Macrophage scavenger receptors are trimeric integral membrane proteins that bind a diverse array of negatively charged ligands. They have been shown to play a role in the pathogenesis of atherosclerosis and in host responses to microbial infections. Earlier mutational studies demonstrated that the distal segment of the collagen domain of the receptor was critically important for high affinity ligand binding activity. In this study, mutations spanning the entire collagen domain were generated and binding was assayed in transfected cells, as well as in assays employing a secreted, receptor fusion protein. Many of the distal, positively charged C-terminal residues in the type II collagen domain of the receptor, previously reported to be essential for binding at 37 °C, were found not to be critical for binding at 4 °C. Conversely, more proximally charged residues of the collagen receptor that have not been previously mutated were shown to have substantial effects on binding that were also temperature-dependent. These data suggest that scavenger receptor ligand recognition depends on more complex conformational interactions, involving charged residues throughout the entire collagen domain, than was previously recognized.

Macrophage scavenger receptors exhibit a breadth of ligand binding specificity that is unusual among cell surface endocytotic receptors. This property may facilitate macrophage participation in a wide variety of host defense and immunologic responses. Although most interest in macrophage scavenger receptors has focused on their role in the uptake and degradation of modified forms of low density lipoprotein (LDL), an event thought to be critical to the earliest stages of atherosclerotic plaque formation, the discovery of scavenger receptor binding interactions with lipopolysaccharide (3, 4), crocidolite asbestos (5), and β-amyloid fibrils (6, 7) suggests that their role in human diseases and host immune responses may be a more expansive one. The discovery that scavenger receptors may also participate in cell adhesion further strengthens this hypothesis (8). An elucidation of the structural features of scavenger receptors that confer their broad but yet restricted binding specificity might, therefore, yield useful insights into a variety of macrophage-associated host defense responses.

The cDNAs encoding two forms of the macrophage scavenger receptor, now termed SR class A type I and type II receptors, were initially isolated using bovine lung mRNA (9, 10). Subsequently, homologous receptors of both types have been cloned from murine, human, and rabbit tissues (4, 11–13). The amino acid sequence of the type I receptor suggested that the protein could be divided into six domains (4, 9, 14) (domain I, an N-terminal cytoplasmic tail (residues 1–50 in the rabbit sequence); domain II, transmembrane domain-(51–76); domain III, a spacer domain-(77–151); domain IV, α-helical coiled-coil domain-(152–272); domain V, collagen domain-(273–344), and domain VI, a cysteine-rich C-terminal region-(345–454) that is a highly conserved domain found in a diverse group of proteins (11, 15, 16)). The type II receptor differs from type I only in that it lacks the cysteine-rich C terminus, instead possessing a 6–17-residue (varying with species) C-terminal truncated tail. The type II receptor was shown to bind the ligands traditionally used to define SR function (10) establishing that the cysteine-rich domain could not be the major ligand binding region of the scavenger receptor. As the known ligands for the SR are all polyanionic, it was initially postulated that the SR collagen sequences might serve as the receptor binding domain, because all 24 Gly-X-Y triplets of the bovine collagen region would be predicted to be neutral, or positively charged, at physiologic pH. Recent experimental work has provided support for this initial hypothesis.

Acton et al. (17) reported that a scavenger receptor mutant lacking the C-terminal 16 Gly-X-Y repeats of the collagen domain of the bovine receptor was incapable of binding the defining ligand for the scavenger receptor, acetylated LDL (AcLDL). This mutation did not appear to disrupt other aspects of scavenger receptor cell biology, such as receptor synthesis, tamerization, post-translational modification, or cell surface stability. It was also demonstrated to inhibit the activity of a co-transfected wild type receptor. Similar findings were reported by Dejager et al. (18) who also showed that collagen truncation mutants could inhibit the activity of endogenous scavenger receptors, when cDNAs encoding the truncation mutants were transfected into the mouse macrophage cell line, P388D1. To identify more precisely the critical amino acids in the collagen domain, Doi et al. (19) generated a series of deletion mutants as well as point mutations affecting the positively charged residues found in the last six Gly-X-Y triplets of the human collagen domain. These receptors were studied in degradation and binding assays utilizing COS cells transfected with cDNAs encoding the mutant forms of the receptors. Binding and degradation of AcLDL was analyzed in these transfected cell lines. Convincing evidence was presented to support
the contention that several positively charged residues in the C terminus of the collagen domain were important for the binding and degradation of modified lipoprotein ligands. The authors (19) of this work concluded that Lys-337 is essential for binding and that the four “lysine cluster in the most C-terminal portion of the collagen is the ligand-binding domain of the scavenger receptor.”

In this paper, we report the results of binding studies, utilizing both transfected cells and a direct receptor protein binding assay, to perform a broader mutational analysis of the collagen domain of the macrophage scavenger receptor. It is demonstrated that a scavenger receptor/human IgG fusion protein can assemble into higher weight oligomers, including the characteristic trimeric form of the native SR. These fusion proteins, after purification by affinity column chromatography, retain the ability to bind SR ligands with affinity comparable to that of the native receptor expressed on the cell surface. Whereas mutational studies of both the fusion proteins and their transmembrane analogues confirm the importance of the C-terminal collagen residues for ligand binding, they also indicate that other residues outside this region are equally critical for receptor binding. Importantly, the restoration of binding at 4 °C to several different receptor proteins with mutations in charged residues, previously thought to be critical to ligand interactions, provides strong evidence that the prevailing paradigm for SR binding interactions is incomplete. Temperature-dependent binding activity alterations, demonstrated in both proximal and distal collagen subdomain mutants, suggest that these mutations substantially alter the conformation of the receptor, rather than simply effect a loss of ionic receptor-ligand interactions. These data make it clear that the current model of SR binding, which postulates an ionic interaction between ligand and receptor, involving the distal segment of the collagen domain, cannot account for the full complexity of the binding behavior of this receptor.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Lipoproteins—**All cell culture incubations were at 37 °C in 5% air and 5% CO₂, COS M7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% calf serum. For cell degradation assays, native and mutant forms of pXRSR2 (13), a plasmid containing the rabbit type II scavenger receptor in pcDNA-1 (Invitrogen, San Diego), were transfected into the COS cells using the DEAE-dextran method described by Aruffo and Seed (20). The ability of the transfected cells to degrade 125I-AcLDL in the presence or absence of inhibitors was measured 72 h after transfections. All degradation were conducted at 37 °C for 5 h as described previously (13). Lipoproteins used in these assays as well as for the ligand blots and binding assays were prepared by methods previously detailed (21). Iodination of the lipoproteins was accomplished by a modified iodine monochloride reaction (22).

