Structure and Function of Class A Macrophage Scavenger Receptors

by

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Abstract

Class A macrophage scavenger receptors (SR-As) have been implicated in a variety of macrophage associated processes, including adhesion, atherogenesis, and host defense. There are two alternatively spliced forms of the Class A receptor, type I and type II. Based on the primary sequence, the receptors were predicted to have 6 domains: an N-terminal cytoplasmic domain, a single transmembrane domain, a spacer domain, an α-helical coiled-coil domain, and a collagenous region. The type II receptor has a short C-terminal domain, and the type I receptor has a 110 amino acid domain which contains a 102 residue cysteine rich (SRCR) domain. Structural and functional studies with transmembrane proteins can be hindered by the difficulties inherent in working with these water insoluble molecules. Accordingly, stable cell lines expressing secreted forms of the type I and II SR-As which had ligand binding activity were generated. Using a solid-phase assay, two new ligands for the SR-A were identified: crocidolite asbestos, a silicate responsible for asbestosis, and lipoteichoic acid, a cell surface lipid that is a Gram-positive analog of endotoxin. Ligand binding was pH dependent, and was shown to be mediated by the collagenous domain. Biophysical and biochemical studies revealed that the secreted receptor is trimeric, with 6 N-linked oligosaccharides per polypeptide. Both receptor isotypes contain significant amounts of hydroxylysine and hydroxyproline, modified amino acids characteristic of proteins containing collagenous triple helices. Circular dichroism studies revealed that both isoforms of the receptor contain amounts of α-helix consistent with the presence of an α-helical coiled-coil, and also that gross pH disruption of structure does not occur, suggesting that pH dependent ligand release does not require major structural change. The pattern of the three intramolecular disulfide bonds in the SRCR domain of SR-AI (Cys^2-Cys^7, Cys^3-Cys^8, Cys^5-Cys^6), which was determined using proteolytic analysis, is probably shared by these domains found in other members of the SRCR superfamily. Negative stain and rotary shadowed electron microscopy of the SR-As revealed that they are highly extended molecules, with a flexible hinge between the collagenous and coiled-coil domains. These studies have helped validate and extend the structural model of the scavenger receptor.

Thesis Supervisor: Dr. Monty Krieger
Title: Professor of Biology

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Figures and Tables

Chapter 1

Introduction: The Scavenger Receptors

Scavenger receptors: introduction and general properties

Scavenger receptors are defined as molecules which bind to modified lipoproteins. They were initially discovered by Brown and Goldstein and co-workers in their efforts to resolve a quandary. Patients with familial hypercholesteremia were found to lack the receptor for low density lipoprotein (LDL), the major carrier of cholesterol in the blood (Goldstein et al., 1995). Despite this, these patients still manage to accumulate massive amounts of cholesterol in macrophages in the arterial wall in atherosclerotic plaques (Goldstein et al., 1995). Obviously these cells were accumulating cholesterol in a LDL-receptor independent fashion. Additionally, when macrophages are exposed to high doses of LDL in culture for extended periods of time, they do not become the cholesterol-laden macrophage-derived foam cells which are observed *in vivo* (Goldstein et al., 1979; Brown et al., 1979). The breakthrough in this area came with the observation that macrophages have a large capacity for the receptor mediated uptake of modified forms of LDL, in particular acetylated LDL (AcLDL), leading to massive accumulation of cholesterol in the cell (Goldstein et al., 1979).

The receptor activity they had identified was soon found to have highly unusual ligand binding properties. The uptake and degradation of AcLDL by macrophages could be competed for by a diverse array of macromolecules, breaking the general paradigm of one receptor - one ligand. The scavenger receptor competitors are summarized in table 1.1 (adapted from Krieger and Hertz, 1994). These include some modified proteins (AcLDL and oxidized LDL), nucleic acids (poly(G) and poly(I)), polysaccharides (dextran sulfate and fucoidin), and other molecules, including polyvinyl sulfate and, notably, endotoxin. The common theme to this array of ligands it that all ligands are polyanionic macromolecules or macromolecular complexes. However, not all polyanions are ligands. Molecules such as poly (D-glutamate), poly(C), and heparin have similar (or even greater) negative charge density and fairly similar structures and properties to molecules which are ligands. The exact features which make a molecule a ligand remain unclear; however in the case of the polynucleotides studies have begun clarify this issue. In order to act as ligands, nucleic acids must form four-stranded structures (Pearson et al., 1992). Poly(G) and (I) can form such structures, while many other polynucletides can not. Furthermore, if the four stranded conformation is disrupted by removal of necessary cations, the ability of poly(I) to function as a ligand is lost. Apparently, the four stranded structures have phosphate groups arranged in a manner which enables these nucleotides to bind to the scavenger receptors. In order to try to understand the function of these unusual receptors, as well as to investigate other issues such as the basis for this broad ligand binding activity, the receptors were purified and cloned as described below.

Purification of scavenger receptors

A number of groups interested in the potential role of the scavenger receptor in atherosclerosis and other macrophage associated properties set out to purify it. Ligand blotting experiments revealed that a 200-260 kDa AcLDL binding protein was expressed in several tissues and cultured cell lines (Wong et al., 1983; Via et al., 1985; Dresel et al., 1987). Kodama, Krieger, and co-workers succeeded in obtaining a purified glycoprotein of 220 kDa (Kodama et al., 1988). Upon reduction, the protein exhibited an electrophoretic mobility of 77 kDa, indicating that the protein was presumably trimeric in its native state. Ligand blotting experiments demonstrated that only the trimeric form of the receptor was capable of binding AcLDL. A filter binding assay was used to show that ^{125}I-AcLDL could bind to the purified protein with high affinity, and that this binding could be blocked by the known scavenger receptor inhibitors fucoidin, maleylated BSA, polyvinyl sulfate, and poly (I:C), but not by LDL. Using a monoclonal antibody raised against the purified receptor to examine expression in the liver, it was found that the receptor was localized in only those cells which had been shown to have AcLDL binding activity. These data strongly suggested that the purified protein was the macrophage receptor for AcLDL initially identified by Brown, Goldstein and co-workers.

Cloning and molecular structure of the macrophage scavenger receptor

Using DNA probes based on the sequences of peptides derived from these purified receptors, two closely related cDNAs were isolated from a bovine lung cDNA library (Kodama et al., 1990; Rohrer et al., 1990). The cDNAs were shown to encode scavenger receptors by a number of criteria. Using Northern-blot analysis, it was found that the receptor was expressed in alveolar macrophages and bovine lungs, but not in brain and muscle. In the human monocyte cell line THP-1, expression of receptor was only detectable after treatment with phorbol esters to differentiate the cells into macrophages. This correlates with scavenger receptor activity, which is essentially absent in the uninduced cells (Hara et al., 1987). Transient transfection of the cloned cDNAs in COS M6 cells conferred the ability to bind AcLDL with high affinity (Kodama et al., 1990; Rohrer et al., 1990). This binding was competible by the classic scavenger receptor ligands, but not by control molecules. Thus, the macrophage scavenger receptor had been cloned.

Sequence analysis of the cloned bovine scavenger receptor cDNAs revealed that they encode modular proteins with some highly unusual features. Based on the primary sequence, the receptors were divided into 6 domains (Kodama et al., 1990; Rohrer et al., 1990; Ashkenas et al., 1993) (Figure 1.1). The receptor has a 50 residue N-terminal cytoplasmic domain which contains several possible phosphorylation sites. This is followed by a hydrophobic stretch of 26 amino acids predicted to form the single transmembrane domain and a 75 amino acid spacer domain whose only notable features are a cysteine and 2 of the 7 potential N-linked glycosylation sites on the receptor. Immediately to the C-terminus of the spacer domain is a 121 amino acid region

containing 2 segments of heptad repeats, with hydrophobic (often aliphatic) residues in the "a" and "d" positions of the repeat. This sequence motif is characteristic of the left handed α-helical coiled-coil (Cohen and Parry, 1990). There is a "skip" in the middle of the domain, interrupting the pattern of heptad repeats. The domain also includes the remaining 5 potential N-linked sites. Following the putative coiled-coil region is a domain containing 24 repeats of a different sort: Gly-X-Y, where X and Y are frequently proline. This sequence characteristically forms a right handed three stranded collagenous structure, and was the first report of such a domain in a transmembrane protein. Following the collagenous domain is an isotype specific C-terminal region. The two different receptor isotypes are the products of alternative splicing of transcripts from a single gene (Freeman et al., 1990; Emi et al., 1993). In the type II receptor, this domain is a 6 residue region. In the type I receptor, it is a 110 amino acid domain containing 6 cysteines. This domain helped reveal an ancient, highly conserved sequence module termed the "scavenger receptor cysteine rich" or SRCR domain (Freeman et al., 1990). The other members of the superfamily of proteins containing the SRCR domain and the characteristic features of this domain are discussed in chapter 5 and appendix B of this thesis. Following the cloning of murine, human, and rabbit homologs of the type I and II scavenger receptors and determination of intron/exon junctions (Ashkenas et al., 1993; Matsumoto et al., 1990; Bickel and Freeman, 1992), the boundaries of these domains were adjusted somewhat to take the additional information into account (Ashkenas et al., 1993). The receptors from different species are highly similar to each other. Notable differences include a loss of one Gly-X-Y repeat from the human receptor and an almost complete lack of homology between the different type II C-terminal domains.

It is rather unusual that a cloned sequence allowed a fairly detailed prediction of the receptors' quaternary structure to be made in the absence of any additional studies (Kodama et al., 1990; Rohrer et al., 1990). A model of the receptor is shown in Figure 1.1. Extended collagenous sequences in proteins invariably form trimers (Miller and Gay, 1987). α-helical coiled-coil regions can be two, three, or four stranded structures (Cohen and Parry, 1994). The model shown in figure 1.1 is most consistent with the ligand blotting data (Kodama et al., 1988), which indicated that the receptor is trimeric and thus probably contains a 3 stranded α-helical coiled-coil. It was predicted that the cysteine rich C-terminal SRCR domain contains some intramolecular or intermolecular disulfide bonds (Kodama et al., 1990). Furthermore, knowledge of the nature of the ligands (polyanionic) and the observation that the collagenous sequence contains significant positive charge led to the hypothesis that the collagenous domain could mediate ligand binding (Rohrer et al., 1990). A major emphasis of this thesis has been to evaluate and expand upon these predictions, yielding a better understanding of receptor structure and function.

Tissue distribution and regulation

The type I and II receptors are primarily expressed in macrophages. Use of non-isotype specific monoclonal antibodies to screen multiple tissues from many different organs for scavenger receptor expression revealed that essentially all monocytes and tissues macrophages express this receptor (Naito et al., 1991; Hughes et al., 1995). The receptors are also expressed by endothelial cells in high-endothelial venules (HEV) (Geng and Hansson, 1995) and under certain conditions by smooth muscle cells (Inaba et al., 1992; Bickel and Freeman, 1992; Li et al., 1995). Differential expression of the type I and II receptors has for the most part not been examined. Metabolic labeling and immunoprecipitation with antibodies that recognize both forms of the receptor demonstrated that the type II receptor is the predominantly expressed form in the macrophage-like P388D1 and RAW264 cell lines (Ashkenas et al., 1993; Fraser et al., 1993). Use of isotype specific antibodies and RT-PCR showed that the type I receptor is expressed at higher levels in HEV cells (Geng and Hansson, 1995). Studies with peripheral blood monocytes revealed that the type I expression levels are increased marked upon differentiation into macrophages, while type II levels remain constant (Geng et al., 1994). As the receptor isotypes have virtually identical ligand binding properties (Ashkenas et al., 1993), the significance of differential expression is presently unclear. Presumably differences in localization of type I and II receptors are based on the requirement of certain cells express the SRCR domain and thus to bind the as yet unknown SRCR domain ligands.

A large number of cytokines and other small molecules can influence the expression levels of the scavenger receptor. These include phorbol esters, endotoxin, poly[I:C], macrophage colony stimulating factor (M-CSF), and TGF-β (reviewed in Krieger and Herz, 1994). Scavenger receptor expression in microglial cells is also observed in response to optic nerve crush (Bell et al., 1994) . Promoter elements which control the macrophage specific expression of these receptors are under investigation (Aftring and Freeman, 1995; Horvai et al., 1995). Horvai and colleagues have shown that PU.1, AP-1, and ets-domain binding sites located in the scavenger receptor's 291 bp promoter can, in concert with a 400 bp upstream enhancer site, direct expression of human growth hormone (hGH) to at least some macrophages in a transgenic animal model.

Physiological and pathophysiological roles for the scavenger receptor

The vast number of molecules which act as scavenger receptor ligands in *in vitro* competition assays has complicated the question of the *in vivo* function of the type I and II receptors. Macrophage associated physiological and pathophysiological functions include roles in host defense, apoptosis, clearance, atherogenesis, and adhesion. Scavenger receptor ligands and binding properties have led to suggestions that these versatile receptors could play a role in some or all of these processes.

The evidence for the involvement of the scavenger receptor in the development of atherosclerotic lesions by causing the massive uptake of OxLDL into resident and recruited macrophages is indirect but compelling (Krieger and Herz, 1994). Evidence for involvement of OxLDL is twofold: 1) antibodies against OxLDL have been used to demonstrated that OxLDL is present in plaques (Haberland et al., 1988; Palinski et al., 1989); 2)*in vivo* studies have shown that probucol, an anti-oxidant, helps defend against atherogenesis in an animal model (Carew et al., 1987). Two different lines of evidence argue for the involvement of type I and II receptors: 1) expression of type I or type II scavenger receptors in CHO cells confers on these cells the ability to take up massive quantities of modified low density lipoprotein, converting them into lipid droplet filled cells reminiscent of the macrophage foam cells observed in plaques (Freeman et al., 1991) and 2) anti-peptide antibodies against an epitope found in both receptor isotypes revealed that macrophage foam cells in atherosclerotic plaques have SR on their surface (Matsumoto et al., 1990), and *in situ* hybridization showed that the mRNA for these receptors is present (Yla-Herttuala et al., 1991). These data are highly consistent with the SRs playing a role in the development of atherogenesis by causing massive accumulation of OxLDL derived cholesterol in macrophages in the developing plaque.

In addition to their putative role in atherosclerosis, SRs may play a role in macrophage adhesion. In the process of trying to identify cation-independent macrophage adhesion receptors by using monoclonal antibodies against surface components, an antibody which recognized both the type I and II scavenger receptors was isolated (Fraser et al., 1993). This monoclonal antibody, 2F8, completely blocked adhesion of macrophages to tissue culture plastic in the presence of serum and EDTA. It is thus possible that the SR plays a role in macrophage recruitment in atherosclerotic plaques and at other sites. These observations were extended with the finding that the divalent cation independent adhesion of macrophages to frozen spleen, lymph node, lung, medulla, and gut tissue sections could be completely blocked by 2F8 (Hughes et al., 1995). Macrophage adhesion to other tissue sections could only be partially blocked with the antibody. This binding interaction occurred in the absence of serum, suggesting that the SRs are directly recognizing surface molecules expressed in these tissues. It is unclear which portion of the receptor is mediating this interaction -- the 2F8 antibody recognizes an epitope in the coiled-coil region, so presumably this domain or an adjacent one is involved (S. Gordon, unpublished). The SRCR domain could also potentially play a role in adhesion. An SRCR domain in another member of the SRCR superfamily, CD6, has been shown to influence the interaction of thymocytes with thymic epithelial cells (Bowen et al., 1995). This raises that possibility that the SRCR domain of the type I scavenger receptor could have a similar function in mediating cell-cell interactions.

The notion that the SRs could play a role in host defense is an attractive one. Their specific localization on the surface of macrophages makes them ideally situated for such a role. Janeway has proposed that an important role in immunity is played by "pattern recognition receptors" (Janeway, 1992). These evolutionarily ancient molecules are non-clonally distributed receptors whose role is to broadly distinguish between "self" and "non-self". Given their ability to recognize a broad array of molecules, it seems possible that the SRs could act as such pattern recognition receptors (Krieger and Herz, 1994). The findings that endotoxin could compete for binding of ^{125}I-AcLDL to CHO cells expressing SRs, and that *in vivo* scavenger receptor ligands could inhibit the clearance of Lipid IVa (an endotoxin core-lipid precursor) provide some support for the notion. (Hampton et al., 1991). Thus, the scavenger receptor could play a role in preventing shock by clearing endotoxin, and could presumably recognize and phagocytose bacteria by this mechanism as well.

An additional role of the macrophage is clearance - that is the removal of foreign materials as well as debris such as apoptotic cells. Scavenger receptors have been suggested to be involved in the clearance of particulates from the lung (Kobzik, 1995). In *in vitro* and *in vivo* experiments, scavenger receptor ligands (Poly(I) and dextran sulfate, but not chondroitin sulfate) have been shown to block the clearance of environmental particulates such as quartz, titanium oxide, and iron oxide. Scavenger receptors have also been implicated in the clearance of type I and III procollagen peptides (Melkko et al., 1994). In *in vivo* experiments, it was found that these peptides are largely cleared by Kupfer cells in the liver. In tissue culture, binding of these propeptides to Kupfer cells was inhibited by SR-A ligands (formaldehyde treated BSA, AcLDL, poly(I), but not heparin or LDL). Studies with SR-II transfected CHO cells revealed that advanced glycation endproducts (AGE) are ligands, suggesting that scavenger receptors could play a role in the recognition and clearance of these molecule (Araki et al., 1995). Studies with microglial cells revealed that SR-A competitors could block uptake of aggregated β-amyloid protein (Paresce et al., 1995). Studies with CHO cells transfected with murine SR-AI revealed that this receptor confers upon these cells the ability to take up these aggregates, suggesting that it is SR-A in the microglial cells that is responsible for the uptake. These studies have shown that the scavenger receptor could play a role in the clearance of proteinaceous and other substances from the body.

Recent studies also indicate that the type I and II receptors could be involved in the recognition of apoptotic cells. Clearance of apoptotic thymocytes by macrophages is specifically inhibited 50% by addition of the anti-SR monoclonal 2F8, arguing strongly that this receptor could be involved in this process (Platt and Gordon, 1995). This clearance is inhibited 90% by the SR ligand poly(I), but is not affected by poly(C). The recognition of these thymocytes is presumably due to the apoptosis induced exposure on their surfaces of some SR ligand.

Other scavenger receptors

Numerous studies have indicated the type I and II receptors described above are not the only "scavenger receptors", i.e. proteins that bind modified low density lipoproteins (Krieger and Herz, 1994). The actual number of such receptors remains unclear. Part of the confusion in the field is undoubtedly attributable to the fact that the type I and II receptors exhibit non-reciprocal cross competition (Freeman et al., 1991). In experiments with CHO cells transfected with these receptors, unlabelled AcLDL and OxLDL compete efficiently for the uptake and degradation of their iodinated counterparts. However while AcLDL competes efficiently for OxLDL, the reverse is not true. This made it appear that there were more receptors than is actually the case. Interestingly, the reverse is seen in macrophages, where OxLDL can efficiently block AcLDL and OxLDL binding, but AcLDL can only partially inhibit OxLDL binding (Sparrow et al., 1989). More recently, the cloning of multiple additional receptors has begun to clarify these issues. The scavenger receptors have been divided into families based on sequence (Acton et al., 1994). The class A receptors, including the type I and II receptors described above, contain a collagenous region. The class B receptors are in the CD36 family of proteins. The class C group currently contains a single multi-domain *Drosophila* protein. The following is a brief description of these SR families and their members.

In addition to SR-AI and SR-AII (on which this introduction and thesis are primarily focused), a third member of the Class A scavenger receptor family has recently been identified. MARCO, or SR-AIII, was isolated in an attempt to use hybridization to clone human type XIII collagen (Elomaa et al., 1995). MARCO is strikingly similar to SR-AI. In addition to an elongated collagenous region which contains positively charged triplets near its C-terminus, it contains an SRCR domain. MARCO is specifically expressed in the spleen's marginal zone macrophages as well as peritoneal macrophages, and binds AcLDL as well as fluorescently labeled Gram-negative bacteria.

The class B receptor family contain proteins homologous to CD36. This family was initially identified as having scavenger receptor activity when human CD36 was isolated in an expression cloning search for additional proteins capable of binding oxidized LDL (Endemann et al., 1993). They found that OKM5, a blocking antibody, inhibited binding of OxLDL to phorbol ester induced macrophage-like THP-1 cells by approximately 50%, suggesting that CD36 may play a significant role in OxLDL recognition by macrophages. Recent studies have demonstrated that CD36 derived from several species has a broad polyanion binding specificity similar to that seen in SR-A (X. Huang, S. Acton, and M. Krieger, unpublished), although human CD36 apparently does not have this property (Acton et al., 1994). In addition to being expressed in macrophages, CD36 is found in some endothelial cells, platelets, and adipocytes (Greenwalt et al., 1992).

Attempts to expression clone the receptor responsible for a CHO cell line's unusual scavenger receptor-like activity resulted in the identification of a new CD36 family member, SR-BI (Acton et al., 1994). SR-BI, which is approximately 32% identical to CD36, has rather different ligand binding properties to those found in the other SRs. In addition to binding AcLDL and OxLDL, it binds unmodified lipoproteins, namely LDL and HDL (Acton et al., 1994; Acton et al., 1996). SR-BI also binds maleylated BSA and negatively charged phospholipids (Rigotti et al., 1995), but does not recognize the myriad polyanions characteristic of the SR-As. Studies with anti-peptide antibodies have revealed that SR-BI is primarily expressed in liver, ovaries, and adrenal glands (Acton et al., 1996). It seems likely that the principle role of SR-BI is in lipid trafficking.

Injection of fluorescently labeled AcLDL into *Drosophila* embryos revealed that hemocytes (macrophage-like cells) could recognize and internalize this ligand (Abrams et al., 1992). Accordingly, an expression cloning strategy was employed to attempt to isolate the receptor responsible for this activity (Pearson et al., 1995). The receptor obtained (SR-CI) had no sequence resemblance to the class A or B receptors, nor, taken as a whole, to any other protein. It contains a single transmembrane domain and domains with homology to complement control proteins (CCP) as well as somatomedin B, MAM, and mucin like domains. Localization of expression by *in situ* hybridization revealed that it was found in hemocytes early in development. Studies with transfected CHO cells revealed that SR-CI has a broad ligand binding specificity similar to that of SR-A.

Additional classes of scavenger receptors could well remain to be discovered. The identification of macrosialin and Fcγ-RII as OxLDL binding proteins has perhaps added two other classes of scavenger receptors. Macrosialin was initially observed as a 94-97 kDa protein which binds OxLDL and phosphatidylserine (Sambrano and Steinberg, 1995), and subsequently purified and identified based on partial amino acid sequencing (Ramprasad et al., 1995). As this protein is predominantly expressed in the lysosome and endosome, it is unclear whether this receptor plays a role in any way similar to the other scavenger receptors. Fcγ-RII, a macrophage expressed immunoglubulin receptor, has also been shown to bind OxLDL in an antibody independent manner when transfected into 293 cells (Stanton et al., 1992). However as antibodies which block binding of OxLDL to transfected 293 cells fail to have any effect on macrophage uptake of OxLDL, the significance of this is questionable.

The increasing number and diverse properties of the scavenger receptors renders it difficult to make blanket statements about this group of receptors. All bind modified (oxidized and/or acetylated) lipoproteins, although

this is probably not be of functional significance in some cases. Many have fairly similar broad polyanionic ligand binding properties. Most, but not all, appear to be predominantly expressed in macrophages or macrophage-like cells. Studies in progress on all of these scavenger receptors should begin to help us understand the structural and functional properties of these unusual molecules. Described here are studies on the properties of the Class A type I and II macrophage scavenger receptors. The primary focus of this thesis has been to use recombinant secreted forms of the receptors to experimentally evaluate and expand upon the structural model of these scavenger receptors and also to identify new ligands to better understand receptor function.

Table 1.1. Ligand binding properties macrophage scavenger receptors

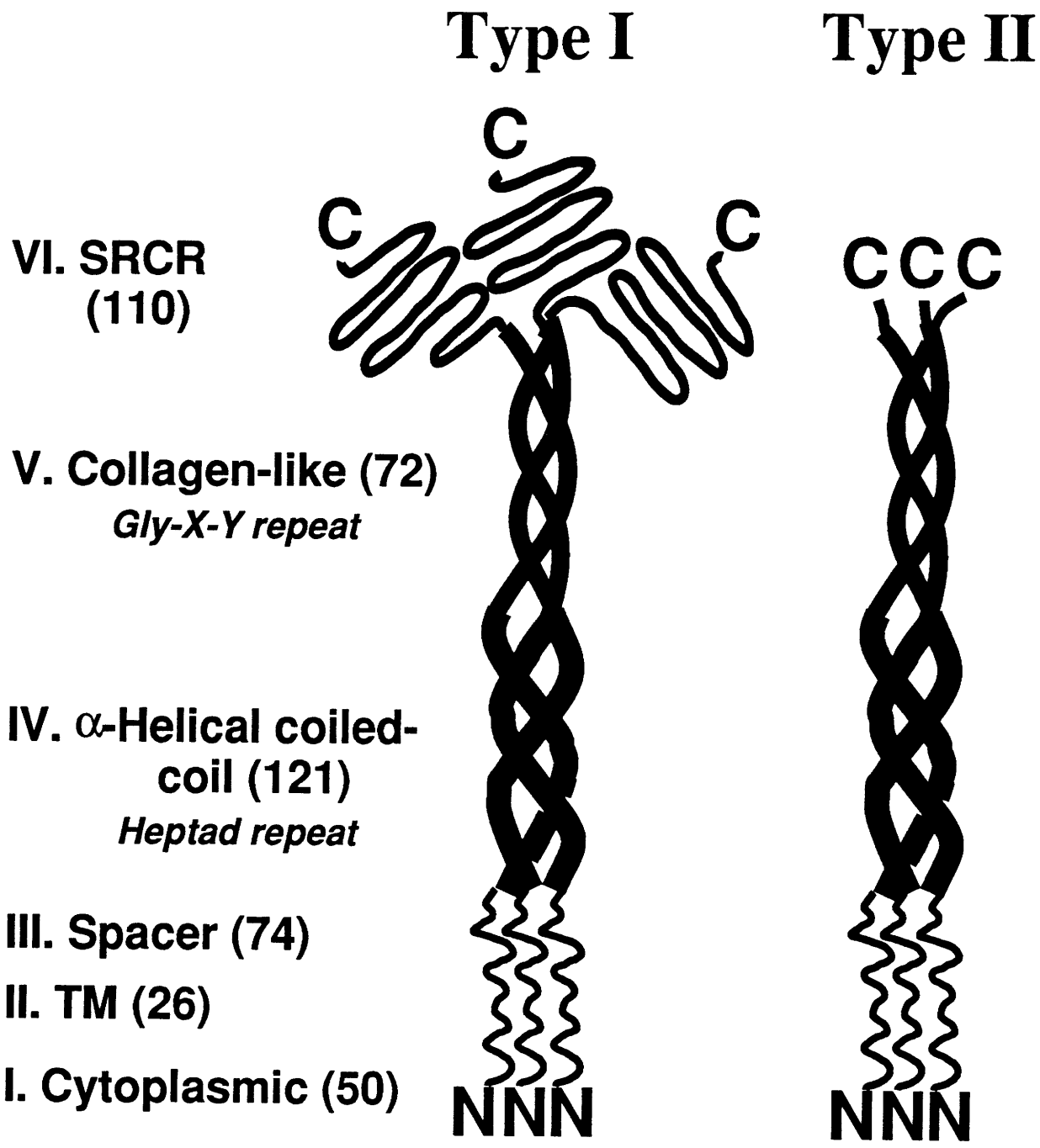
Effective competitors	Ineffective competitors
Modified proteins AcLDL, OxLDL, M-BSA, M-HDL	Native and modified proteins LDL, HDL, BSA, poly (D-glutamate), Ac-HDL, Ac-BSA, fetuin
Nucleic acids Polyinosinic acid (poly(I)), poly(G), telomere models ($d(G_4T_4)$), poly(I•C)	Nucleic acids Poly(A), poly(C), poly(U), single and double stranded DNA
Polysaccharides Dextran sulfate, fucoidin carragheenan	Polysaccharides Heparin, chondroitin sulfate, polysialic acid, mannan
Other molecules Polyvinyl sulfate, bovine sulfatides, endotoxin	Other molecules Polyphosphate

The molecules listed here have been assayed for their ability to block the binding, uptake, and degradation of AcLDL by either cultured macrophages or transfected cell lines. Some of these molecules have also been shown to directly bind the scavenger receptor. Adapted from Krieger and Herz, 1994.

