

Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors

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Abstract Macrophage scavenger receptors have been implicated in various macrophage-associated processes, including atherosclerosis and clearance of bacterial endotoxin. They bind to a wide variety of polyanionic ligands and display complex binding characteristics. cDNAs from the murine macrophage-like cell line P388D1 encoding the full-length type I and type II murine scavenger receptors were cloned, sequenced, and expressed in Chinese hamster ovary cells. A fragment of the corresponding murine genomic DNA was also cloned, partially sequenced, and the positions of the cloned intron/exon boundaries were determined. Comparisons of the murine scavenger receptors' sequences with the bovine, rabbit, and human sequences were used to refine a multidomain model of these trimeric, fibrous, membrane receptors. Metabolic labeling/immunoprecipitation experiments showed that most of the macrophage scavenger receptor protein expressed by P388D1 cells was the N-glycosylated type II receptor; only small amounts of type I receptor were detected. Analysis of the binding properties of the receptors provided evidence that such differential expression of the type I and type II forms may have functional significance. There were substantial receptor-type (I vs. II), as well as receptor-species (bovine vs. murine), differences in the inhibition of ¹²⁵I-labeled AcLDL (acetylated low density lipoprotein) binding by ReLPS, a form of bacterial endotoxin. These differences arose, in part, because these receptors exhibited both high ($K_{dt}(4^{\circ}\text{C}) = 0.05\text{--}0.2\ \mu\text{g protein/ml}$) and low ($K_{dt}(4^{\circ}\text{C}) = 2.5\text{--}12.8\ \mu\text{g protein/ml}$) affinity binding of ¹²⁵I-labeled AcLDL. The ability of ReLPS (1 mg/ml) to inhibit either or both of these two classes of binding interactions varied depending on the species and type of receptor.—Ashkenas, J., M. Penman, E. Vasile, S. Acton, M. Freeman, and M. Krieger. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J. Lipid Res.* 1993. 34: 983–1000.

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Macrophage scavenger receptors are trimeric integral membrane glycoproteins that have been implicated in atherogenesis, endotoxin clearance, and other macrophage-mediated processes (1–6). These receptors bind to a diverse set of polyanionic compounds and display com-

plex ligand binding characteristics (1, 3, 4). Ligands for scavenger receptors include some chemically modified proteins such as oxidized low density lipoprotein (OxLDL), acetylated LDL (AcLDL), maleylated BSA (M-BSA); some polynucleotides, such as poly G and poly I (but not poly A or poly C); acidic phospholipids; and bacterial endotoxin (1, 5–7). Two types of mammalian scavenger receptors have been characterized. Type I receptors are identical to type II receptors except that the type I receptors have an extracellular C-terminal cysteine-rich domain not present in the type II receptors. Scavenger receptor activity similar to that in mammalian macrophages has recently been observed in embryonic *Drosophila melanogaster* macrophages (8). cDNAs encoding the full-length bovine, human, and rabbit type I and type II forms of the macrophage scavenger receptors have been cloned and sequenced (9–12). We have also reported the C-terminal sequences of the type I and type II receptors from the mouse (13), a mammalian species amenable to genetic manipulation (14).

In the current work, we have determined the complete coding sequences of the murine type I and type II macrophage scavenger receptor cDNAs, mapped the intron–exon boundaries for the 5' end of the murine gene, and used these data to revise an earlier model (9) of the domain structure of the receptors. In addition, we have

Abbreviations: LDL, low density lipoprotein; AcLDL, acetylated LDL; bp, base pair; CHO, Chinese hamster ovary; kb, kilobase or kilobase pair; M-BSA, maleylated bovine serum albumin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SRCR domain, scavenger receptor cysteine-rich domain; ReLPS, the Re form of lipopolysaccharide (LPS).

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expressed murine scavenger receptor cDNAs in stably transfected CHO cells and analyzed the receptors' trimeric structures and binding characteristics. These properties were compared to those of bovine scavenger receptors expressed in transfected CHO cells and the endogenous scavenger receptors expressed in the murine macrophage-like cell line P388D1. Unexpectedly, we observed dramatic species-dependent and receptor-type-dependent differences in the interaction of endotoxin with the bovine and murine scavenger receptors. This represents the first substantial difference observed between the binding properties of the type I and type II forms of scavenger receptors. Analysis of the mechanisms underlying these differences showed that the scavenger receptors exhibited both high affinity and low affinity binding of ^{125}I -labeled AcLDL. Depending on the type and species of scavenger receptor, 1 mg/ml of the ReLPS form of endotoxin blocked both classes of binding, only high affinity binding, or neither class of binding. The binding properties of scavenger receptors are discussed in the light of the similarities and differences in the sequences of the murine, bovine, human, and rabbit receptors.

MATERIALS AND METHODS

Materials

Native LDL, AcLDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled AcLDL (DiI-AcLDL), and newborn calf lipoprotein-deficient serum were prepared as previously described (15–18). Medium for tissue culture was from Gibco/BRL or JRH Biosciences and serum was from JRH Biosciences. Oligo dT-cellulose (type 7) was purchased from Pharmacia. Polybrene was supplied by Aldrich. Stock solutions (1–2 mg/ml) of ReLPS (Sigma re595) were prepared by adding the lipids to medium A (Ham's F-12 supplemented with 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM glutamine) containing 10 mM HEPES, pH 7.4, and sonicating twice for 15 sec with a Branson sonifier cell disrupter 185 at a setting of 7. Other reagents were obtained as previously described (17, 19) or as indicated below, or were from standard commercial sources.

Cell culture

All incubations with intact cells were performed at 37°C in a humidified 5% CO_2 /95% air incubator unless specified otherwise. Stock cultures of wild-type CHO cells were grown in medium B (medium A containing 5% (v/v) fetal bovine serum). P388D1 cells were grown in medium C (medium A supplemented with 10% (v/v) fetal bovine serum). To isolate transfectants expressing either the type I or type II murine scavenger receptors, CHO cells (5×10^5) were cotransfected with a DNA mixture con-

taining 0.5 μg pSV2neo, 0.1 μg pSV2dhfr* (20) and 10 μg of either pXmSRI-28 (type I) or pXmSRII-22 (type II) (see below for construction of expression vectors), using the polybrene method (19, 21). Type I scavenger receptor-transfected cells were selected in medium D (medium B containing 0.5 mg/ml of geneticin (G418, Gibco)), and the surviving colonies were screened for the uptake of DiI-AcLDL (3 μg protein/ml in medium D as previously described (19)). Overnight incubation was followed by fluorescence microscopy, as previously described (22). Receptor-positive colonies were harvested and a clone (CHO[mSR-I], clone C5) was isolated by dilution plating, and used as indicated. Type II scavenger receptor-transfectants were selected in medium E (medium A containing 3% (v/v) newborn calf lipoprotein-deficient serum, 0.5 mg/ml geneticin (Gibco), 250 μM mevalonate, 40 μM compactin, and 3 μg protein/ml of AcLDL). Medium E provides nutritional selection for cells expressing functional scavenger receptors (23) as well as a drug selection for transfected cells. We isolated a receptor-positive colony, from which a clone (CHO[mSR-II], clone C8) was generated by dilution plating. This clone was used in all of the murine type II receptor experiments presented below. Stably transfected CHO lines were maintained as stock cultures in medium E. The characterization of transfected CHO cells expressing the type I bovine scavenger receptor (CHO[bSR-I], clone I-B2) and the bovine type II scavenger receptor, CHO[bSR-II], clone II-5, were described previously (19, 23). We have confirmed the findings regarding the differences in ReLPS inhibition of bovine and murine type I scavenger receptor activity using different CHO transfectants that express these receptors.

P388D1 cDNA library construction

Poly A⁺ RNA was prepared from P388D1 cells according to the method of Libermann et al. (24). First strand cDNA synthesis primed with oligo dT was carried out according to Aruffo and Seed (25). Second strand synthesis and ligation to *Eco* RI/*Not* I adaptors were performed using the Pharmacia cDNA synthesis kit (Pharmacia, Piscataway, NJ). Adaptor-ligated cDNAs longer than 500 bp were selected using a 5–20% potassium acetate gradient (25) and selected cDNA was inserted into the λZAP II vector, using the ZAP cDNA Gigapack II Gold cloning kit, following the manufacturer's instructions (Stratagene, La Jolla, CA). A portion of the resulting library was packaged, plated, and amplified, while the remainder was reserved for PCR amplification.

PCR amplification and hybridization cloning of murine scavenger receptor cDNAs

We previously reported the sequences of type I-specific and type II-specific 3' ends of the murine scavenger receptor

cDNAs (13). The 5' end of the murine scavenger receptor cDNA was amplified from the P388D1 cDNA library by PCR, using as primers a cDNA-specific antisense oligonucleotide (5' CTTAAGGGGGTGTGATCCGCC 3'), and a λ ZAP II vector-specific oligonucleotide (5' ATTAACCCCTCACTAAAGGGA 3'). The 1.1 kb amplification product was subcloned into pBluescript KS(+) (Stratagene) and independent subclones were sequenced.