**Plasmid Constructs and Mutagenesis—**The overall structure of the proteins used in this study are schematically depicted in Fig. 1. By using the polymerase chain reaction, a nine amino acid epitope sequence, TETSSQAPA, encoding a C-terminal epitope of the rhodopsin protein was inserted into pXRSR2 (13) DNA as the template. The polymerase chain reaction primers contained 5’ BamHI and 3’ XhoI sites, and these were used to ligate the epitope sequence into wild type pXRSR2 that had been digested with BamHI and XhoI. The epitope sequence encoded a translational stop codon immediately distal to the final alanine of the epitope sequence. The epitope sequence was then transferred to the type II SR COS cell expression plasmid, pXRSR2. These two plasmids, pXRSR2 and pXRSR2, were then used to generate mutant receptor cDNAs by the oligonucleotide site-directed mutagenesis method of Kunkel (24).

Fusion proteins were generated by creating a BamHI site in pXRSR2 immediately distal to the putative receptor transmembrane sequence via the polymerase chain reaction. This fragment was then ligated in frame to the Fc portion of a human IgG cDNA that contained a BamHI site immediately distal to the IgG sequence. This construct was generated in a CD8-8 COS cell expression derivative, pCD5speyAC71, kindly provided by Dr. Brian Seed (Massachusetts General Hospital, Boston). A similar construct was made using the epitope containing scavenger receptor sequences, whose creation is described above.

To generate mutant forms of the fusion protein, mutations were first generated in pXRSR2 or pXRSR2-epl by a modified Kunkel method and then transferred to the human IgG-SR fusion construct by cleavage with ApoLI, an enzyme with a restriction site in the coiled-coil region of the SR and a second site in the origin of replication sequence found in both pCD5speyAC71 and pcDNA-1. All mutations were sequenced from the start of the collagen domain to its terminus using the dideoxy chain termination method and Sequenase (U.S. Biochemical Corp.).

**Antibodies, Immunoblots, and Ligand Binding Studies—**The rhodopsin epitope antibody, ID4 (23), was kindly provided by Frank Kowalkowski (Massachusetts General Hospital, Boston). The monoclonal anti-fusion protein antibody, ASAb-1, was generated in Balb/c mice by injection of purified fusion protein. Splenic fusions and hybridoma generation were performed according to the protocols of Harlow and Lane (25).

Following separation by SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes in a Bio-Rad Mini-Transblot apparatus, using 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol as the transfer buffer. Transfers were performed at 150 mA for 2 h. After transfer, the membrane was blocked for 1 h in PBS containing 1% Tween 20, 10% dried milk (Buffer A) with Buffer A minus the milk (Buffer B), and then incubated for 30 min with first antibody diluted in Buffer A. For ID4 (protein A purified from mouse ascites fluid), a dilution of 1:1000 was employed; the supernatant from hybridoma 7180–112 (ASAb-1) was used at a 1:5 dilution. The unbound first antibody was removed with 4 washes of Buffer B. A 30-min incubation with a 1:1000 dilution of horseradish peroxidase-labeled anti-mouse antibody (Sigma) in Buffer A was then performed. The membrane was then washed 4 × with Buffer B and developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) according to the manufacturer’s (Sigma) instructions.

Ligand blots were performed according to a modification of the procedure of Daniel et al. (26). AcLDL was substituted for LDL, and the binding and wash buffers were substituted 50 mM NaCl for 90 mM NaCl. Rabbit anti-human LDL antibody (Biomedical Technologies, Inc., Stoughton, MA) was used at a 1:5000 dilution in conjunction with a peroxidase-conjugated goat anti-rabbit antibody (Sigma) diluted 1:1000. Development with DAB was done as for the immunoblots. For quantitative ligand binding assays, 125I-AcLDL was used (1 × 10⁶ cpm per filter strip) to bind to nitrocellulose strips on which fusion proteins had been transferred. Equal amounts of protein, as measured by a modified Bradford assay (Pierce), were loaded in each lane of the gel prior to transfer. All binding assays were done in triplicate.

For saturation binding studies, receptor-coated plastic wells (Teraskan plates) were employed. Plates were coated initially with 0.5 μg of fusion protein, in PBS, pH 7.4, for a minimum of 4 h at 4 °C. The wells were then blocked with 0.25% BSA, 0.05% Tween, and 0.02% azide for 30 min, and then incubated with 1:1000 dilution of horseradish peroxidase-labeled anti-mouse antibody (Sigma) in Buffer A was then performed. The membrane was then washed 4 × with Buffer B and developed with 3,3-diaminobenzidine tetrahydrochloride (DAB) according to the manufacturer’s (Sigma) instructions.

**Ligand Binding Analysis—**Data from saturation binding studies were analyzed using a modified version of the Ligand program (RA-DLIG; G.A. McPherson, Biosoft, Ferguson, MO). Curve fitting was done using both a one-site and a two-site binding model. The molecular
The weight of apolipoprotein B (518,000 daltons) was used to estimate the molarity of AcLDL protein.

