was set and total gray level intensity of red fluorescence within a single ROI was recorded. At least 20000 cells were measured for each sample. The assays were conducted in at least three independent experiments.

**Co-immunoprecipitation assay**

Anti-clathrin antibody was well mixed with 2 mg proteins of cell lysates and 20 µl protein A beads. The mixture was then incubated at 4°C overnight. The protein A beads were washed with lysis buffer for three times and eluted into Laemmli sample buffer. The immunoprecipitated cellular proteins were separated by SDS-PAGE and analyzed by Western blot for SR-A and clathrin.

**Statistical analysis**

Results are reported as mean ± SD. Differences were evaluated by t test or analysis of variance, and accepted as significance when p value is less than 0.05.

**References**


Figure legends

**Fig. I. Localization of DiI-AcLDL in CHO cells expressing full-length or N3132LM SR-A**

SR-A transfected or control cells incubated with DiI-AcLDL were examined by fluorescent microscopy for EGFP and DiI fluorescence. A: EGFP-transfected cell; B: SR-A-transfected cell; C: N3132LM-transfected cell. A1, B1, C1: GFP fluorescence; A2, B2, C2: DiI-AcLDL; A3, B3, C3: overlapping of 1 and 2.

**Fig. II. Expression of SR-A in transfected CHO cells**

CHO cells were transiently transfected with different constructed plasmids. After incubation for 24 h, the cells were harvested and assayed. The intensity of green fluorescence measured by FACS was normalized to β-galactosidase activity. Similar results were obtained in three independent experiments.
Fig II

![Bar graph showing relative intensity of green fluorescence for SR-A and N3132LM.]