Charged Collagen Structure Mediates the Recognition of Negatively Charged Macromolecules by Macrophage Scavenger Receptors*

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Macrophage scavenger receptors mediate the recognition of a wide range of negatively charged macromolecules including modified low density lipoproteins (LDL). Truncated bovine receptors lacking residues 330–342, which include the conserved lysine cluster of a collagen-like domain, were unable to degrade modified LDL in spite of their expression on the cell surface. Substitution of lysine 337 into alanine abolished the acetyl-LDL degradation and binding at 37 °C, but did not abolish the 4 °C binding. In contrast, substitution of more than 2 lysines in this region are needed to abolish the oxidized LDL degradation and 37 °C binding. Based on computational modeling of this domain, we propose that a “charged collagen” structure containing a lysine cluster forms a positively charged groove which specifically interacts with negatively charged ligands.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D13381 and D13382.

1 The abbreviations used are: LDL, low density lipoprotein; FH, familial hypercholesterolemia; Ac, acetyl, Ox, oxidized; PBS, phosphate-buffered saline; Dil, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine.
range of ligands are recognized by this cluster in various way. Based on these data and computational modeling using the crystallographic data of the collagen backbone, we propose a "charged collagen" model of the ligand-binding domain of macrophage scavenger receptors.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs and DNA Transfection**

The constructions of mutant genes were carried out as follows. **Truncated Mutants**—pXSR7-derived plasmid pXSRI7, which has an extra PvuI site between SstI and XhoI sites of pXSRI7, was digested with both PvuI and XhoI. The linker, 5'-CGAGATCTAATAC-3', 3'-TAGCTCTAGATGTTGATGTGACT-5', which contains a stop codon in each frame and a BgII site just before the stop codons, was introduced into the PvuII-XhoI-digested vector. This plasmid was digested with BgIII and the 5' protruding ends produced were protected by filling in with n-phosphorothioate deoxyribonucleotides and Klenow DNA polymerase (Putney et al., 1981). After digestion with SstI, the end of the blocked vector was treated with exonuclease III followed by S1 nuclease in successive depletions. Various lengths of blunt-ended DNA fragments self-circularized.

**Point Mutants**—pXSR7 was digested with BamHI and XhoI to produce the vector and the receptor gene. The receptor gene was further cut with either NciI or SpH1 to isolate the BamHII-NciI fragment or the SpH1-XhoI fragment, respectively. The DNA sequence between the NciI and SpH1 sites is the target sequence to mutate. The sequence was changed by replacement with synthetic oligonucleotides. Nonmutated (for positive control) and seven kinds of mutated NciI-SpH1 fragments were synthesized. Each NciI-SpH1 fragment was ligated to the BamHII-NciI fragment. The yield of each BamHI-SpH1 fragment was ligated to the SpH1-XhoI fragment and the BamHII-XhoI vector.

The DNA sequences of all mutant genes were determined by the dyeoxy method.

These plasmid DNAs were transfected into COS cells by the DEAE-dextran method (Sussman and Milman, 1984), and 2 days after transfection these cells were used for assays.

**Western blot Analysis**

Bovine lung macrophage scavenger receptors were partially purified using a combination of maleyl bovine serum albumin affinity chromatography and hydroxyapatite chromatography by the method of Kodama et al. (1988). Aliquots of partially purified receptors (20 mg/lane) were subjected to SDS-polyacrylamide gel electrophoresis with or without a reducing agent, electroblotted onto nitrocellulose filters, and incubated with either 5 mg/ml of 125I-acetyl-LDL (Kodama et al., 1990) or 5 mg/ml of 125I-oxidized-LDL obtained by the incubation of 100 mg/ml of 125I-LDL in Ham's F-10 medium in the presence of 10 mM CuSO4, in a 5% CO2 incubator at 37 °C for overnight (Steinbrecher et al., 1984), was used. In both cases, maleyl bovine serum albumin (200 mg/ml) was used as a competitor. In the case of the 37 °C ligand binding assay, cells were preincubated with 100 μM phenylarsine oxide for 20 min at 37 °C before the ligands were added as described (Wiley and Cunningham, 1982; Teraseki et al., 1989).