Figure 1.1. Domain structure of the type I and II class A scavenger receptors

The receptor has been divided into 6 domains: I) N-terminal cytoplasmic domain; 2) transmembrane (TM) domain; 3) spacer domain; 4) α-helical coiled-coil domain; 5) collagenous domain; 6) variable C-terminal domain. In the type I receptor, the C-terminal region is a scavenger receptor cysteine rich (SRCR) domain, while the type II receptor has a 6 residue C-terminal domain (not labeled in the figure). The number of amino acids in each domain in the bovine receptors is indicated in parenthesis.

Class A Scavenger Receptors



Chapter 2

Secreted extracellular domains of macrophage scavenger receptors form elongated trimers which specifically bind crocidolite asbestos

The overall goal of my thesis was to carry out structural and functional studies of the class A macrophage scavenger receptors. Structural studies in particular can be quite difficult to perform using proteins with transmembrane domains. These proteins are generally insoluble, expressed at low levels, and can be difficult to purify in quantity. Since the domains which we were interested in studying were extracellular, making secreted forms of the receptors was a reasonable way to address these issues. This chapter contains a series of experiments describing the production of secreted forms of the type I and II class A bovine scavenger receptors and the use of metabolically labeled secreted receptors for a variety of structural and functional studies. The questions addressed were as follows: 1) are the secreted receptors functional (i.e. do they have the same ligand binding specificity as the transmembrane forms); 2) is ligand binding pH dependent; 3) what is the extent of glycosylation of the receptor; 4) what is the receptor's degree of oligomerization; and 5) is crocidolite asbestos a SR ligand. This work was published in its entirety in 1993 in *The Journal of Biological Chemistry* **268**, 3538-3545. The only modifications which have been made are that figures have been re-numbered with the prefix "2" and the references have been merged with those of other chapters in the "references" section of this thesis.

The experiments in this chapter were for the most part performed by me, with the exception of the pH dependence of ligand binding (Fig 2.7), which was carried out by Shangzhe Xu. In the early stages of this work, I worked with Dr. Neil Freedman, who initiated the secreted receptor project and generated and sequenced the secretion constructs. Dr. Monty Krieger suggested testing asbestos as a potential ligand, and assisted with the first binding experiment. I wrote the initial draft of the paper, which was subsequently revised and edited with the assistance of Dr. Krieger. Dr. Freedman also provided useful input on the paper.

Secreted extracellular domains of macrophage scavenger receptors form elongated trimers which specifically bind crocidolite asbestos

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Summary

Macrophage scavenger receptors, which have been implicated in the development of atherosclerosis and other macrophage-mediated events, are trimeric integral membrane glycoproteins whose extracellular domains have been predicted to include α-helical coiled-coil, collagenous and globular structures. To elucidate further the structural and functional properties of these receptors, we generated transfected Chinese hamster ovary (CHO) cells which express secreted extracellular domains of the type I and type II bovine scavenger receptors and developed a solid-phase bead-binding assay to assess their ligand binding properties. The secreted receptors exhibited the distinctive, high-affinity, broad polyanionic ligand-binding specificity and the pH dependence of binding which characterize the membrane-anchored, cell-surface forms of the receptors. Both the type I and type II secreted receptors were trimeric glycoproteins comprising disulfide-linked dimers and noncovalently associated monomers. Gel filtration and glycerol-gradient centrifugation established that the type II trimers were highly elongated and did not associate into higher order oligomers at the low concentrations used in these experiments. Crocidolite asbestos, which is phagocytosed by alveolar macrophages and can cause asbestosis and mesothelioma, bound efficiently to secreted type I receptors and less well to the type II receptors. This binding was specific in that it was competed by a variety of well-established scavenger receptor ligands but not by negative controls. These studies have identified a new type of insoluble scavenger receptor ligand, and have raised the possibility that scavenger receptors may play a role in mediating the physiological and pathological interactions of inspired particles with alveolar macrophages.

Introduction

Macrophage scavenger receptors are trimeric integral membrane proteins which exhibit unusual ligand-binding characteristics (Goldstein et al., 1979; Brown and Goldstein, 1983; Kodama et al., 1988; Kodama et al., 1990; Rohrer et al., 1990; Penman et al., 1991). They bind a broad array of polyanionic macromolecules or macromolecular complexes, including modified LDL, acidic phospholipids, endotoxin (Raetz et al., 1988; Nishikawa et al., 1990; Hampton et al., 1991), some polysaccharides (fucoidan and dextran sulfate, but not heparin or chondroitin sulfate), and certain polyribonucleotides (poly I and poly G, but not poly C) and polydeoxyribonucleotides (Brown and Goldstein, 1983; Pearson et al., 1992). A number of studies have suggested that the scavenger receptors may be involved in the accumulation of cholesterol by macrophages in arterial walls during atherogenesis (Brown and Goldstein, 1983; Jurgens et al., 1987; Steinberg et al., 1989; Steinbrecher et al., 1990). These receptors may also participate in other physiologic and pathophysiologic processes, such as macrophage-associated immune responses and inflammation (Freeman et al., 1990; Kodama et al., 1990; Krieger, 1992).

The cDNAs for two forms (type I and type II) of bovine, murine, rabbit, and human macrophage scavenger receptors have been cloned and sequenced (Kodama et al., 1990; Matsumoto et al., 1990; Rohrer et al., 1990; Freeman et al 1990, Ashkenas et al., 1992; Bickel et al., 1992). The predicted amino acid sequences define 6 structural domains in each polypeptide chain (Kodama et al., 1990; Ashkenas et al., 1992): I. an N-terminal cytoplasmic domain, II. a single transmembrane domain, III. a spacer domain, IV. an α-helical coiled-coil domain, V. a collagenous domain, and VI. a variable length C-terminal domain. Domains I-V are identical in the type I and type II receptors. In the type II receptor, domain VI is a short domain (6-17 amino acids) that is poorly conserved (Ashkenas et al., 1992). In the type I receptor, domain VI is 110 residues long and includes a 102 amino acid region designated the scavenger receptor cysteine-rich (SRCR) domain. This domain helped to define an ancient, highly conserved family of cysteine-rich protein domains (Freeman et al., 1990, Aruffo et al., 1991; Krieger, 1992). Both type I and type II receptors exhibit the distinctive, high affinity, broad-specificity ligand binding which is characteristic of scavenger receptors. The functions of the SRCR domain have not yet been established; however, recent studies have suggested that the presence of this domain in the type I receptor can modulate its binding properties relative to those of the type II receptor (Ashkenas et al., 1993).

Detailed analyses of the structures and properties of integral membrane receptors are often complicated by difficulties inherent in working with these water-insoluble proteins, whose natural levels of expression are frequently low. In several cases, these technical problems have been overcome by constructing expression vectors encoding secreted forms of these receptors and by expressing high levels of these soluble proteins in transfected cultured cells

(Watson et al., 1990; Duan et al., 1991; Lax et al., 1991; De Vos et al., 1992). In some cases, the secreted forms are simply truncated versions of the full-length receptors. In others, fusion proteins have been generated (Watson et al., 1990) to facilitate secretion or subsequent characterization of the secreted molecule. In the current study, we have generated Chinese hamster ovary (CHO) cells which express secreted forms of the bovine type I and type II scavenger receptors. We have developed a solid-phase binding assay to characterize the ligand-binding properties and oligomerization state of these secreted receptors. We have also used the secreted receptors to show that crocidolite asbestos can bind to scavenger receptors. This finding potentially defines both a new class of ligands and additional physiologic and pathophysiologic functions of scavenger receptors.

Materials and Methods

Materials - Reagents (and sources) were: pcDNA1 and pRc/CMV (Invitrogen), Poly G, Poly I, and Poly C covalently attached to Sepharose beads (Pharmacia), N-glycanase (Genzyme), and *E. coli* ribonuclease inhibitor (Calbiochem). Rabbit anti-bovine spacer domain IgG, low density lipoprotein (LDL), acetylated LDL (AcLDL) and maleylated bovine serum albumin (M-BSA) were prepared as previously described (Penman et al., 1991; Krieger, 1983; Goldstein et al., 1979). M-BSA and BSA were coupled to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. There were approximately 2.7 mg of M-BSA or 2.85 mg of BSA crosslinked per ml of packed gel. Crocolidite asbestos was a gift from Dr. Joseph Brain (Harvard School of Public Health).

Construction of pRc/CMV-s-bSR-I and s-bSR-II Expression Vectors - To express the secreted extracellular region of the bovine type I and type II scavenger receptors (s-bSR-I and s-bSR-II), we constructed vectors in several steps. First, a segment of DNA including the myelin-associated glycoprotein (MAG) leader sequence (Arquint et al., 1987) was excised from a fibronectin expression vector (P. Johnson and Richard Hynes, manuscript in preparation) with the enzymes *Bam*H I and *Xba* I, and then inserted into the cloning site of pcDNA1. Next, a segment of DNA encoding the scavenger receptor spacer domain and a portion of the coiled-coil domain, which are common to the type I and type II bovine scavenger receptors, was inserted into the MAG leader-containing vector. This segment (codons 78-227) was generated by PCR (primer 1: 5'-CGCGGGCCCGGGTGGGAAACGAAGAATTGCAC-3'; primer 2: 5'-TTTTTCACCTGGAGGTCCA-3') using pXSR7 -- a plasmid encoding the type I bovine scavenger receptor (Kodama et al., 1990) -- as a template (Sambrook et al., 1989), and subsequent digestion with *Sma* I and *Xba* I. The digested PCR product was ligated into the pcDNA1/MAG vector, which was prepared by *Xho* I treatment, Klenow blunting, and digestion with *Xba* I. This construct resulted in the substitution of Arg-Gly for Lys^{77} at the amino terminus of the spacer domain. The sequence of this MAG/scavenger receptor "common domain" fusion was determined in its entirety by the dideoxy method (Sequenase, United States Biochemical). This construct was then digested with *Xba* I, and the remaining 3' portions of the extracellular domains of bSR-I or bSR-II were added by ligation of *Xba* I-*Xba* I fragments from either pXSR7 or pXSR3 (Rohrer et al., 1990), respectively. The inserts were subsequently excised with *Hind* III and subcloned into pRc/CMV.

Cell Culture and Transfections - All incubations with intact cells were performed at 37°C in a humidified 5% CO_2 incubator. As previously described (Krieger, 1983), stock cultures of wild-type CHO cells were grown in medium A (Ham's F-12 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) containing 5% (v/v) fetal bovine serum (medium B).

Transfectants expressing the type I (CHO[s-bSR-I]) and type II (CHO[s-bSR-II]) secreted bovine scavenger receptors were generated using the polybrene method (Sambrook et al., 1989). On day 0, wild-type CHO cells were plated at $5x10^5$ cells/100 mm dish in medium B. On day 1, cells were refed with 3 ml of medium B containing 10 μg/ml polybrene (Aldrich), and either 0.5 μg pSV2dhfr* (Stuhlmann et al., 1989) and 10 μg of pRc/CMV[s-bSR-I] per dish or 0.5 μg of pSV2dhfr (Subramani et al., 1981) and 10 μg of pRc/CMV[s-bSR-II] per dish. After a 6 hr incubation, the polybrene-containing media were removed, and the cells were incubated with medium B containing 30% (v/v) dimethylsulfoxide for 4 min at room temperature. The monolayers were then washed with medium A and refed with medium B. On day 3, cells were harvested with trypsin and plated at 10^5 cells/100 mm dish in medium C (medium B supplemented with 0.5 mg/ml Geneticin(Gibco)). On days 10-12, 48 colonies were picked, and subsequently grown into mass cultures and screened for receptor synthesis as follows. Cells transfected with the s-bSR-I expression vector were pulse-labeled for 30 min. with [^{35}S]methionine (135 μCi per well) and newly synthesized receptors were detected using an anti-spacer domain antibody and an immunoprecipitation-gel electrophoresis-autoradiography assay as previously described (Penman et al., 1991). One positive colony was subsequently cloned, to yield the cell line CHO[s-bSR-I]-A2, which was used for all experiments involving s-bSR-I. Cells transfected with the s-bSR-II expression vector were screened by immunoprecipitation of media from radiolabeled cells as described below. One positive colony, designated CHO[s-bSR-II]-2C3, was used for all experiments involving s-bSR-II.

Preparation of [^{35}S]Methionine-Labeled Conditioned Media - Cells were plated on day 0 in 3 ml of medium B (untransfected CHO cells) or medium C (transfected cell lines) in six-well dishes ($1.5x10^5$ cells/well). On day 2, cells were washed two times with Dulbecco's phosphate buffered saline (PBS, Gibco) and refed with 0.75 ml of labeling medium (methionine-free medium A containing 80 μCi/ml of either [^{35}S]methionine or [^{35}S]Protein labeling mix (New England Nuclear)). After incubation for 5 hours, the media were harvested and pooled if multiple, identical wells were labeled. Phenylmethylsulfonylfluoride and leupeptin were then added to final concentrations of 1 mM and 0.1 mM, respectively, and the media were clarified by centrifugation at 1500xg for 15 minutes.

Bead-Binding Assay and Electrophoresis - Metabolically labeled medium produced as described above (500-750 μl) was added to 250 μl of buffer A (20 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$) containing 2 mg/ml bovine serum albumin and 0.01% (w/v) NaN_3 (Buffer B). We then added 25 μl of a suspension of buffer A containing 50% (v/v) of Sepharose beads coupled to the indicated ligands, and enough additional buffer A to bring the final volume to 1 ml. The mixture was placed on a rotator overnight at 4°C. The beads were then

washed twice with buffer A, and adsorbed proteins were eluted by boiling for 5 minutes in 25 µl of electrophoresis sample buffer (50 mM Tris, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM dithiothreitol, pH 6.8). The eluate was subjected to 10% SDS-polyacrylamide gel electrophoresis. Fixation, staining, and autoradiography were carried out as previously described (Kozarsky et al., 1986). In some experiments, the beads were replaced with 50 µg/sample of crocidolite asbestos. Quantitation of the intensity of the ^{35}S-labeled protein bands in dried gels was carried out using a Molecular Dynamics PhorphorImager according to the manufacturer's instructions.

During the course of these studies, we observed that ribonuclease activity in the metabolically labeled conditioned media could partially degrade single-stranded soluble poly C. It seems possible that the poly C covalently linked to Sepharose beads was also susceptible to ribonuclease-mediated degradation. We did not, however, detect degradation of poly G, presumably because of its quadruplex structure (Pearson et al 1992). In all cases, experiments with soluble or bead-bound poly C were repeated in the presence of 40 units/ml of *E. coli* ribonuclease inhibitor, which effectively prevented the degradation but did not interfere with the assay (data not shown). All results with and without the inhibitor were essentially identical.

Immunoprecipitation of Secreted Receptors - A solution composed of 90 µl of buffer A, 100 µl of 10x detergents (5% (w/v) SDS, 2.5% (w/v) sodium deoxycholate, 10% Triton X-100 (v/v) in water), 50 µl of BSA (10 mg/ml in water), and 10 µl of rabbit anti-bovine spacer domain antibody was added to 750 µl of metabolically labeled sample. After a preincubation at 37°C for 1 hour, 25 µl of a 1:1 suspension of protein A-Sepharose (Pharmacia) in PBS were added. This mixture was placed on a rotator overnight at 4°C. The samples were then washed twice with buffer C (100 mM Tris pH 8.0, 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.25% (w/v) SDS), and proteins were eluted by boiling for 5 minutes in 25 µl of electrophoresis sample buffer (with or without 100 mM dithiothreitol as indicated). N-glycanase treatment was performed as previously described (Penman et al., 1991) using the indicated digestion times and quantities of enzyme. Samples were analyzed by electrophoresis in 3-10% gradient gels as described above. The estimated apparent masses of the unreduced and N-glycanase-treated receptors are from a single experiment and are based on a calibration curve defined by a set of ^{14}C-methylated protein standards (Penman et al., 1991). Masses for the reduced receptor subunits are based on averages from multiple experiments.

Stokes Radius Determination by Gel Filtration - A 200 µl aliquot of conditioned medium from [^{35}S]methionine-labeled CHO[s-bSR-II] cells was loaded onto a Pharmacia FPLC-Superose 6 HR 10/30 column (pre-equilibrated in Buffer A),

which was eluted with Buffer A at a flow rate of 0.25 ml/min. Fractions (250 µl) were diluted to 750 µl with buffer A, and subjected to the bead binding assay using Poly G beads as described above. The calibration of the column, which was monitored by absorbance at 280 nm, was determined with the standards of known Stokes radii (Axelsson, 1978; Peterson et al., 1986) listed in the legend to Figure 8. The value for IgM (11.62 nm) was calculated using equation 1 and previously reported values for $\bar{\upsilon}$, $s_{20,w}$, and M_r (Fasman, 1976). The column volume, V_t, was measured with 5'dGMP, and the excluded volume, V_o, was measured using a 12,358 bp supercoiled plasmid, both of which were monitored by absorbance at 254 nm.

Sedimentation Coefficient Determination by Glycerol Gradient Centrifugation - A 200 µl aliquot of conditioned medium from [^{35}S]methionine-labeled CHO[s-bSR-II] cells to which 10 µl of bromophenol blue (4 mg/ml) were added was layered on the top of an 11 ml linear glycerol gradient (5-20% (v/v) in Buffer A containing 1 mg/ml of BSA). Calibration was performed in a separate gradient prepared with buffer A without the BSA using the standards (Peterson et al., 1986; Kumar et al., 1991) listed in the legend to Figure 8. After centrifugation in a Beckman SW41 rotor for 18 hr at 134,000x*g*, fractions of 500 µl were collected with an Isco Density Gradient Fractionator, model 183, and the s-bSR-II was detected by immunoprecipitation, electrophoresis (8% gels) and autoradiography as described above. Fractions from the calibration gradient were precipitated with trichloroacetic acid (8% (w/v) final concentration). The precipitated proteins were dissolved in electrophoresis sample buffer, and the samples were neutralized by the addition of 1 M Tris HCl, pH 9.5. The samples were then boiled, subjected to 13% polyacrylamide gel electrophoresis and the proteins were detected by Coomassie brilliant blue staining as previously described (Kozarsky et al., 1986).

Calculations - The molecular weight and frictional ratio of the receptor were calculated using the following equations (Siegel and Monty, 1966).

Equation 1: $$M_r = \frac{6\pi N\eta}{1-\bar{\upsilon}\rho} r_s s_{20,w}$$

Equation 2: $$\frac{f}{f_o} = r_s\left(\frac{4\pi N}{3M_r\bar{\upsilon}}\right)^{\frac{1}{3}}$$

where N is Avogadro's number (6.022×10^{23} mol^{-1}), η is the viscosity of water at 20°C (1.0×10^{-3} kg/m/s), $\bar{\upsilon}$ is the partial specific volume, ρ is the density of H_2O at 20° C (0.998 g/ml), r_s is the Stokes radius determined by gel filtration chromatography, and $s_{20,w}$ is the sedimentation coefficient determined by

glycerol gradient centrifugation. The ratio (P) of the length to width (or semimajor axis to semiminor axis) of the receptor was calculated using successive approximations from the frictional ratio, with the receptor modeled as a cylinder (equation 3) or a prolate ellipsoid (equation 4) (van Holde, 1985).

Equation 3: $$\frac{f}{f_o} = \left(\frac{2}{3}\right)^{\frac{1}{3}}\left(\frac{P^{\frac{2}{3}}}{-0.30+\ln[2P]}\right)$$

Equation 4: $$\frac{f}{f_o} = \frac{P^{-\frac{1}{3}}(P^2-1)^{\frac{1}{2}}}{\ln\left(P+(P^2-1)^{\frac{1}{2}}\right)}$$

Results

Stable CHO cell lines expressing secreted forms of the type I (CHO[s-bSR-I]) and type II (CHO[s-bSR-II]) bovine scavenger receptors were isolated as described in Materials and Methods. The predicted domain structures of the wild-type, integral membrane receptors and the secreted forms are shown in Figure 2.1. To generate secreted forms, we replaced the cytoplasmic and transmembrane domains of the wild-type receptors with the myelin-associated glycoprotein ("MAG") leader sequence. This leader sequence was presumably removed by a signal peptidase when the proteins were translated and inserted into the lumen of the endoplasmic reticulum. In addition, this construction resulted in the substitution of an arginine-glycine dipeptide for the first amino acid of the spacer domain (Lys^{77}, indicated by a dot in Figure 2.1). The molecular weight of the predicted, type I secreted scavenger receptor (s-bSR-I), calculated from its amino acid composition is 41.7 kD (378 amino acids) and that of the type II secreted receptor (s-bSR-II) is 30.1 kD (274 amino acids).

Metabolically labeled receptors secreted into the media by transfectants were initially characterized by immunoprecipitation with polyclonal rabbit anti-spacer domain antibodies (Figure 2.2). After reduction, s-bSR-I (left) and s-bSR-II (right) migrated as broad bands with apparent masses of 78 kDa and 68.7 kDa (*lanes 1 and 4*). The unreduced samples (*lanes 2* and *5*) were resolved into monomeric ("1°") and dimeric ("2°") forms. The unreduced type I monomeric form exhibited an electrophoretic mobility which was slightly greater than that of the reduced form. We have reported similar result for the full-length type I receptor (Penman et al., 1991). Initial estimates of the apparent masses of the dimeric forms are 135 kDa for s-bSR-I and 125 kD for s-bSR-II. These results are consistent with our previous studies which showed that the full-length, membrane-anchored bovine scavenger receptors are synthesized as trimers comprising Cys^{83} disulfide-linked dimers, each of which is noncovalently associated with a receptor monomer (Penman et al., 1991). We presume that the reduction-sensitive dimers seen in the unreduced samples were also crosslinked by disulfide bonds at Cys^{83}. Treatment with N-glycanase (*lanes 3 and 6*) sharpened the bands and reduced the apparent masses of s-bSR-I and s-bSR-II to approximately 46 and 35 kDa, respectively. The rather faint 40 kDa band in lane 6 was presumably due to incomplete deglycosylation of s-bSR-II (see below). These N-glycanase-mediated shifts in mass were similar to those previously observed for the full-length membrane-anchored receptors (Penman et al., 1991). Thus, conversion of the receptors to secreted forms did not significantly alter the extent of their N-glycosylation.

To determine how many of the 7 potential N-glycosylation sites on the receptors (Kodama et al., 1990, Rohrer et al., 1990) were occupied with oligosaccharides, we examined the time course of type II secreted receptor digestion by N-glycanase. Figure 2.3 shows that the electrophoretic mobility of

the receptor increased in discrete steps as a function of time of digestion with N-glycanase. There were 5 intermediate forms detected as the apparent mass of the mature form of the receptor decreased from 68.7 kD (*lane 1*) to 35 kD (*lane 15*). While there are several possible interpretations of this result, the simplest is that 6 of the 7 potential N-glycosylation sites were occupied (Green et al., 1985).

The ligand binding activities of the secreted receptors were examined using a solid-phase bead-binding assay. Sepharose beads crosslinked to either scavenger receptor ligands (poly G, poly I, or M-BSA) or to control molecules (poly C or native BSA) were incubated at 4° C overnight with [^{35}S]methionine-labeled conditioned media from either transfected or untransfected cells. Proteins tightly associated with the beads were eluted and reduced by boiling in electrophoresis sample buffer and visualized after electrophoresis by autoradiography. Figure 2.4 shows the results of this assay for conditioned media from CHO[s-bSR-I] (left), CHO[s-bSR-II] (center), and untransfected CHO (right) cells. The conditioned media contained a large number of [^{35}S]methionine-labeled, secreted proteins (not shown). The secreted type I scavenger receptor, which represented only a small fraction of the all of the labeled and secreted proteins (not shown), bound efficiently to beads bearing the ligands poly G ("pG", lane 1), poly I ("pI", lane 3), and M-BSA (lane 5). Under the conditions of these experiments, the poly G and poly I beads bound virtually all of the labeled receptor, whereas the M-BSA beads bound most, but not all of the active receptor molecules (not shown). In contrast, there was little or no receptor binding to the control poly C and BSA beads (lanes 2 and 4). Analogous results were observed for the bead binding of s-bSR-II (lanes 6-10, center). Thus, the secreted forms of the scavenger receptors exhibited the distinctive ligand-binding specificity which characterizes the membrane-anchored, full-length forms.

Unexpectedly, the conditioned media from the transfected cells contained two additional species -- pA and pB -- which bound to the beads bearing scavenger receptor ligands, but not to beads linked to the control molecules. The pA and pB molecules, but not the secreted scavenger receptors, were also found in the media from untransfected CHO cells (lanes 11-15, right). We have observed clonal variations in the amounts of pA and pB in the conditioned media from CHO cells. The binding properties of pB, which often resolved into 2 major bands, were similar, but not identical, to those of the scavenger receptors (see below and unpublished data). Additional experiments will be required to characterize these molecules in greater detail.

To examine further the ligand-binding specificity of the secreted scavenger receptors, we determined the capacity of a variety of water-soluble scavenger receptor ligands and their negative controls to compete with M-BSA beads for receptor binding. Figure 2.5 shows that relatively high concentrations of poly G (100 μg/ml), M-BSA (400 μg/ml), and AcLDL (400 μg/ml) prevented

both type I and type II secreted receptors from binding to M-BSA beads, while poly C, BSA, and LDL at corresponding concentrations did not block binding. Secreted scavenger receptors were not detected in the medium from untransfected cells (lane 1). Again, the ligand-binding specificity observed was entirely consistent with that previously reported for the full-length, cell-surface forms of the receptors. In other experiments (not shown), we observed that the scavenger receptor ligands fucoidin and dextran sulfate could fully block, and lipid IV_A could less effectively block secreted receptor binding in this assay. Heparin and chondroitin sulfate, two control polysaccharides which are not scavenger receptor ligands, did not inhibit this bead binding (data not shown).

Figure 2.6 shows the concentration dependence of binding inhibition by soluble poly I. These quantitative binding values were determined using a Molecular Dynamics PhosphorImager. The ID_{50} for poly I was less than 1 μg/ml for s-bSR-I, and between 1 and 2.5 μg/ml for s-bSR-II. These values are similar to those determined for the inhibition by poly I of receptor-mediated ^{125}I-AcLDL degradation by CHO cells expressing full-length type I or type II bovine scavenger receptors (Pearson et al., 1992 and A. Pearson, Y. Ekkel and M. Krieger, unpublished data). Similar quantitative results were obtained using M-BSA and AcLDL as soluble competitors (data not shown).

Figure 2.7 shows that s-bSR-I binding to poly G beads was pH dependent. Binding decreased to background levels as the pH was lowered from 7.5 to 5.0. This loss in activity was reversible; after an overnight incubation at pH 5.5, binding activity was restored to control values when the pH was increased to 7.5 (not shown). Similar pH dependence was observed for secreted receptor binding to M-BSA beads (not shown), suggesting that the pH dependence was an intrinsic property of the receptor and not the ligands. Ligand binding to scavenger receptors expressed by bovine alveolar macrophages has previously been reported to be pH-sensitive (Naito et al., 1991). Taken together, these bead-binding assays established that the soluble scavenger receptors exhibited quantitative as well as qualitative ligand-binding properties which are similar, if not identical, to those of the full-length, membrane-anchored forms.

The preparation of soluble scavenger receptors provided an opportunity to examine in greater detail the structure of the receptor. For example, the oligomerization state of s-bSR-II was assessed using gel filtration and sedimentation analysis to compare the molecular weight of the intact receptor, M_r, to that of the receptor monomers. The estimated Stokes radius, derived from Superose 6 chromatography (Figure 2.8A), was 10.2 nm . The receptor eluted from the column between thyroglobulin (8.58 nm, standard #3) and fibrinogen (10.7 nm, standard #2). As can be seen in Figure 2.8B, the receptor eluted as a rather broad peak, suggesting that there was heterogeneity in the preparation.

Figure 2.8C shows that the sedimentation coefficient determined by centrifugation through a glycerol gradient was 4.65 S, with the receptor running near the BSA standard (4.31 S, standard #a). The bulk of the receptor was detected in two fractions of the gradient (not shown). In order to calculate a molecular weight (M_r) from the Stokes radius and the sedimentation coefficient, it is necessary to measure or to estimate the partial specific volume of the protein (see equation 1). The partial specific volume of the polypeptide component of the receptor can be calculated (Hinz, 1986) from its sequence to be 0.717 ml/g. This value, however, does not include contributions of the hydroxyl groups on the proline and lysine side chains of the scavenger receptors' collagenous domains (Penman et al., 1991), and, more importantly, does not include the substantial contribution of the N-linked oligosaccharides. The addition of the contributions from these components reduces the calculated value for the partial specific volume. For example, the partial specific volume of a typical N-linked oligosaccharide, $GlcNAc_5Man_3Gal_2Sia_2$, in CHO cells is 0.629 ml/g (Hinz, 1986). Assuming that there are 6 such chains (see above), the partial specific volume of the receptor would be 0.689 ml/g. Substituting this and the experimentally determined values of r_s and $s_{20,w}$ into equation 1 (see Materials and Methods), we find that M_r is 173 kD.

