A naturally occurring isoform of the human macrophage scavenger receptor (SR-A) gene generated by alternative splicing blocks modified LDL uptake[1]

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Abstract The class A macrophage scavenger receptors (SR-A) are macrophage-specific trimeric integral membrane glycoproteins that have been implicated in many macrophage-associated physiological and pathological processes including atherosclerosis, Alzheimer's disease, and host defense. There are two forms of the receptor that have been previously cloned, and both are generated by alternative splicing of a single gene. Here we report the cloning of a third, alternatively spliced isoform of the human SR-A gene (type III hSR-A). The novel isoform is expressed in the human monocytic leukemia cell line THP-1 and also in primary human monocyte derived macrophages. When expressed in CHO-K1 cells, type III hSR-A does not internalize AcLDL despite having the domain shown to mediate this function in type I and II hSR-A. We show that type III protein has altered intracellular processing and is trapped within the endoplasmic reticulum, making it unable to perform endocytosis. Type III protein acts as a dominant negative isoform by reducing modified LDL uptake in CHO cells stably expressing either type I or type II SR-A. The demonstration that a naturally occurring splice variant of SR-A mRNA can act as a dominant negative isoform suggests a novel mechanism for regulation of scavenger receptor activity in macrophages.—**Gough, P. J., D. R. Greaves, and S. Gordon.** A naturally occurring isoform of the human macrophage scavenger receptor (SR-A) gene generated by alternative splicing blocks modified LDL uptake. *J. Lipid Res.* 1998. **39:** 531–543.

Supplementary key words macrophage • scavenger receptor • alternative splicing • atherosclerosis • dominant negative mutant

The class A macrophage scavenger receptors (SR-A) are trimeric integral membrane glycoproteins that show unusually broad ligand binding properties (1–6). The receptor binds a diverse array of macromolecules including modified lipoproteins (acetylated or oxidized low density lipoprotein), bacterial surface lipids (endotoxin and lipoteichoic acid), proteins modified by advanced glycation (advanced glycation end products, AGE), and β-amyloid fibrils (7–10). The SR-A-mediated uptake of modified LDL is not regulated by cellular cholesterol levels, unlike the native LDL receptor, and therefore leads to large intracellular cholesterol accumulation and the formation of foam cells. The generation of macrophage-derived foam cells is hypothesized to be a key step in the pathogenesis of atherosclerosis. The role of SR-A in the development of atherosclerosis has recently been highlighted by a double knockout strategy, with disruption of the SR-A gene resulting in a reduction in the size of atherosclerotic lesions in mice deficient in apolipoprotein E (10). In addition to endocytosis, SR-A has been implicated in a number of other macrophage processes including host defense, adhesion, phagocytosis, and intracellular signaling (10–13).

Two SR-A isoforms have previously been described and are generated by alternative splicing of a single gene (14, 15). Both isoforms contain six predicted structural domains (4, 5, 16): cytoplasmic, transmembrane, spacer, α-helical coiled coil, collagenous, and an isoform-specific carboxyl terminus. Type I SR-A has the 110 amino acid scavenger receptor cysteine-rich domain (SRCR), a highly conserved protein motif found in many other proteins of immunological significance (17). Type II SR-A has a short domain which is relatively unconserved between species. Both receptor iso-

Abbreviations: hSR-A, human macrophage scavenger receptor; FACS, fluorescence activated cell sorter; SRCR, scavenger receptor cysteine rich; SR-A, macrophage scavenger receptor; AcLDL, acetylated low density lipoprotein; LDL, low density lipoprotein; CAT, chloramphenicol acetyl transferase; PCR, polymerase chain reaction; mAb, monoclonal antibody; bp, base pair(s).

[1] The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number AF037351.

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forms mediate the binding and endocytosis of AcLDL with comparable efficiency when transfected into CHO-K1 cells, and both can be detected on murine tissue macrophages (16, 18). The functional significance of the two isoforms remains unclear at present.

Here, we describe a third alternatively spliced variant of the hSR-A mRNA. We show expression of all three alternatively spliced isoforms in cultured human monocyte-derived macrophages. When expressed in transiently transfected CHO-K1 cells this novel isoform of the receptor does not internalize AcLDL despite containing the collagenous domain that mediates ligand binding in type I and II SR-A (19). This third form of SR-A inhibits the function of type I and II SR-A when co-expressed in CHO cells, having a dominant negative effect. We suggest that alternative splicing is a potential mechanism for the regulation of SR-A activity in macrophages.

METHODS

Cell culture and human monocyte isolation

THP-1 cells were cultured in RPMI 1640 medium supplemented with 50 IU/ml penicillin G, 50 μg/ml streptomycin, 2 mm glutamine (PSG) (all from Life Sciences, Paisley, UK) and 10% fetal calf serum (FCS) (Sigma, Poole, UK) in the presence or absence of 200 nm phorbol 12-myristate 13-acetate (PMA) (Sigma) for 4 days. CHO-K1 cells were routinely cultured in Ham's F-12 medium supplemented as above.

Buffy coats were obtained through the National Blood Transfusion Centre at the John Radcliffe Hospital, Oxford. Mononuclear cells were obtained by Ficoll-Paque centrifugation (Pharmacia LKB, Uppsala, Sweden) and were washed five times with phosphate-buffered saline (PBS; 137 mm NaCl, 2.7 mm KCl, 8.1 mm Na_2HPO_4, 1.5 mm KH_2PO_4 at pH 7.3) to remove platelets. Cells were resuspended in RPMI-1640 medium supplemented with 5% heat-inactivated autologous human serum and PSG, and monocytes enriched by adherence for 90 min at 37°C in 75 cm^2 polystyrene cell culture flasks (Falcon, Becton Dickinson Labware, Oxford, UK). Non-adherent cells were removed by washing the flask 6 times with RPMI-1640 pre-warmed to 37°C. The cells were subsequently cultivated at 37°C in X-Vivo 10 (Bio-Whittaker, Reading, UK) supplemented with 1% autologous human serum and PSG for the indicated times.

Reverse transcription (RT) PCR, cloning and sequencing

Total cellular RNA was extracted with RNAzol solution (Biogenesis, Bournemouth, UK) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase and an oligo-dT primer (Life Sciences). Reactions were always set up with and without the reverse transcriptase enzyme to control for DNA contamination in the subsequent PCR reactions. The cDNA obtained served as a template for PCR.