**Immunohistochemical Staining**

The cells expressing mutant genes were washed with PBS and fixed in ice-cold acetone for 15 min. After rehydration with PBS, the fixed cells were incubated with IgG-D2 for 1 h. Then, the cells were washed with PBS three times and treated with peroxidase conjugated anti-mouse IgG for 1 h. After three washes in PBS, the cells were treated with 0.1 N NaOH and the cell-associated counts recovered.

**Cell Surface IgG-D2 Binding**

Transfected cells were chilled to 4 °C. After three washes with ice-cold PBS, the cells were incubated with IgG-D2 antibody for 1 h at 4 °C. The cells were washed with ice-cold PBS three times and treated with 125I-labeled anti-mouse IgG for 1 h at 4 °C. After three ice-cold washes with PBS, the cells were treated with 0.1 N NaOH and the cell-associated counts recovered.

**RESULTS**

**IGG-D2 Recognizes the Trimer Structure of Bovine Scavenger Receptors**—Characterization of IgG-D2 monoclonal anti-bovine scavenger receptor antibody was carried out by Western blot analyses (Fig. 1). Partially purified receptors were subjected to SDS-polyacrylamide gel electrophoresis and were detected on nitrocellulose filters by using either IgG-D2 monoclonal antibody or rabbit anti-scavenger receptor peptide polyclonal antibody (1:100 dilution) (Kodama et al., 1988). The bound IgG was visualized by using goat anti-mouse IgG conjugated with alkaline phosphatase.

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**cDNA Isolation and Sequences**

Poly(A)+ RNA was isolated from a murine macrophage-like cell line P388D1 or rabbit lung and was used to construct a size-fractionated random-primer CDNA library in lambda ZAP II (Kodama et al., 1990). The XhoI-SpH1 fragment of pBSR7 corresponding to the collagen-like domain of the bovine receptor (Kodama et al., 1980) labeled by random priming was used as a hybridization probe to screen 5 × 106 plaques. Positive clones were isolated, and the DNA was excised in vivo and sequenced. The designation of type I or II receptor was carried out according to the presence of a cysteine-rich domain or the absence of cysteine in domain VI (Kodama et al., 1990).

**Structure and Function of the C-terminal Truncated Scavenger Receptors**—13 C terminus deletion mutant genes of the type I receptor were constructed and expressed in COS cells. The plasmid pXSR7, which has the entire cDNA of the bovine type I scavenger receptor, was deleted from the 3′ end of the cDNA by exonuclease III, and translational stop codons were added to yield a series of deletion mutant genes (Fig. 2). These mutant genes were transfected into COS cells by the DEAE-dextran method, and 2 days after transfection the expressed mutant proteins were detected by an immunohistochemical technique with IgG-D2 antibody. This antibody recognized...
FIG. 1. Characterization of IgG-D2 monoclonal anti-bovine scavenger receptor antibody. Partially purified bovine scavenger receptors were subjected to SDS-polyacrylamide gel electrophoresis with dithiothreitol (lanes B and D) or without dithiothreitol (lanes A and C). Western blot analysis of these gel-loaded samples was performed by using IgG-D2 antibody (lanes A and B) or anti-scavenger receptor peptide (RIQYLSDFEANLDASNFQ) antibody.

FIG. 2. Schematic representation of the C-terminal deletion mutants of the scavenger receptors. Each of the five domains is distinguished by a type of shadowing. The ligand uptake activities determined by fluorescent labeled Dil-acetyl-LDL and the trimer formation determined by cell surface IgG-D2 binding of the mutants are shown as positive (+) or negative (−), respectively.

The mutant protein N221 (a deletion mutant protein constructed from 221 amino acids from the N terminus) and longer proteins (Figs. 2 and 3). This indicates that neither the cysteine-rich nor the collagen-like domains are necessary for building the trimeric form but that the α-helical coiled-coil domain is necessary.