RESULTS

Proteins Generated in the Study—The SR fusion proteins created for this study are depicted in Fig. 1. These proteins were encoded by rabbit type II cDNAs from which the N-terminal cytoplasmic tail and transmembrane sequences had been removed. They were replaced with a signal sequence and Fc domain derived from human IgG. Wild type scavenger receptor cDNAs (for use in cell degradation studies) and fusion receptor cDNAs were also modified so as to encode a rhodopsin epitope sequence at the C terminus of the protein (Fig. 1). The epitope tag was originally designed to assay receptor expression on the surface of transfected cells. Although the epitope tag was readily detected under the denaturing conditions used for the immunoblots, its detection on intact cells was unreliable, perhaps indicating that access to this C-terminal tag is blocked when the SR is folded into its native, trimeric structure. The demonstration by Resnick et al. (27) that the extended C terminus of the type I scavenger receptor appears to fold into a globular domain tightly juxtaposed to the fibrous strands of the collagen domain provides some support for this hypothesis. The nine amino acid epitope sequence did serve two other purposes as follows: 1) to identify any unintended frameshift mutation that could have occurred in the mutagenesis process and that might have eluded detection by

FIG. 1. A, schematic representation of proteins employed in this study. The wild type protein for the study is the type II rabbit scavenger receptor encoded by the cDNA cloned by Bickel and Freeman (13). The six domains of the receptor are not depicted to scale. Proteins containing human IgG have had their scavenger receptor cytoplasmic tail and transmembrane sequences replaced by an Fc fragment from human immunoglobulin. The nine amino acid epitope tag, represented by the single letter amino acid code TETSQVAPA, derives from bacterial rhodopsin and is recognized by the monoclonal antibody, ID4 (23). The inset photo shows media collected from COS cells transfected with the expression plasmid, pCDNA-1, containing the wild type fusion cDNA (IgG-SR) or no insert (Mock). Cells were labeled with 587 μCi of [35S]methionine per 10-cm plate, washed in serum-free media, and then incubated in serum-free media for 2 h. Media were bound to 0.5 g of protein A, washed with 10 ml of 0.1 M Tris, pH 8.0, then washed with 10 ml of 0.01 M Tris, pH 8.0, and finally eluted with 0.1 M glycine, pH 3.0, in 0.5-ml aliquots. The aliquots were adjusted to pH 8.0 with 1 ml Tris, pH 8.0, containing the protease inhibitor phenylmethanesulfonyl fluoride. The eluate was electrophoresed under reducing conditions on an 8% SDS-polyacrylamide gel overlaid with a 4% stacking gel, and autoradiography was performed. B, collagen domain alignment of SR proteins. The collagen domains of the bovine (bo), human (hu), murine (ms), and rabbit (ra) are aligned. The collagen triplets (Gly-X-Y) containing charged residues are in bold. Four subdomains, containing positively charged residues, were arbitrarily assigned the names CCM (collagen charge mutation)-1 through CCM-4. The bottom half of the figure lists each of the wild type and mutant proteins used in the study and the mutations they contain. All of the charge mutants converted a positively charged amino acid (Lys or Arg) to an uncharged residue. Proteins that contain the rhodopsin epitope tag have an E appended to their name.
**Change in Scavenger Receptor Binding Induced by Charge Mutants**

**Rabbit SR Fusion Protein Gel Electrophoresis**

**Fig. 2. Oligomerization and glycosylation of fusion proteins.** A. wild type and mutant scavenger receptor fusion proteins assemble into high molecular mass oligomers of approximately 400, 300, and 180 kDa. 5–10 µg of each affinity chromatography purified fusion protein was electrophoresed in a 3–10% SDS-polyacrylamide gel along with protein size markers (Sigma) of the indicated molecular weight. Proteins were fixed in methanol/acetic acid containing Coomassie Blue, destained in methanol/acetic acid without the dye, and then photographed. B, monomeric scavenger receptor fusion proteins migrate with an apparent size of 95 kDa, 30 kDa of which can be removed by deglycosylation. 5–10 µg of each fusion protein was electrophoresed in an 3–10% SDS-polyacrylamide gel in a loading buffer containing 5% β-mercaptoethanol. In lanes 2–5, the fusion proteins were first deglycosylated, prior to electrophoresis, using peptide-N-glycosidase F. Proteins were fixed, stained, and photographed as described in Fig. 3A. The protein migrating between 45 and 66 kDa was not visible on autoradiographs of media collected from [35S]methionine-labeled cells, was recognized by antibodies to bovine IgG, and was absent from cells incubated in serum that had been depleted in bovine IgG by column chromatography (data not shown). B-gal; β-galactosidase; phos.b, phosphorylase b; ovalb, ovalbumin.

DNA sequencing; 2) to permit detection of the epitope-containing proteins by immunoblot. To ensure that the binding of ligands to the SR fusion proteins was dependent only on the presence of the SR sequences within the fusion, a truncated protein (IgGS), containing only the human IgG Fc fragment, was also generated. Mutant receptors cDNAs, corresponding to the fusion proteins, but retaining their cytoplasmic and transmembrane domains, were also generated for use in cellular degradation experiments following transfection into COS cells.