A sense oligonucleotide defining the 5' end of this 1.1 kb product (5' CGCCGAGCGGCCGCGCTGTCTTCTTTTACCAGC 3') was used together with oligonucleotides complementary to the extreme 3' ends of the type I and type II murine scavenger receptor (5' CGCCGGTCTAGAGGTTTCATAATTGTAATTTTC 3' for the type I cDNA; 5' CGCCGGTCTAGATAGAATTTAATAGGATTCTG 3' for the 1.5 kb type II cDNA, i.e., the type II-1 cDNA; see below) to amplify the fully coding cDNAs. These amplification products were subcloned into pRC/CMV (Invitrogen, San Diego, CA), using the *Not* I and *Xba* I restriction sites engineered into these primers, and multiple subcloned isolates were sequenced. Because none of the subcloned products was free of PCR errors, the cDNA library was screened by hybridization to a random hexamer-labeled (26) 5' cDNA fragment (bp -31 to 221) generated by PCR from one of the cloned cDNAs. One clone of 4 kb was isolated and the *in vivo* excision of the insert as a pBluescript-based plasmid was performed following the Stratagene protocol. This insert, which extends from bp -17 to the 3' untranslated region of the 4 kb type II murine scavenger receptor cDNA (designated the type II-3 cDNA; see below), was subcloned into the expression vector pcDNA I (Invitrogen) to generate the type II expression construct pXmSR11-22. The 3' end of the insert in this construct was replaced by the error-free 3' end fragment of a full length type I cDNA PCR product to generate the type I expression construct pXmSR1-28. Because the 5' end of the pXmSR1-28 insert was derived from pXmSR11-22, neither expression construct includes sequence 5' of bp -17.

Genomic cloning

A λ DASH (Stratagene) genomic library derived from murine D3 cells, which was generously provided by Doug Gray and Rudolf Jaenisch, was screened by hybridization using as a probe a mixture of DNAs containing the murine scavenger receptor type I (bp -17 to 1440 in Fig. 1A) and type II (-17 to 1175 in Fig. 1B) cDNA sequences. One clone, λ DASH 29, hybridized both to a 32 P-labeled N-terminal domain-specific oligonucleotide (5' ATGACAGAGAATCAGAGGC 3') and to an oligonucleotide from the coiled coil domain (5' AGTGAGAGTATTGAA-CAACA 3'), and was selected for further study.

Sequence analysis

cDNA inserts in the type I and type II murine expres-

sion constructs and subcloned portions of the genomic clone λ DASH 29 were sequenced using Sequenase following the manufacturer's recommendations (USB, Cincinnati, OH). DNA and protein sequence analyses were performed with the GCG package of programs versions 7.0 and 7.1 (27).

Northern blot analysis

Poly A⁺ RNA prepared from P388D1 cells (2 μ g/lane) was fractionated by electrophoresis through a denaturing formaldehyde 1% agarose gel (21) and transferred to a Genescreen nylon membrane (DuPont NEN, Boston, MA) according to the manufacturer's recommendations. Probes were generated from cloned cDNAs by PCR (probes A through D; see Fig. 3) or by restriction enzyme digestion of pXmSR11-22 with *Not*I and *Xba*I which yielded an approximately 600 bp fragment from the 3' end of the insert (probe E in Fig. 3). Sense and antisense oligonucleotide primers were as follows. For probe A, 5' CGCCGAGCGGCCGCGCTGTCTTCTTTTACCAGC 3' and 5' CGCCGGTCTAGATTATACTGATCTTGATCCGC 3'; for probe B, 5' CCCCTTAAGACAGTTCGAC 3' and 5' GGTTCATAATTGTAATTTTC 3'; for probe C, 5' ACTCCCTGAACATATTGGGG 3' and 5' TAATAGGATTCTGCACAGTTA 3'; for probe D, 5' ATTGATTAAGTATTAGTTCT 3' and 5' CGCCGGATCGATT-TAATATATTAGACTAC 3'. Hybridization was with 50% formamide at 42°C, following the Genescreen protocol. The final wash stringency was 2 \times SSC (21) at 65°C.

125 I-labeled AcLDL degradation assays

Scavenger receptor activity at 37°C was assessed by measuring cellular degradation of 125 I-AcLDL in 24-well culture dishes as previously described (15, 17, 23). On day 0, the indicated transfected CHO cell lines were seeded at a concentration of 60,000 cells/well in medium D while P388D1 cells were seeded at 100,000 cells/well in medium C. On day 2, the monolayers were refed with 0.5 ml of medium B containing the indicated amounts of 125 I-labeled AcLDL and the ReLPS form of bacterial endotoxin in the absence (duplicate determinations) or, in some experiments, the presence (single determinations) of 400 μ g/ml of poly I. After a 5-h incubation at 37°C, the amounts of 125 I-AcLDL degradation products released into the media were measured. The values presented in Fig. 8 were calculated with the program MacLigand as described below. Protein concentrations were determined by the method of Lowry et al. (28). The degradation values represent μ g of 125 I-AcLDL protein degraded in 5 h per mg of cell protein.

4°C 125 I-AcLDL binding assay

The cell surface binding assay was based on the procedure previously described by Goldstein and colleagues (15, 16). On day 0, the indicated cells were seeded into

6-well dishes at a concentration of 250,000 cells/well in 3 ml of medium D. On day 2, the monolayers were prechilled by refeeding with 1.5 ml of ice-cold medium D and incubating at 4°C for 30–45 min. All subsequent steps with the intact cells were performed at 4°C using prechilled reagents. The monolayers were refed with 1 ml of medium F (medium B supplemented with 10 mM HEPES, pH 7.4) containing the indicated amounts of ¹²⁵I-AcLDL, either with or without 1 mg/ml of ReLPS, and in the absence (duplicate or triplicate determinations) or presence (single determinations) of 400 µg/ml of poly I. After a 2-h incubation on an orbital shaker (~ 60 rpm), the cells were washed 3 times rapidly and 2 times slowly with 2 ml/well of Tris-BSA buffer (50 mM Tris-HCl, 0.15 N NaCl, 2 mg/ml BSA, pH 7.4) and then they received 2 additional rapid washes with the Tris buffer without BSA as previously described (16). It is possible that some of the receptor-bound ¹²⁵I-AcLDL, especially that bound to sites with relatively high dissociation rates, may have dissociated during this thorough washing procedure (29). However, comparison of the 4°C binding and 37°C degradation results (see Table 2) suggests that such potential dissociation is not likely to have dramatically altered the calculated ratios of the amounts of low and high affinity binding. The cells were then lysed at room temperature for 15 min with 1.5 ml/well of 0.1 N NaOH and the amounts of ¹²⁵I-AcLDL and protein in the lysates were measured using an LKB gamma counter and the method of Lowry et al. (28).

Data analysis

Analysis of the ¹²⁵I-AcLDL binding data was performed using the program MacLigand, version 1.01, adapted for the Macintosh computer by Robert Williams from the program Ligand (30). Each set of data subjected to analysis was observed in a single experiment and comprised 18–20 observations for the 4°C binding curves and 11–12 observations for the 37°C degradation curves. As suggested by the programs' authors, the specific ¹²⁵I-AcLDL binding and degradation values shown in Figs. 7 and 8 were calculated by subtracting from the total observed values the nonspecific values calculated by the MacLigand program during the regression analyses (30). To independently verify these calculated nonspecific values, we also measured ¹²⁵I-AcLDL binding and degradation in the presence of an excess of the competitive inhibitor poly I. The observed nonspecific values were similar to the calculated values. As described previously (30) and in the manual for MacLigand, we used the F-test criterion on the residual variances to determine whether the two-binding sites model (five-parameter fit) was significantly better than the one-binding site model (three-parameter fit) for each set of data. Statistical analysis of all of the 4°C binding data observed in the absence of ReLPS showed that the two-site model was significantly better than the

one-site model. Except as noted below, all of the 4°C binding and 37°C degradation results shown were confirmed in at least two independent experiments. The 4°C binding curves for the bovine type I and murine type II receptors were determined once. The two-site model for this 4°C binding was confirmed by analysis of dose-response curves for degradation at 37°C (not shown).

Antibodies

A peptide corresponding to the N-terminus of the murine scavenger receptors (MTENQRLCPHEREDADC) was prepared by the MIT biopolymer laboratory and coupled via cysteine to hemocyanin as previously described (31–33). Antibodies (designated anti-mN) were raised in a New Zealand White rabbit (33) and purified from the serum prior to use by Protein-A Sepharose (Pharmacia) chromatography (34). The protein concentration of the purified antibody-preparation was 7.8 mg/ml.

Biosynthetic labeling and immunoprecipitations

On day 0, transfected cells and P388D1 cells were plated in medium B or medium C, respectively, in 6-well dishes (500,000 cells/well). On day 1, each monolayer was rinsed twice with 1 ml of Dulbecco's phosphate-buffered saline (PBS), pulse-labeled for 15 min with 0.5 ml of methionine-free medium A containing 3% (v/v) newborn calf lipoprotein-deficient serum and 300 µCi/ml of ³⁵S-labeled protein labeling mix (DuPont-New England Nuclear), washed once with medium C and chased for the indicated times in medium C supplemented with 1 mM unlabeled methionine. Cells were harvested by scraping each well with 210 µl of lysis buffer (PBS with 1% (v/v) Triton X-100, 0.25% (w/v) SDS, 1 mM methionine, 50 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) with or without 10 mM iodoacetamide to block artifactual disulfide formation (19) as indicated. The lysates were then boiled for 5 min and clarified by centrifugation (10 min, 4°C, 10,000 g). To 90 µl of each lysate we added 50 µl of 1% (w/v) BSA in PBS and 150 µl of detergent buffer (1% (v/v) Triton X-100, 1% (w/v) deoxycholic acid, 0.25% (w/v) SDS, 100 mM Tris-HCl, pH 8.0). This lysate/detergent mixture was then precleared twice with 40 µl of a 1:3 mixture (v/v) of preimmune serum and a 50% (v/v) slurry of protein A-Sepharose in PBS. Scavenger receptors in these specimens were then immunoprecipitated by adding to each specimen 30 µl of a 1:2 mixture (v/v) of anti-mN antibody and a slurry of protein A-Sepharose in PBS (50% v/v). The immunoprecipitated proteins were solubilized by boiling in reducing sample buffer (0.2% SDS, 0.1 mg/ml bromphenol blue, 10% (v/v) glycerol, 0.5% (v/v) β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) and subjected to SDS gel electrophoresis (3–10% polyacrylamide gradients) followed by fixation, staining, and autoradiography, as previously described (33).