These deletion mutants were also examined for their ligand uptake activities. Fig. 3 shows the results of immunohistochemical staining and the ligand uptake assay with the mutants N320 and N342 as examples. The cells expressing N221 and longer proteins were recognized by the IgG-D2 antibody and stained in the same manner as the cells expressing N320. In terms of ligand uptake, all mutants shorter than N320 had no ligand uptake activity, as was seen by using fluorescent-labeled acetyl-LDL (Kodama et al., 1988). On the other hand, all mutants longer than N342 had the same strong uptake activity as that of N342.

To clarify the mutants’ activities quantitatively, we executed a degradation assay. The data shown in Fig. 4b indicate that N342 had the same uptake activity as that of the intact receptor. However, N320 and N280 did not show any significant uptake. Thus, the data from the degradation assay correspond to the result of the fluorescent-labeled ligand uptake experiment.

FIG. 3. Ligand uptake activities and trimer formations of the mutant receptors expressed in COS cells. COS cells transfected with either pcDNA1 (vector plasmid without receptor cDNA) (a and e), N320 cDNA (b and f), N342 cDNA (c and g) or pXSR7 (full-length type I cDNA) (d and h) were used for the ligand uptake experiment (a-d) and for immunohistochemical staining to certify the trimeric structures (e–h).
We also carried out a degradation assay by using OxLDL, a physiological candidate for atherogenic lipoprotein, as another ligand. OxLDL was prepared by the oxidative modification of LDL with CuSO4. The results show the same uptake pattern as that found by using AcLDL, indicating that this C-terminal collagen-like domain mediates not only recognition of AcLDL but also OxLDL (Fig. 4c). All mutants were confirmed for their expression of the trimeric structure on the cell surface by means of IgG-D2 antibody followed by 125I-labeled anti-mouse IgG (Fig. 4a). Based on truncation analysis, the 22 C-terminal part of the collagen-like domain might be essential for ligand binding and uptake.

Comparison of Amino Acid Sequences of the C-terminal Collagen-like Domain among Human, Mouse, Rabbit, and Bovine—In order to compare the structure of scavenger receptors of various animal species and to elucidate the essential structure conserved among these species, we cloned murine and rabbit scavenger receptor cDNAs. Using an XbaI-SphI fragment of bovine scavenger receptor cDNA (which corresponds to the collagen-like domain) as a probe, murine and rabbit scavenger receptor cDNAs were isolated from a murine macrophage-like cell line P388D1 cDNA library and from a rabbit lung cDNA library, respectively. The amino acid sequences deduced from the nucleotide sequence of these cDNAs and bovine (Kodama et al., 1990), human (Matsumoto et al., 1990), and murine domain VI sequences (Freeman et al. 1990) indicated that the six-domain structure originally proposed (Kodama et al., 1990) is well conserved among scavenger receptors of these four animal species. As can be seen in Fig. 5, in the cytoplasmic domain (domain I), except for murine receptor amino acid residues 1-15, the sequences are highly homologous. The position of a possible phosphorylation site and the position of an aromatic residue in the middle of this domain are conserved. In the extracellular domain, two major fibrous domains, the α-helical coiled-coil and collagen-like domains, are highly conserved. In the α-helical coiled-coil domain, the heptad repeats of a hydrophobic amino acid and its interruption by 2 histidines are conserved. The presence of a gap in the middle of the coiled-coil domain is also conserved. In the collagen-like domain, there are 24 Gly-Xaa-Yaa (Xaa and Yaa, variable amino acid) triplet repeats in bovine, murine, and rabbit receptors, whereas the human receptor has 23 Gly-Xaa-Yaa repeats. Receptors of all four animal species have the cluster of basic amino acids in the 22-amino-acid residues, which is essential for ligand binding as indicated above. The positions and intervals of the basic amino acids in the C-terminal part of the collagen-like domain are completely conserved in the four species.

A Point Mutation in the Lysine Cluster Diminishes Ligand Binding and Uptake Activity of the Receptor—Because the lysine cluster in the C-terminal part of the collagen-like domain is completely conserved, it is highly likely that these lysine residues are critical for ligand binding and uptake. We, therefore, investigated the role of these lysine residues by point mutation analyses. Lys337, Lys334, Lys331, and Lys340 were substituted with alanine residues (Fig. 6).