The oligomerization state of the receptors can be determined by dividing M_r by the mass of a single subunit. The apparent mass of the s-bSR-II monomers determined by gel electrophoresis was 68.7 kD. However, glycoproteins run with anomalously high apparent molecular weights on SDS gels compared to unglycosylated standards because of their reduced binding of SDS (Segrest et al., 1972). To calculate the oligomerization state, we estimated the molecular weight of the monomeric form to be 49.2 kD, the sum of the molecular weight of the N-glycanase-digested form (35 kD) determined by gel electrophoresis and the estimated weight of 6 typical N-linked oligosaccharide chains (14.2 kD). The calculated oligomerization state is 3.5 (173/49.2). Although there is some uncertainty in this value because of the assumptions used to account for the receptor's oligosaccharide groups, these results suggest that the soluble receptor was trimeric. This conclusion is consistent with our previous results, which show that the membrane-anchored, full-length bovine scavenger receptors are trimeric (Kodama et al., 1988; Penman et al., 1991). It also indicates that, under the conditions of these experiments, the receptor formed very little, if any, higher-order oligomers. Preliminary crosslinking experiments with ethylene glyco *bis*(succinimidyl-succinate) also suggested that s-bSR-II and s-bSR-I were trimeric (data not shown).

Information about the shape of the receptor can also be derived from these data. Based on the molecular weight of the receptor (173 kD), its Stokes radius (10.2 nm), and an assumed partial specific volume of 0.689 ml/g, the frictional ratio (f/f_0) for s-bSR-II was calculated to be 2.82 (see equation 2). This indicates that s-bSR-II is an elongated molecule. If the receptor is assumed to

be cylindrical in shape, its length would be 53 times its diameter; whereas if it were a prolate ellipsoid, its semimajor axis would be 45 times as long as its semiminor axis (see equations 3 and 4). These values are indicative of a highly elongated molecule and are consistent with our previously proposed models of scavenger receptors (Kodama et al., 1990, Rohrer et al., 1990, Krieger, 1992).

Unfortunately, we were unable to determine the Stokes radius and sedimentation coefficient for s-bSR-I. Recovery of the type I molecule from both the chromatographic column and the glycerol gradients was extremely low and reliable values could not be obtained. The reasons for the low recoveries are not clear at this time.

The metabolically labeled, secreted scavenger receptors and the solid-phase bead-binding assay provide convenient tools to search for additional scavenger receptor ligands. In an initial application of this approach, we examined secreted scavenger receptor binding to asbestos fibers. We performed a solid-phase binding assay in which the Sepharose-ligand beads were replaced with insoluble crocidolite asbestos fibers. Figure 2.9 shows that the incubation of metabolically labeled conditioned medium from CHO[s-bSR-I] cells with asbestos resulted in the binding of s-bSR-I and pB to the fibers (lane 1). Furthermore, the scavenger receptor ligands poly G, poly I, fucoidin, dextran sulfate, AcLDL, and M-BSA all inhibited receptor binding (*lanes 2, 3, 5, 6, 9, 10, and 11*), while the control molecules poly C, LDL, heparin, and chondroitin sulfate did not (*lanes 4, 7, 8, and 12*). The ability of scavenger receptor ligands to block specifically receptor binding to asbestos and the intrinsic negative charge on asbestos suggest that asbestos binding may have occurred at the site(s) in the collagenous domain of the receptor responsible for binding other receptor-ligands (Kodama et al., 1991; Acton et al., 1992; Doi, T., Higashino, K., Kurihara, Wada, Y., Uesugi, S., Imanishi, T., Kawabe, Y., Matsumoto, A., Itakura, H., Yazaki, Y., Nakamura, H., and Kodama, T., manuscript submitted for publication). Asbestos-binding experiments with s-bSR-II gave qualitatively similar results; however, we observed significantly less s-bSR-II binding to the asbestos than s-bSR-I binding (not shown). Additional studies with macrophages *in vitro* and *in vivo* will be required to determine the physiological relevance of this binding.

Discussion

In the current studies, we have examined the structures and binding properties of secreted forms of the type I and type II bovine scavenger receptors. Expression vectors for these proteins were constructed by substituting sequences encoding the myelin-associated glycoprotein leader sequence for those encoding the cytoplasmic and transmembrane domains of the full-length receptors (see Figure 2.1). Stable CHO cell transfectants expressing either the type I or type II secreted receptors were isolated and metabolically labeled conditioned media from these transfectants were used as sources of these soluble receptors.

Both the type I and type II secreted bovine scavenger receptors retained the distinctive, high-affinity, broad-specificity ligand binding which characterizes their full-length, membrane anchored counterparts (Goldstein et al., 1979; Brown and Goldstein, 1983; Kodama et al., 1990; Rohrer et al., 1990; Freeman et al., 1991; Krieger, 1992). Ligand binding to the type I secreted receptor was sensitive to low pH (5.5) as has been observed for scavenger receptor activity in bovine macrophages (Naito et al., 1991). The mass of the intact type II secreted receptor estimated from hydrodynamic data was 173 kD. The mass of the individual monomeric subunits calculated from the experimentally determined mass of the N-glycanase-deglycosylated receptor and an estimate of the masses of the receptor's 6 N-linked oligosaccharides was 49.2 kD. Thus, the ratio of the oligomer to subunit masses was 3.5. In conjunction with electrophoretic analysis of unreduced and chemically crosslinked receptors, this ratio suggests that, as is the case with the membrane anchored receptor (Penman et al., 1991), the secreted type II receptors are trimers comprising disulfide-crosslinked dimers noncovalently associated with monomers. The type I receptors were also composed of monomers and dimers. Thus, the cytoplasmic and transmembrane domains in the full-length receptors apparently do not play an essential role in establishing the ligand-binding properties of these receptors and are not required for the assembly of the receptors into trimers.

At the very low concentrations of receptor used in the gel filtration and gradient centrifugation experiments reported here, there was no evidence of the formation of higher-order oligomers of trimers. This finding was somewhat surprising because scavenger receptors, even though they are normally integral membrane proteins, bear a striking resemblance to the members of a group of secreted oligomeric mammalian proteins which all appear to participate in host defense. Members of this group include lung surfactant apoprotein A and the serum proteins complement factor C1q, mannose binding protein, and conglutinin. All of these proteins have relatively short collagenous domains, are elongated, and form higher-order oligomers in which multiple trimeric collagenous stalks support globular head groups (Thiel and Reid, 1989). The similarities between the scavenger receptor and C1q extend to

their ligand-binding properties. C1q has previously been reported to bind polyanions (Loos, 1983) and we show in the accompanying paper (Acton et al., 1992) that the collagenous domain of C1q exhibits broad ligand binding specificity which is remarkably similar, but not identical, to that of scavenger receptors. Additional studies will be required to determine if, at higher receptor concentrations, the secreted or full-length scavenger receptors form higher-order oligomers of trimers or if ligand binding affects the oligomerization state of the receptors.

The frictional ratio (f/f_o) of the type II secreted scavenger receptor was 2.82. This value indicates that the receptor is highly elongated (f/f_o=1 for a sphere). If it were cylindrical or ellipsoidal, it would be approximately 45-53 times as long as it is wide. By comparison, the frictional ratio of the extended fibrinogen molecule is 2.37. We previously suggested that the scavenger receptors would be elongated, because approximately 49% of the extracellular residues of the type I receptor and 68% of the type II receptor are predicted to fold into fibrous α-helical coiled-coil and collagenous domains (Kodama et al., 1990; Rohrer et al., 1990; Ashkenas et al., 1992; Krieger, 1992). The preparation of substantial amounts of secreted scavenger receptors should permit additional biochemical and biophysical analyses of the receptors' binding properties and structures, including electron microscopic and X-ray diffraction studies.

The metabolically labeled, secreted scavenger receptors and the solid-phase bead-binding assay provide convenient tools to search for additional, potentially physiologically or pathophysiologically relevant ligands. This is especially true for water-insoluble ligands because the insoluble ligands can be used in place of beads in the binding assay. In an initial application of this approach, we investigated the ability of secreted scavenger receptors to bind to fibers of crocidolite asbestos. After inhalation, the majority of the asbestos which is deposited in the lungs is initially accumulated by alveolar macrophages (Rom et al., 1991). Exposure to asbestos can result in asbestosis, malignant mesothelioma, and lung cancer (Mossman and Gee, 1989). Holian and Scheule initially raised the possibility that scavenger receptors might play a role in macrophage recognition of negatively charged, silicate-based asbestos fibers and other particulates in the lung (Holian and Scheule, 1990). We found that crocolidite asbestos, a particularly pathogenic form of asbestos, bound specifically to secreted scavenger receptors and that this binding was inhibited by scavenger receptor ligands, but not their negative controls. This finding raises the possibility that scavenger receptors play a role in the recognition and uptake of asbestos by alveolar macrophages. Additional *in vitro* and *in vivo* studies will be required to test this hypothesis. Similar solid-phase binding assays using soluble scavenger receptors should prove useful in helping to identify other scavenger receptor ligands. Also, the use of secreted

forms of the scavenger receptors in other *in vitro* and *in vivo* experiments may help further define their physiological functions.

Acknowledgments - We thank Dr. Joseph Brain for providing the crocolidite asbestos and for helpful discussions, Drs. P. Johnson and R. Hynes for providing the plasmid encoding the MAG leader sequence and for advice, Dr. Dan Levin for assistance with the Isco Gradient Collector, Alan Pearson and other members of our laboratory for helpful discussions, and Julia Khorana for help with preparation of the figures.

Figure 2.1. Domain structures of full-length and secreted scavenger receptors

The domain structures of the full-length type I (bSR-I) and type II (bSR-II) bovine scavenger receptors were deduced from their cDNA sequences (Kodama et al., 1990; Rohrer et al., 1990; Ashkenas et al., 1992). These domains include a short cytoplasmic domain (Cyto.), a single transmembrane segment (TM), a spacer domain, a region predicted to form an α-helical coiled-coil, and a collagenous domain with 24 Gly-X-Y repeats. The type I receptor has a 110 residue long C-terminal cysteine rich domain (SRCR), while the type II receptor has a 6 amino acid C-terminus (solid boxes). Secreted forms of these receptors (s-bSR-I, s-bSR-II) differ from the full-length forms in that the cytoplasmic and transmembrane domains were replaced with the myelin-associated glycoprotein (MAG) leader sequence (shaded boxes), and Lys^{77} was converted to an Arg-Gly dipeptide (dot at the N-terminus of the spacer domains).

Structures of Wild-type and Secreted Bovine Scavenger Receptors

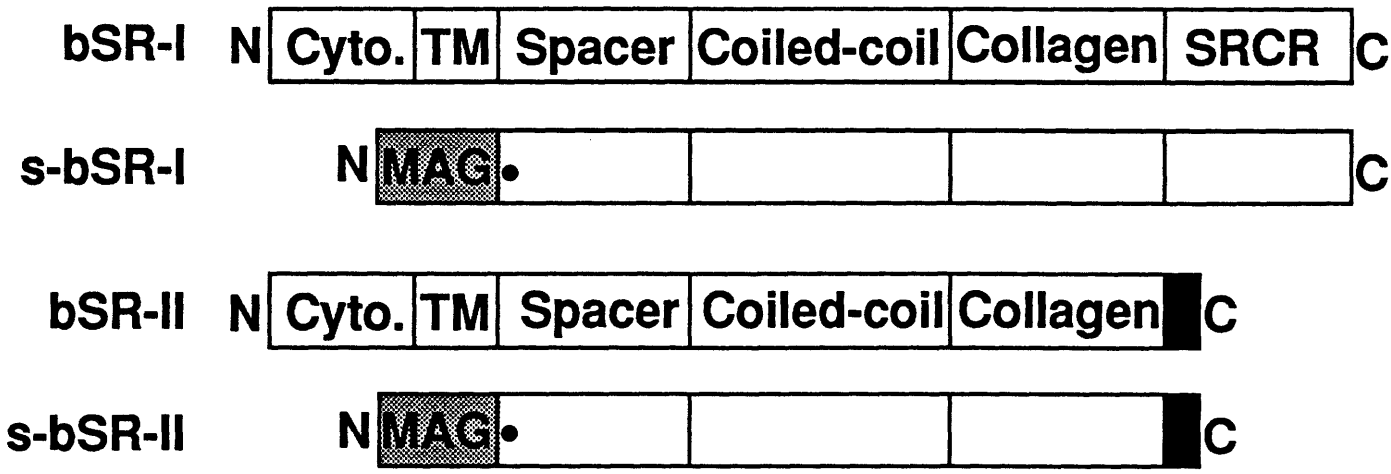


Figure 2.2. Immunoprecipitation of secreted bovine scavenger receptors expressed by transfected CHO cells

The type I (CHO[s-bSR-I]) and type II (CHOI[s-bSR-II]) secreted bovine scavenger receptor-expressing cells were plated on day 0 in 3 ml of medium C into 6 well dishes (150,000 cells/well). On day 2, the cells were labelled for 5 hr with 80 μCi/ml of [^{35}S]methionine and then the radiolabelled media were subjected to immunoprecipitation using a rabbit anti-spacer domain anti-peptide antibody and protein A sepharose. The samples were dissolved in either reducing (lanes 1 and 4) or non-reducing (lanes 2 and 5) sample buffer, or were subjected to treatment with N-glycanase (18 hr, .375 units) prior to addition of reducing sample buffer (lanes 3 and 6) as described in "Materials and Methods". Samples were then subjected to 3-10% polyacrylamide gel electrophoresis and autoradiography as described in "Materials and Methods".

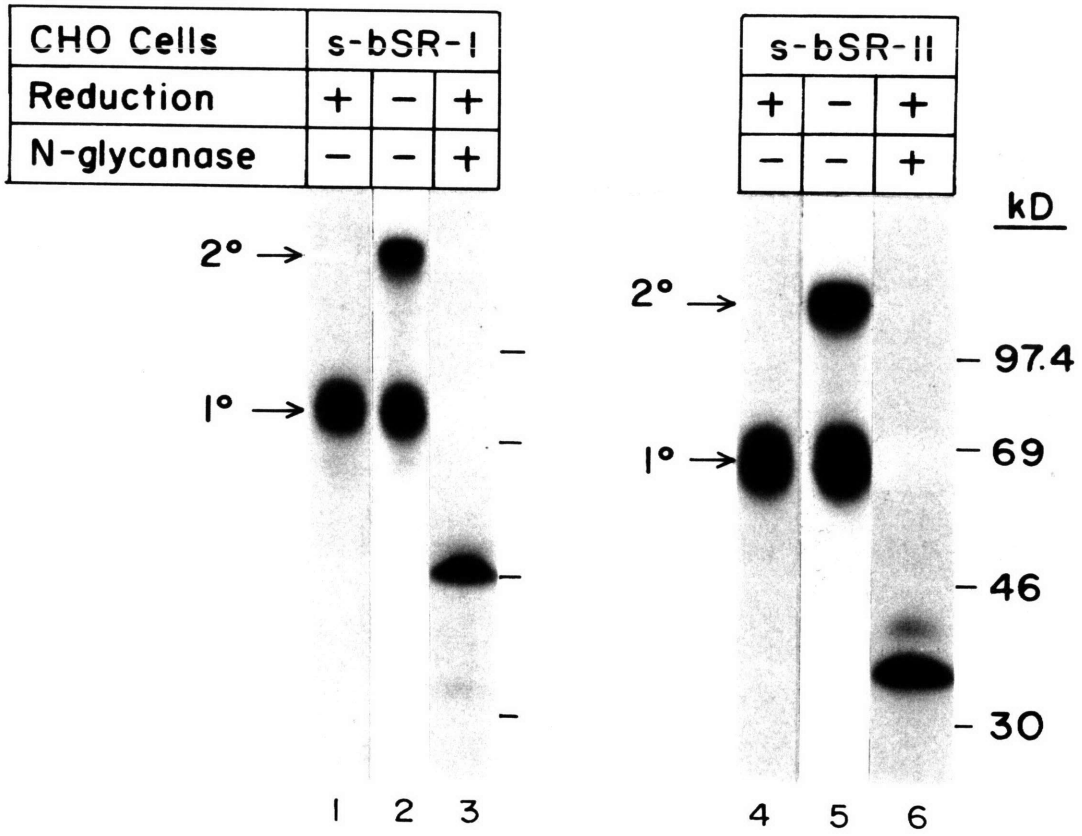

CHO Cells
s-bSR-I
Reduction
N-glycanase
+
−
+
−
−
+
2°
1°
s-bSR-II
kD
97.4
69
46
30
1
2
3
4
5
6

Figure 2.3. Kinetics of s-bSR-II digestion by N-glycanase.

Immunoprecipitates of [^{35}S]methionine-labelled s-bSR-II were prepared as described in the legend of Figure 2. The samples were digested at 37° with the indicated amounts of N-glycanase for the indicated times and subsequently analyzed by reducing 10% polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods.

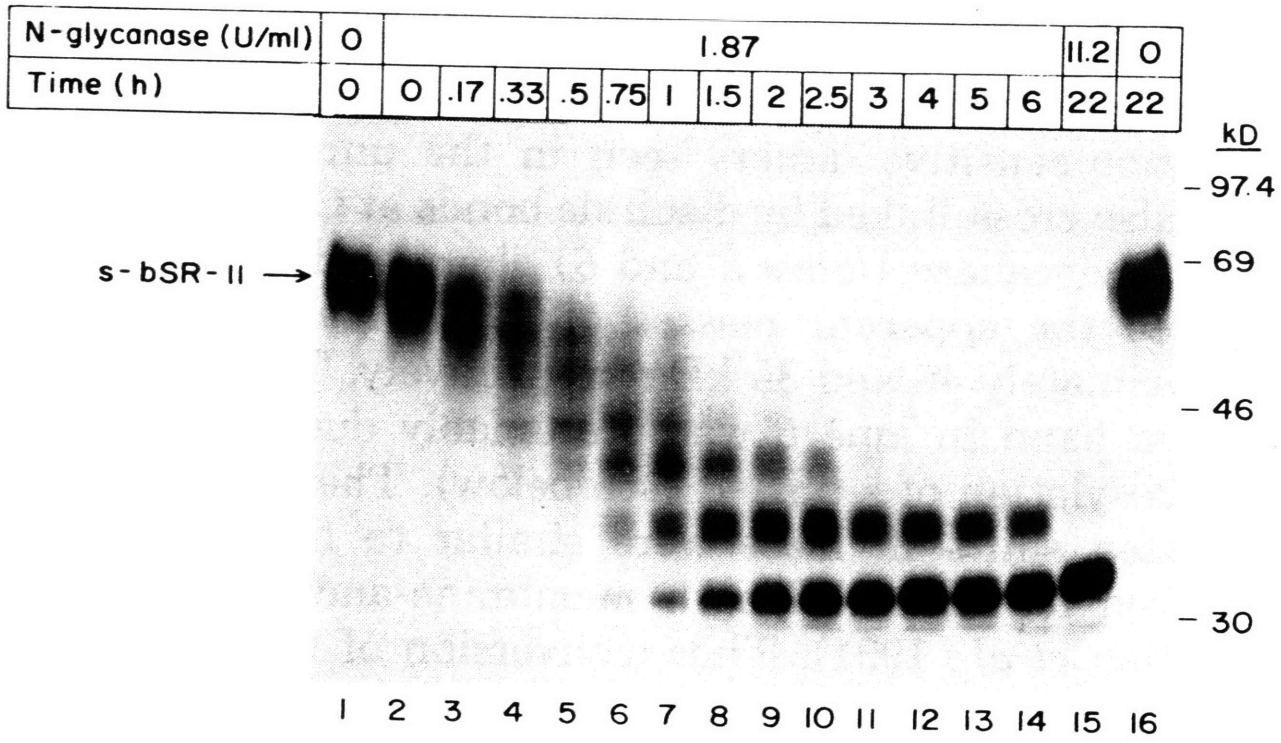

N-glycanase (U/ml)
0
1.87
11.2
0
Time (h)
0
0
.17
.33
.5
.75
1
1.5
2
2.5
3
4
5
6
22
22
kD
97.4
69
46
30
s-bSR-II
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 2.4. Binding of secreted bovine scavenger receptors to ligands coupled to Sepharose beads.

Type I (CHO[s-bSR-I], left) and type II (CHO[s-bSR-II], center) secreted bovine scavenger receptor-expressing cells, as well as untransfected CHO cells (right), were plated on day 0 in 3 ml of either medium B (untransfected cells) or medium C (transfected cells) into 6 well dishes (150,000 cells/well). On day 2, the cells were labelled for 5 hr with [^{35}S]methionine (80 μCi/ml) as described in Materials and Methods. Aliquots of the radiolabelled media were incubated overnight at 4°C with buffer B and sepharose beads coupled to poly G (pG), poly C (pC), poly I (pI), bovine serum albumin (BSA), or maleylated-BSA (M-BSA) as indicated. The beads were then washed, and the bound components were eluted by boiling with reducing electrophoresis sample buffer and then subjected to 10% polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods.

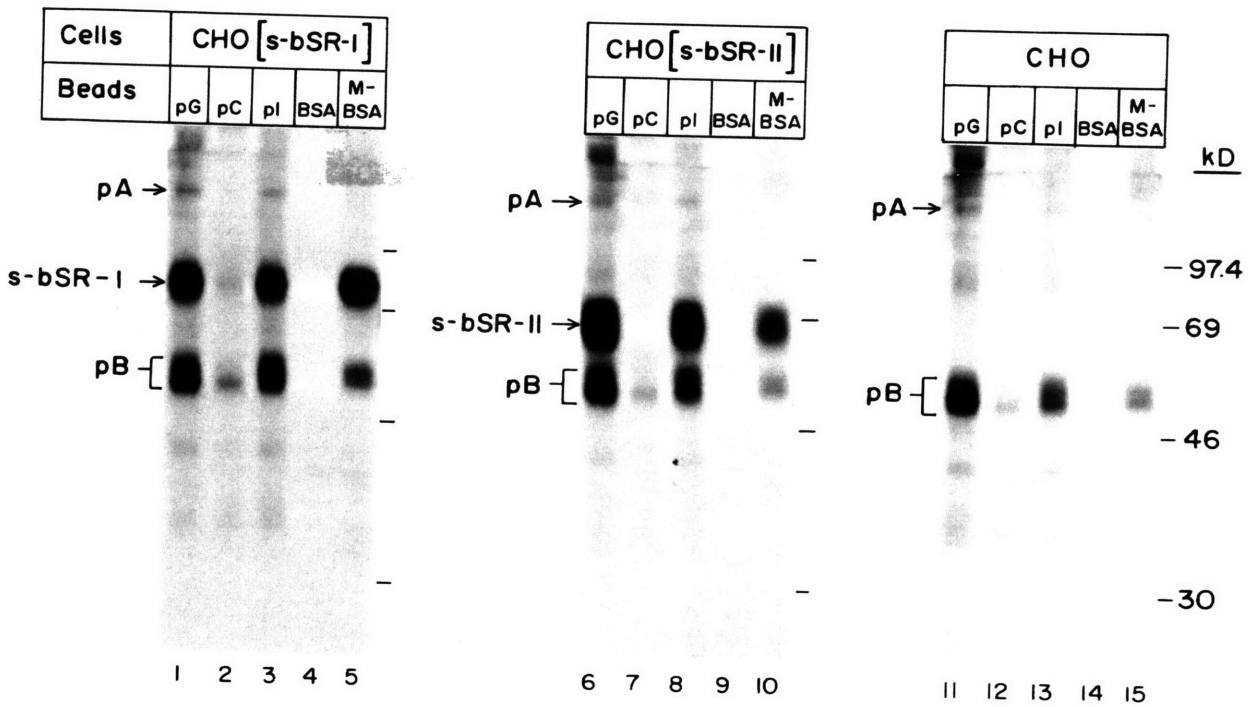

Cells
CHO [s-bSR-I]
Beads
pG
pC
pI
BSA
M-BSA
pA
s-bSR-I
pB
1 2 3 4 5
CHO [s-bSR-II]
pG
pC
pI
BSA
M-BSA
pA
s-bSR-II
pB
6 7 8 9 10
CHO
pG
pC
pI
BSA
M-BSA
kD
pA
pB
97.4
69
46
30
11 12 13 14 15

Figure 2.5. Inhibition of secreted bovine scavenger receptor binding to M-BSA sepharose beads by soluble competitors.

[^{35}S]Methionine-labelled conditioned media from CHO[s-bSR-I], CHO[s-bSR-II] and CHO cells were prepared as described in the legend of Figure 4 and were incubated overnight at 4°C with M-BSA sepharose beads and the following additions: none (-), poly G (pG, 100 μg/ml), poly C (pC, 100 μg/ml), bovine serum albumin (BSA, 400 μg/ml), maleylated BSA (M-BSA, 400 μg/ml), low density lipoprotein (LDL, 400 μg protein/ml), or acetylated LDL (AcLDL, 400 μg protein/ml). The beads were then washed, and the bound components were eluted by boiling with reducing electrophoresis sample buffer and then subjected to 10% polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods.

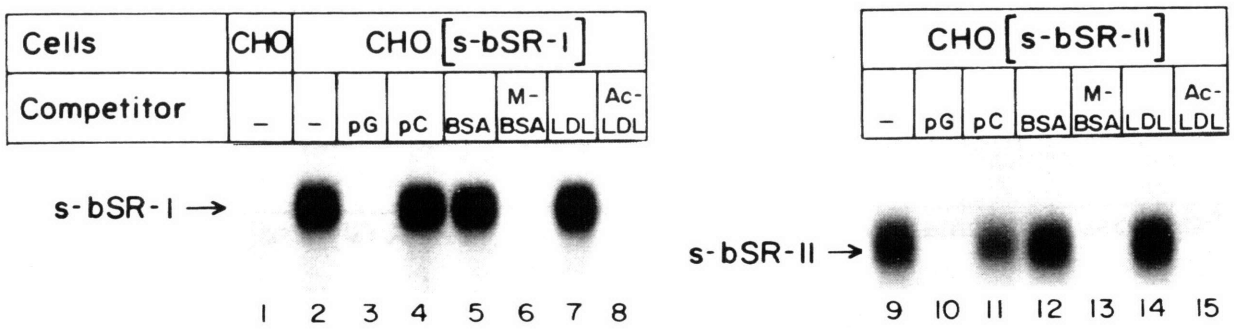

Cells
CHO
CHO [s-bSR-I]
CHO [s-bSR-II]
Competitor
–
–
pG
pC
BSA
M-BSA
LDL
Ac-LDL
–
pG
pC
BSA
M-BSA
LDL
Ac-LDL
s-bSR-I →
s-bSR-II →
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15

Figure 2.6 Concentration-dependence of the inhibition by poly I of secreted bovine scavenger receptor binding to M-BSA sepharose beads.

[^{35}S]Methionine-labelled conditioned media from CHO[s-bSR-I] and CHO[s-bSR-II] cells were prepared as described in the legend of Figure 4 and incubated overnight at 4°C with M-BSA sepharose beads, buffer B, and the indicated concentrations of poly I. The beads were then washed, and the bound components were eluted by boiling with reducing electrophoresis sample buffer and then subjected to 10% polyacrylamide gel electrophoresis as described in Materials and Methods. The intensities of the secreted receptor bands were quantified with a Molecular Dynamics PhosphorImager.