**Protein Purification and Characterization—**After DEAE-dextran transfection of COS cells with the receptor cDNAs, secreted proteins were collected in a serum-free medium and then purified by protein A column chromatography as described under “Experimental Procedures.” Fig. 1 shows an autoradiogram of the column eluate that resulted from this procedure following [35S]methionine labeling of COS cells transfected with the wild type fusion cDNA. Under reducing conditions, a single predominant band of approximate molecular mass of 95 kDa was visualized (Figs. 1 and 2B). The expected molecular weight of the fusion protein is the sum of the mass of the IgG fragments (approximately 30 kDa) plus the mass of the SR's extracellular domains. The extracellular domains of the type II SR would be expected to have a mass of 57–64 kDa (the highly homologous intact bovine type II receptor was previously shown to have a mass of between 65 and 72 kDa (28) and the two SR domains removed in the fusion construct have a calculated mass of 8.3 kDa). Thus, the 95-kDa fusion protein migrated on an SDS-polyacrylamide gel in the expected size range. The yield of fusion protein varied between 4 and 12 µg per 10-cm transfection plate, 72 h after transfection, with most transfections typically yielding 6–8 µg per plate. COS cells continued to secrete fusion proteins for at least 120 h from the time of transfection, making it possible to obtain additional protein with subsequent media collections. Minimal non-scavenger receptor protein contamination of the protein A-purified preparations was visible after [35S]methionine labeling (Fig. 1 and data not shown). A protein of approximately 50 kDa (Fig. 2) was consistently evident on Coomassie staining of electrophoresed proteins. This protein had no ligand binding activity (Fig. 3A) and was subsequently determined to be residual bovine IgG, deriving from the calf serum in which the COS cells were originally cultured. More extensive washing and depletion of calf serum of IgG before use eliminated this contaminant (see Fig. 4).

SDS-polyacrylamide gels, run without (Fig. 2A) or with (Fig. 2B) reducing agent, in which wild type and mutant SR fusion proteins have been separated by electrophoresis are depicted in Fig. 2. Protein was detected by Coomassie staining (A and B). In the unreduced state, there appear to be four major regions of protein staining of the wild type fusion protein. The lack of discrete bands is due to variable glycosylation of the scavenger receptor portion of the fusion proteins. These four regions have approximate molecular masses, in descending order, of 400, 300, 180, and 100–130 kDa. The three higher weight proteins are multiples of the 95-kDa monomeric mass of the SR fusion protein and are presumed to be tetramers, trimers, and dimers. The band migrating between 100 and 130 kDa may represent dimers of poorly glycosylated fusion proteins or proteolytic fragments of the higher molecular weight oligomers. Neither contaminating bovine immunoglobulin nor the control human IgG can bind A-cLDL, as seen by the lack of lipoprotein staining on ligand blots (Fig. 3A, lanes 4 and 7, respectively). As previously shown by Penman et al. (28), the native SR normally runs as a trimer and dimer under non-reducing conditions, and the trimeric form is composed of a non-covalently linked monomer in association with a disulfide-linked dimer.
The putative tetrameric protein present in Fig. 3A could represent the addition of a monomer to the trimer, via a second disulfide linkage between the cysteine residues present in the hinge region of the Fc portion of the fusion protein, or a dimer of dimers. As Penman et al. (28) showed, preparations of purified native SR proteins that do not protect against oxidation frequently result in disulfide linkages between all three chains of the trimer.

Under reducing conditions (Fig. 2B), the native fusion protein migrates with the expected mass of approximately 95 kDa.
Changes in Scavenger Receptor Binding Induced by Charge Mutants

After de-glycosylation, this protein has an apparent mass of 60–65 kDa, indicating that glycosylation contributes approximately 30 kDa to the mass of the fusion protein (lane 4). The epitope-containing fusion proteins (RT2E) migrate somewhat more slowly in both the unreduced and reduced conditions, reflecting the presence of their C-terminal nine amino acid epitope tag. The migration of the human Fc fragment alone (IgGS) and an epitope-containing mutant form of the fusion protein (CCM-2FE) are also shown on these gels. The CCM-2FE migrates in a pattern identical to RT2E. These data indicate that the SR fusion protein assembles into higher molecular weight oligomeric structures, including those typically seen with the native SR protein, and that charge mutations in the collagen domain do not prevent the oligomerization process. These data, in conjunction with the binding data presented below, establish that the native cytoplasmic tail and transmembrane domains of the SR are not essential for the formation of a functional, oligomeric, rabbit scavenger receptor, a finding confirming similar observations made by Resnick et al. (5) in work with bovine and human SRs.

Fusion Protein Function—The function of the SR fusion proteins was tested using several different ligand binding assays. These included ligand blots, in which the proteins were first electrophoresed through polyacrylamide and then transferred to a solid filter support (nitrocellulose). Ligand binding to the proteins could then be measured either using a radiolabeled ligand or using a non-labeled ligand whose binding could be detected immunologically. Fig. 3, A and B, shows ligand blots in which the demonstration of ligand binding was performed using the antibody detection method. When ligand binding was conducted in the presence or absence of the SR binding competitor, polyinosinic acid (poly(I)) (Fig. 3B), the specificity of ligand binding to the wild type SR fusion protein, with and without the epitope sequence, was demonstrated. No anti-AcLDL antibody staining is detected to the IgGS mutant, either in the presence or absence of poly(I), indicating that the human IgG fragment is not responsible for any of the binding of the AcLDL to the SR fusion proteins. Similarly, there is little or no binding evident with the CCM-2FE mutant. Several other mutants appear to have diminished binding (e.g. CCM-3FE, CCM-1-(335,341)) when compared with that seen with the wild type proteins. These results qualitatively demonstrate that charged residues throughout the entire collagen domain of the SR fusion protein are critical for receptor binding activity.

To confirm that the proteins that did not bind ligand were effectively transferred to the nitrocellulose, without undergoing degradation, immunoblots were performed using two antibodies (Fig. 4). ID4, a monoclonal antibody to the rhodopsin epitope, and ASAb-1, a monoclonal antibody raised to the wild type SR fusion protein, were used. Only the epitope containing proteins (RT2E, CCM-2FE, CCM-3FE, and CCM-4FE) were recognized by ID4, whereas all proteins were recognized by ASAb-1, including the IgGS, indicating that its epitope recognition site resides within the human IgG portion of the fusion protein. The immunoblots demonstrate that the failure to detect AcLDL binding to several of the mutant fusion proteins was neither due to a failure of those proteins to be transferred to the nitrocellulose membrane nor to protein degradation of the mutant receptors.