RESULTS

Cloning and sequence analysis of murine scavenger receptor cDNAs and genomic DNA

In previous studies Via and colleagues (35; 36) have described the partial purification and characterization of ~260 kD scavenger receptors from P388D1-derived tumors, and we have reported the cloning and sequencing of portions of the cDNAs encoding the C-terminal ends of the type I and type II murine scavenger receptors (13). These C-terminal sequences were used in conjunction with the PCR technique and standard hybridization screening to clone cDNAs encoding the full-length type I and type II polypeptides from a library generated from P388D1 cells, a murine macrophage-like cell line (37). The cDNA and predicted protein sequences are shown in Fig. 1. The 1457 bp murine scavenger receptor type I cDNA encodes a protein of 454 amino acids with a predicted molecular mass of 49.6 kD. The cDNA sequence surrounding the methionine at position 1 (Fig. 1) conforms to the Kozak consensus (38) and, based on sequence alignment, presumably represents the initiator methionine. However, as the open reading frame extends to the 5' end of our cloned cDNA sequence (Fig. 1 and data not shown), it is possible that this is an internal methionine and that the cytoplasmic domain begins at nucleotide position -12 or at an ATG further upstream. The predicted protein sequence defines six domains, as was first described for the bovine scavenger receptors (9): I, N-terminal cytoplasmic domain (residues 1-51); II, transmembrane domain (52-77); III, spacer domain (78-151); IV, α -helical coiled coil domain (subdomain IVa: 152-211; subdomain IVb: 212-272); V, collagenous domain (273-344); and VI, a C-terminal domain (345-454) comprising an eight-residue hinge (subdomain VIa) and the 102-residue scavenger receptor cysteine-rich, SRCR, domain (subdomain VIb). The type I receptor contains two cysteines in intracellular domain I, and two and six each in the extracellular domains III and VI, respectively. It also has three potential Asn-linked (N-linked) glycosylation sites (Asn-X-Thr/Ser) in domain III and three in domain IV.

The 4-kb murine type II scavenger receptor cDNA encodes a 350 amino acid polypeptide (predicted molecular mass, 38.2 kD), which is identical to the type I receptor from amino acids 1 to 347 (domains I-V and three residues in domain VI). At residue 348, the sequences diverge (arrows in Fig. 1A and 1B) and the type II cDNA encodes three additional C-terminal amino acids followed by a long 3' untranslated region. Thus, as we reported previously (13), the murine type II receptor has a six-(345-350) amino acid C-terminal domain in place of the 110-residue SRCR domain. Additional features of the coding sequences of the type I and type II murine scavenger receptor cDNAs are described below.

We used cDNA-derived probes to clone the scavenger receptor gene from a mouse genomic library. One 15-kb genomic fragment includes four coding exons. Sequence analysis of this clone, designated λ DASH 29, confirmed the cDNA sequence from nucleotides 1-817 and established the positions of four intervening sequences (indicated by triangles in Fig. 1A). These introns divide the exons encoding the N-terminal five domains of the receptor. Three of these are located at or near the junctions between domains or subdomains. For example, an intron after nucleotide 220 is near the proposed border between transmembrane domain II and spacer domain III. The positions of the introns in the murine gene are similar to the corresponding introns reported for the bovine and human genes (39, 40).

Comparison of the sequences of the murine, bovine, human, and rabbit scavenger receptors

Fig. 2 illustrates sequence comparisons for each of the domains and subdomains of the murine, bovine, human, and rabbit scavenger receptors. At those positions where all, or all but one, of the corresponding residues are identical, the amino acids are boxed and shaded. Of the 454 amino acids in the murine type I receptor, 248, or 55%, are identical to corresponding residues in all of the other three species. The percent sequence identities⁴ for each domain are: domain I, 70%; domain II, 79%; domain III, 59%; domain IVa, 61%; domain IVb, 78%; domain V, 73%; domain VIa (type I), 23%; domain VIb (type I), 81%; and domain VI (type II), 22%.

In addition to the conserved domain organization, there are a number of structural features common to all four species of receptor. For example, six of the seven potential N-glycosylation sites in the bovine, rabbit, and human receptors are present at similar or identical locations in the murine receptors. The cytoplasmic domains in all of the receptors share three potential phosphorylation sites and one cysteine residue. Seven other cysteines are conserved, including one in the spacer domain (see immunochemical analysis below), and six that help to define the SRCR domain (5, 13, 41). In addition, there is a conserved proline, Pro67, in the middle of the transmembrane domains that might affect their secondary structures and influence their packing in the trimeric receptors. There are also a number of noteworthy interspecies differences. With the exception of the methionine in position 1, all of the 12 N-terminal amino acids in the murine receptors differ from the corresponding, well-conserved residues in the other three species. This is the largest single segment of poor homology among the four

⁴Calculated by averaging the percent identities from all six pairwise comparisons among the four species.

A

1	ATGACAGAGAATCAGAGGCTCTGCCCTCATGAACGAGAGGATGCTGACTGCAGTTCAGAA	60
1	M T E N Q R L C P H E R E D A D C S S E	20
61	TCCGTGAAATTTGACGCACGTTCAATGACAGCATCCCTTCCTCACAGCACTAAAAATGGC	120
21	S V K F D A R S M T A S L P H S T K N G	40
121	CCCTCCGTTTCAAGGAGAGTTGAAGTCCTTCAAGGCTGCCCTCATTGCTCTCTACCTCTT	180
41	P S V Q E K L K S F K A A L I A L Y L L	60
181	GTGTTTGCAGTACTAATACCTGTTGTTGGAATAGTGACAGCTCAGCTTTTGAATTGGGAA	240
61	V F A V L I P V V G I V T A Q L L N W E	80
241	ATGAAGAACTGCTTAGTTTGTTCACGTAACACAAGTGATACATCTCAAGGTCCTATGGAA	300
81	M K N C L V C S R (N T S) D T S Q G P M E	100
301	AAAGAAAATACCAGTAACGTGGAAATGAGATTACAATTATCATGGCACACATGAAGGAC	360
101	K E (N T S) N V E M R F T I I M A H M K D	120
361	ATGGAGGAGAGAATCCAAAGCATTTCAAACTCAAAGCCGACCTTATAGACACGGGACGC	420
121	M E E R I Q S I S N S K A D L I D T G R	140
421	TTCCAGAATTTTCAGCATGGCAACTGACCAAGACTTAATGATATTCTTCTGCAGTTAAAT	480
141	F Q (N F S) M A T D Q R L N D I L L Q L N	160
481	TCCTTGATTTTGTCTAGTCCAGGAACATGGGAATTCAGTGATGCAATCTCCAAGTCCTTG	540
161	S L I L S V Q E H G N S L D A I S K S L	180
541	CAGAGTCTGAATATGACACTGCTTGATGTTCAACTCCATACAGAAACACTGCATGTCAGA	600
181	Q S L (N M T) L L D V Q L H T E T L H V R	200
601	GTCCGTGAATCTACAGCAAAGCAACAGGAGGACATCAGTAAATTGGAGGAACGTGTGTAC	660
201	V R E S T A K Q Q E D I S K L E E R V Y	220
661	AAAGTATCAGCAGAAGTCCAGTCTGTGAAAGAAGAACAAGCGCACGTGGAACAGGAAGTA	720
221	K V S A E V Q S V K E E Q A H V E Q E V	240
721	AAACAGGAAGTGAGAGTATTGAACAACATCACAACGACCTCAGACTGAAGGACTGGGAA	780
241	K Q E V R V L N (N I T) N D L R L K D W E	260
781	CACTCACAGACACTGAAAAACATCACCTTCATTCAAGGGCCTCCTGGACCCCAAGGTGAA	840
261	H S Q T L K (N I T) F I Q G P P G P Q G E	280
841	AAGGGAGACAGAGGGCTTACTGGACAAACTGGTCCACCTGGTGCTCCAGGAATAAGAGGT	900
281	K G D R G L T G Q T G P P G A P G I R G	300
901	ATTCCAGGTGTTAAAGGTGATCGGGGACAAATTTGGCTTCCCTGGAGGTCGAGGAAACCCA	960
301	I P G V K G D R G Q I G F P G G R G N P	320
961	GGAGCACCAGGAAGCCAGGGAGGTCGGGATCTCCTGGACCTAAAGGACAAAAGGGAGAG	1020
321	G A P G K P G R S G S P G P K G Q K G E	340
1021	AAGGGAGTGTAGCGGATCAACCCCCCTTAAGACAGTTCGACTGGTTGGTGGTAGTGGA	1080
341	K G S V G G S T P L K T V R L V G G S G	360
1081	GCCCATGAGGGCCGAGTGGAGATCTTCCACCAAGGCCAGTGGGGCACAACTCTGTGATGAT	1140
361	A H E G R V E I F H Q G Q W G T I C D D	380
1141	CGCTGGGATATACGGGCTGGACAAGTTGTCTGCCGGAGTCTAGGATACCAAGAAGTTCTA	1200
381	R W D I R A G Q V V C R S L G Y Q E V L	400
1201	GCTGTGCACAAGAGAGCTCACTTTGGACAAGGTACTGGTCCAATATGGCTGAATGAAGTG	1260
401	A V H K R A H F G Q G T G P I W L N E V	420
1261	ATGTGTTTTGGAAGAGAATCATCTATTGAGAAGTGTAAATCAACAGTGGGGAGTACTA	1320
421	M C F G R E S S I E N C K I N Q W G V L	440
1321	AGCTGTTACATTTCAGAAGATGCTGGGGTCACTTGTTACTTCATAAgtatcatattttca	1380
441	S C S H S E D A G V T C T S *	460
1381	atggcatttatgaaattacaattatgaaaccccccccccccccccccccccccccccccccc	1440