Using the following oligonucleotide pairs, fragments were amplified containing the full-length coding sequence of type I and type II human SR-A: a common sense primer 5′ CCC **AAG CTT** GGG ATG GAG CAG TGG GAT CAC TTT CAC 3′, and type-specific antisense primers: Type I 5′ CGA GA**T CTA GA**G CAT TAT AAA GTG CAA GTG ACT CCA GCA 3′ and Type II 5′ CGA GA**T CTA GA**G TTA AGA GGG CCC TGC CCT AAT ATG 3′. The PCR conditions were as follows: 50-μl final reaction volume containing 1.5 mm $MgCl_2$, 200 μm dNTP, 0.5 μg each primer, 5 μl 10× reaction buffer, 2.5 units of Taq DNA polymerase (Life Sciences) and 2 μl THP-1 treated with PMA cDNA as template using the following cycling parameters: 94°C/1 min, 60°C/1 min, 72°C/1.5 min for a total of 30 cycles in a model PTC-100 MJ Research, Inc. thermocycler. PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Bands of the predicted size were excised and purified using Qiaex II (Qiagen, Dorking, UK) prior to cutting with Xba I and Hind III restriction enzymes (restriction enzyme sites incorporated in PCR primers; sites shown in bold) and cloning into the similarly cut pcDNA3 expression vector (Invitrogen, San Diego, CA). Clones were analyzed by a combination of restriction mapping and double-stranded DNA sequencing using the PRISM ready reaction Dyedeoxy terminator cycle sequencing kit and an ABI model 373A stretch DNA sequencer (both from Applied Biosystems, Perkin Elmer, Warrington, UK).

PCR fragments encoding the full length SR-A open reading frame incorporating the 8 amino acid FLAG epitope (DYKDDDDK) immediately after the initiator methionine were amplified by PCR with Vent DNA polymerase (New England Biolabs, Beverly, MA) using 100 ng of the purified plasmids constructed above as template, the sense primer 5′ CCC **AAG CTT** GGG ATG GAC TAC AAG GAC GAC GAT GAC AAG GAG CAG TGG GAT CAC TTT CAC AAT (coding sequence for the FLAG epitope is underlined, and restriction site in bold) and the antisense primer sets described above, the type I antisense primer being used to generate the coding sequence for the type III construct. The inserts were cloned into the pcDNA3 vector and sequenced as described above.

Analysis of scavenger receptor isoform expression was performed by RT-PCR using the following primer pair sets (sense/antisense): HPRT: 5′ GCTACCTGC TGGATTACAT 3′ and 5′ CCAGTTTCACTAATGACA

CAA 3′ (predicted PCR product size 410 bp). Type I hSR-A: 5′ TGGGAACATTCTCAGACCTTGAG 3′ and 5′ TTGTCCAAAGTGAGCTGCCTTGT 3′ (predicted PCR product size 447 bp). Type II hSR-A: sense as for type I and antisense 5′ TGCCCTAATATGATCAGT GAGTTG 3′ (predicted PCR product size 291 bp). Type III hSR-A: 5′ GTGGAAACACATTAAGTACTG 3′ and 5′ CCCTTGGCCTTTGTAATCTGGAAGC 3′ (predicted PCR product size 439 bp). PCR reactions were performed as described above.

Transfection and quantitation of AcLDL uptake

CHO-K1 cells (3×10^6) were plated in 9-cm tissue culture plastic petri dishes (Nunc, Denmark) 2 days prior to transfection. Transfection was performed with Lipofectamine (Life Sciences) using an adapted version of the manufacturer's protocol. Briefly, 25 μl of Lipofectamine and 5 μg of plasmid DNA were added to 5 ml of serum free Optimem (Life Sciences), mixed by inversion, and allowed to stand for 15 min. The cells were washed twice in PBS to remove serum and the transfection mix was added. The cells were incubated at 37°C for 4 h after which the transfection mix was aspirated and replaced with growth medium. Twenty-four hours post-transfection, cells were harvested using PBS, 5 mm EDTA, and 0.1% trypsin, washed once in PBS, and plated in either 6-well clusters or 24-well plates (Falcon) at a density of 5×10^5 and 2×10^5 cells per well, respectively.

To assay AcLDL uptake, 48 h post-transfection, cells in 24-well plates were washed once with PBS and then incubated in Ham's F-12 supplemented with PSG and 2% lipoprotein-deficient fetal calf serum (LPD-FCS; Sigma) in the presence or absence of 5 μg/ml DiI (1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) labeled AcLDL (DiI-AcLDL) (Biogenesis) for 3 h at 37°C. Cells were washed five times in PBS, harvested with PBS, 5 mm EDTA, and 0.1% trypsin, fixed in a 4% (w/v) solution of paraformaldehyde in PBS, and analyzed on a FACScan (Becton Dickinson & Co., Mountain View, CA) using the FL2 photomultiplier.

Confocal microscopy and FACS analysis of transfected CHO-K1 cells

Cells for indirect immunofluorescence were plated 24 h post-transfection at a density of 2×10^5 cells per well in a 24-well plate containing 13 mm acid-washed glass coverslips. Forty-eight hours post-transfection, cells were washed twice with PBS and fixed in a 4% (w/v) solution of paraformaldehyde in PBS. Cells for FACS analysis were harvested using PBS, 5 mm EDTA, and 0.1% trypsin prior to fixation. Cells were stained with anti-FLAG mAb M2 (IBI Ltd, Cambridge, UK), or isotype matched control mAb OX-45 (mouse IgG_1; anti-rat CD 48; a kind gift from Mike Puklavec, Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford) diluted to 10 μg/ml in PBS containing 10% (v/v) normal goat serum (Sigma) and 0.25% (w/v) saponin by incubation at room temperature for 60 min. Cells were washed 3 times in PBS containing 0.25% saponin before incubation with FITC-conjugated goat anti-mouse IgG ($F(ab')_2$) (Chemicon, Harrow, UK) diluted 1:100 in PBS containing 0.25% saponin. Confocal microscopy was performed using a Bio-Rad MRC-1024, mounted on a Nikon Diaphot 200 microscope equipped with a 60:1 planapochromat NA 1.4 objective. A 15 mW air-cooled krypton-argon ion laser at wavelengths 488 nm, 568 nm, and 647 nm was used. After selecting the correct focal plane under direct scan, images were recorded and analyzed using Lasersharp software (Bio-Rad, UK). FACS analysis was performed with a FACScan using the FL1 photomultiplier.

Generation of stably transfected CHO-K1 cell lines

CHO-K1 cells were transfected with lipofectamine as above and 48 h post-transfection selected in Ham's F-12 medium supplemented with 10% FCS, PSG, and 500 μg/ml Geneticin (Life Sciences). Stably transfected cells were cultured for 2 weeks before further selection in "MAC-medium": Ham's F-12 containing PSG, 3% lipoprotein-deficient fetal calf serum, 250 μm mevalonate, 40 μm mevastatin (all from Sigma), and 3 μg/ml AcLDL (Biogenesis). This medium provides nutritional selection for cells expressing functional scavenger receptors (20). Cells expressing high levels of type I and II SR-A were obtained by culture of cells in MAC-medium for 9 months.