Whereas the ligand blotting methods permitted a qualitative assessment of SR fusion protein binding activity, they did not permit any quantitative assessment of function to be made. Two other binding assays were employed in an effort to establish more quantitative estimates of fusion protein function. In the first assay, equal amounts of receptor proteins were electrophoresed and then transferred to nitrocellulose filters. Radiolabeled AcLDL was then bound to the filters at varying temperatures. Equally sized filter strips representing each lane of the gel were then excised, and the bound 125I AcLDL was subsequently counted. A second assay was used in which the SR fusion proteins were bound to plastic wells (Terasaki plates), with binding of 125I-AcLDL, and then measured in the presence or absence of competing, unlabeled AcLDL. Saturation binding studies were conducted to determine the affinity of radiolabeled AcLDL for the purified proteins.

Filter binding assays were conducted at three different temperatures (4, 22, and 37 °C) and are shown in Fig. 5A–C. At the lowest temperature, only the IgG control and CCM-2FE proteins had binding activity less than 10% of the wild type proteins. All of the CCM-1 proteins, as well as CCM-4FE and CCM-2-(317,325) retained greater than 60–70% of wild type binding activity. The CCM-3FE protein, however, did have significantly less binding activity than the RT2E protein, indicating that mutations in this more proximal region of the SR collagen domain could dramatically affect receptor binding. Somewhat surprisingly, the effect of many of the mutations proved to be temperature-dependent. As the binding reactions were carried out at progressively higher temperatures, more of the mutants lost their activity. At 22 °C, all of the CCM-1 mutations fell to less than 50% wild type binding, and at 37 °C only CCM-2-(317,325) retained more than one-quarter of the wild type activity. These data indicate that mutations in charged residues throughout the collagen domain can dramatically affect ligand binding but that the effects of most of those mutations are temperature-dependent. Only the triple charge mutant, CCM-2FE, was incapable of binding ligand at any of the three temperatures, establishing the critical importance of residues 317, 325, and 328 in receptor function. The temperature-dependent effects of the other mutations suggest that the receptor conformation in the collagen domain is a critical feature of ligand binding and that the substitution of non-charged residues for positively charged residues in this domain can disrupt that conformation.

An alternative explanation for the temperature-dependent loss in protein binding seen with many of the mutant receptor proteins was that these proteins were more readily susceptible to protein degradation at the higher temperatures. To test that possibility, a temperature shift binding experiment was conducted. In this study, one of the mutants (CCM-1-(335,341)) with the most dramatic temperature-dependent losses in binding was employed. Binding was preceded by a 4-h incubation at either 37 or 4 °C, followed by binding at either 37 or 4 °C. As seen in Fig. 5D, the mutant CCM-1-(335,341) had less than 20% of its binding activity retained when both incubations were carried out at 37 °C, consistent with the data shown in Fig. 5C. When the preincubation at 37 °C was followed by binding at 4 °C, however, the protein had a similar binding activity to that seen when both the preincubation and the binding were performed at the cooler temperature. Temperature shifts had little or no effect on the two wild-type proteins. This experiment demonstrated that the results of the 4-h binding experiments conducted at 37 °C (Fig. 5C) were not due to irreversible denaturation or degradation of the mutant receptor protein.

Saturation binding curves for all of the mutants that retained significant binding activity at 4 °C were performed (data not shown). The ligand binding dissociation constants for these purified proteins, presented in Table I, are in close agreement to those established for the wild type receptor protein expressed on Chinese hamster ovary cells (4). Modeling of the number of receptor binding sites for AcLDL did not show a statistically significant better fit of the data for a two-site
model versus a one-site model. There was also a very high error rate in the estimate of the \(k_d\) of a potential second binding site. In addition, the ligand used for these studies, iodinated AcLDL, has not been demonstrated to represent a single molecular species, making lower affinity interactions with the receptor difficult to interpret. Thus, based on these studies, we cannot

**Fig. 5.** Binding of \(^{125}\)I-AcLDL at varying temperatures to fusion proteins immobilized on nitrocellulose membranes. A, 4 °C binding. Equal amounts of fusion proteins were electrophoresed through SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described in Figs. 2–3 and under "Experimental Procedures." Individual lanes were excised and separated and then bound to \(^{125}\)I-AcLDL (1 × 10^6 cpm) in Buffer A (50 mM Tris, pH 8.0, 2 mM CaCl, 50 mM NaCl, 50 mg/ml BSA) for 4 h at 4 °C. Strips were washed four times in Buffer A containing only 10 mg/ml BSA and then counted in a gamma counter. The difference between the binding to identically sized filter strips containing fusion proteins and control strips, cut from the same filter but in lanes lacking any protein, was determined, and this value is depicted as specific binding in the graphs. Values represent the mean ± S.D.

**B**, 22 °C binding. Binding studies carried out as described in A were performed at 22 °C. C, 37 °C binding. Binding studies carried out as described in A were performed at 37 °C. D, loss of binding to a mutant protein at 37 °C is not due to degradation or irreversible denaturation. The mutant CCM-1-(335,341), which showed a marked temperature-dependent loss in ligand binding, was used to assess the reversibility of this loss. Wild type fusion proteins, which showed no loss of binding at increased temperatures, were included for comparison. Fusion proteins transferred to filters as described in the previous figures were incubated for 4 h at either 4 or 37 °C. Following this preincubation period, a 4-h binding with \(^{125}\)I-AcLDL (1 × 10^6 cpm), at either 4 or 37 °C, was then performed. Binding was assayed as described in A.