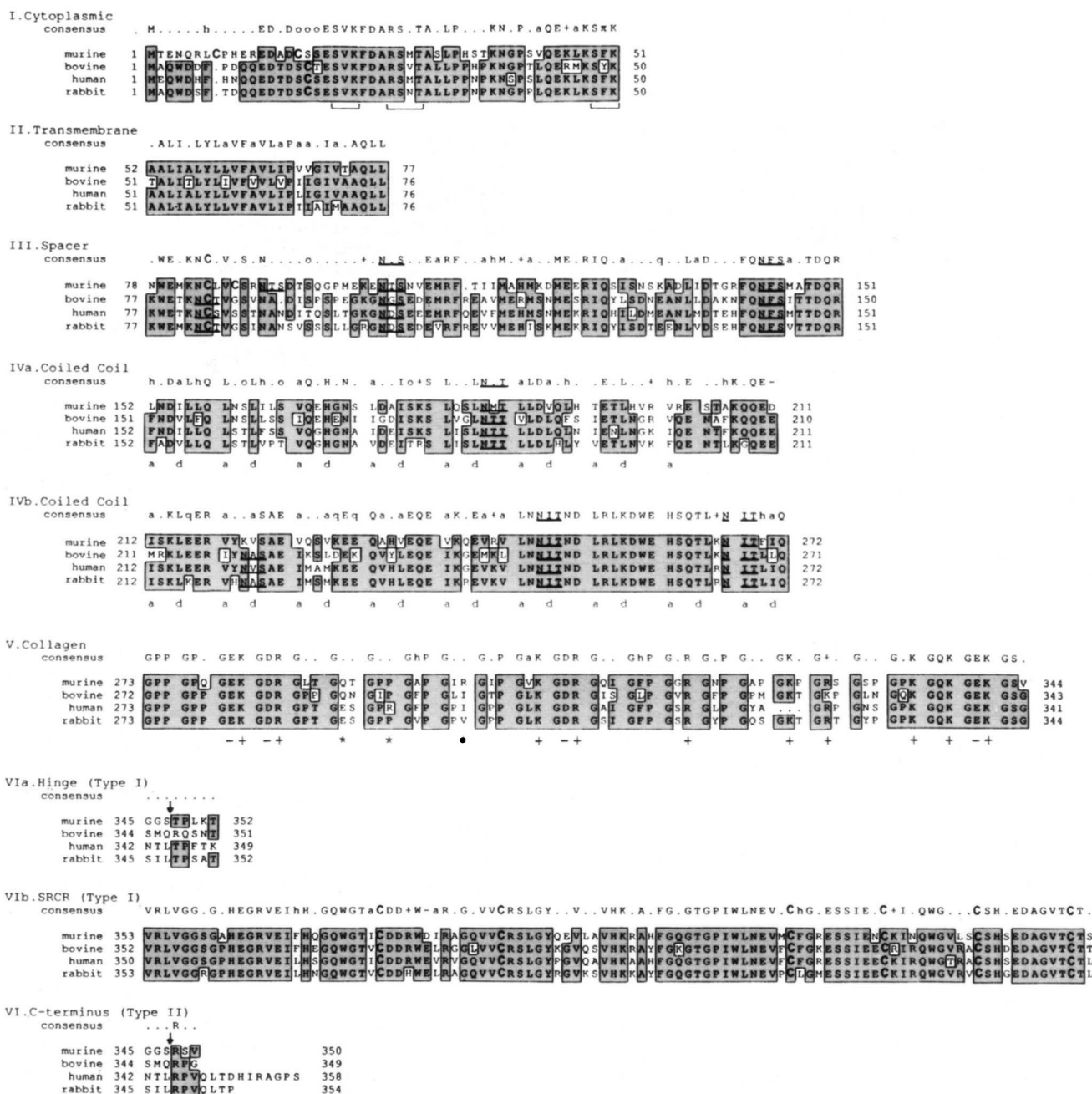


Fig. 2. Comparison of the predicted protein sequences of the murine, bovine, human, and rabbit macrophage scavenger receptors. The sequences (single letter code) were aligned using the program PILEUP (27) and adjusted manually. At those positions where all or all but one of the corresponding residues are identical, the amino acids are boxed and shaded. Consensus residues (top lines) are indicated when individual amino acids (capital letters) or classes of residues (lower case letters or symbols) are present at all sites for a given position. Classes of amino acids are defined as follows: "a", A, I, L, M and V; "π", F, W and Y; "+", H, K, and R; "-", D and E; "o", C, S and T; "h" denotes sets of residues in which all residues at a given position are from either the "a" or "π" classes; "q" denotes sets of residues in which all residues at a given position are from either the "+" or "-" classes. Cysteines are shown in bold and predicted N-linked glycosylation sites are underlined. In domain I, predicted phosphorylation sites conserved among all four species for protein kinase C (S/T-X-K/R) (61) and multifunctional calmodulin-dependent protein kinase II (R-X-X-S/T) (62, 63) are indicated by brackets. In the consensus sequence of heptads in α -helical coiled coils, the first and fourth positions ("a" and "d") are occupied by hydrophobic, usually aliphatic, residues while the other positions are often polar (64, 65). In domain IV, heptad repeats are separated and the a and d positions are indicated. In domain V, the G-X-Y triplets are separated and conserved positively ("+") and negatively ("-") charged positions are indicated. Charged triplets which are not conserved are indicated by an asterisk ("**"). The points at which the type I and type II sequences diverge are indicated by arrows in domain VIa (type I) and domain VI (type II).

a single exon. In addition, the same PCR amplifications using total P388D1 cDNA as a template generated single bands of the expected sizes. This suggests that the differ-

ent mRNAs did not arise as a consequence of internal deletions. The functional significance, if any, for the generation of different classes of type II mRNA is currently unknown.

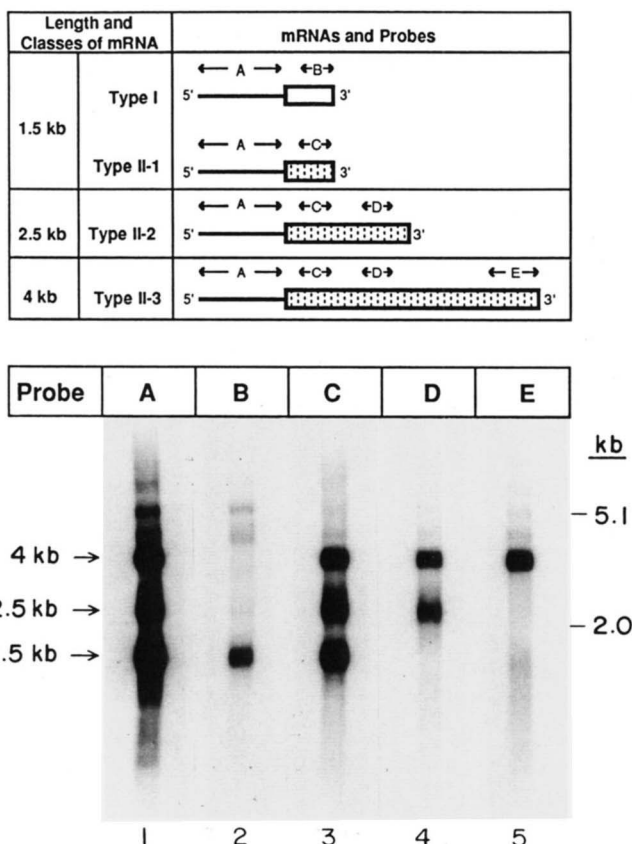


Fig. 3. Northern blot analysis of scavenger receptors in P388D1 cells. Poly A⁺ RNA from P388D1 cells was prepared and subjected to Northern blot analysis as described in Materials and Methods. Replicate filters were incubated with ³²P-labeled probes (designated A-E) derived from the type I and type II murine scavenger receptor cDNAs as indicated and hybridization was visualized by autoradiography. The positions of the probes relative to the cDNA sequences that are common to the type I and type II forms (line) or specific to the type I (open box) or type II (stippled boxes) forms are indicated in the diagram above the autoradiograms. We have cloned cDNAs corresponding to the type II-1 (13) and the type II-3 (this study) mRNAs, but not the type II-2 mRNA.

Immunochemical analysis of protein structure. The structures of the murine type I and type II receptors expressed in stably transfected CHO (CHO[mSR-I] and CHO[mSR-II]) cells were examined in a series of pulse/chase metabolic labeling/immunoprecipitation experiments using a rabbit polyclonal antipeptide-antibody raised against the murine receptors' N-terminus. In the experiment shown in **Fig. 4**, the cells were pulse labeled with ³⁵S-labeled protein labeling mix for 15 min and harvested either immediately (0 chase) or after a 4-h incubation in unlabeled medium (4-h chase). The immunoprecipitated receptors were reduced prior to electrophoresis and autoradiography. Both the type I and type II receptors were synthesized as precursors with apparent masses of approximately 66 and 57 kD, respectively (lanes 1 and 3), which were converted to mature forms during the chase (lanes 2 and 4). The conversion of the type I receptor to the mature form was not as efficient as that of the type II recep-

tor (compared the relative amounts of precursor and mature forms after 4 h of chase in lanes 2 and 4). The apparent masses of the type I and type II receptors were reduced to approximately 53 and 44 kD after treatment with N-glycanase (lanes 7 and 8). Thus, as previously described for the bovine receptors (19), the murine receptors were synthesized as glycoproteins containing N-linked oligosaccharide chains.