Metabolic labeling and endoglycosidase H treatment

Day 1 post transfection, cells were plated in 6-well clusters (Falcon) at a density of 5×10^5 cells per well. On day 2 cells were washed 3 times with PBS and pulse-labeled for 30 min with 0.5 ml of methionine and cysteine-deficient DMEM supplemented with 5% dialyzed FCS and 500 μCi/ml ^{35}S-Trans label (ICN, Thame, UK). Cells were washed 3 times with PBS and incubated in "chase" medium consisting of Ham's-F12, 10% FCS supplemented with 1 mm unlabeled methionine and 4 mm unlabeled cysteine. After incubation for the indicated times, cells were washed 3 times and lysed on ice in 150 mm NaCl, 10 mm EDTA, 10 mm NaN_3, 10 mm Tris (pH 8.0), 1 mm PMSF, 5 mm iodoacetamide, and 1% NP-40. Lysates were centrifuged at 15,000 *g* for 10 min to remove debris before preclearing with protein G-Sepharose (Pharmacia LKB) by precipitation at 4°C. Antigen was precipitated overnight by incubation with 10 μg/ml anti-FLAG mAb M2 and protein G-Sepharose, washed 6 times with 10 mm Tris pH 8.0 buffers at 4°C; 3 times in 500 mm NaCl, 0.5% (v/v) Triton

X-100, 0.5% (w/v) deoxycholate, 0.05% (w/v) SDS; twice with 150 mm NaCl, 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.05% (w/v) SDS; once with 0.05% (w/v) SDS, then boiled for 5 min in 96 μl of 100 mm Na citrate phosphate (pH 5.5), 0.2% SDS, 0.1 m 2-mercaptoethanol and 1 mm PMSF. The boiled sample was split into equal aliquots and incubated at 37°C overnight in the presence or absence of 2 mU of endoglycosidase H (Endo H) (Oxford Glycosystems, Oxford, UK) prior to separation under reducing conditions by 10% SDS-PAGE. Gels were fixed and impregnated with EN^{3}HANCE (DuPont NEN, Stevenage, UK) before drying and exposure to film at −70°C.

RESULTS

In order to study the ligand binding properties of type I and type II hSR-A we generated expression constructs for the full-length protein. Using RT-PCR, with cDNA from THP-1 cells treated with PMA serving as template, we amplified the coding regions of type I and type II receptors. Whilst cloning the RT-PCR fragment for the coding region of type I hSR-A into the pcDNA3 expression vector, several plasmids were detected containing an insert smaller than the predicted size. Diagnostic restriction enzyme digests localized this difference to the 3′ region of the coding sequence (data not shown). Sequencing of the clones revealed a novel form of hSR-A mRNA (**Fig. 1A**) generated by alternative splicing of the primary hSR-A transcript. This form of the receptor, which we have called the type III SR-A, has exon 8 spliced directly onto exon 11 (Fig. 1B). This maintains the open reading frame and produces a change in amino acid residue 345 from threonine to serine compared to type I amino acid sequence and gives a novel C-terminal domain containing 47 amino acids and four of the six cysteines found in the SRCR

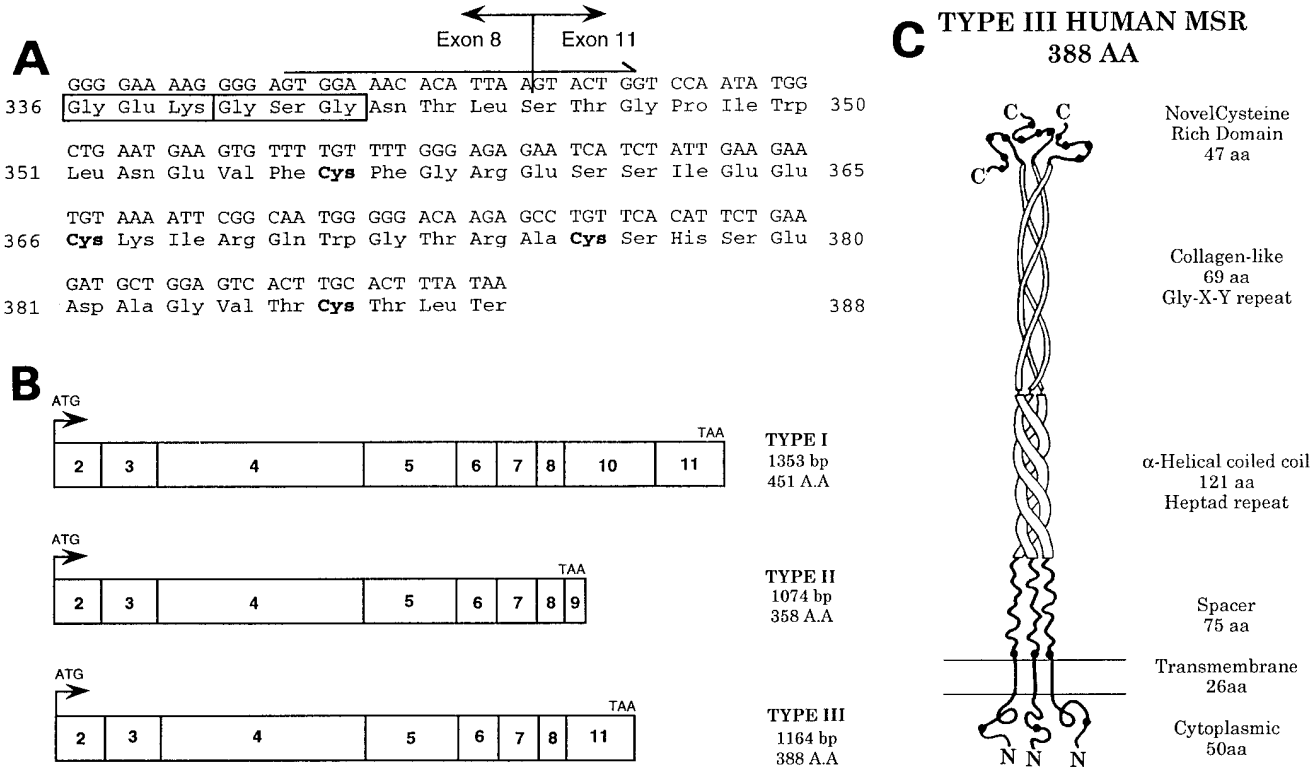

Fig. 1. (A) Nucleotide (upper line) and predicted protein (lower line) sequence of the carboxyl terminal region of the type III human scavenger receptor. The sequence of amino acids 1–335 has been omitted and is identical to the previously published sequence of type I and II hSR-A (36), the point of divergence is indicated by the vertical line and arrows marking the boundary of exons 8 and 11. The collagen-like Gly-X-Y repeats are indicated by boxes, cysteine residues are shown in bold, and the sequence of the type III-specific RT-PCR sense oligonucleotide is marked by the horizontal arrow. (B) Exon organization of the coding regions of the SR-A splice isoforms. The numbers in the boxes refer to exon numbers. All three isoforms contain common exons 2–8; type I has exons 10 and 11, type II has the type specific exon 9 and type III has exon 11. (C) Schematic model of the predicted structure of type III human scavenger receptor. The receptor contains six domains: N-terminal cytoplasmic, transmembrane, spacer, α-helical coiled coil, collagen-like, and the novel cysteine-rich domain. The number of amino acids in each domain is shown. Cysteine residues in the cytoplasmic, spacer, and cysteine domains are shown by small filled circles. Adapted from (5).

motif. The novel transcript contains the same 3′ untranslated region as the type I receptor which is encoded by exon 11. The predicted structure of the type III protein is shown in Fig. 1C, and contains the domains shown to be responsible for receptor trimerization and ligand binding in type I and II SR-A.