Point mutant genes were constructed by ligation between DNA fragments chemically synthesized for mutation sites and a vector including other natural sequences. These mutant genes were transfected into COS cells, and the expressed receptors were analyzed in the same manner as the truncated receptors had been. The results of the surface binding experiment showed that all mutants except the negative control (pcDNA1) were expressed on the cell surface as trimeric structures and that the expression levels were almost the same (Fig. 7a). On the other hand, the results of degradation assays showed that only one substitution, Lys307 to alanine, abolished AcLDL degradation completely. However, in the case of OxLDL degradation, one substitution was not sufficient to remove full uptake activity. For abolishing this degradation, more than 2 lysine substitutions were required (Fig. 7, b and c).

4 and 37 °C Ligand Binding—Degradation experiments revealed that Lys307 or the lysine cluster in the C-terminal part...
of the collagen-like domain was essential for ligand uptake activity of the receptor. However, degradation assays examine several different activities, such as the internalizing and degrading activities as well as ligand binding. In order to determine the ligand-binding domain of scavenger receptor, we, therefore, carried out a direct ligand binding experiment. Point mutant genes were transfected into COS cells, and mutant receptors expressed on the cell surface were used for a direct ligand binding assay at 4°C. The data on both AcLDL and OxLDL binding at 4°C indicated that their binding patterns were not identical with the degradation assay patterns (Fig. 7, d and e). The obvious difference between them can be seen in mutant K337A (a mutant protein whose Lys337 is substituted for by Ala). That is, this mutant has poor degradation activity but almost full ligand binding activity at 4°C. To clarify the reason for this difference, we executed 37°C ligand binding experiments using an endocytosis inhibitor, phenylarsine oxide (Fig. 7, f and g). Interestingly, the results revealed that the pattern of 37°C ligand binding was not identical to that of 4°C ligand binding, but was identical to that of the degradation assay. This indicates that the alanine substitution mutants change their function at different temperatures and that the lysine cluster is essential for AcLDL and OxLDL binding at 37°C, and that Lys337 is the most important residue for recognition of the modified LDLs.

**DISCUSSION**

**The Ligand-binding Domain of the Scavenger Receptor**—In order to exhibit its ligand binding activity, the scavenger receptor must form a trimeric structure (Kodama et al., 1988). Immunohistochemical study of deletion mutants by means of an IgG-D2 monoclonal antibody recognizing the trimeric structures revealed that an α-helical coiled-coil domain was necessary and a collagen-like domain was not for constructing the trimer structure. Therefore, N221 and longer deletion mutants are worth examining for their ligand binding and uptake activities. The results of this experiment with the deletion mutants suggest that the 22 C-terminal amino acids
Fig. 8. Computer graphics model of the ligand-binding domain. Stereo drawing van der Waals model of the ligand-binding domain of the scavenger receptor. Lys\textsuperscript{337} and other Lys and Gln side chains are indicated by red, orange, and yellow-green, respectively. Peptide bond backbone and other side chains are shown by blue and light blue, respectively.

Fig. 9. Computer graphics model of the interaction between the charged collagen structure of the scavenger receptors and polynucleic acids. The complex of ligand-binding domain with poly(G) (a), and high magnification around the Lys\textsuperscript{337} side chains of the complex (b and c). One of the possible interactions of 6-carbonyl groups of guanine or inosine bases and glutamine residues is indicated by a dotted line. This interaction cannot occur with poly(A), (C), or (U), which may explain the selective recognition of polynucleic acids by macrophage scavenger receptors. The amino group of Lys\textsuperscript{337} can interact with the phosphate group of polynucleic acids shown by another dotted line. Yellow-green values indicate the distances (\AA). The colors of amino acids are shown in the same way as in Fig. 8. Nucleic acids are indicated by a wire model in yellow, light blue, red, and purple, as C, N, O, and P atoms, respectively.
of the collagen-like domain are essential for ligand binding and uptake. That is, these 22 amino acids are not related to the trimer formation but only to ligand binding and uptake activity.