**Table I**

| RT2  | RT2E  | CCM-1-(335) | CCM-1-(335,338) | CCM-1-(335,341) | CCM-2-(317,325) | CCM-3F | CCM-4F |
|------|-------|-------------|----------------|----------------|----------------|--------|--------|
| \(k_d\) 4 °C | 3.2 ± 0.2 | 2.9 ± 0.9 | 5.3 ± 1.3 | 1.0 ± 0.4 | 1.1 ± 0.2 | 1.9 ± 0.2 | 1.6 ± 0.2 |
| \(B_{max}\) 4 °C | 1.6 ± 0.05 | 1.2 ± 0.07 | 2.1 ± 0.3 | 0.8 ± 0.1 | 0.22 ± 0.03 | 0.27 ± 0.02 | 0.86 ± 0.04 | 1.7 ± 0.09 |
| \(k_d\) 22 °C | 1.0 ± 0.04 | 0.87 ± 0.07 | 2.49 ± 0.5 | 4.10 >1000 |
| \(B_{max}\) 22 °C | 1.27 ± 0.3 | 0.87 ± 0.1 | 5.9 >1000 |
establish unequivocally the number of receptor ligand binding sites, although there does appear to be only one high affinity binding region. These data indicate that the purified proteins, with nanomolar binding affinities virtually identical to those found for receptors expressed in vivo, are a useful surrogate for receptor studies in vivo.

As the fusion proteins were also capable of assembling into oligomers not typically formed by the wild type scavenger receptor, it was important to confirm that the results obtained with the purified proteins were not due to any anomalies of the fusion protein structure. Cell binding and degradation studies were, therefore, conducted using transmembrane scavenger receptor mutants identical (in their extracellular domains) to the SR portions of the fusion proteins studied in vitro (Fig. 6). The binding and degradation of AcLDL at 37 °C in COS cells transfected with these mutant proteins provide corroborative evidence for the importance of temperature on receptor activity. The fusion proteins that bound poorly at 37 °C also produced transmembrane proteins with markedly reduced AcLDL degradation or binding at the same temperature. When cellular binding activity was measured at 4 °C, the binding was reconstituted in those cells expressing proteins whose fusion analogue also reconstituted binding at the lower temperature. The reconstitution of binding at 4 °C indicates that intracellular mishandling or degradation of the mutant proteins cannot account for the reduced cellular binding and degradation demonstrated at 37 °C. Thus, the cellular experiments provide an important corroboration of the validity of the results obtained with the purified fusion proteins.

**DISCUSSION**

In these studies, we have generated mutants spanning the entire collagen domain of the type II macrophage scavenger receptor. These mutants were expressed as integral membrane proteins on the surface of transfected COS cells, as well as purified, secreted fusion proteins. The removal of the cytoplasmic tail and transmembrane domains of the SR fusion proteins did not prevent the assembly of the receptor into functional oligomers that retain wild type binding affinity. AcLDL bound to the fusion proteins, containing wild type extracellular do-
mains of the scavenger receptor protein, when the receptor proteins were either transferred to nitrocellulose membranes or used to coat plastic wells. Saturation binding studies and Scatchard analysis suggested that the receptor fusion proteins possessed a single high affinity binding site with an apparent $K_d$ of approximately 1–4 nM (1.0 μg/ml = 1.93 nM), consistent with the estimates of SR binding affinities suggested by previous studies conducted in transfected cell lines (4). This high affinity binding is destroyed in a single mutant receptor protein that simultaneously replaces the three basic residues at positions 317, 325, and 328 of the C terminus of the collagen domain with three non-charged amino acids. Table II shows a comparison of the binding activities of all the SR charge mutants reported to date (the data from this paper combined with those reported by Doi et al. (19)). Mutations in the subdomains of the collagen region that we termed CCM-3 and CCM-4, which had not been previously altered in SR binding studies, also resulted in a significant decline in ligand binding at 37 °C that was at least as profound as that found with the distal collagen mutants. Several mutations in the collagen domain demonstrated a clear dependence on temperature for their effect. This progressive loss in binding, seen as temperatures were raised from 4 to 37 °C, occurred without irreversible dissociation of the oligomeric structure of the receptor, as binding could be restored by lowering the temperature again to 4 °C. The temperature-dependent loss in binding did suggest, however, that important conformational shifts in the protein occurred at the higher temperatures.

The data presented in this paper call into question the prevailing model of SR binding. Doi et al. (19) concluded that their mutational analysis demonstrated that Lys-337 (equivalent to Lys-338 in the rabbit sequence) was essential for AεLDL binding and that “a lysine cluster in the C-terminal collagen-like domain is the ligand-binding domain of the scavenger receptor.” The authors of this work constructed a computer graphic model that suggested that the receptor might form a three-coil, positively charged groove responsible for the interaction with polyanionic ligands. They suggested that residues 327, 334, 337, and 340 (comparable to 328, 335, 338, and 341 in this study) were the critical ligand binding amino acids and that they formed a region of positive charges that bound to the negatively charged moieties on SR ligands. This conclusion, now widely accepted, has led to several subsequent studies by different investigative groups in which peptide models of SR binding have been constructed using only the three or four terminal lysine cluster residues to generate synthetic receptor analogues (29–32). Although these peptides can bind to SR ligands, the affinities of the binding interactions appear to be at least 10-fold lower than for the native receptor or for the fusion protein reported in this work (32). Earlier work by Acton et al. (17), using C1q collagen sequences that are unrelated to the SR collagen domain, also showed that these collagenous molecules retained considerable capacity for binding SR ligands. Thus, the demonstration of lower affinity binding of SR ligands to synthetic collagen peptides is not sufficient to establish that any one region constitutes the authentic binding locus of the receptor. Finally, established SR ligands, such as polyanionic acid, have failed to bind to peptide models based on the lysine cluster model (30), a finding that does not support some of the concepts presented in the initial description of the positively charged groove hypothesis (19).