Inhibition of s-bSR Binding to M-BSA Beads by Poly I

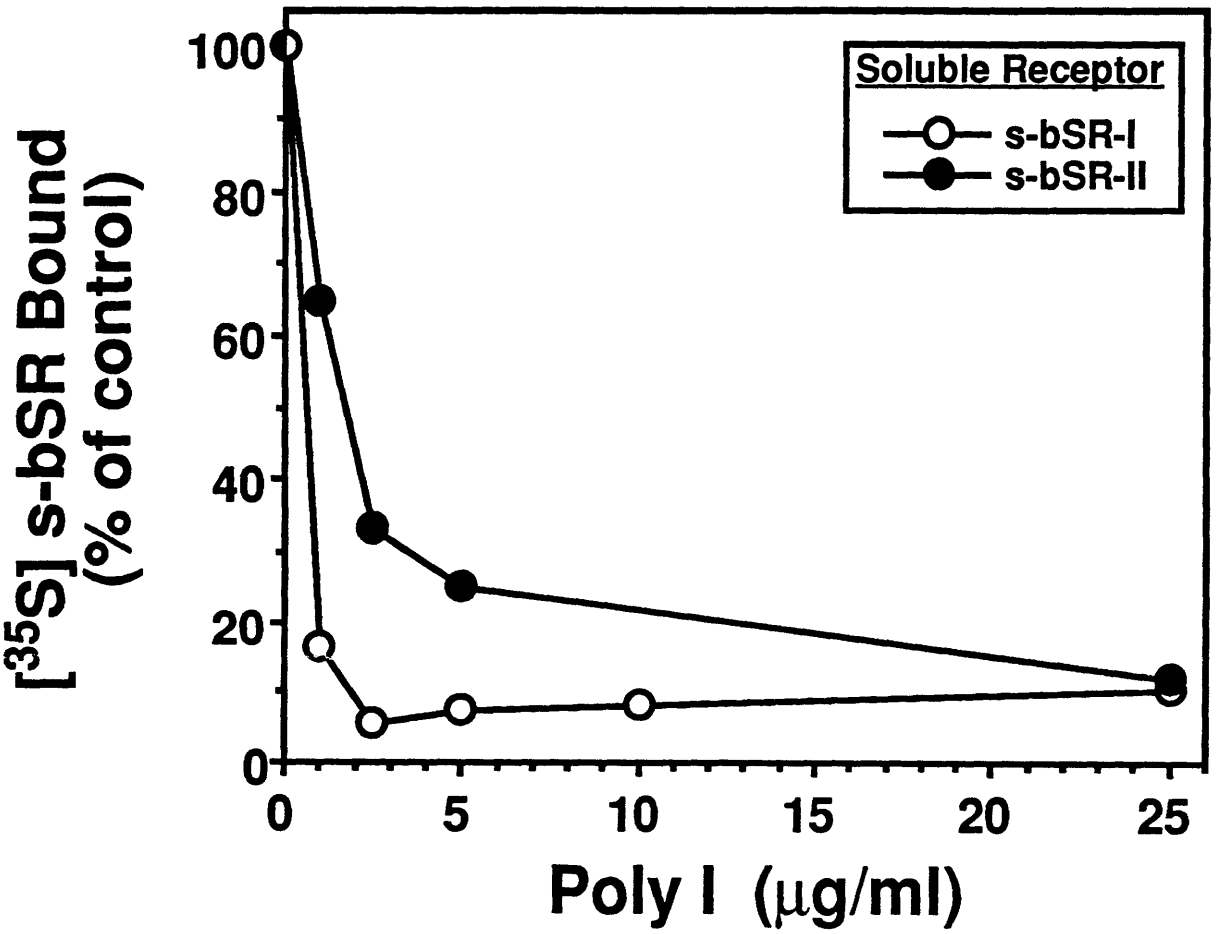


Figure 2.7. Effects of pH on s-bSR-I binding to poly G sepharose beads.

Aliquots of [^{35}S]methionine-labelled conditioned medium from CHO[s-bSR-I] cells were prepared as described in the legend of Figure 4 and the pH of each was adjusted to the indicated values by adding buffer (50 mM 2-[N-morpholino]ethanesulfonic acid, 50 mM Tris, 50 mM sodium acetate, 1 mM $CaCl_2$, 150 mM NaCl) of the appropriate pH. After addition of BSA (final concentration: 0.5 mg/ml), the samples were incubated overnight at 4°C with poly G beads and binding of the secreted receptor to the beads was evaluated by electrophoresis and autoradiography as described in Materials and Methods.

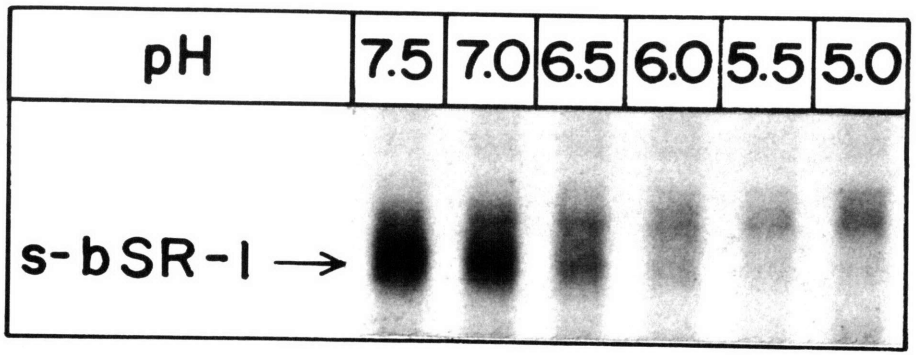
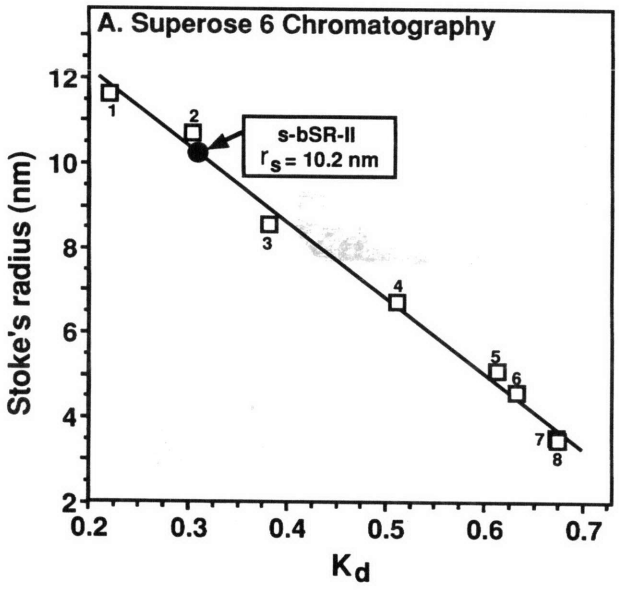
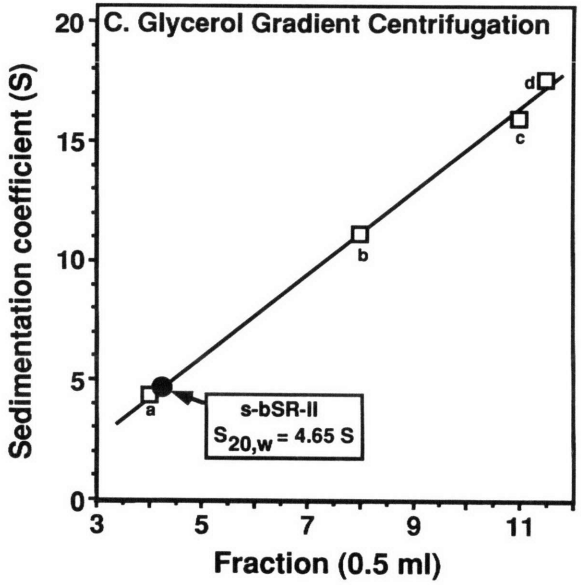

pH
7.5
7.0
6.5
6.0
5.5
5.0
s-bSR-1 →

Figure 2.8. Determination of the Stokes radius (r_s) and sedimentation coefficient of s-bSR-II by gel filtration and glycerol gradient sedimentation.

[^{35}S]Methionine-labelled conditioned medium from CHO[s-bSR-II] cells was prepared as described in the legend of Figure 4 and 200 µl aliquots were subjected to either superose 6 gel filtration chromatography (panels A and B) or glycerol gradient centrifugation (panel C) as described in Materials and Methods. Panel A: For chromatography, the calibration standards and their Stokes radii were: 1, IgM, 11.62 nm; 2, fibrinogen, 10.7 nm; 3, thyroglobulin, 8.58 nm; 4, apoferritin, 6.73 nm; 5, catalase, 5.12 nm; 6, alcohol dehydrogenase, 4.6 nm; 7, transferrin, 3.55 nm; and 8, bovine serum albumin, 3.48 nm. The Stokes radii of the calibration standards are plotted against their distribution coefficients, K_d, where $K_d = (V_e - V_o)/(V_t - V_o)$ and V_e = elution position of the sample, V_o = void volume of column, and V_t = total volume of the column. The K_d of s-bSR-II is indicated by the solid circle. Panel B shows a portion of the elution profile measured by poly G sepharose bead binding, electrophoresis and autoradiography as described in Materials and Methods. Panel C: For glycerol gradient centrifugation, the calibration standards and their sedimentation coefficients ($s_{20,w}$) were: a, BSA, 4.31; b, catalase, 11.35; c, β-galactosidase, 15.93; and d, apoferritin, 17.6. The location of s-bSR-II in the gradient (solid circle) was determined by immunoprecipitation, electrophoresis, and autoradiography as described in Materials and Methods.


A. Superose 6 Chromatography
Stoke's radius (nm)
12
10
8
6
4
2
0.2
0.3
0.4
0.5
0.6
0.7
K_d
s-bSR-II
r_s = 10.2 nm
1
2
3
4
5
6
7
8


C. Glycerol Gradient Centrifugation
Sedimentation coefficient (S)
20
15
10
5
0
3
5
7
9
11
Fraction (0.5 ml)
s-bSR-II
$S_{20,w}$ = 4.65 S
a
b
c
d

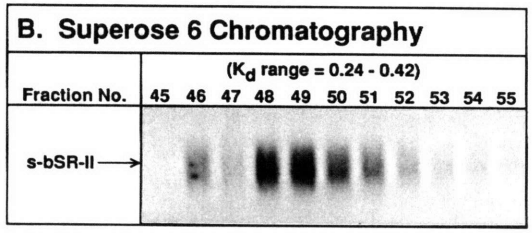
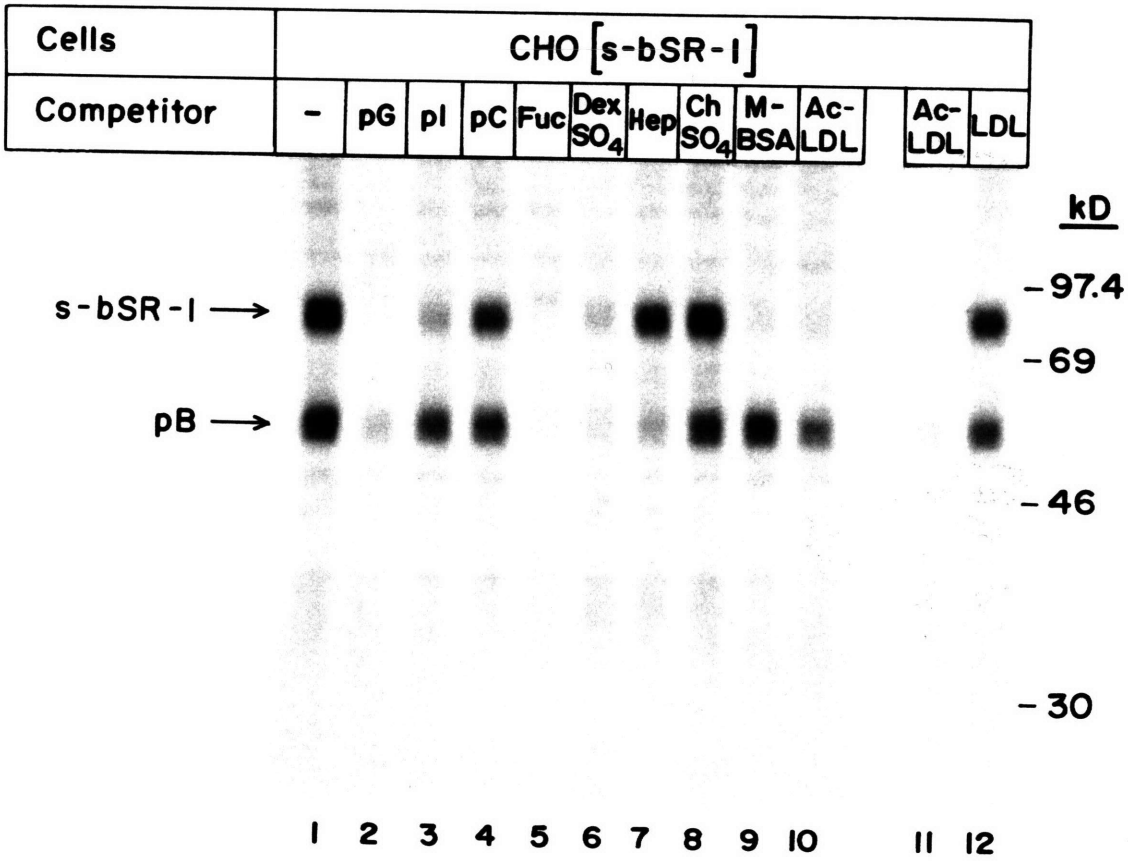

B. Superose 6 Chromatography
(K_d range = 0.24 - 0.42)
Fraction No.
45 46 47 48 49 50 51 52 53 54 55
s-bSR-II

Figure 2.9. Binding of s-bSR-I to crocolidite asbestos.

[^{35}S]Methionine-labelled conditioned medium from CHO[s-bSR-I] cells was prepared as described in the legend of Figure 4 and incubated overnight at 4°C with crocolidite asbestos (50 μg/tube) and the following soluble competitors: none (-), poly G (pG, 25 μg/ml), poly I (pI, 25 μg/ml), poly C (pC, 25 μg/ml), fucoidin (Fuc, 300 μg/ml), dextran sulfate (Dex SO_4, 300 μg/ml), heparin (Hep, 300 μg/ml), chondroitin sulfate (Ch SO_4, 300 μg/ml), maleylated BSA (M-BSA, 300 μg/ml), low density lipoprotein (LDL, 300 μg protein/ml), or acetylated LDL (AcLDL, 300 μg protein/ml). Ribonuclease inhibitor (40 U/ml) was added to the poly C incubations to prevent hydrolysis during the incubation. The asbestos fibers were then washed, and the bound components were eluted by boiling with reducing electrophoresis sample buffer and then subjected to 10% polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. The autoradiograms shown in lanes 1-10 and 11-12 were from two separate experiments.


Cells
CHO [s-bSR-I]
Competitor
-
pG
pI
pC
Fuc
Dex SO4
Hep
Ch SO4
M-BSA
Ac-LDL
Ac-LDL
LDL
kD
s-bSR-I
pB
97.4
69
46
30
1 2 3 4 5 6 7 8 9 10 11 12

Chapter 3

The type I macrophage scavenger receptor binds to Gram-positive bacteria and recognizes lipoteichoic acid

The physiological role of the class A scavenger receptors remains an open question. Among the veritable plethora of scavenger receptor ligands (see Table 1.1 in the introduction), perhaps the most intriguing is lipopolysaccharide (LPS), the ubiquitous endotoxin located on the surface of Gram-negative bacteria (Hampton et al., 1991). The finding that SR-A binds LPS raised the possibility that not only could the receptors play a role in clearance of free LPS from the serum, but they might also potentially recognize bacteria directly by means of this or related ligands. This chapter contains a series of experiments designed to address this question. It describes the use of metabolically labeled secreted scavenger receptor as a probe to study receptor binding to intact bacteria and to identify potential bacterial surface components involved in this binding. This work was published in its entirety in 1994 in *Proceedings of the National Academy of Science USA* **91**, 1863-1867. The only modifications which have been made are that figures have been re-numbered with the prefix "3" and the references have been merged with those of other chapters in the "references" section of this thesis.

The work in this paper was carried out in collaboration with Dr. Dana Dunne and Dr. Jordan Greenberg, who were postdoctoral associates in Dr. Keith Joiner's laboratory. I did some preliminary experiments evaluating whether secreted scavenger receptors could bind Gram-negative bacteria -- perversely enough, they apparently do not bind, despite the fact that the SRs recognize endotoxin. Perhaps the endotoxin liposomes present the LPS in a different context than the bacterial surface. I also performed the lipoteichoic acid binding assays shown in Figure 3.3, and provided considerable advice and assistance in getting the bacterial binding assays to work. The initial draft of the paper was written by Dr. Dunne, however I provided considerable input into the final published document.

The Type I Macrophage Scavenger Receptor Binds to Gram-positive Bacteria and Recognizes Lipoteichoic Acid

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Classification: Microbiology
Communicated by Maclyn McCarty

Abbreviations: LTA, lipoteichoic acid; poly G, poly guanosine; M-BSA, maleylated bovine serum albumin; CHO, Chinese Hamster Ovary cells; s-bSR-I, soluble type I bovine scavenger receptor.

Abstract

Macrophage scavenger receptors exhibit unusually broad binding specificity for polyanionic ligands and have been implicated in atherosclerosis and a variety of host defense functions. Using a radiolabelled, secreted form of the type I bovine macrophage scavenger receptor in an *in vitro* binding assay, we have found that this receptor binds to intact Gram-positive bacteria, including *Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus aureus, Enterococcus hirae, and Listeria monocytogenes.* Competition binding studies using purified lipoteichoic acid (LTA), an anionic polymer expressed on the surface of most Gram-positive bacteria, show that LTAs are scavenger receptor ligands and probably mediate binding of the receptor to Gram-positive bacteria. LTAs, for which no host cell receptors have previously been identified, are implicated in the pathogenesis of septic shock due to Gram-positive bacteria. Scavenger receptors may participate in host defense by clearing LTA and/or intact bacteria from tissues and the circulation during Gram-positive sepsis. Since scavenger receptors have been previously shown to bind to and facilitate bloodstream clearance of Gram-negative bacterial endotoxin (lipopolysaccharide), these receptors may provide a general mechanism for macrophage recognition and internalization of pathogens and their cell surface components.

Introduction

Macrophage scavenger receptors exhibit unusual broad ligand binding specificities (reviewed in Brown and Goldstein, 1983; Krieger et al., 1993). Their diverse, high affinity, polyanionic ligands include 1) chemically modified proteins, such as acetylated and oxidized low density lipoprotein (LDL) and maleylated bovine serum albumin (M-BSA), but not their unmodified counterparts, 2) certain polysaccharides, such as dextran sulfate, but not chondroitin sulfate, 3) four-stranded, but not one- or two-stranded, polynucleotides, including poly G and poly I, 4) and others, such as anionic phospholipids and crocidolite asbestos. The broad ligand binding specificity of scavenger receptors is mediated by a short positively charged collagenous domain in the extracellular region of the receptor (Doi et al., 1993; Acton et al., 1993). The two isoforms of the macrophage scavenger receptor exhibit similar binding properties, despite the presence of a cysteine rich C-terminal domain of unknown function on the type I isoform which is not present on the type II molecule (Kodama et al., 1990; Rohrer et al., 1990).

The physiologic and pathophysiologic functions of macrophage scavenger receptors have not yet been established with certainty. Scavenger receptor-mediated uptake of modified lipoproteins into macrophages has been suggested to play a key role in the deposition of lipoprotein cholesterol in artery walls during the formation of atherosclerotic plaques (reviewed in Brown and Goldstein, 1983; Steinberg et al., 1989; Krieger, 1992). Because of their broad binding specificity, macrophage scavenger receptors may also participate in host defense activities by recognizing and mediating the endocytosis of a wide variety of pathogenic substances (Krieger et al., 1993; Krieger, 1992). The recent demonstration that scavenger receptors can bind a precursor of Gram-negative bacterial lipid A provided the first support for this proposal (Hampton et al., 1991). These findings suggest that scavenger receptors help define a set of proteins which all contain short collagenous domains and participate in host defense by binding a variety of pathogens (Acton et al., 1993; Sastry and Ezekowitz, 1993). Other members of this family include complement component C1q, mannose binding protein, pulmonary surfactant apoproteins A and D, and conglutinin.

To further investigate the potential role of scavenger receptors in recognizing pathogens, we developed a method for measuring the binding of scavenger receptors to intact bacteria. We report here that a soluble form of the type I bovine scavenger receptor binds to an wide array of Gram-positive bacteria. We also show that lipoteichoic acid (LTA), a virtually ubiquitous Gram-positive bacterial cell surface component, is a scavenger receptor ligand and probably mediates receptor binding to intact bacteria. Thus, scavenger receptors may participate in host defense by clearing LTA and/or Gram-positive bacteria from both tissues and the bloodstream.

Materials and Methods

Buffers and reagents - Buffers used the experiments were as follows: Buffer A - 20 mM Tris base pH 8.0/150 mM NaCl/1 mM $CaCl_2$; Buffer A + BSA - Buffer A containing 2 mg/ml BSA and 0.05% NaN_3; Buffer B - 20 mM Tris base pH 8.0/150 mM NaCl/0.05% NaN_3; Buffer C - 0.1 M sodium acetate pH 4.7. Phenylmethylsulfonylfluoride (PMSF), leupeptin, pepstatin, L-methionine, polyGuanosine, salicylic acid and geneticin disulfate were purchased from Sigma (St. Louis, MO). Ham's F-12 media, L-glutamine, and penicillin/streptomycin were purchased from Gibco BRL (Gaithersburg, MD). [^{35}S]methionine (Trans ^{35}S-label with 70% methionine) was from ICN Biomedicals, (Irvine, CA). Maleylated BSA (M-BSA) coupled to CNBr-activated Sepharose (Pharmacia) (approximately 3 mg M-BSA/ml hydrated resin) was prepared as described previously (Resnick et al., 1993) .

Purified LTA from S. *pyogenes,* isolated as previously described (Simpson et al., 1980), was generously provided by James Dale and Harry Courtney, Memphis, TN. Purified LTA was suspended in LTA buffer (0.25% deoxycholate, 0.2 M NaCl, 1 mM EDTA, 0.02% NaN_3, 10 mM Tris HCl, pH 8.0) prior to use.

Bacterial strains - *Streptococcus pyogenes* strains T1/195/2, S43/192/4, J17E/165/3, T2/44/RB4/119, and T22/76/2 in addition to a spontaneous M-protein negative mutant (T28/51/4-4) with a large deletion in the *mry/emm* operon (Scott et al., 1984) and its parent wild type (T28/150A/5) were from Vincent Fischetti (Rockefeller University, New York City, NY). A Tn916 mutant (JRS75) of *S. pyogenes* which lacks both M protein and carboxypeptidase (Caparon et al., 1991), and its type 6 parent strain (JRS4) were donated by June Scott, Atlanta, GA. *S. pyogenes* wild- type strain 87-282 and its acapsular Tn916 mutant, TX-4, were kindly provided by Mike Wessels (Channing Laboratory, Boston, MA) (Wessels et al., 1991). *Streptococcus agalactiae* strains A909 (type Ia), and COH-31-15 (unencapsulated Tn 918 mutant of type II strain COH 15) were also from M. Wessels (Rubens et al., 1987). *S. agalactiae* strains 110 and 181 were from Steve Mattingly, San Antonio. *Staphylococcus aureus* capsular type 5 strain Reynolds and its transconjugate mutants, strains JL 236 (Tn-918-induced capsule deficient mutant) and JL 240 (EMS-derived capsule negative mutant) in addition to type 8 strain Becker and its mutant, JL 252 (Tn-551-induced capsule negative mutant) were generously donated by Jean Lee (Channing Laboratory, Boston, MA)(Albus et al., 1991). *Streptococcus mutans* (ATCC 25175), *Enterococcus hirae* (ATCC 9790), *Listeria monocytogenes* (ATCC 43251), and *Bacillus subtilis* (ATCC 6633) were obtained from the American Type Tissue Collection (Rockville, MD). Strains of *Streptococcus pneumoniae* (rough, type 6, type 8) were from our laboratory.

Growth of Bacteria - Gram-positive bacteria were grown overnight either on LB agar plates (*L. monocytogenes, B. subtilis)* or 5% sheep blood agar plates (for all remaining organisms). Fresh colonies were inoculated into Todd Hewitt Broth (DIFCO) and were grown to stationary phase (16-18 hours; $1x10^9$ CFU/1 ml) at 37°C without shaking. Initial experiments indicated greater binding of scavenger receptor to organisms grown to stationary phase compared to organisms used at log phase. Thus stationary phase organisms were used in all experiments presented here, although results were similar for log phase organisms. Bacterial strains were harvested by centrifugation at 3500 x g for 10 minutes and washed twice in cold Buffer B before resuspending to a final density of approximately 1×10^9 bacteria/ml ($O.D._{600} = 2.0$).

FITC-labelled S. pyogenes - Stationary phase organisms were suspended in PBS to $O.D._{600}$= 1.0. To 5cc of organisms, 5µl of fluorescein isothiocyanate (100mg/ml in DMSO) was added, and the mixture was incubated at 37°C for 60 minutes with periodic vortexing. Organisms were washed twice in PBS, then suspended in Ham's F12 containing 10% fetal calf serum.

Preparation of metabolically labeled soluble bovine scavenger receptor, type I (s-bSR-I) - Untransfected Chinese hamster ovary (CHO) cells and transfected CHO cells (CHO[s-bSRI]-A2) expressing a truncated, secreted form of the type I bovine scavenger receptor (s-bSR-I) were described previously (Resnick et al., 1993). The trimeric s-bSR-I receptor comprises all four extracellular domains of the intact receptor and exhibits the same broad binding specificity as the full length integral membrane form of the receptor. CHO cells were grown in medium A (Ham's F-12 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine) containing 5% (vol/vol) fetal calf serum (Medium B). Transfected cells were grown in Medium B containing 0.5 mg/ml G418. [^{35}S] methionine- labelled conditioned medium was prepared from transfected CHO[s-bSRI]-A2 cells and untransfected CHO cells as previously described (Resnick et al., 1993), and designated [^{35}S]s-bSR-I medium and [^{35}S]control medium, respectively. Protease inhibitors added to conditioned media were 1mM PMSF, 1 µM leupeptin, and 1µM pepstatin.

Microbial binding assay - Thirty minutes prior to binding assays, unlabelled L-methionine (10 mM final concentration) was added to suspensions of microorganisms grown as described above to minimize incorporation of free [^{35}S]methionine into microbial proteins during the assays. One ml assay mixtures comprising 200 µl of bacterial suspension (approximately 2×10^8 bacteria), 50 µl of Buffer A and 750 µl of either [^{35}S]s-bSR-I or [^{35}S]control media were prepared in microcentrifuge tubes and incubated overnight on a rotator at 4°C. Suspensions of maleylated BSA-coupled Sepharose beads (M-BSA beads) (25 µl) were used in place of microorganism suspensions as positive controls. In the experiment shown in Figure 3, the total assay volume

was 0.25 ml instead of 1.0 ml and each assay contained 50 µl of LTA buffer with the indicated amounts of LTA and 10 µl of M-BSA beads. The amounts of scavenger receptor binding shown in Figure 3 were measured with a Molecular Dynamics PhosphorImager.

After the overnight incubation, the bacteria or beads were pelleted by centrifugation in a microcentrifuge at 12,500 x g for 5 minutes at 4°C and washed twice with cold buffer A. In experiments examining the ability of LTA to inhibit the binding of the s-bSR-I to M-BSA beads, the beads were allowed to settle by gravity before each washing step, to prevent pelleting of LTA micelles. The washed pellets were then resuspended in SDS-PAGE sample buffer containing 2% ß-mercaptoethanol, boiled for 5 min., and insoluble material was removed by centrifugation at 12,500 x g for 2 minutes. The reduced samples were fractionated by electrophoresis through 10% polyacrylamide gels (Resnick et al., 1993) and the labeled proteins were visualized by autoradiography with Kodak X-AR film. Prior to autoradiography, the gels were impregnated with either 0.125 M sodium salicylate in 30% (v/v) methanol or autofluor (National Diagnostics).

Cell Binding Assay - P388D1 cells were maintained in dishes in Ham's F12 with 10% fetal calf serum, 100units/ml penicillin and 100µg/ml streptomycin. For experiments, approximately 12,500 cells were plated on 1 cm glass cover slips, in media lacking antibiotics. Cells were allowed to adhere for at least 24-36 hours, then washed in Ham's F12 containing 10% fetal calf serum. *S. pyogenes*, either unlabelled or FITC-labelled, was added (final dilution of 1:250 from a stock solution of $O.D._{600}$= 1.0) in the presence or absence of varying concentrations of poly G. Mixtures were incubated for 30 minutes at 37°C in 5%CO_2, then washed three times in PBS, and fixed with 100% cold methanol. Samples containing non-FITC labelled organisms were Gram-stained, then cover slips were mounted with Moviol and DAPCO. Slides were viewed in blinded fashion by two observers using fluorescence and phase contrast microscopy and a Nikon Microphot FXA epifluorescence microscope. The results presented below (bacteria bound per 100 P388D1 cells) represent the means +/- standard deviations determined from three experiments in which at least 100 P388D1 cells were counted for each condition.

Results

The type I bovine scavenger receptor (s-bSR-I) binds to *S. pyogenes*. To determine if a secreted form of the type I bovine scavenger receptor (s-bSR-I) could directly bind to the surface of whole bacteria, we incubated bacteria at 4°C overnight with [^{35}S]methionine-labeled conditioned medium either from transfected CHO cells expressing the secreted receptor ([^{35}S]s-bSR-I medium) or from control untransfected CHO cells ([^{35}S]control medium). The bacteria and any bound protein were then solubilized with detergents and the bound metabolically labeled proteins were visualized after gel electrophoresis by autoradiography. Figure 3.1A shows that when *Streptococcus pyogenes* strain T1/195/2 was incubated with [^{35}S]s-bSR-I medium, the most prominent of the labeled proteins bound to the bacteria was ~78 kD (lane 1), the mass of s-bSR-I (Resnick et al., 1993). This protein was absent from *S. pyogenes* incubated with [^{35}S]control medium (see figure 3.1B below) and these binding data were similar to data for the binding of [^{35}S]s-bSR-I to sepharose beads covalently derivatized with the scavenger receptor ligand maleylated BSA (M-BSA beads, not shown). Therefore, we conclude that the 78 kD binding protein was [^{35}S]s-bSR-I. Because the labeled scavenger receptor in [^{35}S]s-bSR-I medium represented only a small fraction of the total labeled protein (not shown), yet it was the major component of the bound proteins (lane 1), [^{35}S]s-bSR-I binding to *S. pyogenes* appears to have been specific. In preliminary studies we also observed the binding of a soluble form of the type II bovine scavenger receptor as well as soluble forms of human type I and II scavenger receptor to Gram-positive bacteria (not shown).