Comparison of the amino acid sequences among human, mouse, rabbit, and bovine receptors revealed that this region is highly conserved, and in particular, the C-terminal 10 amino acids are identical. The other conspicuous feature is that five basic amino acids exist in the six Gly-Xaa-Yaa repeats. Considering that this receptor recognizes a wide range of negatively charged macromolecules as ligands, these positively charged amino acids should be very important. 3 lysines at positions 334, 337, and 340 on the bovine receptor make a positive charged lysine cluster in the 10 conserved C-terminal amino acids. A basic amino acid at position 327 in bovine is also conserved. On the other hand, in the case of lysine at position 324 in the bovine receptor, there is no lysine or arginine at the same position as in the human receptor, and therefore this lysine may not be critical for ligand uptake activity. From these results, we speculate that the 4 lysines at positions 327, 334, 337, and 340 are critical for ligand binding and uptake. In order to verify this hypothesis, we prepared point mutant receptors whose lysines at the above positions were replaced with alanines and investigated their ligand binding and uptake activities. The results of these degradation assays indicate that, in general, the replacement of more than 2 lysines decreased ligand uptake activities but only 1 Lys<sup>337</sup> replacement abolished AcLDL binding and uptake activity completely. In addition, the following 37 °C ligand binding experiments showed the same results as the degradation assay. We, therefore, conclude that Lys<sup>337</sup> is essential for AcLDL binding and that a lysine cluster in the C-terminal collagen-like domain is the ligand-binding domain of the scavenger receptor.

**Charged Collagen Model of Ligand-binding Domain**—From the amino acid sequence, we can estimate that the backbone structure of this domain is essentially the same as that of collagen. By data from crystallography studies of poly(l-prolyl-glycyl-L-proline) (Yonath and Traub, 1969), we built a computer graphic model of this domain, which we named the charged collagen structure (Fig. 8). In this model, glysine residues are buried in the center of the triple helix, and the side chains of other residues grow on the surface. If these amino acid side chains are extended outside, a coiled groove surrounded by conserved lysine residues can be detected. Among conserved lysines, the Lys<sup>337</sup> side chain in each strand occurs in the center of this coiled groove structure, and, therefore, Lys<sup>337</sup> plays a critical role in making a positively charged three turn coil space. In the case of K337A, this mutant receptor could not form the obvious three coiled groove structure because the key molecules, 3 Lys<sup>337</sup>s, were missing. This could be a reason why this molecule cannot bind to AcLDL. Scavenger receptor binds and uptakes both AcLDL and OxLDL, but its recognition sites are a bit different. AcLDL is recognized in a narrow region of this binding domain which includes Lys<sup>337</sup>s. On the other hand, in case of OxLDL, the substitution of Lys<sup>337</sup> to Ala in the receptor was not sufficient to abolish its ligand binding activity, indicating that a wide region on the lysine cluster is necessary for high affinity binding to OxLDL. This observation is consistent with reported cross-competition studies (Freeman et al., 1991), which indicate that AcLDL is able to bind more tightly than OxLDL and that unilateral cross-competition occurs.

From the competition assays of AcLDL using many kinds of molecules, macrophage scavenger receptors bind to a wide range of negatively charged macromolecules, such as modified proteins (maleyl bovine serum albumin), nucleic acids (poly(I)), and carbohydrates (fucoidan), as well as modified lipoproteins. It is an interesting observation that when a phosphate backbone of poly(G) is put onto the modeled ligand binding groove, alternative phosphate groups are fitted to interact with lysine residues, and the poly (G) chain winds around the collagen structure along the three turn coiled groove (Fig. 9). Since the bases of the nucleotide chains seem to be recognized by the receptor, syn orientation about the glycosyl bond is necessary for the proposed phosphate backbone structure so that the bases interact with the peptides. It is well known that anti conformers dominate over syn for poly pyrimidines and that syn and anti are about equally abundant for poly purines (Saenger, 1984). This may explain why poly pyrimidines are not binding competitors to AcLDL in our model. Considering the structural difference between poly(G) or (I) and poly(A), 6-carboxyl groups of guanine and inosine base could be recognized by the lysine and the preceding glutamine residues (Fig. 9).