To explore the significance of the novel isoform we examined the expression of the three SR-A splice variants by human monocyte-derived macrophages. We designed RT-PCR primer pairs that specifically detect each SR-A isoform mRNA. We cultured monocyte-derived macrophages and prepared RNA at various time points which served as a template for cDNA synthesis and the subsequent RT-PCR reaction. Primers specific for the house-keeping gene hypoxanthine phosphoribosyl transferase (HPRT) were used to confirm that equal amounts of cDNA were present in each of the reverse transcriptase reactions. Primer sets specific for type I and II were identical to those used previously for investigation of SR-A isoform expression (21). As type III SR-A contains no unique sequence, a number of primer pairs specific for type III SR-A were tested on cDNA from transiently transfected CHO-K1 cells for type III hSR-A specific PCR amplification (data not shown). The primer pair used depends on the sense primer for its specificity, running across the junction between exons 8 and 11 (Fig. 1A). **Fig. 2A** shows equal amounts of the HPRT message for all of the samples from two donors at the specified time points. Fig. 2B shows that type I SR-A message is ubiquitous throughout macrophage differentiation in both donors with a slight increase in signal from day 1 to day 3 after which there is no further increase in levels. Type II SR-A message (Fig. 2C) is also present throughout differentiation, levels showing no obvious fluctuation. Type III SR-A message has a different expression pattern (Fig. 2D), mRNA appearing first on day 7 and remaining at the same level for donor 1 and decreasing for donor 2. The levels of type III hSR-A mRNA in monocyte-derived macrophages appear to be lower than those seen for the other two isoforms. A similar pattern of SR-A isoform expression was seen during the PMA-induced differentiation of THP-1 cells (data not shown).

To investigate the functional properties of this novel form of the receptor, we decided to assay the uptake of the fluorescently labeled SR-A ligand DiI-AcLDL in CHO-K1 cells transiently transfected with type III hSR-A. In multiple experiments performed with several independent expression plasmids, no uptake of the fluorescent ligand was measurable by either FACS analysis or fluorescence microscopy (data not shown). To ensure that type III transfected cells were expressing protein, we analyzed hSR-A protein expression by FACS analysis. Due to the absence of suitable antibodies recognizing hSR-A, we decided to generate a series of expression constructs incorporating the 8 amino acid FLAG epitope (DYKDDDDK) between the initiator methionine and the second residue glutamate of each of the three isoforms of hSR-A. In transiently transfected CHO-K1 cells FACS analysis using anti-FLAG antibody M2 showed that cells transfected with any of the FLAG-tagged hSR-A constructs have a similar pattern of total SR-A expression, with the transfected subpopulation of cells having approximately equal levels of fluorescence (**Fig. 3A**). FACS analysis of DiI-AcLDL uptake in the same transfected cells shows that cells transfected with either the type I or type II hSR-A expression vector efficiently endocytose the fluorescent ligand, compared to control cells transfected with a construct containing the CAT reporter gene (Fig. 3B). This indicates that addition of the FLAG epitope does not impair hSR-A endocytic function. Cells transfected with type III hSR-A have a fluorescence profile similar to that for the control cells transfected with the CAT reporter gene, indicating no uptake of DiI-AcLDL. Hence, the absence of DiI-AcLDL uptake by CHO-K1 cells transiently transfected with type III hSR-A is not due to lack of protein expression.

To confirm that transfection with the type III expression construct does not confer the ability to internalize AcLDL, CHO-K1 cells stably transfected with type III hSR-A, generated by culture in G418 for 2 weeks post-transfection, were selected in "MAC-medium" (see Materials and Methods for details). This culture medium makes cells dependent upon the expression of a functional scavenger receptor able to take up AcLDL as a sole source of cholesterol (20). The CHO-K1 cells stably transfected with the type III receptor died in this medium while those transfected with either type I or type II receptor proliferated (data not shown).

These results show that the type III hSR-A receptor, unlike the other SR-A isoforms, does not take up AcLDL. This could be due to different ligand binding properties of type III SR-A despite having the same ligand binding domain or a difference in the receptor's intracellular processing. The latter point was examined by the pulse/chase experiment shown in **Fig. 4**. Cells transfected with either type I, II, or III SR-A were pulse-labeled for 30 min, and immunoprecipitates from each of the indicated chase times were treated with or without endoglycosidase H (Endo H). Conversion from an Endo H-sensitive form to a resistant form indicates that a protein contains N-linked oligosaccharides that are processed from a high mannose, Endo H-sensitive precursor form to a complex, Endo H-resistant mature form in the medial Golgi compartment (22). Figure 4 shows that for both type I and II hSR-A the Endo H-sensitive precursor forms of the receptor (p) were