The loss in receptor binding seen in our CCM-3 and CCM-4 mutants establishes that residues as proximal as position 284, and no more distal than 308, are potentially as important to SR binding as those in the more C-terminal lysine cluster. Furthermore, the restoration of binding to several mutants at 4 °C, in both the proximal and distal collagen domain, is very strong evidence that the loss in ligand binding seen at the higher temperatures was not due only to disruption of ionic interactions between the receptor and ligand, but rather that multiple, charged residue interactions within the receptor are required for its binding conformation at higher temperatures. The similarity of the apparent binding dissociation constants ($K_d$) for the mutant receptors and the wild type receptor at 4 °C (Table I) further suggests that the mutant receptors that can reassemble at the lower temperature are capable of presenting a binding domain that does not differ very substantially from that of the wild type protein. The lower $B_{max}$ for the mutant receptors suggests that not all of the mutant molecules can successfully re-establish a binding conformation when the temperature is reduced. Interestingly, the study by Doi et al. (19) also contains two mutants, both involving the amino acid they propose is critical to binding (Lys-337), that substantially reconstitute binding activity at a lower temperature (Table II). This observation led them to conclude that binding studies to the SR need to be conducted at 37 °C. Whereas we agree that the relevant binding temperature for understanding SR function in vivo is 37 °C, the binding studies at 4 °C are essential to any understanding of the structural determinants of receptor function. The slight increase in binding affinity we noted in wild type receptor interaction at higher temperatures (Table I) does provide some support for the concept by Doi et al. (19) that the collagen strands become more closely packed at increasing temperature and that this could account for improved binding.

It should be noted that for the two mutants that were common to our work and that of Doi et al. (19), the binding data are not entirely concordant. Binding to the single 335 mutant at 4 °C and to the double (335,338) mutant at 37 °C gave similar results, but the 335 mutant binding at 37 °C and the double mutant activity at 4 °C yielded discordant results. Whereas there is no definitive explanation for these discrepancies, it is possible that the differences in amino acid substituted (Doi et al. (19) replaced 335 and 338 charged residues with alanines) could account for the difference. The failure of the alanine substitutions to show consistently better retention of binding

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**TABLE II**

**Comparison of binding of proximal and distal collagen mutants at 37 and 4 °C**

Values in the table represent the percentage of binding compared to the appropriate wild type control. As the bovine SR (used in Doi et al. (19) study) has one less amino acid than the rabbit SR, proximal to the collagen domain, the numbers used in this table have been converted to the rabbit numbering system, e.g., bovine Lys-337 is homologous to Lys-338 in the rabbit sequence. The data from Doi et al. (19) derives from the graphic data presented in their paper.

| Mutant            | 4 °C | 37 °C |
|-------------------|-----|-------|
| 281/284<sup>a</sup> | 94  | 26    |
| 305/308<sup>b</sup> | 25  | 16    |
| 317/325<sup>c</sup> | 63  | 61    |
| 317/325/328<sup>d</sup> | <5  | 55    |
| 328<sup>e</sup> | 80/70 | 75/28 |
| 335<sup>f</sup> | 69  | 21    |
| 335/338<sup>g</sup> | 33  | 20    |
| 335/338/341<sup>h</sup> | 78  | 20    |
| 338<sup>i</sup> | 60  | 20    |
| 338/341<sup>j</sup> | 80  | 75    |

<sup>a</sup> Data are from this article.
<sup>b</sup> Data from Doi et al. (19).
<sup>c</sup> Data for mutants generated in both studies. The first value in each pair is derived from the current study.
activity in the two mutant receptors makes this explanation, perhaps, somewhat less compelling. Alternatively, a defect in endocytosis might explain the discrepant values at 37 °C, as our cellular binding studies were not performed in the presence of an endocytosis inhibitor. Thus, the cellular binding values we report at 37 °C also include the uptake of some ligand that has been internalized. If any of the collagen mutants lowered the rate of endocytosis, this would result in an apparent lower binding compared with wild type receptor. As our cell binding experiments recapitulated the binding measured in the purified protein assays, and as a wide range of mutants, studied at both 37 and 22 °C, share the temperature-dependent binding behavior, we think the data are unlikely to differ because of a difference in endocytosis. An endocytosis defect would also not account for the difference in binding measured at 4 °C with the double mutants. Thus, there is no entirely satisfactory explanation for the differences in the binding behavior of the two mutants reported in these two studies.

Currently available data make it challenging to propose a precise model for SR ligand binding. It is clear that the collagen domain is required for binding and that positively charged residues in both the proximal and distal regions of that domain are necessary to maintain binding at physiologic temperature. Inactivating charge mutants in both regions can be silenced by reducing the temperature of binding, a finding that is most consistent with the hypothesis that the mutated residues are critical for stabilizing receptor conformation at higher temperatures. The simplest explanation for this result is that the trimeric collagen strands must adopt a precise conformation to achieve ligand binding and that changes in charge, in either the proximal or distal collagen domain, disrupt that conformation. In lower energy states, the necessary binding conformation can be maintained without all of the interactions that are mediated by the charged residues, but at physiologic temperature, these interactions are essential to hold the receptor binding domain together. It is possible that SR ligands interact directly with many of the charged residues, spanning the collagen domain, or only a few, clustered in one region. The effects of mutations in one region may serve merely to disrupt the conformation of contact residues at a distant site. An alternative explanation for the data presented in this study is that the temperature shifts employed induced an alteration in the ligand conformation, either in conjunction with or instead of alterations in the receptor conformation. Whereas this remains a formal possibility, the diversity of receptor mutants that restore activity, their widespread spacing across the entire collagen domain, and the similarity of the affinity constants for binding to mutant and wild type receptors at the lower temperatures all suggest that this explanation is unlikely.