Fig. 4 also shows the synthesis and processing of endogenous scavenger receptors in P388D1 macrophages (lanes 5, 6, and 9). One relatively weak and one strong receptor precursor band in the pulse-labeled cells (lane 5) comigrated with the precursor forms of the type I and type II receptors expressed in the CHO cells (lanes 1 and 3). After the chase, the electrophoretic mobilities of the somewhat heterogeneous receptors in the P388D1 cells (lane 6) fell between the mature type I and type II receptors in the CHO cells. To determine whether these differences were due to cell-type dependent differences in N-glycosylation, we compared the electrophoretic mobilities after treatment with N-glycanase (lanes 7-9). The bulk of the receptor protein in P388D1 cells observed after a 4-h chase and N-glycanase treatment comigrated with the type II receptor from CHO[mSR-II] cells. We conclude that both type I and type II scavenger receptors were synthesized in P388D1 cells; however, the amount of type I

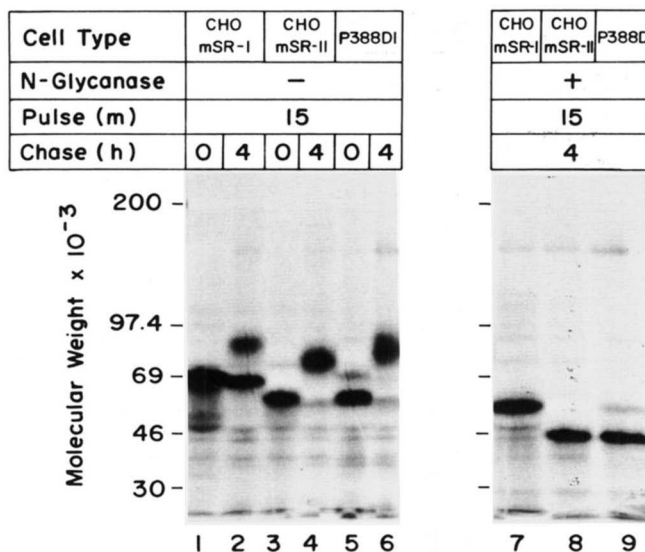


Fig. 4. Biosynthesis of murine scavenger receptors in transfected CHO cells and P388D1 macrophages. On day 1, the indicated cells were plated into 6-well dishes at a concentration of 0.5×10^6 cells/well. On day 2, the cells were pulse-labeled with ³⁵S-labeled protein labeling mix for 15 min and either harvested immediately (0 chase) or chased in medium C containing 1 mM unlabeled methionine for 4 h. The cells were lysed and scavenger receptors were immunoprecipitated with the polyclonal anti-murine scavenger receptor antibody anti-mN. Immunoprecipitates were reduced, subjected to electrophoresis in an 8% SDS-polyacrylamide gel, and visualized by autoradiography as described in Materials and Methods. For N-glycanase treatment (lanes 7-9), immunoprecipitates were incubated with N-glycanase overnight at 37°C, prior to electrophoresis and autoradiography.

receptor detected after the 15-min pulse-labeling was substantially less than that of the type II receptor. Although rapid degradation of newly synthesized type I receptors relative to that of type II receptors might contribute to this difference, it seems likely that P388D1 cells synthesized substantially more type II than type I receptors. Additional evidence for the predominant expression of type II receptors in P388D1 cells is described below.

We have previously shown that the Cys84 in the spacer domain, which is found in all scavenger receptors sequenced to date (see Fig. 2), participates in an intermolecular disulfide bond covalently linking two of the three chains in the trimeric bovine scavenger receptor (19). The third chain noncovalently associates with the disulfide-linked dimer. The existence of an additional cysteine, Cys87, in the murine receptor's spacer domain, which is not present in the other species, raised the possibility that the murine scavenger receptor, unlike its bovine homolog, might form trimers in which all three chains were covalently linked by disulfide bonds at residues 84 and 87. This possibility was examined in the experiment shown in Fig. 5. In this experiment, we compared the electrophoretic mobilities of reduced and unreduced murine scavenger receptors expressed in CHO[mSR-I], CHO[mSR-II], and P388D1 cells and those of the bovine

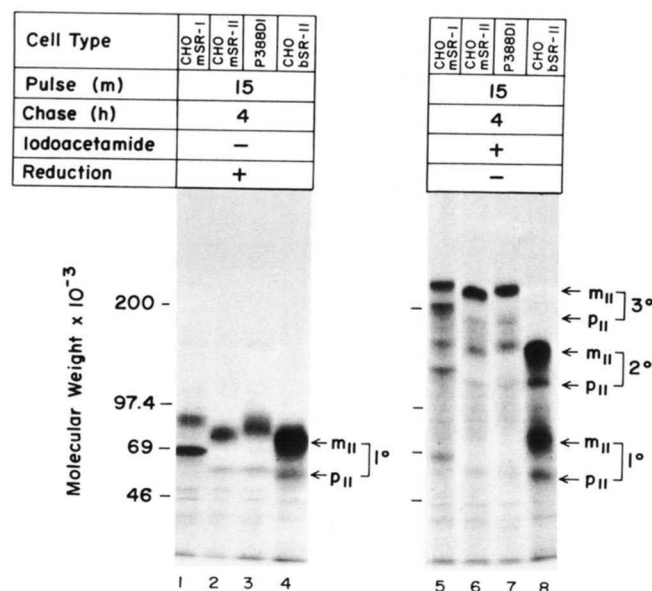


Fig. 5. Oligomerization of murine or bovine scavenger receptors in transfected CHO cells and P388D1 murine macrophages. On day 1, the indicated cells were plated into 6-well dishes at a concentration of 0.5×10^6 cells/well. On day 2, the cells were pulse-labeled with ^{35}S -labeled protein labeling mix for 15 min and chased in medium C containing 1 mM unlabeled methionine for 4 h. Cells were lysed either in the presence or absence of 10 mM iodoacetamide as indicated. Lysates were then immunoprecipitated. Reduced or unreduced samples were subjected to 3–10% gradient SDS-polyacrylamide gel electrophoresis, and the receptors were visualized by autoradiography (p_{II} and m_{II} denote precursor and mature forms of the type II receptors, and 1° , 2° , and 3° denote monomers, dimers, and trimers, respectively).

TABLE 1. Ligand specificity of murine scavenger receptors expressed in CHO cells

Addition (400 $\mu\text{g/ml}$)	CHO[mSR-I] ^{125}I -AcLDL Degradation	CHO[mSR-II] ^{125}I -AcLDL Degradation
% of control		
None	100	100
AcLDL	4	5
Poly I	7	11
M-BSA	10	13
LDL	91	118
BSA	89	117

The indicated cells were plated on day 0 at a concentration of 60,000 cells per well in a 24-well dish in medium D. On day 2, ^{125}I -AcLDL (3 μg protein/ml, 393 cpm/ng) was added in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated competitors and degradation was measured after a 5-h incubation at 37°C as described in Materials and Methods. The 100% of control values for the CHO[mSR-I] and CHO[mSR-II] cells were each 1.6 $\mu\text{g}/5$ h per mg of cell protein.

type II scavenger receptor expressed in CHO[bSR-II] cells. The cells were pulse-labeled for 15 min with ^{35}S -labeled protein labeling mix, chased for 4 h, and then lysed and boiled in a buffer containing SDS and 10 mM iodoacetamide as indicated. These conditions prevent the artifactual formation of disulfide bonds by Cys17 in the cytoplasmic domain of the bovine receptors when the cells are lysed (19; also Fig. 5, lane 8 and data not shown). It seems likely that these lysis conditions also prevented artifactual disulfide bond formation by the two cytoplasmic cysteines in the murine receptors. After reduction (lanes 1–4), only monomeric forms of the precursor and mature receptors were observed. As previously reported, the unreduced bovine type II receptor (lane 8) comprised monomers (1°) and disulfide linked dimers (2°), but no covalently linked trimers. In contrast, both the type I and type II murine scavenger receptors in both CHO and P388D1 cells formed reduction-sensitive trimers (3°) as well as dimers (lanes 5–7). Thus, it appears that both Cys84 and Cys87 in domain III participate in intermolecular disulfide bonding which covalently crosslinks at least some of the murine type I and type II receptors into trimers.

Analysis of receptor binding. The binding properties of the type I and type II murine scavenger receptors expressed in the transfected CHO cells were studied using either an ^{125}I -AcLDL degradation assay at 37°C or an ^{125}I -AcLDL cell surface binding assay at 4°C . Table 1 shows that both types of murine receptor exhibited ^{125}I -AcLDL degradation activity that was inhibited by classic scavenger receptor ligands (AcLDL, poly I, maleylated BSA) but not by corresponding negative controls (native LDL and BSA). A similar pattern of ligand specificity was previously observed for the cloned bovine type I and type II scavenger receptors (9, 10, 23) and is a hallmark of macrophage scavenger receptor activity (1). Thus, we were surprised to find dramatic differences in the ability of another

scavenger receptor ligand, bacterial endotoxin, to inhibit ^{125}I -AcLDL degradation mediated by the murine and bovine type I and type II receptors.

Fig. 6. shows the effects of increasing concentrations of the ReLPS form of endotoxin (45) on the receptor-mediated degradation of ^{125}I -AcLDL by CHO cells expressing either the murine type I (mSR-I), murine type II (mSR-II), bovine type I (bSR-I), or bovine type II (bSR-II) scavenger receptors and by P388D1 cells. Untransfected, wild-type CHO cells exhibited essentially no scavenger receptor activity (23 and data not shown). The inhibitory effects of ReLPS in the transfected cells were both receptor-type and species-dependent. Although there was very little inhibition of bovine type I receptor activity (open circles) at concentrations as high as 1 mg/ml, ReLPS did inhibit bovine type II receptor activity (shaded circles). This inhibition, however, was incomplete: the estimated 50% inhibitory dose (ID_{50}) was 260 $\mu\text{g}/\text{ml}$, yet at a concentration of ReLPS of 1000 $\mu\text{g}/\text{ml}$, the activity was inhibited by only 61%.