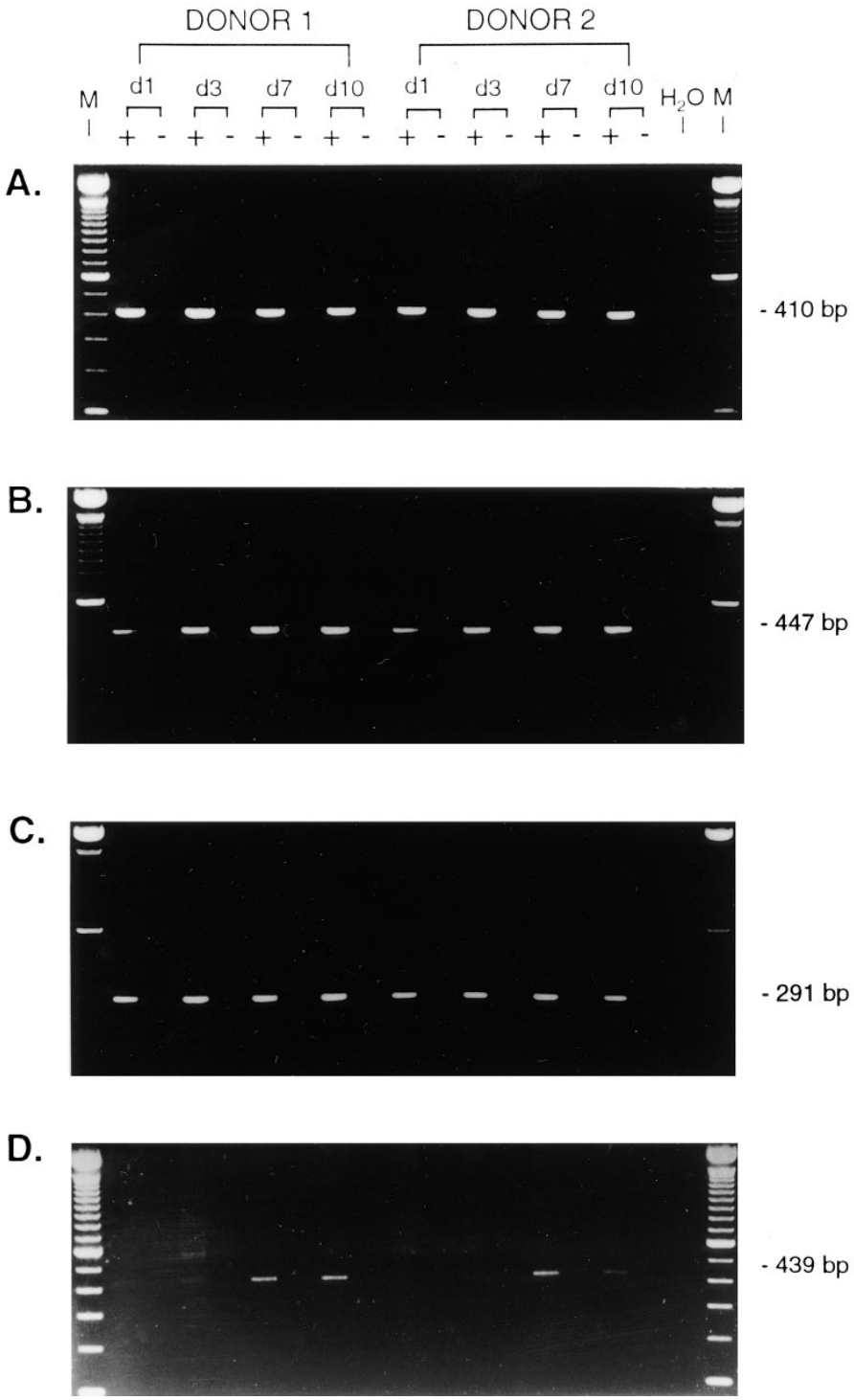

Fig. 2. RT-PCR analysis of SR-A isoform expression by primary monocyte-derived macrophages. Each panel shows an ethidium bromide-stained 1.5% agarose gel. cDNA used in each PCR reaction was synthesized as described in Materials and Methods from 50 ng of total RNA obtained from monocyte-derived macrophages cultured for the indicated times, + and − indicate the presence or absence of reverse transcriptase enzyme in the reaction mixture, respectively. The panels show results using cDNA obtained from two different donors and are representative of results from experiments from three other donors. M-100 bp ladder (Life Sciences), H_2O, reaction containing no cDNA template. (A) HPRT; (B) SR-A type I; (C) SR-A type II; (D) SR-A type III.

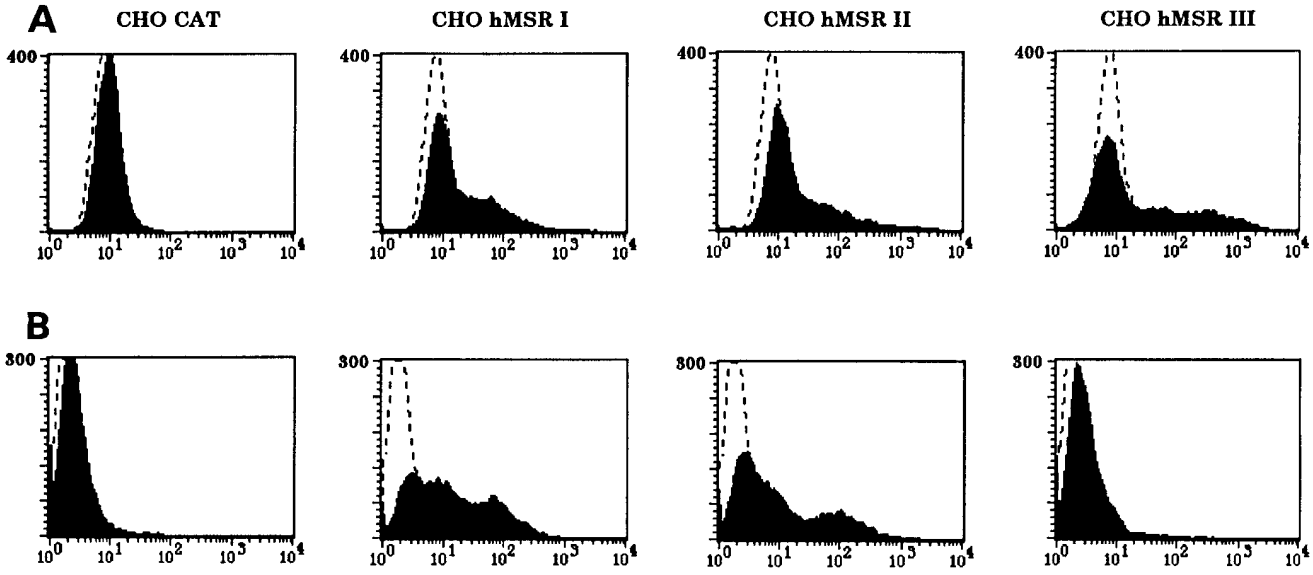

Fig. 3. (A) FACScan analysis of hSR-A protein expression in transiently transfected CHO-K1 cells by staining with anti-FLAG mAb M2. Cells were transfected with expression constructs containing the full-length coding sequence of each of the three types of SR-A including the FLAG epitope and also an identical expression vector containing the CAT reporter gene, described in Materials and Methods. MRC-OX45 control staining is shown as dashed lines. Results are from a single experiment performed in duplicate and are representative of three independent experiments. (B) FACScan analysis of DiI-AcLDL uptake by transiently transfected CHO-K1 cells. The histograms depict fluorescence per cell on the x-axis in arbitrary fluorescent units and numbers of cells in a given fluorescence channel on the y-axis. Dotted lines indicate autofluorescence of cells not incubated with DiI-AcLDL. Results are from a single experiment performed in duplicate and are representative of five independent experiments.

processed into the Endo H-resistant mature form of the receptor (m). However, for type III hSR-A there was no maturation from a precursor form, the protein remaining Endo H-sensitive at all times investigated. Thus it appears that type III hSR-A has different intracellular processing within CHO-K1 cells compared to type I and II hSR-A. This difference in oligosaccharide maturation suggests that the type III protein is trapped inside the endoplasmic reticulum of the cell. Cell surface biotinylation of transfected CHO cells confirmed that the type III form of the receptor does not reach the cell surface, unlike type I and type II isoforms (data not shown).

Confocal microscopy was performed on transfected CHO-K1 cells to further analyze the subcellular localization of hSR-A. Cells transfected with type I or type II expression constructs show similar patterns; diffuse staining throughout the cell characteristic of endoplasmic reticulum, a peri-nuclear halo typical of protein in the trans-Golgi apparatus, and numerous vesicles suggesting active endocytosis involving the protein (**Fig. 5D** and **F**). Type III hSR-A, however, only shows a diffuse staining pattern, indicating that the protein is not being processed through the exocytic pathway and is being retained within the endoplasmic reticulum (Fig. 5H). Studies using immunofluorescence microscopy revealed similar results (data not shown).