The polynucleotide ligand poly G is an efficient competitive inhibitor of the binding of other polyanionic ligands (Brown and Goldstein, 1993; Resnick et al., 1993) to the cationic collagenous binding domain on the type I and type II scavenger receptors. Figure 3.1A (lane 2) shows that the binding of *S. pyogenes* to s-bSR-I resembled binding of other ligands in that poly G was an effective competitor (compare to lane 1). This binding was not restricted to the T1/195/2 strain of *S. pyogenes*. Figures 3.1 and 3.2 and Table 3.1 show that a total of 11 *S. pyogenes* strains representing 7 different M protein types bound [^{35}S]s-bSR-I. Thus, [^{35}S]s-bSR-I binding is a general characteristic of *S. pyogenes* which is not strain specific.

The ability of poly G to inhibit *S. pyogenes* binding was used in experiments to examine the interaction of FITC-labelled *S. pyogenes* (strain T1/195/2) with a cultured murine macrophage-like cell line, P388D1, shown previously to express macrophage scavenger receptors (Ashkenas et al., 1993). After a 30 minute incubation at 37°C, we observed binding of 72 +/- 8 bacteria/100 P388D1 cells. When poly G at concentrations of 2, 20, or 200 μg/ml was included in the incubation medium, the number of cell associated

bacteria/100 P388D1 cells was reduced to 43 +/- 4, 28 +/- 9, and 22 +/- 5, respectively. Similar results were obtained when binding of unlabelled bacteria was assessed by Gram staining (See Methods). These results raise the possibility that association of *S. pyogenes* with intact cultured murine macrophages may be mediated in part by their full length, cell surface scavenger receptors. Additional studies will be necessary to determine the role of scavenger receptors in the internalization and processing of bacteria by macrophages in culture and *in vivo*.

s-bSR-I binds to *S. pyogenes* strains lacking M protein or hyaluronic acid capsules. To determine which cell surface component(s) on *S. pyogenes* might be responsible for scavenger receptor binding, we used mutant strains of *S. pyogenes* to assess the role in receptor binding of two well characterized virulence determinants, M protein and the hyaluronic acid capsule (Wessels et al., 1991; Phillips et al., 1981; Lancefield, 1962; Beachey et al., 1980; Kendall et al., 1937; Whitnack et al., 1981; Hosein et al., 1979). Figure 3.2 (lanes 1 and 2) shows that a type 28 *S. pyogenes* strain and its spontaneous M protein-negative mutant bound s-bSR-I equivalently. Similar results were obtained when assays were performed using a type 6 M protein-negative transposon mutant of *S. pyogenes* and its wild type parent strain (Table I, strains JRS75 and JRS4). Thus, the M protein is not necessary for binding. Figure 3.2 (lanes 3 and 4) also shows that the wild type *S. pyogenes* strain 87-282 and its acapsular, hyaluronic acid-negative, transposon mutant TX4, bound [^{35}S]s-bSR-I equally well. Therefore, it is unlikely that the hyaluronic acid capsule played a significant role in binding to s-bSR-I.

***S. pyogenes* lipoteichoic acid (LTA) is a ligand for s-bSR-I.** These results show that some other surface component must mediate binding of [^{35}S]s-bSR-I to *S. pyogenes*. Another major surface component of *S. pyogenes* is the polyanionic polymer lipoteichoic acid (LTA) (Ofek et al., 1975; Kessler et al., 1979). A direct, genetics based, test of the role of LTA in binding is not possible because no naturally occurring or genetically engineered strains of *S. pyogenes* lacking LTA are available. However, because binding of [^{35}S]s-bSR-I to *S. pyogenes* apparently occurs via interaction with the receptor's cationic collagenous ligand binding domain (see above), it is possible to examine the potential role of LTA in mediating binding. This can be done by determining if LTA can function as a competitive inhibitor of [^{35}S]s-bSR-I binding to other ligands, e.g., M-BSA beads (Resnick et al., 1993). Figure 3.3 shows the results of an experiment in which increasing amounts of *S. pyogenes* LTA were added to mixtures of [^{35}S]s-bSR-I medium and M-BSA beads and the amount of [^{35}S]s-bSR-I binding was quantitated as described in Methods. *S. pyogenes* LTA was an effective inhibitor of binding (IC_{50} = ~4 µg/ml). LTA also inhibited binding of [^{35}S]s-bSR-I to poly G beads, with a similar IC_{50} (not shown). These data suggest that LTA may be the major

scavenger receptor binding determinant on *S. pyogenes*.

s-bSR-I binds to an array of Gram-positive bacteria. The finding that LTA probably mediates binding of scavenger receptors to *S. pyogenes* raised the possibility that many, if not all, Gram-positive bacteria might bind to scavenger receptors because LTA or analogous molecules are ubiquitous cell surface components on these microorganisms (Reviewed in Wicken and Knox, 1975; Fischer, 1988). Figure 3.4 shows that [^{35}S]s-bSR-I bound to *Enterococcus hirae* and *S. agalactiae*. Binding was competible by poly G. In binding studies using a wide variety of Gram-positive bacteria, we observed that all species and strains of bacteria tested bound [^{35}S]s-bSR-I (Table 3.1). Binding was competible with poly G although the extent of competition varied slightly between organisms. In addition to the species shown in Figure 3.4, [^{35}S]s-bSR-I bound to *Staphylococcus aureus*, *Streptococcus mutans*, *Listeria monocytogenes* and *Bacillus subtilis*. Isogenic capsule-minus transposon mutants of *S. agalactiae* and *S. aureus* also bound [^{35}S]s-bSR-I equivalently or better than the parent strains, again suggesting that the capsule plays little, if any, role in [^{35}S]s-bSR-I binding. In contrast to the results with all other Gram-positive organisms examined, only minimal binding was observed to two encapusulated and one rough strain of *S. pneumoniae,* organisms with atypical LTAs (Behr et al., 1992).

Discussion

In a series of *in vitro* binding studies using a truncated, secreted form of the type I bovine macrophage scavenger receptor, we have shown that the scavenger receptor can bind to the surface of *S. pyogenes* and to a wide variety of other Gram-positive bacteria. The binding to *S. pyogenes* was inhibited by poly G, a classic scavenger receptor ligand (Brown and Goldstein, 1983). Thus, the binding was probably mediated by interactions of negatively charged sites on the bacterial surface with the positively charged collagenous ligand binding domain of the receptor. Experiments using *S. pyogenes* mutants showed that the binding was independent of the hyaluronic acid capsule and the M protein, two major surface virulence determinants specific for *S. pyogenes* (Wessels et al., 1991; Phillips et al., 1981; Lancefield, 1962; Beachey et al., 1980; Kendall et al., 1937; Whitnack et al., 1981; Hosein et al., 1979) . Lipoteichoic acid (LTA), a polyanionic surface component found on most Gram-positive bacteria, was shown by competition assays to be a scavenger receptor ligand. It seems likely that LTA is at least one of the major determinants of scavenger receptor binding to *S. pyogenes*.

LTAs are amphipathic molecules typically consisting of a 1-3 phosphodiester-linked polymer of glycerophosphate linked covalently to either a glycolipid or a phosphatidyl glycolipid (reviewed in Wicken and Knox, 1975; Fischer, 1988; Fischer et al., 1990). The resulting structures possess a backbone of repeating negative charges, a common feature of other scavenger receptor ligands, such as poly G and lipopolysaccharide micelles. Although there is immunological evidence that LTAs are absent from some Gram-positive bacteria, the presence of alternative polymers (atypical LTA's) in most of these organisms indicates that this class of molecules is a nearly invariant component in the Gram-positive cell wall (reviewed in Fischer, 1988; Sutcliffe and Shaw, 1991). The inability to isolate LTA-negative strains of Gram-positive bacteria further suggests that this surface component is critical for these organisms. It is likely that some atypical LTA's might not tightly bind to scavenger receptors, as suggested by the weak interaction with *S. pneumoniae*, which expresses an unusual LTA containing choline and ribitolphosphate (Briles and Tomasz, 1973; McCarty, 1959).

Bacteremia with Gram-positive organisms can result in septic shock which is often indistinguishable from that caused by Gram-negative bacteria (reviewed in Danner et al., 1989). It is generally accepted that lipopolysaccharide (endotoxin)-mediated release of cytokines, including IL-1 and TNF, is responsible for the syndrome of septic shock with Gram-negative organisms. It is likely that LTA serves a similar role in Gram-positive sepsis. (reviewed in Danner et al., 1989). We have shown previously that the rapid scavenger receptor-mediated hepatic uptake of endotoxin *in vivo* can play a quantitatively significant role in endotoxin clearance and probably does not play a role in endotoxin induced activation of macrophages (Hampton et al., 1991).

These results suggested that scavenger receptors presumably help in protection from endotoxic shock during Gram-negative bacterial sepsis. The current study raises the possibility that scavenger receptors might play a similar role during Gram-positive sepsis in recognizing and removing from tissues and the bloodstream both intact Gram-positive organisms and LTA released from their surfaces.

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Figure 3.1. Binding of secreted bovine scavenger receptor type I (s-bSR-I) to S. pyogenes.

[^{35}S]Methionine labeled media from transfected CHO cells expressing the truncated, secreted bovine type I scavenger receptor (s-bSR-I) or from untransfected controls (Cont.) were prepared and incubated overnight at 4°C with *S. pyogenes* T1/195/2 (panel A) or the indicated *S. pyogenes* strains (panel B) as described in Methods. In lane 2 of panel A, the mixture was supplemented with 400 μg/ml of the scavenger receptor ligand poly G. After the incubation, the bacteria were washed, dissolved by boiling in reducing sample buffer, and bound [^{35}S]labeled components were resolved by 10% polyacrylamide gel electrophoresis and visualized by autoradiography.

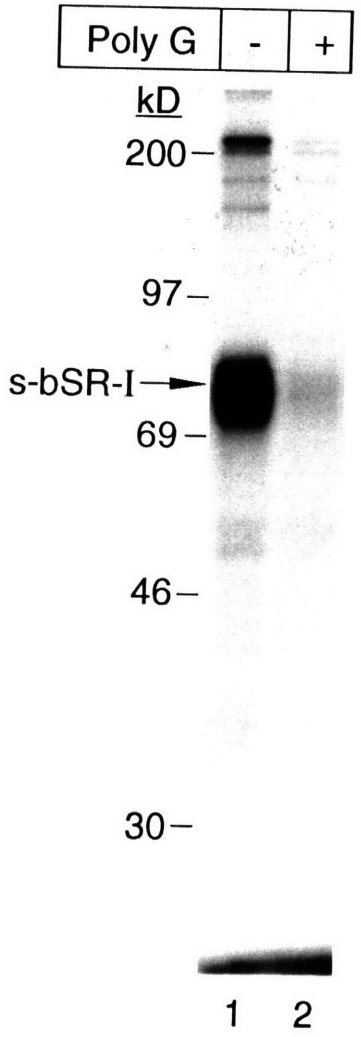
Poly G
-
+
kD
200
97
s-bSR-I
69
46
30
1
2

Figure 3.2. Role of the M-protein and the hyaluronic acid capsule in [^{35}S]s-bSR-I binding to *S. pyogenes*.

The indicated bacteria and [^{35}S]methionine labeled medium from cells expressing the truncated, secreted bovine type I scavenger receptor (s-bSR-I) were incubated together and bound receptor was visualized by electrophoresis and autoradiography as described in Methods. The bacteria used were *S. pyogenes* M-protein positive (T28/150A/5) and negative (T28/51/4-4) strains (left, lanes 1 and 2), and the hyaluronic acid capsule positive (wild type 87-282) and negative (TX-4) strains (right, lanes 3 and 4).

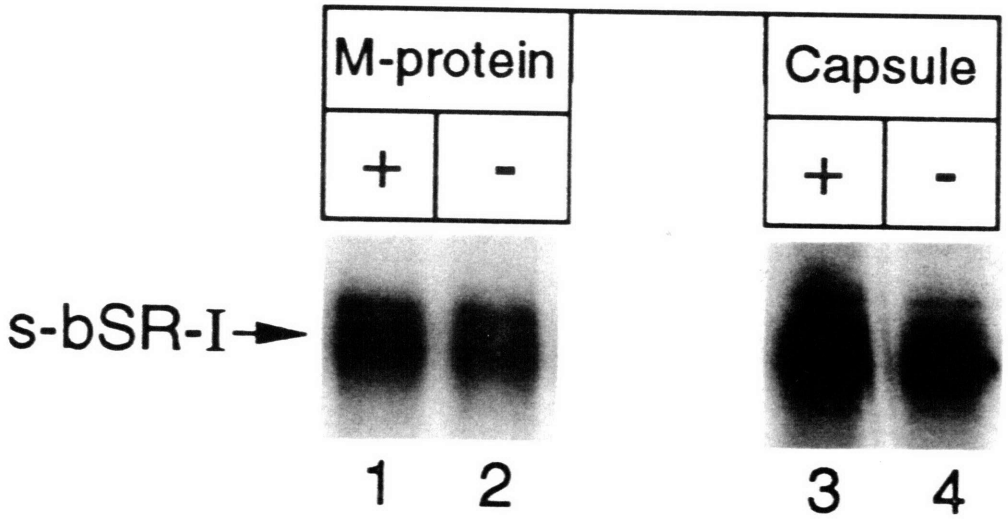
M-protein
Capsule
+
-
+
-
s-bSR-I
1
2
3
4

Figure 3.3. Inhibition of [^{35}S]s-bSR-I binding to M-BSA beads by LTA from *S. pyogenes.*

Binding of [^{35}S]s-bSR-I to M-BSA beads in the presence of the indicated amounts of LTA was performed as described in Methods. After an overnight incubation at 4°C, the adherent proteins were eluted from the beads by boiling in reducing sample buffer, bound [^{35}S]s-bSR-I was resolved by 10% polyacrylamide gel electrophoresis and the relative amounts of bound [^{35}S]s-bSR-I were determined using a Molecular Dynamics Phosphorimager. All of the values shown were corrected for "nonspecific" background binding by subtracting the value determined in the presence of 400 μg/ml poly G.

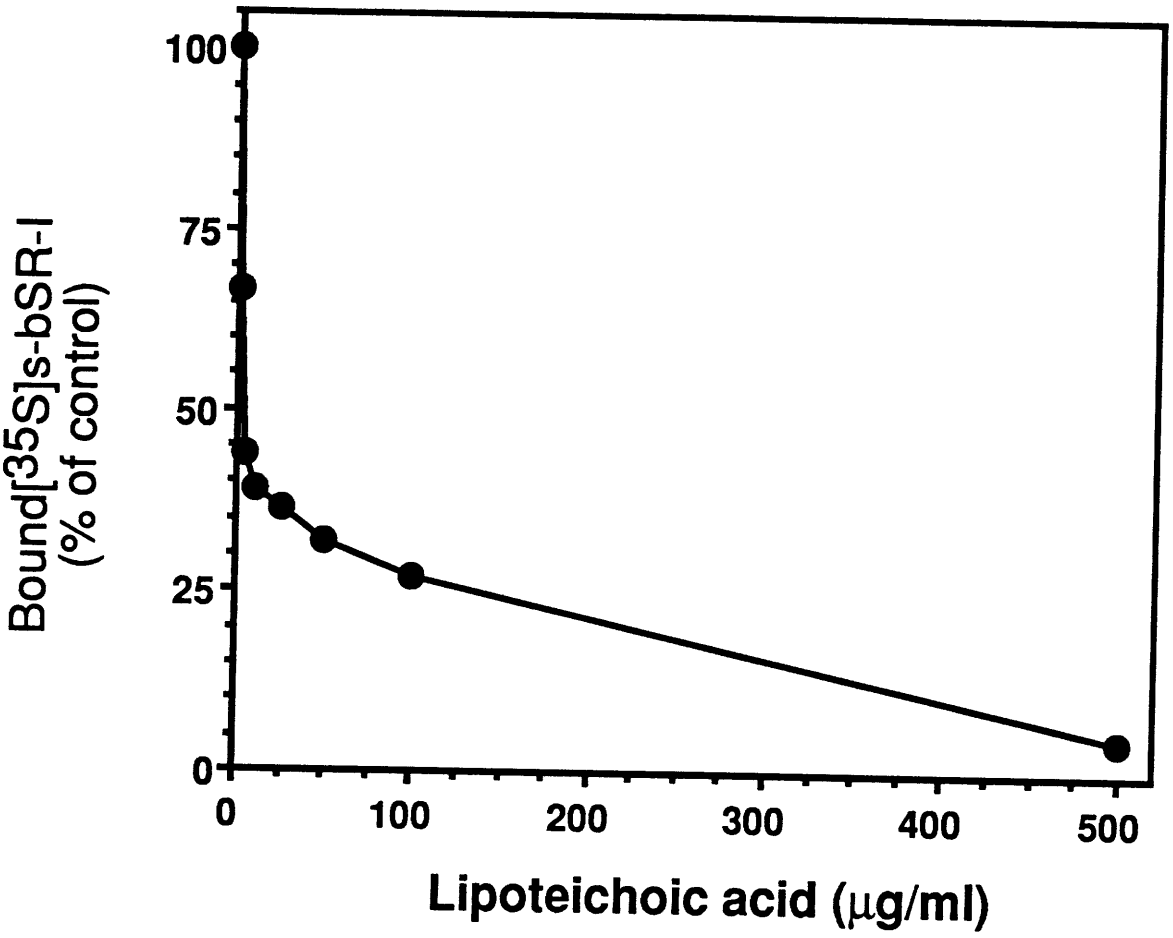
Bound[35S]s-bSR-I
(% of control)
100
75
50
25
0
0
100
200
300
400
500
Lipoteichoic acid (μg/ml)

Figure 3.4. Binding of [^{35}S]s-bSR-I to *Enterococcus hirae* and *Streptococcus agalactiae*

The indicated bacteria and [^{35}S]methionine labeled medium from cells expressing the truncated, secreted bovine type I scavenger receptor (s-bSR-I) were incubated in the presence or absence of 400 μg/ml of poly G and bound receptor was visualized by electrophoresis and autoradiography as described in Methods. The bacterial strains used were *Enterococcus hirae* 9790 and *Streptococcus agalactiae* CHO-31-15.

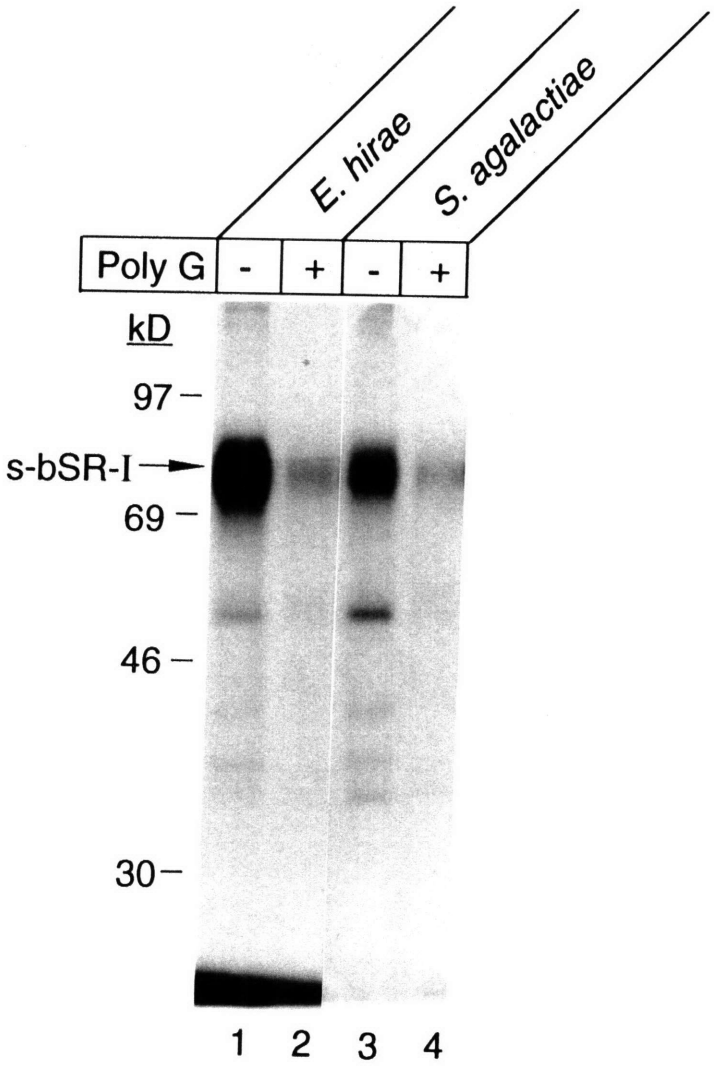
E. hirae
S. agalactiae
Poly G
-
+
-
+
kD
97
s-bSR-I
69
46
30
1
2
3
4

Table 3.1. Strains of Gram-positive bacteria which bound [^{35}S]secreted type I bovine scavenger receptor.

Streptococcus.pyogenes
- T1/195/2
- S43/192/4
- J17E/165/3
- T2/44/RB4/119
- T22/76/2
- T28/51/4-4
- T28/150A/5
- JRS75
- JRS4
- 87-282
- TX4

Staphylococcus aureus
- Reynolds
- JL236
- JL240
- Becker
- JL252

Streptococcus agalactiae
- A909
- COH 31-15
- 110
- 181

Streptococcus mutans ATCC 25175
Enterococcus hirae ATCC 9790
Listeria monocytogenes ATCC 43251
Bacillus subtilis ATCC 6633

Chapter 4

The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificites for polyanionic ligands

One of the more interesting questions pertaining to SR-A structure and function is how a molecule predicted to form a rod can bind such a broad array of ligands with high affinity and yet maintain specificity. After the cloning of the receptor, attention was focused on the collagenous domain as a potential ligand binding site. The cluster of positively charged residues at the C-terminal end of the domain was a particularly attractive possibility (Kodama et al., 1990; Rohrer et al., 1990), as the unifying theme of scavenger receptor ligands is that they are all polyanionic (see Table 1.1, introduction). This work experimentally addresses the question of the ligand binding domain by two means. It evaluates whether the collagenous domain is necessary for ligand binding by examining the effects of the truncation of this region on receptor activity. It indirectly examines the issue of whether the collagenous domain may be sufficient for ligand binding by determining whether a related collagenous sequence (the collagenous stalks of C1q) is capable of mediating a similar broad binding activity. This work was published in its entirety in 1993 in *The Journal of Biological Chemistry* **268**, 3530-3537. The only changes which have been made are that figures have been re-numbered with the prefix "4" and the citations have been merged with those of other chapters in the "references" section of this thesis.

My contribution to this work is limited to the experiments involving C1q, namely those shown in Table 4.4 and Figure 4.6. These experiments were of considerable importance in this work, as they allowed us to draw the conclusion that the collagenous domain of the scavenger receptor is probably sufficient for ligand binding. The paper was primarily written by Dr. Susan Acton and Dr. Monty Krieger; however I provided some assistance with editing and revision of the text.

The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands

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Running title: Scavenger receptor ligand binding domain

Summary

Macrophage scavenger receptors have been implicated in the development of atherosclerosis and other macrophage-associated functions, including host defense. The mechanism by which these receptors bind a wide array of polyanions, such as acetylated LDL, with high affinity has not yet been elucidated; however, it has been proposed that the positively charged extracellular collagenous domain of scavenger receptors plays a key role in ligand binding. To test this proposal, we generated truncation mutants of the bovine and murine scavenger receptors and studied their expression in transiently transfected COS cells. These mutants contain only 8 (bovine) or 5 (murine) of the 24 Gly-X-Y tripeptide repeats found in the collagenous domains of the full-length receptors. Immunochemical analyses established that the truncation of the bovine scavenger receptor did not interfere significantly with its synthesis, trimerization, post-translational processing, intracellular transport, surface expression, or stability. However, unlike their full-length counterparts, the truncated bovine and murine receptors were unable to bind acetylated LDL. Thus, the collagenous domain was necessary for normal ligand binding. In addition, cotransfection of the expression vector for the truncated bovine scavenger receptor with that for the full-length receptor resulted in dramatically reduced activity of the full-length construct (dominant negative effect). A ligand-bead binding assay was used to show that the isolated collagenous domain from a different protein, complement component C1q, could bind a wide variety of polyanions with a specificity which was similar, but not identical, to that of scavenger receptors. These results suggest that the collagenous domain of the scavenger receptor is both necessary and sufficient to determine the broad binding specificity that characterizes this unusual receptor. Scavenger receptors and C1q, along with the mannose binding protein, conglutinin and lung surfactant apoprotein A, help define a set of proteins which all contain short collagenous domains and which all appear to participate in host defense. Their short collagenous domains may contribute significantly to their host-defense functions.

Introduction

The type I and type II macrophage scavenger receptors are trimeric integral membrane glycoproteins which exhibit unusually broad ligand specificity (Goldstein et al., 1979; Brown and Goldstein, 1983; Kodama et al., 1988; Kodama et al., 1990; Rohrer et al., 1990; Penman et al., 1991). They bind a wide variety of polyanions, including chemically modified proteins (e.g, acetylated low density lipoprotein, AcLDL), acidic phospholipids, endotoxin (Raetz et al., 1988; Nishikawa et al., 1990; Hampton et al., 1991) and certain polyribonucleotides (poly I and poly G, but not poly C). Numerous studies have suggested that the scavenger receptors may be critically involved in cholesterol deposition in artery walls during atherogenesis (Brown and Goldstein, 1983; Jurgens et al., 1987; Steinberg et al., 1989; Steinbrecher et al., 1990). The broad binding specificity of the scavenger receptors and their expression in macrophages (Brown and Goldstein, 1983; Nagelkerke et al., 1983; Pitas et al., 1985; Kodama et al., 1990) suggest that they may also play a role in other physiologic and pathophysiologic systems, such as macrophage-associated host-defense and inflammation (Freeman et al., 1990; Kodama et al., 1990; Krieger, 1992).

The cDNAs for the bovine, murine, human, and rabbit type I and type II scavenger receptors have been cloned (Kodama et al., 1990; Rohrer et al., 1990; Ashkenas et al., 1992; Matsumoto et al., 1990; Bickel and Freeman, 1992). The sequence of the type I bovine scavenger receptor cDNA predicts a 453 amino acid protein with the following domains (Kodama et al., 1990): I. N-terminal cytoplasmic (amino-acid residues 1-50), II. transmembrane (51-76), III. spacer (77-108), IV. α-helical coiled-coil (109-271), V. collagenous (272-343), and VI. C-terminal cysteine-rich (344-453), designated SRCR (scavenger receptor cysteine-rich). The SRCR domain helped to define a previously unrecognized family of remarkably well conserved cysteine-rich protein domains (Freeman et al., 1990). The bovine scavenger receptor type II (bSR-II) is identical to the type I receptor, except that the 110-amino-acid SRCR domain is replaced by a six-amino acid C-terminus (Rohrer et al., 1990) (see Figure 1, top). Despite its truncated C-terminus, the type II scavenger receptor mediates the endocytosis of modified LDL with essentially the same affinity and is inhibited by the same group of polyanions as the type I receptor (Rohrer et al., 1990; Freeman et al., 1991). The observation that all of the 24 Gly-X-Y triplets in the collagenous domain of the bovine scavenger receptors were either positively charged or neutral at physiologic pH suggested that this domain might play a critical role in establishing the broad ligand binding specificity of scavenger receptors (Kodama et al., 1990; Rohrer et al., 1990).

In the current study, we have used two approaches to examine the role of the collagenous domain in ligand binding. First, mutant truncated forms of the bovine (t-bSR) and murine (t-mSR) scavenger receptors with only either 8 or 5 Gly-X-Y tripeptide repeats in the collagenous domains, respectively, were

generated. Studies with the bovine mutant established that the intact collagenous domain was not required for receptor synthesis, trimerization, post-translational modification, intracellular transport, or cell surface stability. The collagenous domains in both the bovine and the murine scavenger receptors were, however, necessary for ligand binding. Second, analysis of ligand binding to the isolated collagenous domain of complement component C1q showed that a different short collagenous domain can have broad polyanionic ligand binding specificity similar to that of scavenger receptors. Thus, the collagenous domain of the scavenger receptor may be not only necessary, but also sufficient to confer on this receptor its characteristic broad ligand binding specificity for polyanions.