The inhibition of the activity of the murine type I receptor (open squares) by 1 mg/ml of ReLPS was also incomplete ($\text{ID}_{50} \sim 115 \mu\text{g}/\text{ml}$). In contrast, almost all of the activity of murine type II receptors (solid squares) was inhibited by ReLPS ($\text{ID}_{50} \sim 45 \mu\text{g}/\text{ml}$). The inhibition by ReLPS of scavenger receptor activity in the murine P388D1 cell line (shaded triangles) was also virtually com-

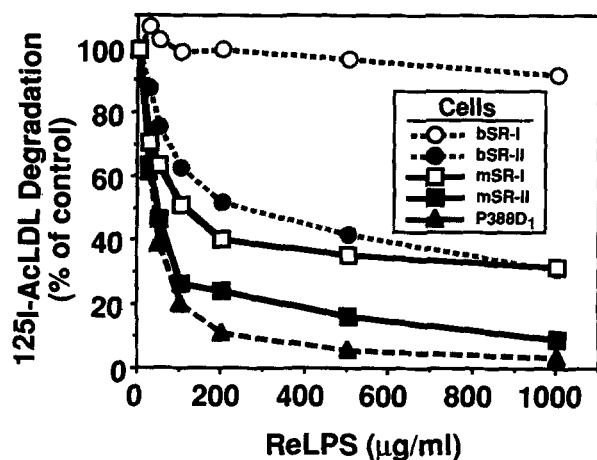


Fig. 6. Concentration dependence of ReLPS inhibition of ^{125}I -AcLDL degradation by transfected CHO cells expressing the bovine (bSR) and murine (mSR) type I and type II macrophage scavenger receptors and by murine P388D1 macrophages. CHO cells stably transfected with cDNA expression vectors for the indicated scavenger receptors and P388D1 cells were plated on day 0 at a concentration of 60,000 cells per well in medium D (transfected CHO cells) or 100,000 cells per well in medium C (P388D1 cells) in the wells of 24-well dishes. On day 2, ^{125}I -AcLDL (3 μg protein/ml, 282 cpm/ng) was added in medium B with the indicated amounts of ReLPS and ^{125}I -AcLDL degradation was measured after a 5-h incubation at 37°C as described in Materials and Methods. The data were from two separate experiments. The percent of control values represent the averages of duplicate determinations. The 100% of control values (ng/5 h/mg) were: bSR-I, 2078; bSR-II, 2269; mSR-I, 1582; mSR-II, 2064; P388D1, 3924.

plete at the higher concentrations of ReLPS ($\text{ID}_{50} \sim 40 \mu\text{g}/\text{ml}$) and most resembled the inhibition of the murine type II receptors in the transfected CHO cells. This is consistent with our immunoprecipitation studies (see Figs. 4 and 5 above) which showed substantial type II and very little type I receptor expression in P388D1 cells. Thus, type I scavenger receptors appear to contribute little to the scavenger receptor activity in this murine macrophage cell line.

The species and receptor type dependences of ReLPS inhibition of scavenger receptors were complex and unexpected. One particularly intriguing feature of the data in Fig. 6 is that the relatively high concentration of 1 mg/ml ReLPS could only partially inhibit the activities of the bovine type II and murine type I receptors. A simple explanation for these partial inhibitions is that there may be two classes of binding of ^{125}I -AcLDL in these cells, only one of which was effectively inhibited by ReLPS at concentrations $\leq 1 \text{ mg}/\text{ml}$. To test this hypothesis, we measured the concentration dependence of ^{125}I -AcLDL binding to the transfected CHO cells at 4°C . Under these conditions surface binding to receptors can occur but receptor internalization is inhibited (15, 46, 47). As was the case at 37°C , we observed that 1 mg/ml of ReLPS did not inhibit ^{125}I -AcLDL binding at 4°C to bovine type I receptors, only partially inhibited binding to bovine type II and murine type I receptors, and almost completely inhibited binding to murine type II receptors (see below and data not shown). Fig. 7 shows the binding data for the four different scavenger receptors expressed in transfected CHO cells. The upper panels in each pair (A-B, C-D, etc.) illustrate the binding as a function of increasing ^{125}I -labeled AcLDL concentrations. The lower panels of Fig. 7 and the values in Table 2A show Scatchard analyses of the same data. The binding parameters in Table 2 were calculated using the MacLigand program as described in Materials and Methods.

For each species and type of receptor, 4°C binding of ^{125}I -AcLDL in the absence of ReLPS (solid circles) was high affinity and saturable (Fig. 7, upper panels). The corresponding Scatchard curves were all nonlinear (Fig. 7, lower panels). The shapes of the Scatchard curves suggested that the transfected cells exhibited two classes of ^{125}I -AcLDL binding: high and low affinity. Models that can account for this finding are discussed below. The dissociation constants (K_{d1}) for the high affinity binding (Table 2A) were similar for all four transfectants, with values ranging from 0.05 to 0.21 μg protein/ml. Because of the relatively limited number of degrees of freedom in the regression analyses, the uncertainties in all of the calculated dissociation constants were fairly high (see Table 2A). The dissociation constants (K_{d2}) for the low affinity binding were approximately 30–60 times larger than those for the high affinity binding, with values ranging from 2.7 to 12.8 μg protein/ml. In every case, the calcu-

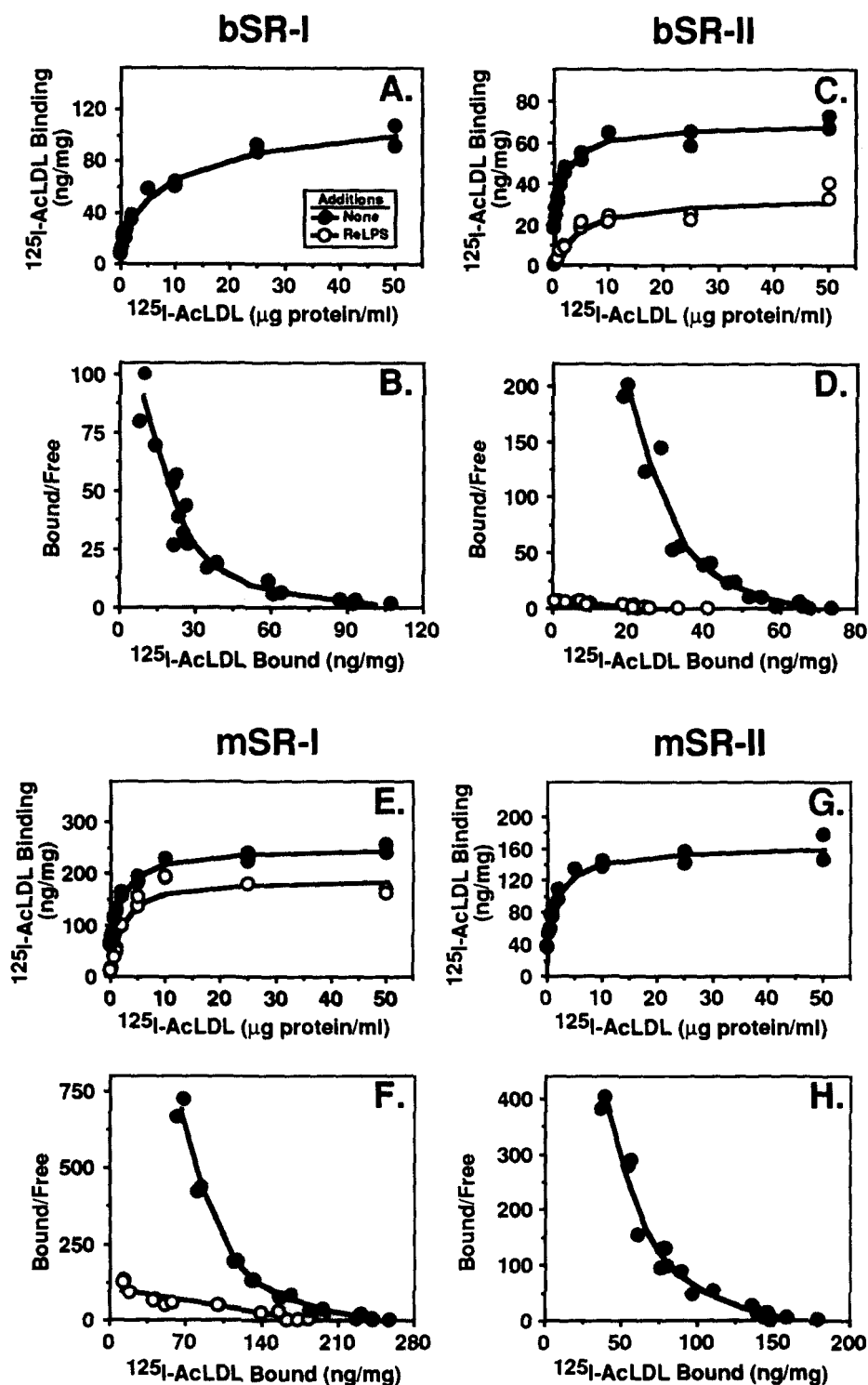


Fig. 7. Concentration dependence (A, C, E, G) and corresponding Scatchard analysis (B, D, F, H) of ^{125}I -AcLDL binding at 4°C to transfected CHO cells expressing the bovine (bSR) and murine (mSR) type I and type II macrophage scavenger receptors. Cells were plated on day 0 at a concentration of 250,000 cells per well in medium D in the wells of 6-well dishes. On day 2, the cells were chilled and the indicated amounts of ^{125}I -AcLDL (duplicate or triplicate determinations) in the absence (solid circles) or presence (open circles) of 1 mg/ml ReLPS were added and cell surface binding was measured after a 2-h incubation at 4°C as described in Materials and Methods. The effects of ReLPS on binding to bSR-I and mSR-II were not determined in these experiments (see text). Nonspecific background was calculated as described in Materials and Methods and subtracted from each datum. Solid lines represent the values calculated using the parameters shown in Table 2A.