The trimeric nature of the SR-A protein suggested that type III hSR-A could alter the function of type I or type II hSR-A when co-expressed in the same cell due to the formation of inactive heterotrimers. To investigate this possibility, we transiently transfected CHO cells stably expressing high levels of either type I or type II SR-A with expression vectors encoding FLAG-tagged SR-A isoforms. Transient SR-A expression was examined by anti-FLAG staining, and SR-A endocytic activity was measured by DiI-AcLDL uptake. Transient transfection of either type I or type II hSR-A into cells stably expressing type I hSR-A leads to an increase in DiI-AcLDL uptake in the transfected subpopulation (**Fig. 6B** and **C**, upper right quadrant). The increase in uptake of DiI-AcLDL in the transfected cells is dependent upon the level of transient hSR-A expression, indicating that SR-A protein levels are the limiting factor for endocytosis in these experiments. In comparison, transfection of type III hSR-A into the cells stably expressing type I hSR-A gives a reduction of DiI-AcLDL uptake, which is proportional to the amount of type III protein expressed (Fig. 6D, lower right quadrant). This dominant negative effect of type III SR-A was also seen when cells stably expressing type II SR-A were transiently transfected with the novel isoform (data not shown). The reduction in DiI-AcLDL uptake in cells

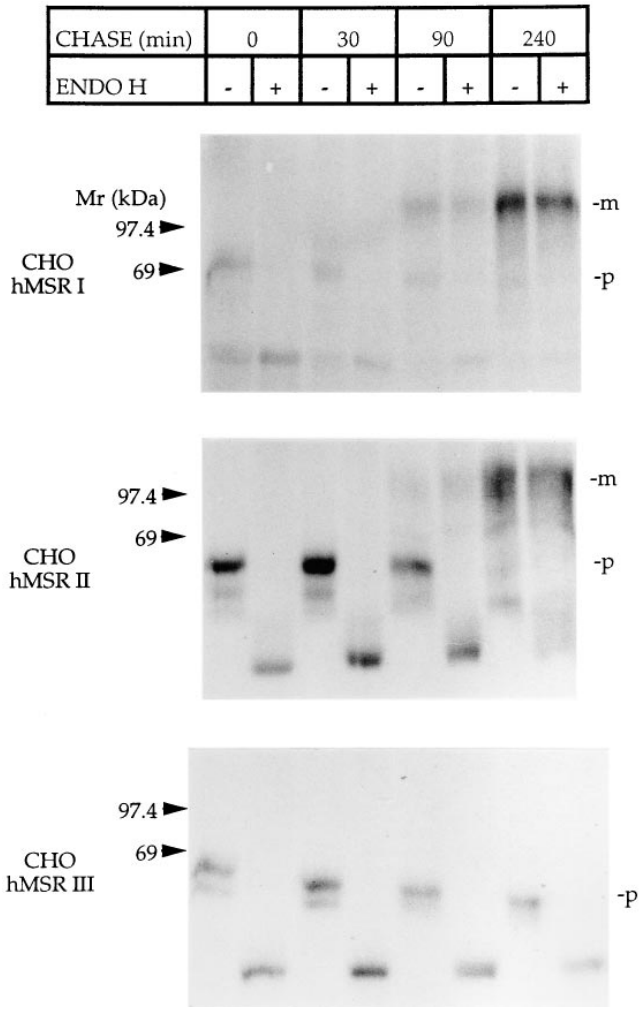

Fig. 4. Biosynthesis of hSR-A in transiently transfected CHO-K1 cells. Cells were pulse-labeled as described in Materials and Methods and chased for the indicated times prior to lysis, immunoprecipitation, and incubation at 37°C in the presence or absence of Endo-H. The samples were separated on a 10% SDS-PAGE gel under reducing conditions prior to visualization by autoradiography; m and p denote mature and precursor forms of the protein, respectively.

stably expressing the type II isoform was not as large as for type I SR-A expressing cells. This was presumably due to type II transfected cells expressing higher levels of SR-A than their type I counterparts, as transient expression of FLAG-tagged type I or type II SR-A did not increase DiI-AcLDL uptake (data not shown).

The dominant negative properties of type III SR-A were confirmed using a co-transfection approach similar to that used previously to investigate the function of truncated bovine scavenger receptors (19, 23). CHO cells were transiently transfected with mixtures of type II and type III SR-A expression vectors and receptor activity was measured by DiI-AcLDL uptake. The total amount of plasmid DNA in the transfection was held constant while the ratio of type II SR-A to type III SR-A and control plasmid DNA was varied. In mixtures of type II SR-A and control plasmid, the receptor activity increased approximately linearly with the amount of type II SR-A plasmid transfected (**Fig. 7**). In contrast, type III SR-A encoding plasmid caused a 60% inhibition of DiI-AcLDL uptake when co-expressed with type II SR-A. The mechanism of inhibition of type I and II SR-A activity by type III SR-A is currently under investigation.

DISCUSSION

In this paper we have described the cloning of a novel isoform of the human SR-A gene generated by alternative splicing. We have designated this new form of this receptor as type III hSR-A and have shown its expression in the THP-1 cell line and human monocyte-derived macrophages. When expressed in CHO-K1 cells, type III hSR-A does not mediate endocytosis of AcLDL despite having the collagenous domain shown to be essential for this function in type I and II hSR-A. We have shown that type III protein has altered intracellular processing and is trapped within the endoplasmic reticulum, making it unable to perform endocytosis. Type III protein acts as a dominant negative isoform, reducing scavenger receptor function in CHO cells stably expressing either type I or type II SR-A.

The recent cloning of several novel genes expressed by macrophages that show scavenger receptor activity led Acton et al. (24) and Pearson, Lux, and Krieger (25) to define three independent classes of scavenger receptor. Under this nomenclature system, type I and II SR-A are designated as SR-AI and SR-AII, and the MARCO antigen, which shows large structural similarity to SR-AI (26), is proposed to be called SR-AIII. We feel that it would be more appropriate to designate this novel protein as SR-AIII, as it is a product of the same gene as SR-AI and SR-AII, despite the fact it does not exhibit scavenger receptor activity.