Furthermore, this charged collagen model explains the result that the molecular weight of the poly nucleotide has to be more than 8300 for effective competitive binding (Brown et al., 1980). In the charged collagen model, 7 bases wind around the coiled groove for one turn. Therefore, more than 21 nucleotides are necessary to make three turns and form a tight complex. Based on this recognition model, we might expect the amino acid sequence of lipoprotein to be responsible for binding, and hence be able to design a new compound to prevent modified LDL binding.

The results of direct binding experiments show that the binding activities of certain mutants at 4 °C were different at 37 °C. This indicates that the structure of the ligand-binding domain is temperature sensitive. What makes this domain temperature sensitive? In general, collagen molecules dissolve in solution at 4 °C and aggregate at 37 °C because the hydrophobic interaction at 37 °C is stronger than at 4 °C. From this feature, one possibility is that this receptor is assembled by the interaction of the collagen-like domain at 37 °C to form a dimer or trimer complex, and this complex is essential for organizing the ligand binding space. The other possibility is a conformational change of the collagen-like domain. The trimer structure of the scavenger receptor is supported by the hydrophobic interactions in the a-helical coiled coil domain. The decrease of temperature from 37 to 4 °C weakens the tight trimer form and extends the distances between the three coils. It is possible that this force generates some conformational change in the backbone of collagen-like domain. In any case, further experiments are needed to answer this question. By our results here, we also proposed the importance of doing 37 °C binding experiments in comparison to 4 °C binding experiments.

**Macrophage Function, Atherosclerosis, and Scavenger Receptor**—The uptake of modified lipoproteins by macrophages is of particular interest because this process is related to foam cell formation and the development of atherosclerosis. In human atherosclerotic lesions, considerable amounts of scavenger receptor proteins have been detected (Matsumoto et al., 1990). As the initial event of atherosclerosis, lipoproteins can be seen in the extracellular matrix attached to collagen fibrils in the artery wall (Frank and Fogelman, 1989). The binding of LDL to collagen is enhanced by modifications that increase its negative charge (Hoover et al., 1988). The results of the present study indicate that the collagen-like structure which contains a cluster of basic amino acids is essential for the binding and degradation of modified LDLs. Macrophages can compete for lipoproteins with the arterial
collagens by means of the collagen-like domain with its cluster of positively charged amino acids. Detailed immunopathological studies indicate that macrophage scavenger receptors are exclusively expressed in cells of macrophage lineage throughout the body. This receptor recycles efficiently (Naito et al., 1991, 1992). The scavenger receptor is a kind of recycling collagen-like molecule between the macrophage cell surface and cytoplasm and may scavenge a wide range of denatured and toxic molecules including modified lipoproteins in vivo. In the case of protein, nucleic acids, or carbohydrate substances, macrophages have the capacity to degrade them quickly, but in the case of cholesterol, only the liver and adrenal glands have the enzyme that catabolizes the cholesterol structure. This may lead to cholesterol accumulation in the macrophage. In FH patients, cholesterol deposition is prominent in organs rich in collagen, such as tendons. Recent experiments have indicated that the LDL antioxidant "probufol" prevents atherosclerosis in Watanabe-heritable hyperlipidemic rabbits, an animal counterpart of FH (Kita et al., 1987; Steinberg et al., 1988). LDL antioxidant therapy has been reported as highly effective in regressing the accumulation of cholesterol from tendon xanthomas of FH patients (Matsuzawa et al., 1988; Yamamoto et al., 1986). These results suggest that the prevention of modification of LDL and the inhibition of accumulation of modified LDL in macrophages may be new ways to prevent atherosclerosis. Further study on the mechanism of ligand-receptor interaction in macrophage scavenger receptors will provide us important information on the function of scavenger cells, and provide new insights into the prevention and treatment of atherosclerosis.

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Charged Collagen Structure 2133