Although the relevant conformational interactions necessary to establish SR ligand recognition could all reside within the collagen domain, recent data by Resnick et al. (27) suggest the intriguing possibility that high affinity SR binding might require the cooperation of two SR domains. In an electron microscopy study, they presented evidence indicating that the SR adopts a jackknife configuration, with the collagen domain bent back in close apposition to the α-helical coiled-coil domain (27). Our data, and the earlier work cited above, are also consistent with the hypothesis that this jackknife configuration is essential for the highest affinity binding. Deletion of the collagen domain, or mutations in its charged residues, could disrupt this two domain interaction, resulting in an unfolding or dismating of the jackknife and a consequent loss in binding activity. This hypothesis is directly testable. As the ability of scavenger receptors to bind a multitude of ligands, some of which are thought to be critical to the pathogenesis of human diseases, is the defining trait of this family of proteins, further studies of its distinct mechanism of ligand recognition are warranted in order to clarify this remarkable property.

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REFERENCES

1. Brown, M. S., Basu, S. K., Falck, J. R., Ho, Y. K., and Goldstein, J. L. (1980) J. Supramol. Struct. 13, 67–81
2. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 333–337
3. Hampton, R. Y., Golenbock, D. T., Pennman, M., Krieger, M., and Raetz, C. R. (1991) Nature 352, 343–346
4. Ashkenas, J., Pennman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1993) J. Lipid Res. 34, 983–1000
5. Resnick, D., Freedman, N. J., Xu, S., and Krieger, M. (1993) J. Biol. Chem. 268, 3538–3545
6. Christie, R. H., Freeman, M., and Hyman, B. T. (1996) Am. J. Pathol. 148, 399–403
7. Parece, D. M., Ghosh, R. N., and Maxfield, F. R. (1996) Neuron 17, 553–565
8. Fraser, J., Hughes, D., and Gordon, S. (1993) Nature 364, 343–346
9. Kodama, T., Freeman, M., Rohrer, L., Zacrebsky, J., Matsudaira, P., and Krieger, M. (1990) Nature 343, 531–535
10. Rohrer, L., Freeman, M., Kodama, T., Pennman, M., and Krieger, M. (1990) Nature 343, 579–572
11. Freeman, M., Ashkenas, J., Rees, D. J., Kingsley, D. M., Copeland, N. G., Jenkins, N. A., and Krieger, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 87, 8810–8814
12. Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asahoka, S., Hayakawa, I., Kamonari, H., Aburatani, H., Takaku, F., Suzuki, H., Kobi, Y., Miyai, T., Takahashi, K., Cohen, E. H., Wydro, R., Housman, D. E., and Kodama, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9133–9137
13. Bickel, P. E., and Freedman, M. W. (1992) J. Clin. Invest. 90, 1450–1457
14. Krieger, M., Acton, S., Ashkenas, J., Pearson, A., Pennman, M., and Resnick, D. (1993) J. Biol. Chem. 268, 4569–4572
15. Mayer, W. E., and Tichy, H. (1995) Gene (Amst.) 164, 267–271
16. Starling, G. C., Llewellyn, M. B., Whitney, G. S., and Aruffo, A. (1997) Tissue Antigens 49, 1–6
17. Acton, S., Resnick, D., Freeman, M., Ekkel, Y., Ashkenas, J., and Krieger, M. (1993) J. Biol. Chem. 268, 3530–3537
18. Dejager, S., Mietus-Snyder, M., Friera, A., and Pitas, R. E. (1993) J. Clin. Invest. 92, 894–902
19. Doi, T., Higashino, K., Kurihara, Y., Wada, Y., Miyazaki, T., Nakamura, H., Uesugi, S., Imanishi, T., Kaswabe, Y., Itakura, H., Yazaki, H., Matsumoto, A., and Kodama, T. (1993) J. Biol. Chem. 268, 2126–2133
20. Aruffo, A., and Seed, B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8573–8577
21. Krieger, M. (1993) Cell 73, 413–422
22. Billeimker, D. W., Eisenberg, S., and Levy, R. I. (1972) Biochim. Biophys. Acta 260, 212–221
23. Hodges, R. S., Heaton, R. J., Parker, J. M., Molday, L., and Molday, R. S. (1988) J. Biol. Chem. 263, 11768–11775
24. Kunkel, T. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
25. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 196–218, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Daniel, T. O., Schneider, W. J., Goldstein, J. L., and Brown, M. S. (1983) J. Biol. Chem. 258, 4686–4691
27. Resnick, D., Chatterton, J. E., Schwartz, K., Slattery, H., and Krieger, M. (1990) J. Biol. Chem. 271, 26924–26930
28. Pennman, M., Lux, A., Freedman, N. J., Rohrer, L., Ekkel, Y., McKinstry, H., Resnick, D., and Krieger, M. (1993) J. Biol. Chem. 268, 23985–23993
29. Anachi, R. B., Siege1, D. L., Baum, J., and Brodsky, B. (1995) FEBS Lett. 368, 551–555
30. Mielewczyk, S. S., Breslauer, K. J., Anachi, R. B., and Brodsky, B. (1996) Biochemistry 35, 11396–11402
31. Tanaka, T., Nishikawa, A., Tanaka, Y., Nakamura, H., Kodama, T., Imanishi, T., and Doi, T. (1996) Protein Eng. 9, 307–313
32. Yamamoto, K., Nishimura, N., Doi, T., Imanishi, T., Kodama, T., Suzuki, K., and Tanaka, T. (1997) FEBS Lett. 414, 182–186
33. Freeman, M., Ekkel, Y., Rohrer, L., Pennman, M., Freedman, N. J., Chiossoli, G. M., and Krieger, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4931–4935