The RT-PCR analysis of hSR-A isoform expression during human monocyte differentiation shows that type I SR-A mRNA levels increase with differentiation, while type II SR-A transcript remains approximately constant. Previous studies into isoform expression in human monocytes have shown conflicting results. Geng, Kodama, and Hansson (21) reported that peripheral blood monocytes have approximately similar levels of type I and type II hSR-A mRNA, but upon differentiation there is a selective increase in type I SR-A mRNA, type II SR-A message remaining at the same level. In agreement with this Ando et al. (27) have recently shown a similar selective up-regulation of type I

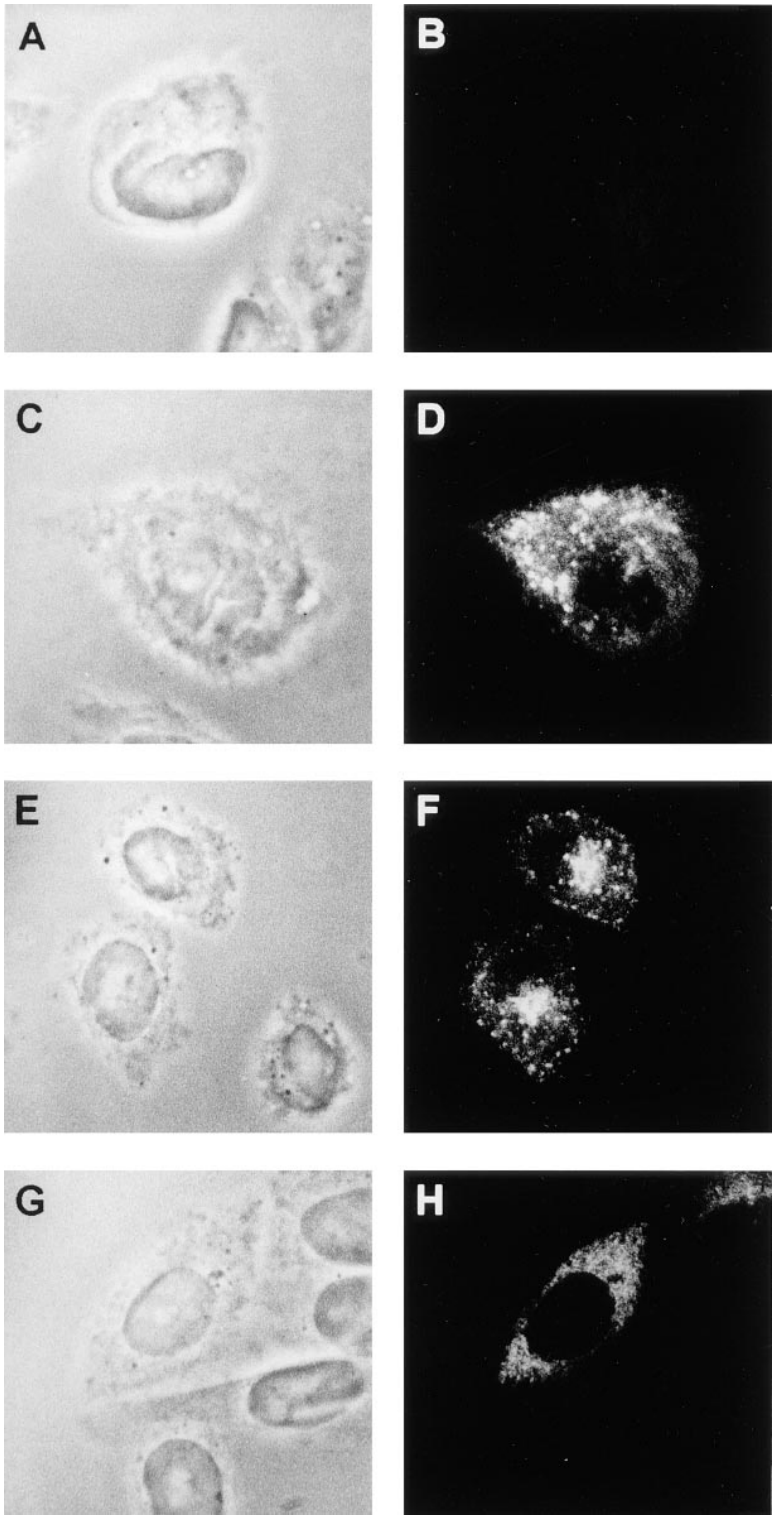

Fig. 5. Confocal microscopy of transiently transfected CHO-K1 cells. Cells were transfected with expression constructs containing the FLAG-tagged full-length coding sequence of each of the three isoforms of hSR-A and also an identical expression construct containing the CAT reporter gene. Cells were stained with anti-FLAG mAb M2 and viewed using confocal microscopy as described in Materials and Methods. Pictures show transmission (A, C, E, G) and fluorescence (B, D, F, H) images of the same field. Magnification ×150. (A + B) CAT-reporter gene. (C + D) SR-A type I. (E + F) SR-A type II. (G + H) SR-A type III.

hSR-A during monocyte differentiation. In contrast, Giry et al. (28) showed an approximately 80-fold increase in total hSR-A expression during a 12-day differentiation, of which half was accounted for by an increase in type II SR-A mRNA. Some of these discrepancies can be accounted for by variations in culture conditions for each of the experiments. A recent report documented wide inter-individual variations in the absolute isoform expression levels (29) which may mask a variety of genetic and environmental factors.