Materials and Methods

Materials - Anti-N-terminal bovine scavenger receptor polyclonal antibodies were prepared against a peptide from the amino-terminus of the bovine scavenger receptor as previously described (Penman et al., 1991). LDL and AcLDL were prepared and iodinated with specific activities of 100-400 cpm/ng, and newborn calf and human lipoprotein-difficient sera were prepared as described previously (Goldstein et al., 1983; Krieger, 1983). ^{125}I-C1q collagenous tails were a gift from Dr. Andrea Tenner (UC Irvine, CA) and were prepared as previously described (Guan et al., 1991). Polyriboguanylic acid (poly G), polyriboinosinic acid (poly I), polyribocytidylic acid (poly C), fucoidin, dextran sulfate, chondroitin sulfate, and methionine were obtained from Sigma. Maleylated BSA (M-BSA) was prepared as described (Goldstein et al., 1979). Poly G, poly I, and poly C covalently attached to Sepharose were purchased from Pharmacia. M-BSA was coupled to CNBr activated Sepharose (Pharmacia) according to the manufacturer's instructions yielding a final concentration of 2.7 mg M-BSA/ml of packed gel.

Preparation of the expression vectors -The t-bSR mutant was generated using the polymerase chain reaction (PCR) and the bovine type II scavenger receptor in pBluescript. The primer used for the 5' end (5' CGC GGG GGA TCC ATG GCA CAG TGG GAT GAC TTT 3') encoded the initiator methionine (underlined) and the 3' primer (5' CGC GGG CTC GAG TTA TTG CAT GCT CCG ATC ACC TTT AAG ACC TGG AGT ACC 3') was antisense and was intended to encode a stop in the middle of the collagenous region. A base omission error at nucleotide 884 (CCAGGT -> C_AGGT) generated during the PCR resulted in a ^{295}Pro -> ^{295}Gln frameshift mutation that terminated the polypeptide after translation of only 8 of the 24 Gly-X-Y repeats (...G-F-^{295}Q-V-stop). The product was excised with *Bam* H1 and *Xho* I and ligated into pcDNAI (Invitrogen, La Jolla, CA) and sequenced using Sequenase 2.1 (USB) according to manufacturer's instructions. The mutant murine scavenger receptor cDNA was also generated by PCR. A 1084 bp murine scavenger receptor PCR product was synthesized from an amplified P388D1 cDNA library (Ashkenas et al., 1992) using a sense oligonucleotide primer in the 5' leader of the cDNA (5' CGC CGA GCG GCC GCG CTG TCT TCT TTA CCA GC 3') and an antisense primer at the 3' end of the coding domain of the type II murine receptor (5' CGC CGG TCT AGA TTA TAC TGA TCT TGA TCC GC3'). This product was cloned into pRc/CMV (Invitrogen) using the *Not* I and *Xba* I restriction sites engineered into the primers. One of the products, pJA20-1, had a base omission error at nucleotide 888 (CTTACT -> C_TACT) causing a ^{287}Thr ->^{287}Leu frameshift. This resulted in a sequence encoding a truncated murine scavenger receptor containing only five Gly-X-Y triplets in its collagenous domain followed by a 10 residue hydrophobic tail at the mutant receptor's C-terminus (^{273}GPP-GPQ-GEK-GDR-GLL-DKLVHLVLQE-stop). The insert from pJA20-1 was cloned into pcDNAI, using *Not* I and *Xba* I, to generate the truncated mutant murine

scavenger receptor (t-mSR) expression vector, pJA27.

Cell culture and transfections - All cell culture incubations were at 37°C in a humidified 95% air, 5% CO_2 incubator unless otherwise noted. COS M6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (medium A) . For transfections, 1.5 x 10^6 cells/dish were set in 100 mm dishes in medium A on day 0. For each dish on day 1, plasmid DNA (2 - 10 µg in 50 µl H_20) was mixed with 200 µl of 10 mg/ml DEAE-dextran (Pharmacia) and then added to 4 ml of medium A in which the FBS was replaced with 10% (v/v) Nuserum (Collaborative Research) and supplemented with 100 µM chloroquine. The entire mixture was then added to the cells. The cells were incubated for 3.5 hrs, the medium was removed and the cells were shocked with 2 ml of 10% (v/v) dimethylsulfoxide in phosphate buffered saline (PBS). After 2-3 minutes the buffer was quickly removed by aspiration and replaced with 10 ml of medium A. On day 2 the cells were harvested with trypsin and replated in medium A at 0.5-1 x 10^6 cells/well in 6-well dishes. Cell labeling and degradation assays were typically performed on day 3. For cotransfection assays, DNAs were combined as indicated prior to preparation of the DEAE-dextran complexes.

Biosynthetic labeling -Transfected cells in 6-well dishes were rinsed twice with 1 ml of PBS per well and incubated with methionine-free Ham's F12 medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 3% (v/v) newborn calf lipoprotein-deficient serum and [^{35}S]methionine or [^{35}S]protein labeling mix (New England Nuclear) (0.5 ml/well, 300 µCi/ml for pulse-chase experiments and 0.75 ml/well, 80-100 µCi/ml for 5 hr labeling). For pulse-chase experiments, cells were labeled for the indicated times, then washed once with warm medium A and incubated in 3 ml of chase medium (medium A supplemented with 1 mM unlabeled methionine) as indicated. Cells were harvested by scraping in lysis buffer (PBS with 1% (v/v) Triton X-100, 1 mM methionine, 50 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) with or without 10 mM iodoacetamide as indicated). For pronase digestions, labeled cells were washed once 20 min before the end of the chase period with 1 ml/well of warm hepes buffered saline (10 mM hepes, pH 7.4, 0.15 M NaCl, 2 mM $CaCl_2$) and incubated for 20 min at 37°C in 0.75 ml of hepes-buffered saline with or without 60 µg/ml pronase (Sigma). The cells were chilled to 0°C and 0.5 ml per well of ice-cold pronase quench were added (PBS with 25% (v/v) human lipoprotein-deficient serum, 1 mM EDTA, and 1 mM PMSF). The cells were scraped from the dish using a rubber policeman, harvested by centrifugation at 2000 x *g* for 5 min at 4°C, washed twice with PBS at 4°C, and lysed in 220 µl of lysis buffer.

Immunoprecipitation, electrophoresis, and autoradiography -
Immunoprecipitations of radiolabeled scavenger receptors were performed as

previously described (Penman et al., 1991), except that unlabeled, untransfected COS cells were used in place of Chinese hamster ovary (CHO) cells to reduce non-specific background and 20 µl of a 50% (v/v) slurry of protein A-Sepharose in PBS were used in place of the goat-anti-rabbit antibody. For endoglycosidase H (Endo H) digestions, immunoprecipitates were suspended in 20 µl of Endo H buffer (30 mM sodium citrate, 0.75% (w/v) SDS, 2% (v/v) ß-mercaptoethanol, 1 mM PMSF, 0.02% (w/v) NaN_3, pH 5.5) and boiled for 5 min. After cooling, the Sepharose was removed by centrifugation at 1000 x *g* for 5 min at 4°C and the supernatant was transferred to 0.5 ml tubes. Endo H (5 µl) was added to a final concentration of 50 mU/ml. After overnight incubation at 37°C, 8 µl of reducing sample buffer (0.25 M Tris pH 6.8, 8% (w/v) SDS, 0.02% (w/v) bromophenol blue, 25% (v/v) glycerol, and 20% (v/v) ß-mercaptoethanol) were added and the samples were boiled for 5 min. SDS gel electrophoresis (8% polyacrylamide and 3-10% polyacrylamide gradients) followed by fixation, staining and autoradiography were carried out as previously described (Kozarsky et al., 1986).

^{125}I-AcLDL and ^{125}I-LDL Degradation Assays -Scavenger receptor and LDL receptor activities were measured by a ligand degradation assay as described previously (Krieger, 1983; Freeman et al., 1991) using 5 µg protein/ml of either ^{125}I-AcLDL or ^{125}I-LDL. Assays were performed in triplicate in 6-well dishes which were prepared as described above. Non-specific degradation was determined by addition of 400 µg/ml of unlabeled competitor ligands (AcLDL or polyinosinic acid for the scavenger receptor, and LDL for the LDL receptor) to one of the three wells. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). To improve the sensitivity of the assays of transfected human LDL receptor activity in the presence of the endogenous COS cell LDL receptor activity, the endogenous receptor activity was suppressed, but not completely inhibited, by preincubating the cells with 25-hydroxycholesterol (1 µg/ml) and cholesterol (10 µg/ml) for 24 hrs (Brown and Goldstein, 1975). For cell-surface binding assays, cells were prechilled on ice for 15 min, refed with 20 µg/ml ^{125}I-AcLDL in ice-cold medium A with or without 400 µg/ml unlabeled AcLDL and incubated 2 hr at 4°C on a shaker. Cells were then washed rapidly three times with Tris wash buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4) containing 2 mg/ml BSA, followed by two 5 min washes, and two rapid washes with Tris wash buffer without BSA. The cells were solubilized in 1 ml of 0.1 N NaOH for 20 min at room temperature on a shaker, 50 µl were removed for protein determination, and the radioactivity in the remainder was determined using a LKB gamma counter. Degradation activity is expressed as ng of ^{125}I-AcLDL or ^{125}I-LDL protein degraded in 5 hours per mg of cell protein.

Binding assays with ^{125}I-C1q collagenous tails -Bead binding assays were conducted at 4°C and were based on an assay for soluble scavenger receptor-binding described by Resnick et al (1992). A mixture consisting of 498 µl Buffer A (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$), 250 µl Buffer B (Buffer A

supplemented with 2 mg/ml of BSA and 0.01% (w/v) NaN_3), and 2 µl of ^{125}I-C1q tails was placed in a 1.5 ml tube. Soluble competitors and 25 µl of a 50% (v/v) suspension of ligand-coupled Sepharose beads in Buffer A were then added, along with enough buffer A to bring the total volume to 1 ml. The mixture was incubated overnight with slow rotation. The beads were then washed twice with buffer A, and the bead-associated radioactivity was measured. Although potential degradation of poly C by ribonucleases was not determined in the experiments shown in Table IV and Figure 6, we have previously found that poly I and poly G, but not poly C, are resistant to contaminating ribonuclease activities (A. Pearson, D. Resnick, S. Acton and M. Krieger, unpublished data). However, when *E. Coli* ribonuclease inhibitor (40 U/ml, Calbiochem) was added to the assay buffer, we found that there was no significant degradation of soluble poly C and that the binding specificities for bead bound and soluble molecules were the same as those shown in Table IV and Figure 6 (not shown). The results presented here were determined using one of three independent preparations of ^{125}I-C1q collagenous tails. The absolute binding activities varied for the three preparations; however, the ligand specificities remained essentially the same.

Results

To test the importance of the scavenger receptor's collagenous domain for ligand binding, we generated a cDNA expression vector encoding a truncated form of the bovine scavenger receptor, t-bSR (Fig. 4.1). This vector contains a frame-shift mutation which converts the codon for Pro^{295}, the 24th of 72 residues in the collagenous domain, to one for Gln^{295}. This is followed by a codon for the C-terminal Val^{296} and a stop codon. Thus, while the normal type II bovine scavenger receptor (bSR-II) is 349 amino acids in length and its collagenous domain comprises 24 Gly-X-Y repeats (Rohrer et al., 1990), the t-bSR is 296 residues long and its collagenous domain contains only 8 Gly-X-Y repeats. An analogous truncated murine mutant (t-mSR, 297 a.a.) with only 5 Gly-X-Y repeats in its collagenous domain was also constructed. Its sequence is identical to that of the normal type II murine scavenger receptor (mSR-II, 350 a.a.) up to residue 286 (Ashkenas et al., 1992) and terminates with a divergent, 11 residue extension (L-D-K-L-V-H-L-V-L-Q-E-stop). Expression vectors containing these mutants and their wild-type counterparts were individually transiently transfected into COS cells. Receptor activity was measured 48 hours after transfection using an ^{125}I-AcLDL degradation assay (Table 4.I). While cells transfected with the full-length type II bovine (bSR-II) or murine (mSR-II) scavenger receptor expression vectors were able to degrade ^{125}I-AcLDL specifically, cells transfected with expression vectors for the truncated receptors exhibited no specific receptor-mediated ligand degradation.

To determine if the mutants' inability to mediate ^{125}I-AcLDL degradation was due to defective binding, we measured the binding of ^{125}I-AcLDL to the cell surface of transfected cells at 4°C. At this temperature, scavenger receptor mediated endocytosis and degradation of ligands is inhibited (Goldstein et al., 1979). Table 4.II shows that cells transfected with vectors encoding the full-length type II receptors exhibited substantial cell surface ^{125}I-AcLDL binding while those transfected with vectors for the truncated receptors exhibited only the background levels of binding observed in control cells. Thus, truncation of the collagenous domains of the bovine and murine scavenger receptors abolished their binding activities.

The above result could have been caused directly by disruption or deletion of the ligand binding sites on the receptors or indirectly as a result of abnormal synthesis, processing, intracellular transport, cell-surface expression or stability of the truncated receptors. To characterize the properties of the truncated bovine receptor, we performed a series of [^{35}S]methionine pulse-chase metabolic labeling/immunoprecipitation experiments in which the receptors were visualized by autoradiography after SDS polyacrylamide gel electrophoresis. In a previous study using stably transfected Chinese hamster ovary (CHO) cells, we found that the full-length bSR-II was initially synthesized

as an 176 kDa trimeric precursor comprising somewhat heterogeneous 55 kDa Endo H sensitive monomers (Penman et al., 1991). The precursors were processed to Endo H resistant 219 kDa trimeric mature forms composed of 69 kDa monomers during their transport to the cell surface. The synthesis and processing of [^{35}S]methionine-labeled full-length bSR-II in transiently transfected COS cells were similar to that seen in CHO cells. For example, Figure 4.2 (left panel) shows that in transfected COS cells the 0.5 hour pulse-labeled monomeric, bSR-II precursors ("p") were efficiently converted to mature forms ("m"). The precursors were Endo H sensitive and were intracellular (resistant to extracellular pronase) while the mature forms were Endo H resistant and mostly expressed on the cell surface (pronase sensitive) (data not shown).

Similar results were obtained with the truncated bovine scavenger receptor, t-bSR. The right panel in Figure 4.2 shows that the amount of synthesis of the t-bSR precursor ("p_t") was similar to that of the full-length bSR-II receptor (0.5 h pulse, 0 h chase) and that the precursor was post-translationally processed to a mature form ("m_t"). As expected, the apparent masses of the precursor and mature forms of the truncated mutant were smaller than those of the full-length bSR-II. The stabilities of the mature forms of the full-length and truncated receptors were similar up to 24 hours. Figure 4.3 shows that, as was the case for the full-length receptor, the truncated precursor was Endo H sensitive (lanes 1 and 2) and pronase-resistant (lanes 3 and 4), while the mature form was Endo H resistant and highly susceptible to pronase digestion. This indicates that the precursor was intracellular and that most of the mature form was expressed on the cell-surface. Thus, the truncated receptor followed a post-translational processing and transport pathway which was strikingly similar to that of the full-length receptor (Penman et al., 1991) and which is typical for normally folded integral membrane cell surface glycoproteins. The only significant difference between the synthesis, post-translational processing and intracellular transport of the full-length and truncated receptors was that the rate of post-translational processing of the truncated mutant was somewhat slower than that of the full-length molecule (Figure 4.2). However, it seems unlikely that this modest difference could account for the dramatic differences in the ligand-binding activities of the receptors (Tables I and II).

We have previously shown that bovine scavenger receptors are trimeric and have suggested that trimerization may play an important role in ligand binding (Kodama et al., 1988; Penman et al., 1991; Resnick et al., 1992; for an alternative view, see Via et al., 1992). This raised the possibility that truncation might prevent activity by interfering with trimerization. In stably transfected CHO cells, the trimeric form of the full-length bSR-II comprises a Cys^{83}-disulfide-linked dimer noncovalently associated with one monomer (Penman et al., 1991). When the cells were lysed in detergent for immunoprecipitations in the absence of a sulfhydryl-trapping reagent, some artifactual interchain disulfides

at Cys^{17} in the cytoplasmic domains were formed, crosslinking the receptor into covalently associated trimers (Penman et al., 1991). Figure 4.4 shows electrophoretic analysis of [^{35}S]methionine-labeled (5 hrs) unreduced full-length and truncated receptors immunoprecipitated from cells lysed in the presence (left) or absence (right) of sulfhydryl-trapping reagent iodoacetamide. The results were virtually identical to our previous findings for full-length receptors expressed in CHO cells. In the presence of iodoacetamide (lanes 1 and 2), monomers ("1°") and dimers ("2°") but not trimers of the precursor and mature forms of both truncated and full-length receptors were observed. In the absence of iodoacetamide (lanes 3 and 4), reduction-sensitive trimeric ("3°") precursor and mature forms were also observed. In every case, the truncated species exhibited lower apparent masses than their full-length counterparts. Thus, for the truncated as well as the full-length receptors, disulfide-linked dimeric precursors associated noncovalently with monomeric precursors in the endoplasmic reticulum to form trimers. These trimers were processed normally to mature forms in the Golgi complex and were subsequently stably expressed on the cell surface. Taken together, these results indicate that the truncated receptor was inactive because it does not have the membrane distal portion of the collagenous domain which is essential for ligand binding.

The self oligomerization of the truncated receptor suggested that this inactive form might act as a dominant-negative mutant by forming inactive heterotrimers with full-length receptors when both types of polypeptide were simultaneously expressed in the same cells. To test this possibility, we transfected mixtures of the bSR-II and t-bSR expression vectors into COS cells and measured receptor activity. In the experiment shown in Figure 4.5, the amount of DNA in each transfection was held constant (10 μg/dish) while the ratio of bSR-II to t-bSR DNA varied. As a control, the t-bSR DNA in the mixtures was replaced with the vector pcDNAI lacking a cDNA insert. In the control mixtures of bSR-II and pcDNAI (Figure 4.5, open squares), the receptor activity increased linearly with the amount of bSR-II in the transfection mixtures. In comparison, receptor activity was partially or completely inhibited when plasmid encoding bSR-II was cotransfected with plasmid encoding t-bSR, even with ratios of full-length to truncated DNAs of 3:1 (solid squares). The inhibition of bSR-II activity by the t-bSR plasmid was seen repeatedly in independent experiments using different amounts and ratios of the vectors. Preliminary results suggest that cotransfection with the t-bSR expression vector resulted in a reduction in the amount of full-length receptor precursor (data not shown). This indicates that there was either a rapid degradation of the full-length polypeptide chain or a substantial reduction in its rate of synthesis.

To determine if the inhibition of bSR-II activity by the t-bSR plasmid was specific for scavenger receptors, we compared the effects of cotransfecting the t-bSR plasmid with expression vectors for either the scavenger receptor or the human low density lipoprotein receptor (hLDLR). Because COS cells express

endogenous LDL receptors, it was possible to evaluate the effects of the t-bSR plasmid on both endogenous and transfected LDL receptor activities. Table III shows that, as before, cotransfection with the t-bSR plasmid virtually completely inhibited bSR-II receptor activity. However, it had only a small effect on the endogenous COS cell and transfected human LDL receptor activities. In other experiments we observed similar reciprocal small reductions in both LDLR and bSR-II activities (20-25%) when their expression vectors were cotransfected. Thus, the minor inhibition of LDL receptor activity by cotransfection with t-bSR may have been due in part to an intrinsic limitation in the capacity of COS cells to express transfected gene products simultaneously.

Comparisons of the sequence and binding properties of the scavenger receptor with those of other proteins containing collagen-like regions previously led us to suggest that the collagen-like region might play a role in the broad ligand-binding specificity of the scavenger receptor (Tall et al., 1978; Ross et al., 1986; Rosenberg et al., 1988; Kodama et al., 1990). For example, C1q has been shown to bind a variety of substrates including fibrinogen, heparin, laminin and DNA (Raepple et al., 1976; Hughes-Jones and Gardner, 1978; Bohnsack et al., 1985; Entwistle and Furcht, 1988; Rosenberg et al., 1988; Jiang et al., 1991). To determine if the collagenous region of another protein might exhibit ligand binding specificities similar to those of the scavenger receptor, we examined the binding properties of the collagenous, or "tail", domain of complement component C1q ("C1q tails") (Guan et al., 1991). The ability of C1q tails to bind directly to several ligands of the scavenger receptor was determined (Table 4.4). In these direct, solid-phase binding studies, Sepharose beads covalently cross-linked to four different polyanionic molecules were tested for their ability to bind to ^{125}I-C1q tails. C1q tails bound efficiently to beads carrying the polyribonucleotides poly I and poly G, and less efficiently to maleylated BSA (M-BSA) beads, but did not bind poly C beads. We have reported similar results for the binding of detergent solubilized bovine lung scavenger receptors (Kodama et al., 1988) and soluble forms of the bovine scavenger receptors (Resnick et al., 1992) to polyribonucleotide beads. Thus, the collagenous tails of C1q appeared to have a ligand-binding specificity similar to that of scavenger receptors (Brown and Goldstein, 1983).

To more thoroughly compare the binding specificities of C1q tails and scavenger receptors, we determined the capacity of a larger panel of ligands to inhibit the binding of ^{125}I-C1q tails to M-BSA beads (Figure 4.6). A variety of molecules previously shown to inhibit AcLDL binding to scavenger receptors (shaded bars) and some of their corresponding negative controls which do not inhibit scavenger receptors (hatched bars) were tested. The scavenger receptor competitors poly G, poly I, the sulfated polysaccharides fucoidin and dextran sulfate, and M-BSA all inhibited binding of ^{125}I-C1q tails to the M-BSA beads. The ID_{50} values for poly I and poly G were determined to be in the range of 10-25 ng/ml in this assay when approximately 34 μg of M-BSA coupled

to the beads was used (data not shown). Poly G and poly I bound more tightly to ^{125}I-C1q tails (Table 4.IV) than to either membrane bound or soluble scavenger receptors (Pearson et al., 1992, Resnick et al., 1992). Unlike the other scavenger receptor ligands, AcLDL, the prototypical scavenger receptor ligand, did not compete for ^{125}I-C1q tail binding to M-BSA beads (see discussion below). The scavenger receptor negative controls chondroitin sulfate, native LDL and poly C did not block ^{125}I-C1q tail binding to M-BSA beads. A high concentration of native BSA (500 μg/ml) was maintained throughout the binding assay to stabilize the ^{125}I-C1q tails. Thus, native BSA, unlike M-BSA, did not inhibit the binding detected in these experiments. These results show that the collagenous tail domain of C1q exhibited a broad binding specificity similar, but not identical, to that of scavenger receptors. Together with the results using the t-bSR mutant, these experiments suggest that the collagenous domain of the scavenger receptor is necessary, and may be sufficient, to determine the broad binding specificity which characterizes this unusual receptor.

Discussion

One of the most distinctive features of scavenger receptors is their broad ligand binding specificity (Brown and Goldstein, 1983; Krieger, 1992). Typical cell surface receptors, such as the LDL or EGF receptors, bind their ligands with high affinity and great specificity. Macrophage scavenger receptors also bind ligands with high affinity; however, they bind an extraordinarily wide variety of ligands. Their ligands include modified proteins such as acetylated LDL (AcLDL) and maleylated bovine serum albumin (M-BSA), but not native LDL or native BSA, certain polyribonucleotides (poly I and poly G but not poly C), and certain polysaccharides (dextran sulfate but not chondroitin sulfate), phospholipids, polyvinyl sulphate, and bacterial lipopolysaccharide (Brown and Goldstein, 1983; Nishikawa et al., 1990; Hampton et al., 1991). The only common feature of scavenger receptor ligands recognized to date is that they are polyanionic macromolecules or complexes; however, many polyanions fail to act as ligands.

We have previously suggested that the receptor's collagenous domain may play a crucial role in ligand binding and receptor function (Kodama et al., 1990; Rohrer et al., 1990) To test this hypothesis, we generated frameshift mutations in the cDNAs for the bovine and murine type II scavenger receptors which result in the premature termination of the proteins within the collagenous domains. These bovine and murine truncation mutants, which contain only the N-terminal 8 of 24 (bovine) or 5 of 24 (murine) Gly-X-Y triplet repeats, exhibited no ligand-binding activity when they were transiently expressed in COS cells. Metabolic labeling/immunoprecipitation experiments established that the synthesis, post-translational processing, oligomerization, cell-surface expression, and stability of the truncated bovine scavenger receptor were essentially identical to those of the full-length bovine type II scavenger receptor. Since the truncation of the bovine scavenger receptor did not prevent its assembly into trimers, this suggests that only little (8 Gly-X-Y triplets), if any, of the collagenous domain is required for trimerization and raises the possibility that the other domains of the receptor, especially the alpha-helical coiled-coil domain, trimerize independently of the collagenous domain. The rate of processing of the truncated receptor was somewhat slower than that of the full-length type II receptor; however, this apparently minor difference seems unlikely to be responsible for the complete inactivity of the truncated receptor. Kodama, Doi and colleagues have independently analyzed similar truncation mutants and point mutants and found that positively charged residues in the 22 C-terminal amino acids of the collagenous domain are required for ligand binding (Kodama et al., 1991, Doi et al., 1993).

To determine indirectly if the collagenous domain might be sufficient to account for the broad binding specificity of the receptor, we examined the binding of scavenger receptor ligands and their negative controls to the collagenous N-terminal tails of complement component C1q. C1q is a complex

heterooligomeric molecule. The individual C1qA, C1qB and C1qC polypeptide chains assemble into $[AB]_2C_2$ hexamers creating two collagenous tails. Each collagenous tail is composed of two short segments of Gly-X-Y triplets, and each has a globular domain at its C-terminus (Reid, 1982). Three $[AB]_2C_2$ hexamers associate into the complete C1q molecule, whose ultrastructure resembles a bouquet of tulips with bent collagenous stems (the tails) and globular flowers (Reid, 1982; Loos, 1983). The C1qA subunit contains a cluster of positively charged residues at the N-terminus of the collagenous region that has been shown to bind DNA and heparin (Jiang et al., 1992). The scavenger receptor also has a conserved, highly positively charged region at the C-terminus of its collagenous domain (Ashkenas et al., 1992). In a series of binding and competition assays, we observed that C1q tails exhibited a broad binding specificity for polyanions which was strikingly similar, but not identical, to that of the scavenger receptors. The binding of C1q to polyanions, some of which include known scavenger receptor ligands, has been reported previously (Raepple et al., 1976; Hughes-Jones and Gardner, 1978; Bohnsack et al., 1985; Entwistle and Furcht, 1988; Rosenberg et al., 1988; Jiang et al., 1991); however, the similarity of the binding specificities of the C1q collagenous region and scavenger receptors has not been previously considered in detail. The current results suggest that the collagenous region of the scavenger receptor is not only necessary, but may also be sufficient, for establishing the characteristic broad binding specificity of the scavenger receptor.

The most notable difference between the specificities of the C1q tail and the scavenger receptors observed in the current studies was that AcLDL, the prototypical high-affinity ligand of the scavenger receptor, did not inhibit the binding of C1q tails to M-BSA beads. AcLDL does inhibit the binding of soluble scavenger receptors to M-BSA beads (Resnick et al., 1992). Thus, while a collagen triple helix containing multiple positively charged residues can exhibit broad polyanionic ligand binding properties similar to those of scavenger receptors, the detailed binding characteristics will almost certainly depend on the particular amino acid sequence of the Gly-X-Y triplets, and perhaps will also depend on the higher order oligomeric structure of the collagenous domains. It is also possible that other domains of the scavenger receptor, especially the adjacent coiled-coil and SRCR domains, may either directly or indirectly modulate the receptors' ligand specificity (e.g., see Penman and Krieger, 1992).