TABLE 2. Scatchard analysis of 4°C binding and 37°C degradation of ¹²⁵I-AcLDL by transfected CHO cells^a

Experiment	1/K _{d1} ml/μg protein	1/K _{d2} ml/μg protein	K _{d1} μg protein/ml	K _{d2} μg protein/ml	R1 ng/mg	R2 ng/mg
A. 4°C Binding						
bSR-I	5 ± 1	0.08 ± 0.09	0.21	12.8	27 ± 5	93 ± 72
bSR-II	15 ± 7	0.37 ± 0.28	0.07	2.7	31 ± 7	40 ± 8
bSR-II + 1 mg/ml ReLPS	—	0.22 ± 0.04	—	4.5	—	34 ± 6
mSR-I	19 ± 6	0.4 ± 0.1	0.05	2.5	93 ± 12	163 ± 14
mSR-I + 1 mg/ml ReLPS	—	0.6 ± 0.1	—	1.7	—	194 ± 28
mSR-II	13 ± 5	0.4 ± 0.2	0.08	2.5	63 ± 13	103 ± 15
B. 37°C Degradation ^b						
	ml/μg protein	ml/μg protein	μg protein/ml	μg protein/ml	μg/5 h/mg	μg/5 h/mg
bSR-II	0.6 ± 0.3	0.03 ± 0.02	1.6	30	2.2 ± 0.8	9 ± 1
bSR-II + 0.5 mg/ml ReLPS	—	0.05 ± 0.01	—	20	—	7 ± 2
mSR-I	0.9 ± 1.3	0.037 ± 0.033	1.1	27	1.3 ± 1.3	7.7 ± 1.6
mSR-I + 0.5 mg/ml ReLPS	—	0.040 ± 0.004	—	25	—	6.6 ± 0.4

^aThe binding at 4°C (A) and degradation at 37°C (B) of ¹²⁵I-AcLDL by CHO cells transfected with expression vectors for the indicated macrophage scavenger receptors were determined in the absence or presence of the indicated concentrations of ReLPS as described in the legends to Figs. 7 and 8 and in Materials and Methods. The data shown in Figs. 7 and 8 were subjected to Scatchard analysis using the program MacLigand as described in Materials and Methods. K_{d1} and K_{d2} represent the high and low affinity dissociation constants, respectively. For the 4°C binding data (A), R1 and R2 represent the calculated maximal amounts of high and low affinity cell surface binding (ng of ¹²⁵I-AcLDL bound per mg cell protein) and are proportional to the number of surface binding sites. The dashes replace numerical values when single-site rather than two-site binding models were used to analyze the data. Because of the relatively limited number of degrees of freedom in the regression analyses, the calculated uncertainties were fairly high.

^bFor the 37°C degradation data (B), R1 and R2 represent the calculated maximal ¹²⁵I-AcLDL degradation (μg degraded in 5 h per mg cell protein, see text). Caution should be exercised in drawing conclusions from the 37°C values for all four parameters, K_{d1}, K_{d2}, R1, and R2. They should be considered *apparent* or estimated values because the data represent ligand degradation values observed at steady state rather than equilibrium.

lated amount of high affinity binding (R1) was less than the low affinity binding (R2); however, some of these differences were not statistically significant.

In the presence of 1 mg/ml of ReLPS, the Scatchard curves for the bovine type II and murine type I receptors (Fig. 7, open circles, panels D and F) were linear with K_d values of 4.5 and 1.7 μg protein/ml, respectively. Both the K_d values and the number of binding sites in the presence of ReLPS were not significantly different from the low affinity values in the absence of ReLPS (Table 2A). The simplest explanation of these data is that ReLPS at concentrations ≤ 1 mg/ml efficiently blocked high affinity, but not low affinity, binding. This would explain the observation in Fig. 6 that 1 mg/ml of ReLPS was unable to completely inhibit ¹²⁵I-AcLDL degradation at 37°C for cells expressing the bovine type II and murine type I scavenger receptors.

To determine whether the effects of ReLPS on the degradation of ¹²⁵I-AcLDL at 37°C were similar to those on binding at 4°C, we measured the concentration dependence of ¹²⁵I-AcLDL degradation by transfectants expressing the bovine type II and murine type I scavenger receptors. Measurements were made in the absence and presence of 0.5 mg/ml of ReLPS (Fig. 8 and Table 2B). Note that the parameters calculated from these degradation assays represent metabolism of the ligand at steady state (15) rather than binding at equilibrium. Scatchard binding analyses are not generally applicable under such nonequilibrium conditions; therefore, caution must be exercised in the interpretation of the calculated parameters.

The *apparent* dissociation constants described below should not be considered to represent *equilibrium* dissociation constants. Nevertheless, previous studies of both scavenger and LDL receptors have shown that at 37°C the *apparent* dissociation constants (the ligand concentrations at which there is half maximal activity) are essentially the same for binding, uptake and degradation. Thus the shapes of the dose-response curves for degradation reflect the first step in the process, binding to the receptors (8, 15, 48). Furthermore, the results described below for degradation at 37°C are consistent with those for binding at 4°C.

Fig. 8 shows ¹²⁵I-AcLDL degradation as a function of increasing ¹²⁵I-AcLDL concentration (panels A and B, left) and the corresponding Scatchard analyses (panels C and D, right, also see Table 2B). At 37°C, the shapes of the curves and the effects of ReLPS were qualitatively similar to those at 4°C (Fig. 7). In the absence of ReLPS (solid circles), the Scatchard curves were nonlinear, suggesting the expression of two classes of receptor binding, while in the presence of ReLPS (open circles) the curves were linear. As has previously been observed for LDL and scavenger receptors (15, 46), the *apparent* dissociation constants for degradation at 37°C were approximately 10-fold higher than the corresponding dissociation constants for binding at 4°C (Table 2; also see discussion below). Taken together, the 4°C and 37°C data strongly suggest that there are two classes of ¹²⁵I-AcLDL binding that have species- and type-dependent differences in their sensitivities to inhibition by ReLPS.

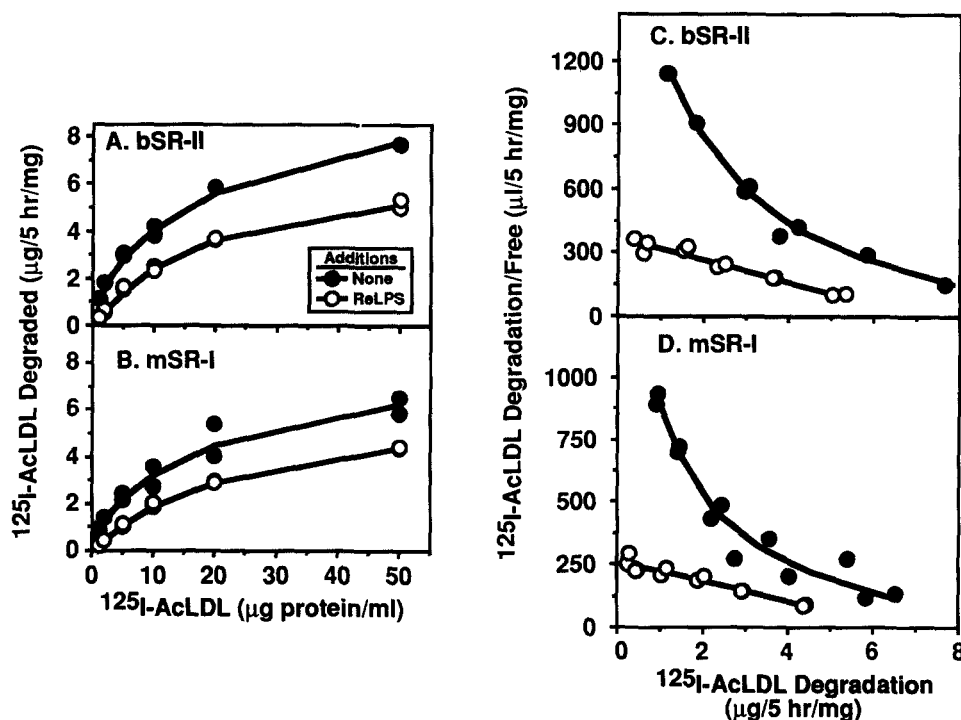


Fig. 8. Concentration dependence and Scatchard analysis of ^{125}I -AcLDL degradation at 37°C by transfected CHO cells expressing the bovine type II (bSR-II) and murine type I (mSR-I) macrophage scavenger receptors. The indicated cells were plated on day 0 at a concentration of 60,000 cells per well in medium D in the wells of 24-well dishes. On day 2, the indicated amounts of ^{125}I -AcLDL (duplicate determinations) in the absence (solid circles) or presence (open circles) of 0.5 mg/ml ReLPS were added and degradation was measured after a 5-h incubation at 37°C as described in Materials and Methods. Nonspecific background was calculated as described in Materials and Methods and subtracted from each datum. Solid lines represent the values calculated using the parameters shown in Table 2B.