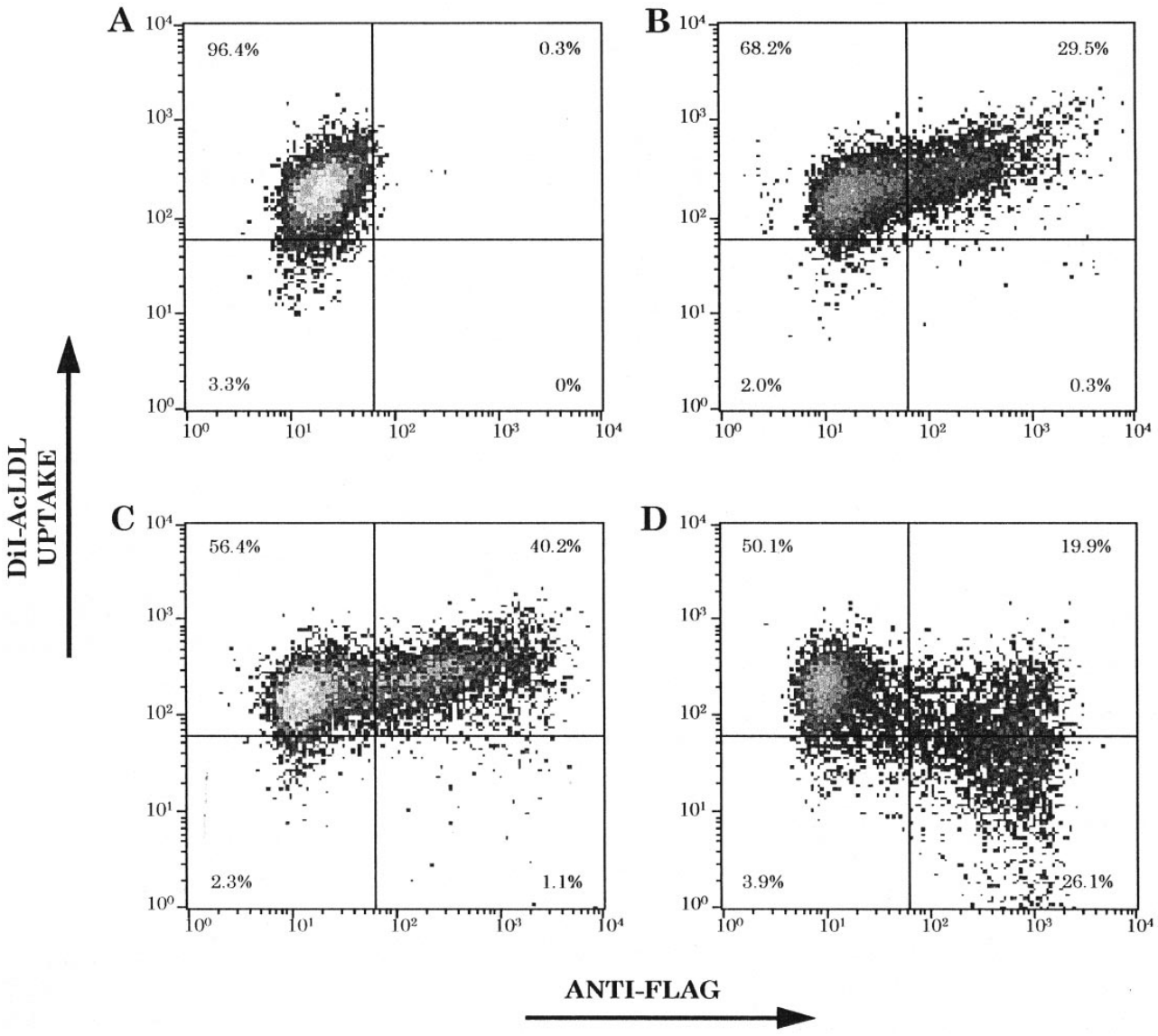

Fig. 6. FACScan analysis of DiI-AcLDL uptake and SR-A expression in transiently transfected CHO cells stably expressing type I SR-A. Cells stably expressing type I SR-A were transfected with expression constructs containing the FLAG-tagged full-length coding sequence of each of the 3 types of hSR-A and also an identical expression vector containing the CAT reporter gene, as described in Materials and Methods. Cells were labeled with DiI-AcLDL prior to staining with anti-FLAG mAb M2. Dot-plots depict DiI-AcLDL uptake (y-axis) versus anti-FLAG M2 staining (x-axis) both in fluorescence units. Indicated quadrant lines were placed during data analysis to quantify cells positive for DiI-AcLDL uptake and FLAG-tagged SR-A expression. Percentages indicate the fraction of cells present in each quadrant (as a percentage of total cells analyzed). Experiments were performed in duplicate and are representative of four similar experiments. (A) CAT-reporter gene. (B) SR-A type I. (C) SR-A type II. (D) SR-A type III.

The pulse–chase / Endo H sensitivity and immunofluorescence microscopy experiments suggest that type III protein is retained within the endoplasmic reticulum. The endoplasmic reticulum exhibits strong "quality control" characteristics, retaining incorrectly folded proteins and, therefore, it is likely that the type III hSR-A protein is misfolded. As the only difference between the type I and III SR-A proteins is the truncated version of the SRCR domain, it is probable that this causes the misfolding of the protein. The pattern of disulfide linkages of the SRCR domain in the human SR-A has recently been determined using proteolytic analysis (30). Three disulfide bonds are formed in the SRCR domain; Cys^2–Cys^7, Cys^3–Cys^8, and Cys^5–Cys^6. In the truncated

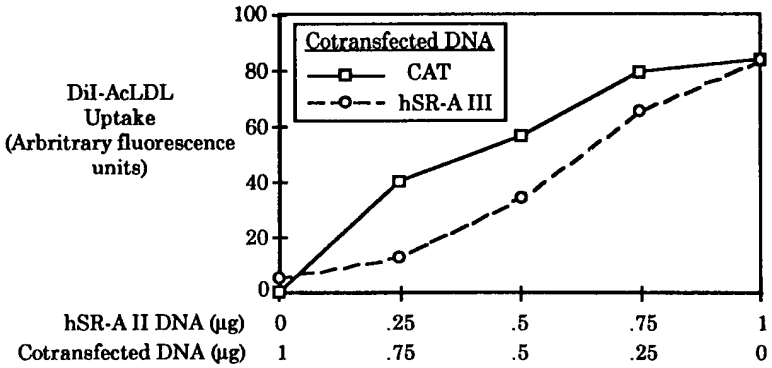

Fig. 7. Effects of co-expression of type III SR-A on type II SR-A function in transiently transfected CHO cells. The indicated amounts of plasmid DNA were cotransfected in addition to 4 μg of CAT reporter gene expression construct using the protocol described in Materials and Methods, and DiI-AcLDL uptake was measured 48 h post transfection. The results are from a single experiment performed in duplicate and are representative of three similar experiments.

form of the SRCR domain found in the type III SR-A protein, Cys^2 and Cys^3 residues are absent. This means that the remaining part of the SRCR domain will be unable to adopt the preferred conformation, and it is this that is likely to lead to the retention of the protein within the endoplasmic reticulum. The disturbance in the disulfide linkage pattern also gives the potential for formation of inter-chain disulfide bonds, or even inter-trimer bonds that would lead to receptor oligomerization.

At present the ability to generate the novel truncated form of the SRCR domain appears unique to SR-A. All other proteins containing SRCR domains for which the genomic organization is know are arranged such that each SRCR domain is encoded by a separate exon (31–33). This rules out the possibility of generating the truncated form of the SRCR domain by alternative splicing in these genes. Expression of the type III SR-A isoform is not restricted to humans and can be detected in rabbit tissues using a RT-PCR approach similar to that used here (P. Gough and S. Ylä Herttuala, unpublished results). Database searches have also failed to reveal any proteins containing the truncated SRCR motif.

SR-A accounts for a large proportion of modified LDL uptake by macrophages, and has been implicated as a key step in the pathogenesis of atherosclerosis (10). The accumulation of intracellular cholesterol via SR-A is thought to be unregulated by a feedback mechanism and SR-A activity continues until the macrophage is transformed into a lipid-laden foam cell (2, 34). This is in contrast to the precise homeostatic regulation of the receptor for native LDL which is down-regulated by excess levels of intracellular cholesterol (35). By using alternative splicing to generate the type III form of the receptor, the cell not only decreases the amount of primary transcript able to generate the type I and II SR-A isoforms, but also generates an SR-A isoform with dominant negative properties. Hence, production of the type III SR-A protein has the capacity to decrease SR-A activity. We hypothesize that type III splicing could provide a novel mechanism for regulation of SR-A activity which may be important in the pathogenesis of atherosclerosis. To test this hypothesis we are currently looking at genetic, in vitro and in vivo factors that regulate type III SR-A expression.

Dr. James Mahoney and Ms. Leanne Peiser are thanked for their assistance with metabolic labeling and confocal microscopy experiments, respectively. PJG holds an Arthritis and Rheumatism Council Studentship and a Senior Scholarship from Wadham College, University of Oxford. DRG is supported by Glaxo Wellcome; work in the laboratory of SG is funded by the British Heart Foundation and the Medical Research Council.

Manuscript received 7 August 1997 and in revised form 17 November 1997.

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