The importance of the full-length collagenous domain for the function, but not the oligomerization, of scavenger receptors suggested that truncated receptor subunits might act as dominant negative mutants by assembling into nonfunctional heterotrimeric complexes with full-length receptor subunits. The expression vector encoding the truncated bovine scavenger receptor mutant did specifically abolish scavenger receptor function when it was cotransfected with an expression vector for the full-length bovine type II receptor. It is possible that this dominant negative effect, although receptor-specific, may be a

consequence of cell-type specific properties of the transient COS cell transfection system used. Further studies will be required to determine whether this construct will be useful for stably reducing scavenger receptor levels in macrophages *in vitro* and *in vivo.*

Collagenous domains are present in several mammalian proteins, including scavenger receptors, complement component C1q, mannan binding protein, lung surfactant apoprotein and conglutinin (Drickamer et al., 1986; Voss et al., 1988; Thiel and Reid, 1989). All of these proteins are thought to be involved in clearing the extracellular space of debris, including pathogenic material. Organisms may have employed collagenous regions in some of their proteins to perform rudimentary host-defense functions, possibly before the development of the humoral immune system. The binding activities and functions of these proteins may have been expanded and/or refined by variations in the sequences of the collagenous domains and by the addition of other functional domains. For example, the globular heads on the collagenous stalks of the mannan binding protein are C-type lectin domains which bind molecules with exposed mannose/N-acetyl-D-glucosamine residues, including yeast mannans and virulent gram-negative bacteria (Drickamer et al., 1986; Ezekowitz et al., 1991). The globular heads of C1q bind to antibodies engaged by their antigens. The functions of the presumably globular SRCR domains on the collagenous stalk of the type I scavenger receptor have not yet been defined. Thus, analysis of the structure and function of these and other proteins suggests that, in addition to the set of collagenous proteins which form the extracellular matrix, there is a distinct set of proteins containing short collagenous triple helical domains which participate in host defense.

Acknowledgements - We thank Dr. Andrea Tenner for the generous gift of ^{125}I-labeled C1q tails, Dr. Douglas Buechter for critical comments, and Lorna Andersson for technical assistance. We also thank Julia Khorana for help with preparation of the figures.

Figure 4.1. Domain structures of the full-length (bSR-II) and truncated (t-bSR) bovine type II scavenger receptors

The domain structure of the type II scavenger receptor (top) was deduced from its cDNA sequence (Rohrer et al., 1990). The N-terminal cytoplasmic tail (Cyto.) is followed by a single transmembrane domain (TM), a spacer domain (Spacer), an alpha-helical coiled-coil domain (Coiled-coil), a collagenous domain (Collagen, shaded), and a six amino acid C-terminal domain. The mutant t-bSR is truncated in its collagenous domain such that it contains only 8 of the 24 Gly-X-Y tripeptide repeats which are normally present.

Domain Structure of Wild-type and Truncated Bovine Scavenger Receptors

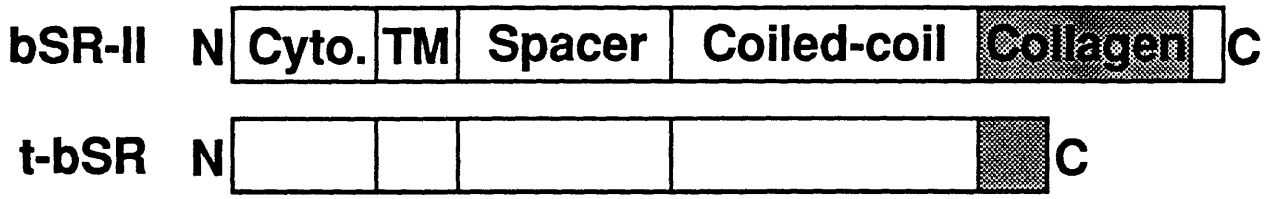


Figure 4.2. Biosynthesis, processing, and stability of full-length and truncated type II bovine scavenger receptors expressed in transiently transfected COS cells

Expression vectors containing either the full-length (bSR-II, left) or truncated (t-bSR, right) bovine type II scavenger receptors were transiently transfected into COS cells (10 μg/100 mm plate) on day 1 and the cells were replated on day 2 into 6-well dishes at 1 x 10^6 cells/well. On day 3, the cells were pulse-labeled with [^{35}S]methionine for 30 min and chased in medium A containing 1 mM unlabeled methionine for the indicated times. After cell lysis, scavenger receptors were immunoprecipitated, reduced, subjected to electrophoresis in an 8% SDS-polyacrylamide gel, and visualized by autoradiography as described in Materials and Methods ('p' and 'p_t' denote precursors of wild-type and truncated receptors, respectively; 'm' and 'm_t' denote mature forms of wild-type and truncated receptors, respectively).

Biosynthesis and Processing of Full-length and Truncated Type II Bovine Scavenger Receptors in Transiently Transfected COS Cells

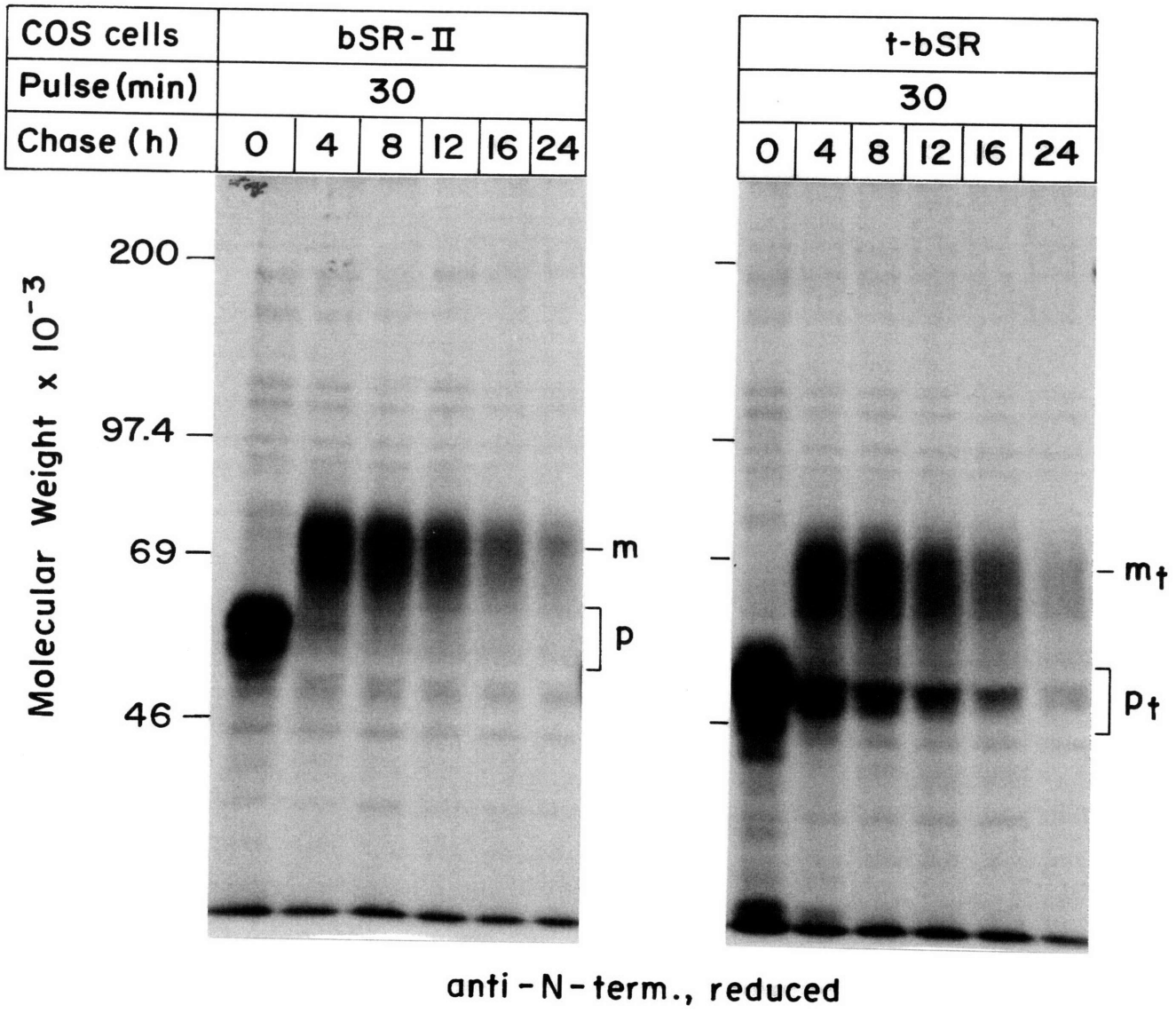
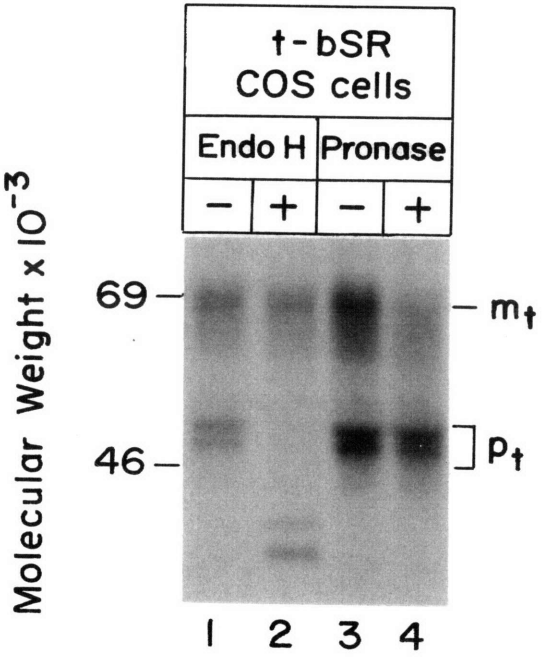


Figure 4.3. Endoglycosidase H- and pronase-sensitivities of truncated bovine scavenger receptors in transiently transfected COS cells

The expression vector for the truncation mutant (t-bSR) was transiently transfected into COS cells (10 μg/100 mm plate) on day 1 and the cells were replated on day 2 into 6-well dishes at 1 x 10^6 cells/well. On day 3 the cells were pulse-labeled for 30 min with [^{35}S]methionine and chased in medium A containing 1 mM unlabeled methionine for 2 hrs. Some of the cells were then incubated with or without pronase (lanes 3 and 4) as described in Materials and Methods. All of the monolayers were lysed and the truncated scavenger receptors were immunoprecipitated, reduced, subjected to electrophoresis in an 8% SDS-polyacrylamide gel, and visualized by autoradiography as described in Materials and Methods. For endoglycosidase H (Endo H) treatment (lanes 1 and 2), immunoprecipitated protein was incubated with Endo H or control buffer overnight at 37°C, prior to electrophoresis and autoradiography.

Endoglycosidase H and Pronase Sensitivity of Truncated Scavenger Receptors in Transiently Transfected COS Cells

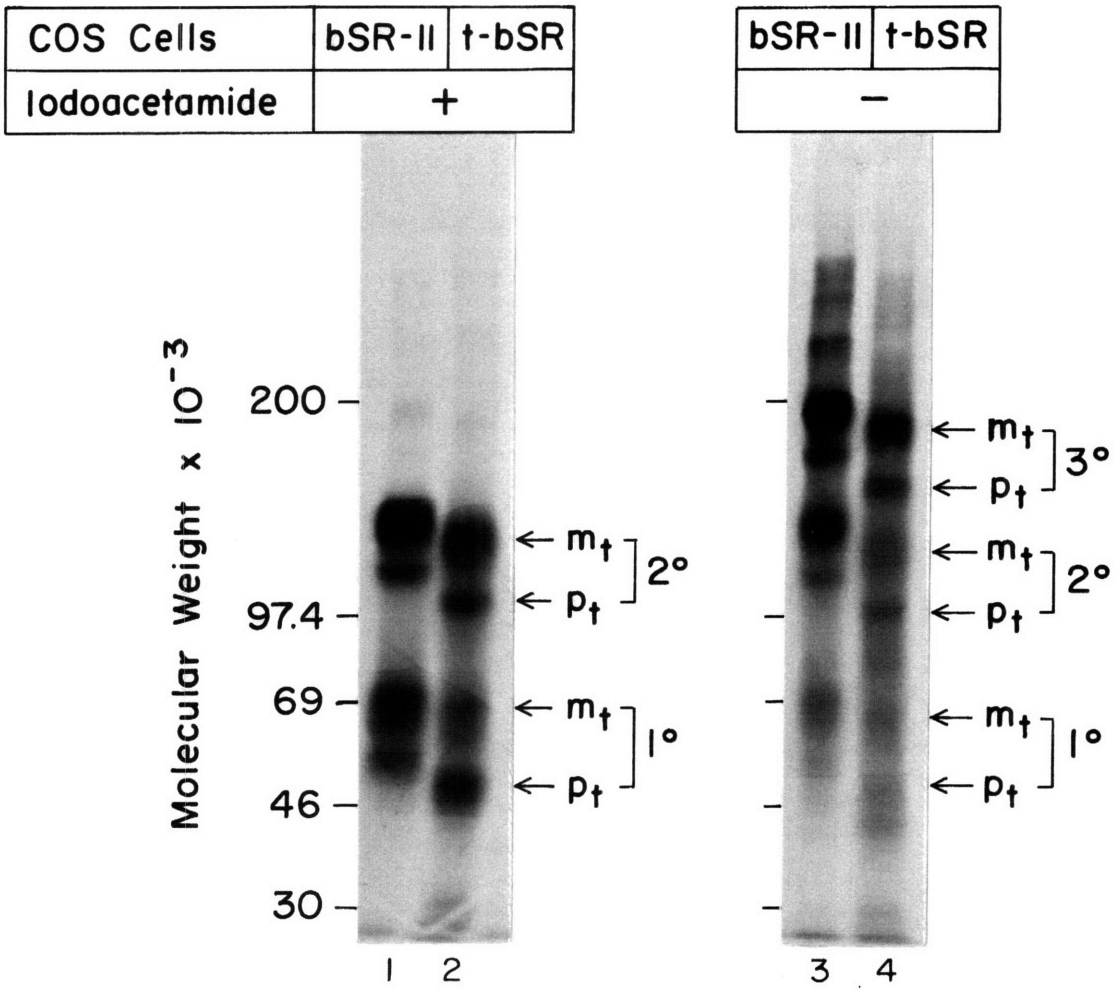


0.5 h pulse - 2h chase
anti-N-term., reduced

Figure 4.4. Oligomerization of full-length and truncated type II bovine scavenger receptors in transiently transfected COS cells

COS cells were transiently transfected with 10 μg/100 mm dish of either the bSR-II or t-bSR expression vectors on day 1, replated at 1 x 10^6 cells/well in 6-well dishes on day 2, and metabolically labeled for 5 hrs with 100 μCi/ml of [^{35}S]protein labeling mix (New England Nuclear) on day 3. Cells were lysed either in the presence or absence of 10 mM iodoacetamide as indicated. Lysates were then immunoprecipitated, and, without reduction, were subjected to 3-10% gradient SDS-polyacrylamide gel electrophoresis, and the receptors were visualized by autoradiography. ('p_t' and 'm_t' denote precursor and mature forms of the truncated receptor, and '1°', '2°' and '3°' denote monomers, dimers, and trimers, respectively)

Oligomerization of Full-Length and Truncated Type II Bovine Scavenger Receptors in Transiently Transfected COS Cells



5 hr label, 10 mM IA, anti-N-term., unreduced

Figure 4.5. Effects of the cotransfection of control and t-bSR expression vectors on the scavenger receptor activity of bSR-II transfected COS cells

On day 1, the indicated amounts of the expression vector for bSR-II were cotransfected with the indicated amounts of either a control vector without a cDNA insert (open squares) or the t-bSR expression vector (solid squares). The cells were replated in 6-well dishes (1×10^6 cells/well) on day 2, and then scavenger receptor activity was measured using an ^{125}I-AcLDL degradation assay on day 3 as described in Materials and Methods.

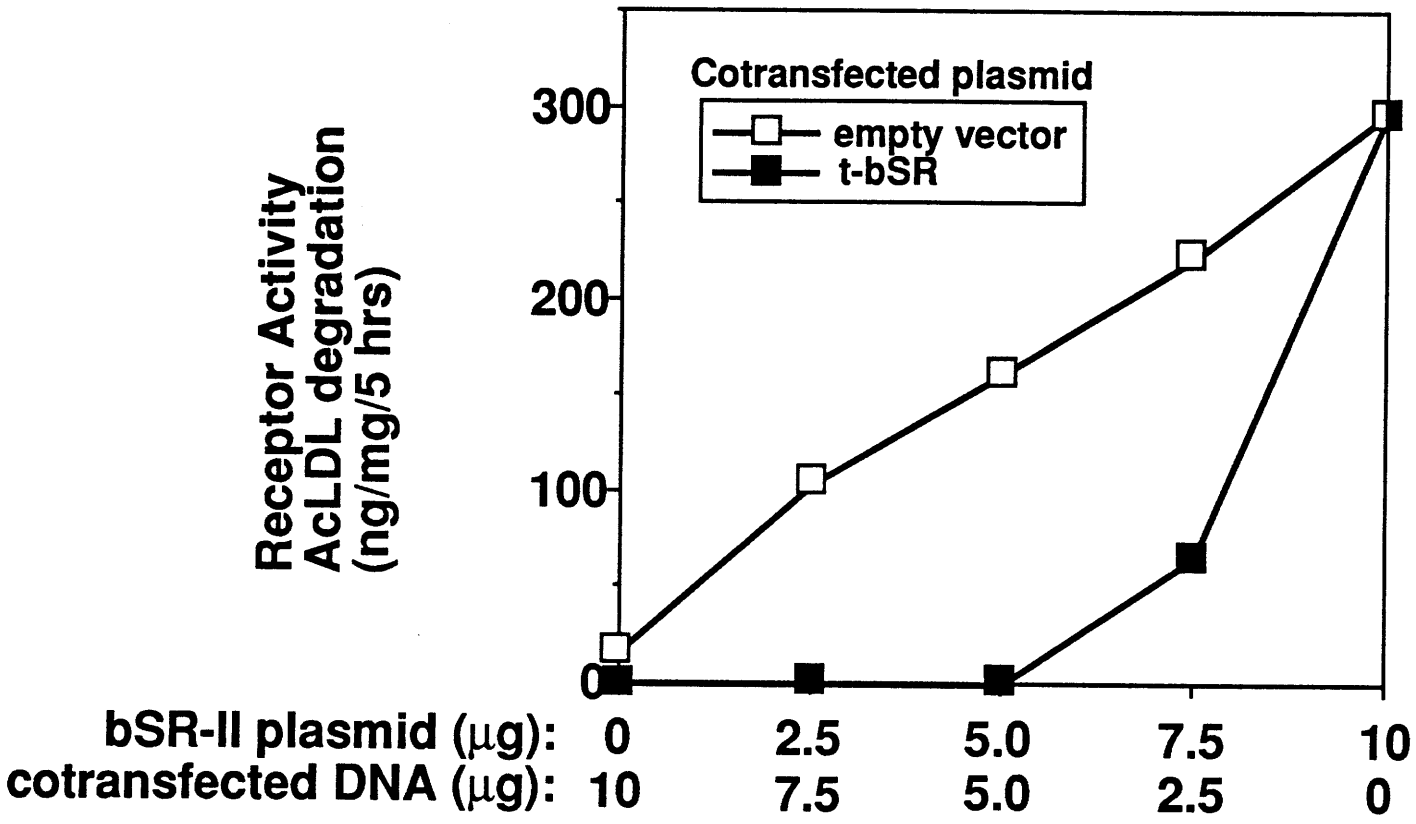
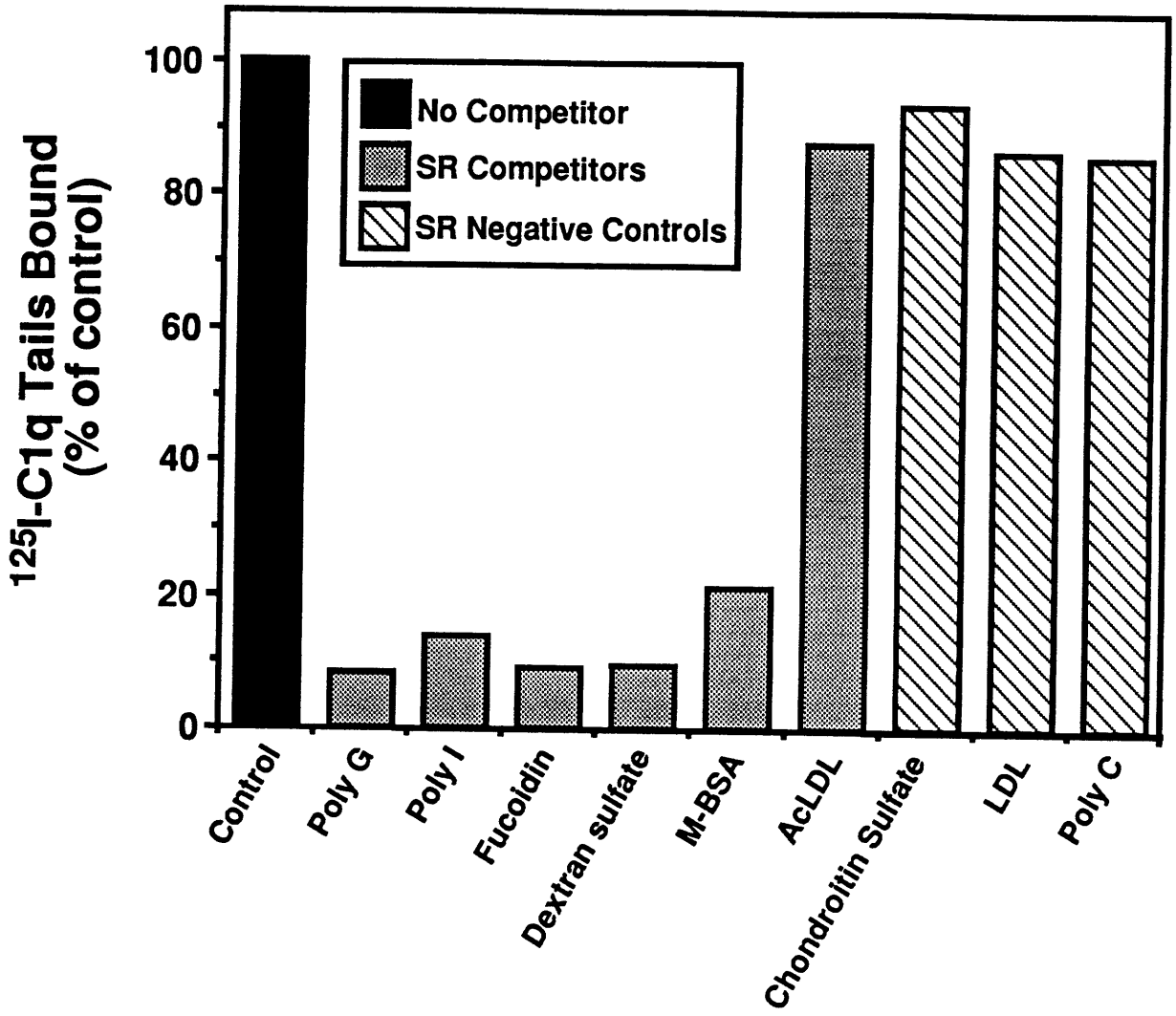

Receptor Activity
AcLDL degradation
(ng/mg/5 hrs)
300
200
100
0
Cotransfected plasmid
empty vector
t-bSR
bSR-II plasmid (μg): 0 2.5 5.0 7.5 10
cotransfected DNA (μg): 10 7.5 5.0 2.5 0

Figure 4.6. Effects of soluble competitors on the binding of ^{125}I-C1q collagenous tails to M-BSA Sepharose beads

The average values for the binding of ^{125}I-C1q collagenous tails to M-BSA Sepharose beads was determined after incubating duplicate specimens overnight at 4° C in buffers containing no additions (control, solid bar) or the indicated scavenger receptor competitors (shaded bars) or negative controls (hatched bars) as described in Materials and Methods. The poly G, poly I, fucoidin, dextran sulfate, chondroitin sulfate, and poly C concentrations were 10 µg/ml while the concentrations of M-BSA, AcLDL, and LDL were 400 µg protein/ml. The 100% of control value was 2020 cpm and represented 12% of the total ^{125}I-C1q collagenous tails included in the incubations.

Binding of ^{125} I-C1q Tails to M-BSA Beads: Effect of Soluble Competitors



Polyribonucleotides/polysaccharides, 10 μg/ml; proteins, 300 μg/ml

Table 4.1. Activities of full-length and truncated type II bovine and murine scavenger receptors in COS cells

transfected scavenger receptor		^{125}I-AcLDL degradation (ng/5 hr/mg protein)
bSR-II	(full-length bovine type II)	275
t-bSR	(truncated bovine type II)	0
mSR-II	(full-length mouse type II)	268
t-mSR	(truncated mouse type II)	0
none		7

COS cells in 100 mm dishes were transiently transfected on day 1 with 10 μg of plasmid pcDNAI with one of the indicated cDNAs inserted into the expression site. The cells were replated on day 2 into 6-well dishes at 1 x 10^6 cells/well in triplicate and receptor activity was assayed on day 3 using an ^{125}I-AcLDL degradation assay as described in Materials and Methods. The values represent averages of duplicate determinations corrected for nonspecific activity (single determinations).

Table 4.2. Cell-surface binding of ^{125}I-AcLDL at 4°C by COS cells transfected with expression vectors for the full-length and truncated type II bovine and murine scavenger receptors

transfected scavenger receptor	^{125}I-AcLDL binding (ng/mg protein)
bSR-II	66
t-bSR	8
mSR-II	109
t-mSR	9
none	8

COS cells in 100 mm dishes were transiently transfected on day 1 with 8 μg of plasmid pcDNAI with one of the indicated cDNAs inserted into the expression site. The cells were replated on day 2 into 6-well dishes at 1 x 10^6 cells/well and assayed on day 3 for ^{125}I-AcLDL binding activity (20 μg protein/ml) at 4°C as described in Materials and Methods. The values represent averages of duplicate determinations corrected for nonspecific activity (single determinations).

Table 4.3. Effects of expression of the truncated scavenger receptor on the activities of the full-length bovine scavenger receptor and the endogenous and transfected human LDL receptors

		ligand degradation (ng/5 hr/mg)		Inhibition by t-bSR
Cotransfected Receptor	Ligand	+control vector	+ t-bSR	(%)
bSR-II	^{125}I-AcLDL	179	8	96
None*	^{125}I-LDL	130	117	10
hLDLR**	^{125}I-LDL	417	323	23

*Endogenous LDL receptor activity measured

**Endogenous LDL receptor plus transfected human LDL receptor activity measured

COS cells in each 100 mm dish were transiently transfected on day 1 with 3 μg of the indicated cotransfected receptor vectors and 5 μg of either the control vector without a cDNA insert or the expression vector for t-bSR. The cells were replated at 1 x 10^6 cells/well in 6-well dishes on day 2, and then assayed for ^{125}I-AcLDL or ^{125}I-LDL degradation activity on day 3. For ^{125}I-LDL degradation assays, cells were preincubated with 25-hydroxycholesterol (1 μg/ml) plus cholesterol (10 μg/ml) for 24 hours prior to the assay as described in Materials and Methods.

Table 4.4. Binding of ^{125}I -C1q collagenous tails to ligands coupled to Sepharose beads

Ligand	^{125}I -C1q tails Bound *(% of total)*
Poly I	103.5%
Poly G	81.6%
M-BSA	28.2%
Poly C	3.5%

The binding at 4°C of ^{125}I-C1q collagenous tails to the indicated ligands coupled to Sepharose beads was determined as described in Materials and Methods. Total counts of ^{125}I-C1q collagenous tails used in each incubation was 14,500 cpm.

Chapter 5

The SRCR Superfamily: A collection of host-defense related proteins reminiscent of the immunoglobulin superfamily

The function of the SRCR domain on the type I receptor has been a mystery since the initial cloning of the receptor. It has little effect on the ability of the scavenger receptor to bind its polyanionic ligands, as the type II receptor (lacking this domain) has essentially identical ligand binding properties (Kodama et al., 1990; Rohrer et al., 1990; Ashkenas et al., 1993). In the course of doing periodic searches of the Genbank database for proteins homologous to the SR-A, I noticed that novel SRCR domain containing proteins were appearing at an increasing rate. This chapter contains a review of the scavenger receptor cysteine rich (SRCR) superfamily. This work was published in its entirety in 1994 in *Trends in Biochemical Sciences* **19**, 5-8. After the publication of this work, I discovered that we had incorrectly aligned CD6 domain 1, resulting in the loss of a cysteine. Several typographical errors in the published version were also corrected prior to inclusion in this thesis, and all citations have been merged into the "references" section of this thesis.

There have been several significant advances in our knowledge of the SRCR domains since the publication of this review. These include the identification of 5 new superfamily members and the discovery of a ligand for CD6. I have summarized many of these findings in appendix B. A map of the disulfide bonding pattern of the SR-AI SRCR domain was determined using proteolytic mapping and HPLC separation of fragments. This work is included in chapter 6 of this thesis. Additional studies are presented in appendix C on progress in efforts to determine the high resolution structure of a single SRCR domain.

I generated the sequence alignment and wrote the initial draft of this review. Alan Pearson noticed the internal repeat in the WC1 and M130, and both Alan and Dr. Monty Krieger provided assistance with finalizing the sequence alignment and the editing and revising of the manuscript.