DISCUSSION

In the current work, we have cloned and sequenced the type I and type II murine scavenger receptors. As a consequence, we have been able to express murine scavenger receptor cDNAs in transfected CHO cells and analyze the receptors' trimeric structures and binding characteristics. These properties were compared to those of bovine scavenger receptors expressed in transfected CHO cells and the endogenous scavenger receptors expressed in the murine macrophage-like cell line P388D1. In addition, we have performed a detailed comparison of the sequences of the murine, bovine, rabbit, and human scavenger receptors,⁵ mapped the intron-exon boundaries for the 5' end of the murine receptor's gene, and incorporated these data into a revised model of the receptors' domain structure.

In each of the four species examined to date, there are two types of scavenger receptor mRNAs which are the products of alternative splicing (10-13, 39). Both types have a short N-terminal cytoplasmic domain followed by

a single transmembrane domain, a spacer domain, an α -helical coiled coil domain, and a collagenous domain. Type I receptors have a 110-residue C-terminal domain (hinge subdomain plus scavenger receptor cysteine-rich, SRCR, subdomain) which is replaced by a 6-17-residue C-terminus in type II receptors. SRCR-like domains are found in a wide variety of proteins and represent a new family of cysteine-rich protein domains (5, 13, 41).

P388D1 macrophage-like cells express both type I and type II receptor mRNAs. However, the bulk of the immunoprecipitable scavenger receptor protein was type II; very little type I receptor protein was detected. These findings are consistent with the results of radiation inactivation target size analysis recently reported by Via and coworkers (36). Molecular sizes determined using radiation inactivation analysis appear to correspond well to the masses of the polypeptide portions of glycoprotein receptors. Via et al. (36) found that the apparent subunit mass was $35 \pm 7 \text{ kD}$. This value is in good agreement with our predicted polypeptide monomer size of 38.2 kD for the type II murine scavenger receptor, but not with the predicted size of the type I receptor (49.6 kD). We cannot exclude the possibility that P388D1 cells might also express other, as yet uncharacterized, scavenger receptors.

⁵Murine and rabbit scavenger receptors have recently been independently cloned by Doi et al. (58).

These receptors, however, would have to have activities (see below) and molecular masses (as determined by radiation inactivation) similar to those of the type II receptor.

The difference in the expression levels of the type I and type II proteins in P388D1 cells may be functionally significant. Although both the type I and type II murine scavenger receptors expressed in transfected CHO cells exhibited broad polyanion binding specificity, which is a hallmark of scavenger receptors (1, 5), their binding properties were not identical. We unexpectedly found that inhibition by bacterial endotoxin of scavenger receptor-mediated binding and subsequent catabolism of ^{125}I -AcLDL were dependent on the type of receptor (type I vs. type II). The ReLPS form of endotoxin essentially completely inhibited murine type II receptor activity at a concentration of 1 mg/ml ($\text{ID}_{50} \sim 45 \mu\text{g/ml}$). In contrast, at that concentration, inhibition of murine type I receptors was incomplete ($\text{ID}_{50} \sim 115 \mu\text{g/ml}$). As expected from the immunoprecipitation studies described above, ReLPS inhibition of receptor activity in P388D1 cells was similar to that for murine type II receptors; the ID_{50} was $\sim 40 \mu\text{g/ml}$.

Scatchard analysis of ^{125}I -AcLDL binding to the receptors at 4°C accounts for the incomplete inhibition of murine type I receptors by 1 mg/ml of ReLPS. Both type I and type II murine scavenger receptors exhibited two classes of ^{125}I -AcLDL binding. The high affinity binding (K_d s of 0.05 and $0.08 \mu\text{g protein/ml}$, respectively) was 30–50 times tighter than the low affinity binding (K_d s of $2.5 \mu\text{g protein/ml}$). In the case of the murine type I receptor, ReLPS at concentrations $\leq 1 \text{ mg/ml}$ inhibited essentially all of the high, but little of the low, affinity binding; thus, 1 mg/ml of ReLPS only partially inhibited the type I form of the receptor. In contrast, 1 mg/ml of ReLPS could block both classes of binding to murine type II receptors. The differential inhibition by ReLPS of high and low affinity binding to murine type I receptors strongly supports the conclusion from Scatchard analysis that there are two distinct classes of ^{125}I -AcLDL binding to these receptors. Indirect evidence for the expression by murine type I receptors of these two classes of binding at 37°C was obtained using an ^{125}I -AcLDL degradation assay. As was previously reported for LDL receptors and scavenger receptors in murine peritoneal macrophages (15, 46, 49), the *apparent* dissociation constants at 37°C (see Results) were 10-fold higher than the corresponding equilibrium dissociation constants at 4°C . This temperature dependence may reflect either true differences in binding affinities (15, 46, 49, 50), or complex kinetic effects due to endocytosis and the absence of equilibrium at 37°C (29), or both.

The simplest explanation for the observation of two classes of binding of ^{125}I -AcLDL is that each scavenger receptor molecule has multiple, nonidentical, binding sites with two different affinities for this ligand. Other, more complex, explanations are possible. For example,

the data are consistent with multiple identical sites on each receptor exhibiting negative cooperativity of binding. Our findings are also compatible with a lattice model previously proposed to account for strikingly similar observations with the LDL and asialoglycoprotein receptors (51, 52). In a lattice model, initial binding of large ligands to an assembly of receptors with closely spaced binding sites (either on a single receptor molecule or adjacent molecules) sterically hinders the subsequent binding of additional ligands (53–55). The resulting negative cooperativity of binding does not require changes in the structure or intrinsic binding affinity of the receptors themselves. It is a property of the ensemble. Both AcLDL and ReLPS, which forms large micelles (56), are large ligands that could sterically hinder adjacent binding sites. Alternatively, the transfected cells might synthesize two (or more) nonidentical forms of each type of receptor, some with a single class of high affinity ^{125}I -AcLDL binding sites and others with only low affinity sites. Heterogeneous posttranslational processing, e.g., variations in glycosylation, proline/lysine hydroxylation, or heterogeneous receptor oligomerization (5, 19, 36), could be the source of nonidentical receptors with differing binding properties.

The ReLPS inhibition data represent the first dramatic differences reported in the binding properties of the type I and type II forms of scavenger receptors. ReLPS was a significantly poorer inhibitor of ^{125}I -AcLDL metabolism mediated by type I receptors than by type II receptors. This difference was almost certainly a consequence of the presence of the large SRCR domain in the type I receptor and its absence in the type II receptor. It seems possible that the large SRCR domains may have interfered with the access of ReLPS micelles to some ^{125}I -AcLDL binding sites. These findings will help direct future studies of the functional significance of the expression of these two different types of receptor. It will be of interest to determine whether macrophages *in vivo* express type I and type II receptors differentially, whether there are differences in receptor expression between different types of macrophages (e.g., Kupffer cells and foam cells), and whether macrophages can regulate the relative expression levels of the two types of receptor to modulate their sensitivities to different scavenger receptor ligands.

In addition to the receptor type dependence of the effects of ReLPS on ^{125}I -AcLDL binding, there was a striking species dependence. For type II receptors, 1 mg/ml of ReLPS inhibited both high and low affinity binding to murine receptors, but only high affinity binding to bovine receptors. In the case of type I receptors, there was efficient inhibition of only high affinity binding to murine receptors, and virtually no inhibition of either class of binding to bovine receptors. It appears that the Gly-X-Y triplet repeats in the collagenous domains of the scavenger receptors, in particular, the conserved positive charges at the C-termini of these domains (4, 5, 9, 10, 57,

58), are responsible for the binding of polyanionic ligands to the receptors. These and other conserved residues in the collagenous domains may define adjacent binding sites which might account for both high and low affinity ^{125}I -AcLDL binding. However, at 19 of the 48 X and Y positions in these domains, the amino acids are not conserved between the bovine and murine sequences. Indeed, the murine collagenous domains contain one positively charged triplet which is not charged in the bovine receptors (Gly-Ile-Arg, asterisk in Fig. 2). These sequence differences presumably play particularly important roles in determining the species specific differences reported here. It should be possible to perform site-directed mutagenesis, (e.g., see refs. 57 and 58) or domain switching experiments, using the sequence comparisons in Fig. 2 as a guide, to further define the mechanisms underlying the unusual binding properties of the collagenous domains. Sequence variations in the other domains, most notably the coiled coil and SRCR domains, also may contribute to the species dependent binding differences.

The physiological functions of macrophage scavenger receptors have not yet been established. Because of their unusual broad binding specificities, it has been suggested that they participate in a variety of physiologic and pathophysiologic processes. These include the accumulation of cholesterol in artery walls during atherogenesis (1, 2, 23), the recognition and clearance of pathogens, including endotoxin, for host defense (5, 6), the recognition of inhaled particles, such as asbestos, in the lungs (59, 60), and possibly the engulfment of dead cells or cell fragments in development or disease (3, 4, 8). The cloning of a murine scavenger receptor genomic fragment should permit the construction of scavenger receptor-deficient macrophage cell lines and strains of mice (14) in which many of these ideas may be examined directly. ■

Note added in proof: Otnad et al. have recently examined hepatic membrane proteins which bind scavenger receptor ligands and obtained evidence for three classes of binding (66).

The nucleotide sequences for the murine scavenger receptors reported in this paper have been submitted to GenBank with accession numbers L04274 (type I) and L04275 (type II). We thank Chris Raetz for generously providing reagents, Jonathan Wallach for oligonucleotide syntheses, Yelena Ekkel for performing transfections, Dr. Endo for the gift of compactin, Rudolf Jaenisch and Doug Gray for the use of the D3 mouse genomic library, C. Raetz, R. Hampton, P. Schimmel, R. Rosenberg, D. Resnick, A. Pearson, S. Podos, and members of our research group for many helpful discussions and suggestions, Julia Khorana for expert help in preparing figures, and Kathy Sweeney for administrative assistance. This work was supported by Grant HL-41484 from the National Institutes of Health-National Heart, Lung, and Blood Institute.

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