The SRCR Superfamily: A collection of host-defense related proteins reminiscent of the immunoglobulin superfamily

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Analysis of the cysteine-rich domain in the type I macrophage scavenger receptor (SRCR domain) has helped define an ancient and highly conserved motif. Single and multiple tandem SRCR domains appear in a number of secreted and integral membrane proteins associated with cell surface recognition and host defense.

Many proteins are molecular mosaics composed of a wide variety of conserved sequence motifs which encode structurally distinct domains (Doolittle, 1985). In many cases, these motifs are characterized by short, disulfide stabilized domains present in the extracellular portions of membrane proteins and in secreted proteins (Doolittle, 1985; Krieger, 1986). Examples include the immunoglobulin repeat (Hunkapiller and Hood, 1989; Williams and Barclan, 1988), kringle domains, epidermal growth factor (EGF)-like repeats, complement C9/LDL receptor domains, Ly-6 repeats (Williams, 1991; Palfree, 1991) and the P-domain (Hoffman and Hauser, 1993). These protein domains are well suited for a variety of biochemical tasks, including ligand binding, and are readily combined with themselves or with other types of domains for the construction of complex mosaic proteins. Numerous proteins containing as few as one and as many as 36 copies of any one single type of domain have been reported (Wharton et al., 1985; McLean et al., 1987).

One ancient and highly conserved family of cysteine-rich protein domains was recognized during the analysis of the structure of the type I macrophage scavenger receptor (Freeman et al., 1990; Krieger, 1992). This class of domain, designated SRCR (scavenger receptor cysteine-rich) domain, was initially defined by the presence of one to four copies per polypeptide chain of an approximately 101 amino acid long motif in the type I scavenger receptor, the speract receptor, CD5/Ly1 and complement factor I (CFI) (total of 13 sequences, including 9 independent sequences and 4 sequences of homologs from different species). The recent cloning of genes encoding 24 additional, independent SRCR domains, and 10 additional homologs, has allowed us to revise our initial description of the consensus SRCR domain and to define two distinct subgroups. The recently available sequences, which were identified using the programs BLASTN and TBLASTN (Altschul et al., 1990), are those of CD6 (Aruffo et al., 1991), the cyclophilin C binding protein (Friedman et al., 1993) and its homolog the MAC2-binding protein (Koths et al., 1993) (CyCAP/MAC2-BP), the WC1 antigen (Wijngaard et al., 1992), M130 (Law et al., 1993), a new CFI homolog (Muglia et al., 1991), and two new homologs each of CD5/Ly1 (Yu et al., 1990; Murakami and Matsuura, 1992) and the scavenger receptor (Matsumoto et al., 1990; Bickel and Freeman, 1992). Thus, the superfamily of SRCR domain containing proteins includes 8 different members derived from five mammalian (human, bovine, murine, rat and rabbit), one amphibian (*Xenopus laevus*) and one invertebrate (sea urchin) species (Table 5.1). The sequences of the SRCR domains are shown in Figure 5.1.

An alignment of all 33 independent SRCR domains (total of 47 sequences) is shown in Figure 5.1. Positions at which there is greater than 50% sequence identity are shaded. We have divided the SRCR superfamily into two groups, A and B, based primarily on the differences in the spacing pattern between their cysteine residues (Figure 5.2). All of the group A domains contain six cysteines, while most of those in group B have eight cysteines. Although several members of group B contain only six (WC1-1, M130-8, CD5-2, and bovine CD5-1) or seven (CD6-1) cysteines, the presence of cysteines at the C^1 and C^4 positions and other sequence features clearly suggest that they are members of group B.

Independent consensus sequences for groups A and B, as well as a combined overall consensus sequence, are shown at the top of Figure 5.1. The overall consensus sequence includes residues at 41 out of 101 possible positions. The principle differences between the group A and B sequences are: 1) cysteines are not present at sites C^1 and C^4 in group A, aromatic residues almost invariably replace the C^4 cysteines, and 2) group B contains a conserved glycine adjacent to the C^4 position and a moderately well conserved tryptophan two residues before position C^6 which are not found in group A.

The sequences of the SRCR domains in CFI and CD5, particularly the CD5-2 domains, are the least well conserved members of the superfamily, and are responsible for much of the gapping seen in the alignment (Figure 5.1). In the case of CD5-2, although the N-terminal half of this SRCR domain clearly conforms to the consensus sequence, most of the residues in the C-terminal half of the domain differ substantially from the consensus (Figure 5.1). Because the CFI and CD5 sequences played important roles in the initial analysis of the SRCR domain (Freeman et al., 1990), the alignment of sequences and the definition of the SRCR consensus shown in Figure 5.1 differs from those proposed previously.

All but one of the 33 independent SRCR domains have six or eight cysteines. Analysis of the structure of the type I macrophage scavenger receptor (Penman et al., 1991) suggests that some, and possibly all, of its SRCR domain's cysteines participate in intradomain disulfide bonds. Furthermore, the cysteines in CyCAP/MAC2-BP do not participate in intermolecular disulfide bonds (Friedman et al., 1993; Koths et al., 1993). Based on the known structures of other cysteine-containing domains (e.g., immunoglobulin, kringle, Ly-6 repeat, P-domain), we assume that in most SRCR domains the conserved cysteines participate in intradomain disulfide bonds. The pattern of cysteine pairing into disulfides has not been established experimentally; however, sequence analysis suggests two likely disulfide pairs. The cysteines at positions C^1 and C^4 in group B domains may be disulfide bonded, because this pair of cysteines is always present in group B but not in group A (Figure 5.2). A similar argument suggests that the cysteines at positions C^2 and C^7 form a disulfide bond. These cysteines are present in the human, murine, and

rat CD5-1 domains but not in their bovine counterpart. This pair of cysteines is also absent in WC1-1 and M130-8. On the other hand, in CFI, WC1-2, and WC1-7, only one member of this pair is present; the other is apparently substituted by a cysteine at a different site. If these proposed C^1-C^4 and C^2-C^7 pairs are correct, the remaining two potential disulfide pairs are either C^3-C^5/C^6-C^8, C^3-C^6/C^5-C^8, or C^3-C^8/C^5-C^6. It is important to note, however, that not all of the cysteines need participate in intradomain disulfide bonds, as is clearly the case for at least one of the seven cysteines in CD6-1.

A schematic representation of the known SRCR domain containing proteins is shown in Figure 5.3. All of the members of this group are cell surface or secreted polypeptides containing between 1 and 11 SRCR domains. Other than the scavenger receptor trimer (Penman et al., 1991), the oligomeric states of these proteins are unknown; although MAC2-BP forms a large multimer (Koths et al., 1993). Proteins containing multiple SRCR domains in a single polypeptide chain have short interdomain spacers whose median length is 6 residues. Our dendrigraphic (Devereux et al., 1984) and visual analysis of two such proteins, WC1 and M130, revealed an interesting higher order repeat, which had previously been identified as an internal repeat in WC1 at the DNA sequence level (Wijngaard et al., 1992) and was independently identified in a somewhat different form by Law and colleagues (Law et al., 1993). This repeat is defined by a cassette of four SRCR domains, designated [b-c-d-e]. The N-to-C terminal ordering of the SRCR domains in WC1 and M130 can be represented as follows: WC1 (domains 1-11), a-[b-c-d-e]-d-[b-c-d-e]-f; and M130 (domains 1-9), h-i-j-k-[b-c-d-e]-l. The [b-c-d-e] cassette contains an unusually long, well conserved, 35 residue spacer between the c and d domains and appears three times in highly conserved, but not identical, forms: twice in WC1 and once in M130. Apparently, WC1 and M130 are derived from a common cassette-bearing ancestor. This cassette may impart some common function to these integral membrane proteins.

The biochemical functions of SRCR domains have not been established with certainty; however, it seems possible that most, if not all, of these domains are involved with binding to other cell surface or extracellular molecules. In the case of the speract receptor, the SRCR domains compose 91% of the extracellular portion of the molecule, and thus probably mediate the binding of its ligand, the sperm activating peptide speract (Dangott et al., 1989). Similarly, the SRCR domains compose most of CD5's extracellular sequence, and probably define the binding site for CD72, the only known ligand for CD5 (Van de Velde et al., 1991). The C-terminal SRCR domain of the type I macrophage scavenger receptor is not required for the protein's assembly, intracellular transport, cell surface expression or binding of its known polyanionic ligands; a second natural isoform of the scavenger receptor, type II, is virtually identical to the type I receptor except that it does not have the C-terminal SRCR domain

(Krieger, 1992). Thus, in scavenger receptors, the SRCR domain may impart an additional, but as yet undefined, binding capacity.

All of the known mammalian SRCR domain-containing proteins are expressed on the surfaces of cells associated with the immune system and host defense functions (T-cells, B-cells, macrophages) or are secreted and known or suspected of being involved with host defense (CFI, CyCAP/MAC2-BP). This is strikingly reminiscent of the immunoglobulin superfamily of cysteine containing protein domains. Single or multiple tandem copies of immunoglobulin domains also appear in diverse secreted and membrane associated proteins. These domains participate in a variety of binding interactions which play critical roles in host defense (Hunkapiller and Hood, 1989; Williams and Barclay, 1988). Additional studies will be required to determine the detailed molecular structures of SRCR domains and their functions.

Acknowledgments - We thank Jason Seaman at the Whitaker College Computing Facility, MIT, for his assistance with computer analysis and P. Schimmel for advice. This work was supported by a grant from the National Institutes of Health-National Heart, Lung and Blood Institute (HL41484). DR is a Howard Hughes Medical Institute Predoctoral Fellow.

Table 5.1. Proteins containing SRCR domains [a]

Protein (abbr.)	Number of SRCR domains per chain	Source	Functions	Location
Macrophage scavenger receptor, type I (MSR)	1	Mammalian macrophages	Binding and endocytosis of diverse ligands	Cell surface
Cyclophilin C (CyCAP) or MAC2 binding protein (MAC2-bp)	1	Murine bone marrow stromal cell line, murine macrophages, and human breast carcinoma cells	Binds cyclophilin C and MAC2, a lactose/galactose specific lectin	Secreted fluids, e.g. breast milk, tears, plasma
Speract receptor (SperactR)	4	Sea urchin sperm	Binds speract, a sperm-activating peptide	Cell surface
Complement factor I (CFI)	1	Mammalian and amphibian plasma	Protease, regulation of the complement cascade	Secreted into the plasma
WC1	11	Mammalian CD4-, CD8-, γδ T cells	Unknown	Cell surface
M130	9	Human macrophages	Unknown	Cell surface
CD6	3	Mammalian T cells and some specialized B cells	Unknown	Cell surface
CD5	3	Mammalian T cells and some specialized B cells	Binds CD72, a B-cell surface protein	Cell surface

[a]Adapted from Krieger, 1995

Figure 5.1. Comparison of the sequences of 47 SRCR domains from 8 proteins.

The sequences were aligned using both the program PILEUP ([24]) and manual adjustment . Only 33 of the 47 SRCR sequences listed represent independent SRCR domains, the remaining sequences are homologs from other species (e.g., four species of scavenger receptor, two species of CFI, etc.). Residues are shaded when at least 17/33 independent sites are identical (when there are four homologs, each sequence contributed .25 to the total count; for two homologs, each sequenced contributed .5 to the total). Consensus sites are indicated in the top row (Overall consensus) when ≥22.25 of the 33 positions are occupied by a single amino acid (capital letter) or a single class of residue (a, aliphatic (A, I, L, V); π, aromatic (F, W, Y); h, hydrophobic (a, π, M); +, positively charged (H, K, R); -, negatively charged (D, E); ±, charged (-, +); o, S or T). Consensus sequences for Group A domains (≥4.75/7 sites) and Group B domains (≥17.5/26 sites) are also shown. In addition, the consensus cysteine positions are identified by number. For multiple SRCR domains in individual proteins, the domains are numbered from the amino terminus. Abbreviations used are as follows: MSR, macrophage scavenger receptor ; CyCAP, cyclophilin C associated protein; MAC2bp, MAC2 binding protein; SperactR, speract receptor; CFI, complement factor I; and WC1, WC1 antigen. In the cases where multiple species homologs are presented, the following abbreviations for species designation are used: b, bovine; h, human; m, murine; r, rat; l, rabbit (lapin); and x, *Xenopus laevus*.

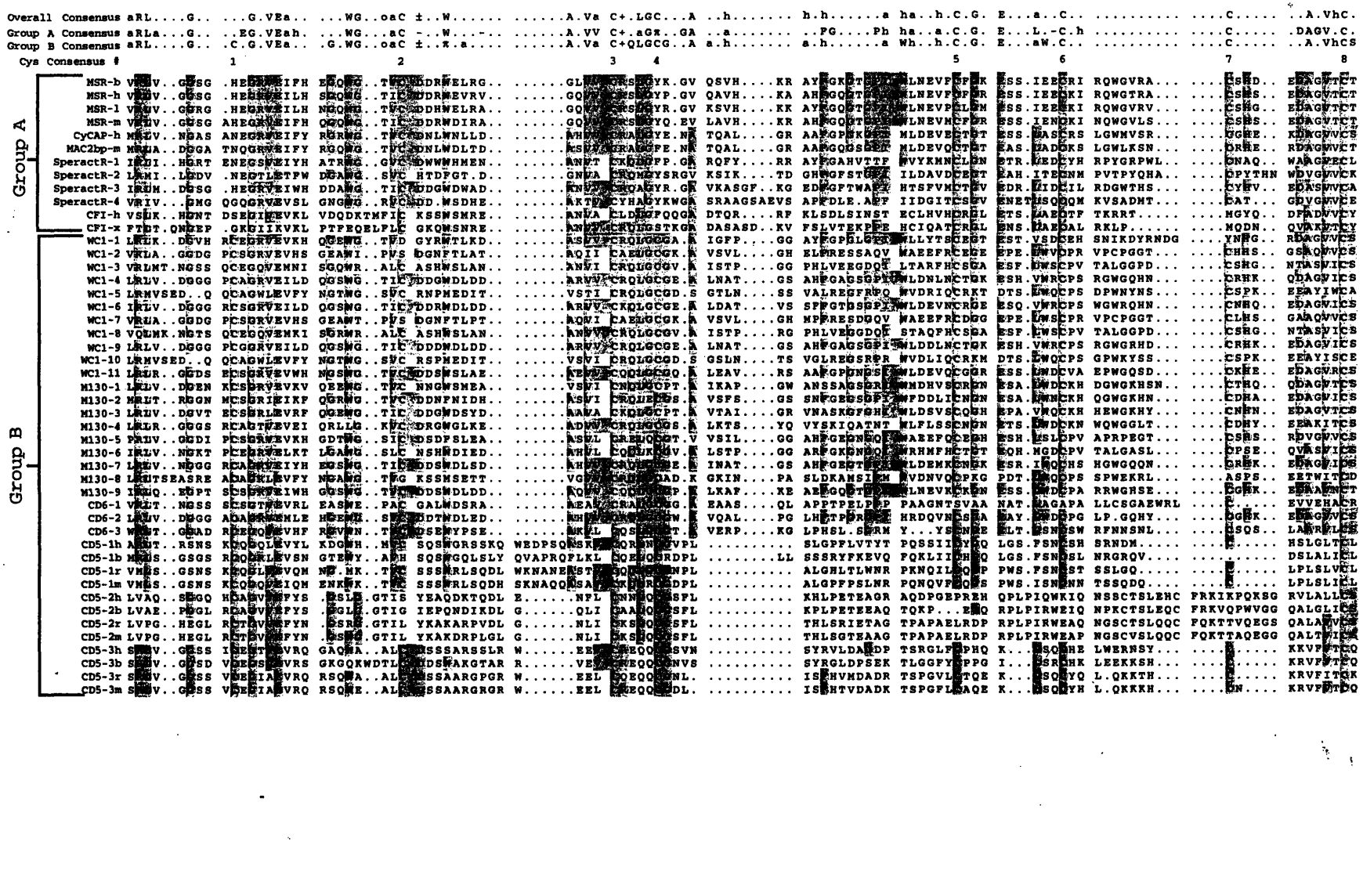

Overall Consensus
Group A Consensus
Group B Consensus
Cys Consensus #
Group A
Group B
MSR-b
MSR-h
MSR-l
MSR-m
CyCAP-h
MAC2bp-m
SperactR-1
SperactR-2
SperactR-3
SperactR-4
CFI-h
CFI-x
WC1-1
WC1-2
WC1-3
WC1-4
WC1-5
WC1-6
WC1-7
WC1-8
WC1-9
WC1-10
WC1-11
M130-1
M130-2
M130-3
M130-4
M130-5
M130-6
M130-7
M130-8
M130-9
CD6-1
CD6-2
CD6-3
CD5-1h
CD5-1b
CD5-1r
CD5-1m
CD5-2h
CD5-2b
CD5-2r
CD5-2m
CD5-3h
CD5-3b
CD5-3r
CD5-3m

Figure 5.2. Consensus spacing of cysteines based on the alignment in part A

The average number of residues between the cysteines is indicated.

Consensus Spacing of Cysteines in SRCR Domain Subtypes

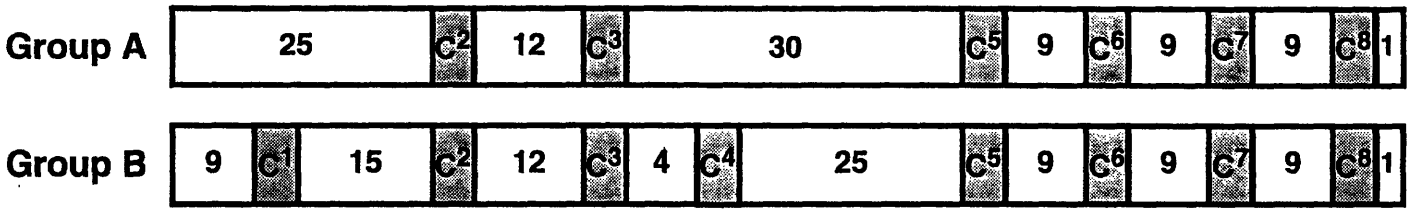
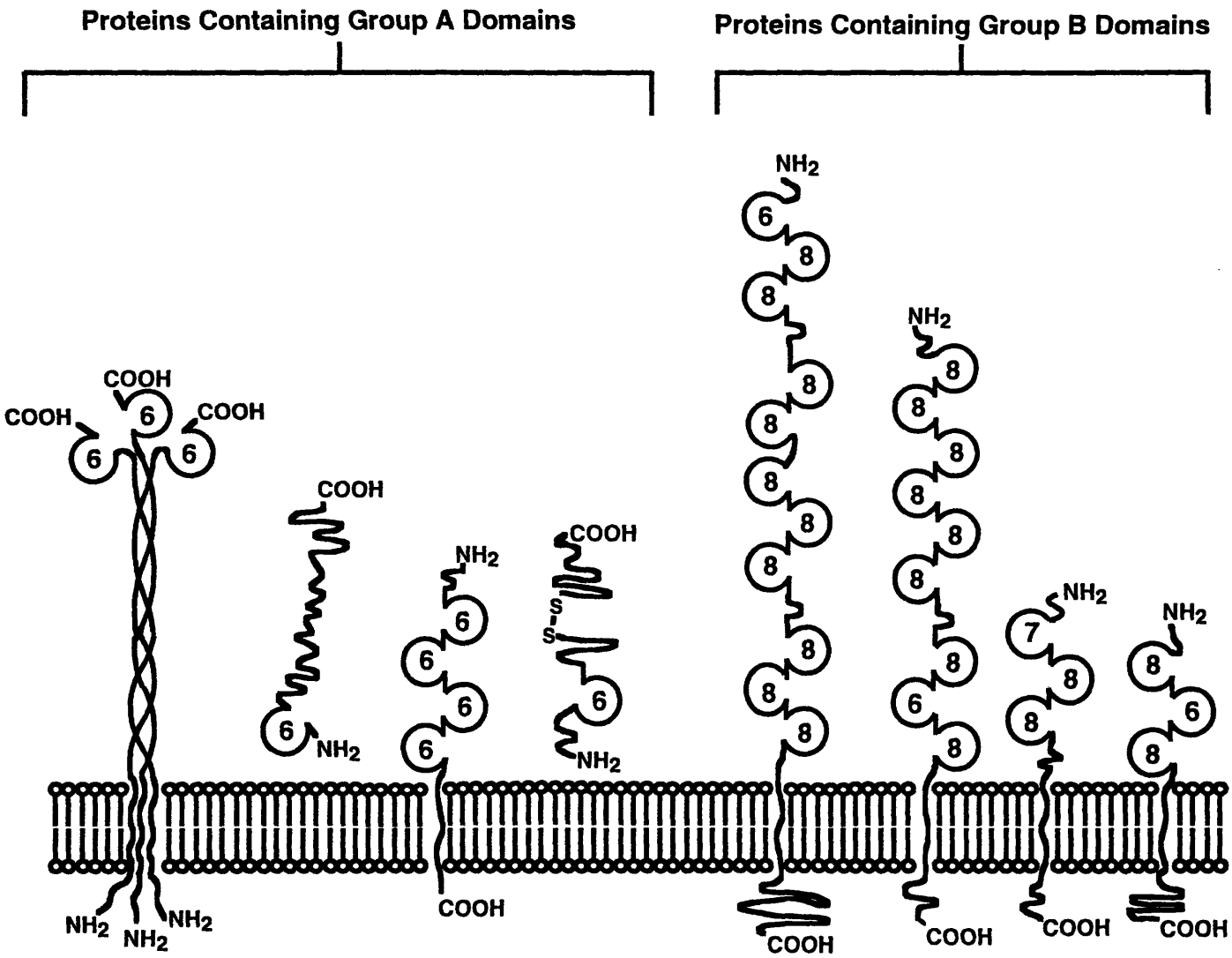


Figure 5.3. Models of proteins in the SRCR domain superfamily

Models illustrating the number and relative positions of SRCR domains (semicircles) in eight proteins are shown, with the names and sources of each protein indicated. Also shown are the numbers of cysteines in each domain.

Members of the SRCR Domain Superfamily



Scavenger Receptor, Type I	CyCAP/ MAC2-BP	Speract Receptor	Complement Factor I	WC1 Antigen	M130	CD6	CD5
Mammalian macrophages	Secreted fluids	Sea urchin sperm	Mammalian and amphibian plasma	Mammalian $CD4^-$, $CD8^-$, γδ T-cells	Human macrophages	Mammalian T cells and some types of B-cells	

Chapter 6

Biochemical, spectroscopic and electron microscopic analysis of the structures of the human class A macrophage scavenger receptors

The preceding chapters have begun to address some structural questions about the scavenger receptor, including the determination of its oligomeric state and generally extended shape, and the identification of its ligand binding domain. However much of the structural model of the scavenger receptor presented in the introduction (diagrammed in Figure 1.1) remains unproven. This chapter contains a series of experiments aimed at testing this model, and at further refining it. In particular, it asks the following questions: 1) does the collagenous sequence contain modified residues associated with collagenous structure; 2) is the gross level of α-helix in the protein consistent with the proposed structure of the coiled-coil domain; and 3) what is the disulfide bonding pattern in the SRCR domain. It also examines the question of pH dependence of receptor structure, in particular with regard to a previously proposed mechanism of pH dependent ligand release. Finally, it begins to address the question of overall receptor structure by using two electron microscopic imaging techniques to visualize individual type I and II receptor molecules.

This chapter is a draft of a manuscript which, when completed, will be submitted to *The Journal of Biological Chemistry*. There remains an unresolved question with regard to the interpretation of the electron microscopic images of the SRs. Different conformations of the receptor have been observed at low and high pH by rotary shadowed EM. Efforts are being made to reproduce this effect with negative stain EM, and to also see whether hydrodynamic data supports such an alteration of structure. The basis for this observation and its significance remain unclear.

I carried out most of the experiments presented in this chapter. The expression constructs for the secreted receptors were generated by Dr. Karen Schwartz, as was the amplified cell line expressing the type II receptor. Shangzhe Xu produced the amplified type I expressing cell line, and provided assistance with tissue culture. Drs. Henry Slater and John Chatterton performed the electron microscopy using purified receptor that I provided. I wrote the initial draft of the paper, and edited and revised it with input from both Dr. Monty Krieger and Dr. Chatterton.

Abstract

Type I and type II class A macrophage scavenger receptors (SR-AI and SR-AII) have been implicated in the development of atherosclerosis and other macrophage-mediated processes. They are predicted to be highly elongated trimers, containing fibrous α-helical coiled-coil and collagenous triple helical domains. The SR-AI trimer also includes three SRCR (scavenger receptor cysteine-rich) domains, each presumably containing three intramolecular disulfides. Transfected Chinese hamster ovary cells expressing secreted forms of the human receptors (s-hSR-AI and s-hSR-AII) were generated, and their highly purified products were used for biochemical and biophysical analysis of these receptors' structures. The pattern of the three intramolecular disulfide bonds in the SRCR domain of SR-A (Cys^2-Cys^7, Cys^3-Cys^8, Cys^5-Cys^6) was determined using proteolytic analysis. Both s-hSR-AI and s-hSR-AII contain significant amounts of hydroxylated lysine and proline, modified amino acids characteristic of proteins with collagenous triple helices. Circular dichroism (CD) studies revealed that both isoforms of the receptor contain amounts of α-helix consistent with the predicted model, that much of the secondary structure is identical at pH 7.4 and 5.7, and that the stabilities of the α-helices are greater at the lower pH (apparent T_ms for both were 64°C (pH 7.4) and 74°C (pH 5.7)). Thus, the dissociation of ligands from these receptors at low pH is unlikely to be a consequence of major structural disruptions in the α-helical domain. Both metal shadowing and negative staining techniques were used for the electron microscopic visualization of these elongated molecules. The negative-stained molecules (~440 Å long) are composed of two rod-like segments (collagenous and α-helical coiled-coil domains, ~200 Å each) connected by a flexible hinge. In s-hSR-AI, the SRCR domains were identified as distinct globular structures at the end of the collagenous domains. These studies have confirmed the previously predicted structural models for the class A scavenger receptors.

Introduction

Three structurally distinct classes of modified lipoprotein-binding scavenger receptors have been identified to date. These include the class A (SR-A), class B (SR-B), and class C (SR-C) receptors (Krieger and Herz, 1994; Elomaa et al., 1995; Endemann et al., 1993; Acton et al., 1994; Pearson et al., 1995). In addition, macrosialin has recently been shown to be an oxidized LDL binding protein (Ramprasad et al., 1995). Class A macrophage scavenger receptors are trimeric integral membrane glycoproteins which exhibit unusual ligand binding properties (Krieger and Herz, 1994). They bind a diverse array of macromolecules or macromolecular complexes, whose only common property is that they are polyanionic. These ligands include modified lipoproteins (acetylated or oxidized LDL), bacterial surface lipids (endotoxin and lipoteichoic acid), certain polynucleotides (poly(G) and poly(I), but not poly(C)), and some sulfated polysaccharides (fucoidan and dextran sulfate, but not heparin or chondroitin sulfate). A number of studies have suggested that class A scavenger receptors may play a role in a wide variety of macrophage associated physiologic and pathophysiologic processes, including cholesterol deposition in arterial walls during atherosclerosis (Krieger and Herz, 1994; Brown and Goldstein, 1983; Steinberg et al., 1989), adhesion (Fraser et al., 1993), and immune responses and inflammation (Hampton et al., 1991; Dunne et al., 1994).

The cDNAs encoding two closely related isoforms of class A macrophage scavenger receptors have been cloned from bovine, murine, rabbit, and human tissues (Kodama et al., 1990; Matsumoto et al., 1990; Rohrer et al., 1990; Freeman et al., 1990; Bickel and Freedman, 1992) These isoforms, designated class A type I (SR-AI) and type II (SR-AII), are the alternative splicing products of a single gene (Freeman et al., 1990; Emi et al., 1993). Based on the amino acid sequence, SR-AI and SR-AII were predicted to contain six distinct structural domains (Kodama et al., 1990; Rohrer et al. 1990; Ashkenas et al., 1993): I, an amino terminal cytoplasmic domain; II, a single transmembrane domain; III, a spacer domain; IV, an α-helical coiled-coil domain; V, a collagenous domain; and VI, an isotype specific C-terminal domain of variable length. Domains I-V are identical in SR-AI and SR-AII. In SR-AII, domain VI consists of a short (6-17 amino acid), poorly conserved sequence with no remarkable features. In SR-AI, domain VI is 110 residues long, and includes an 8 residue hinge followed by a 102 residue region called the scavenger receptor cysteine-rich (SRCR) domain. This domain helped to define an ancient, highly conserved superfamily (Freeman et al., 1990) which currently includes 13 proteins containing 45 independent SRCR domains (Resnick et al., 1994; Li and Snyder, 1995; Kitamoto et al., 1994; Elomaa et al., 1995; Mayer and Tichy, 1995; Nunes et al., 1995). The functions of the SRCR domains in these diverse proteins are, for the most part, undefined; however it has been proposed that SRCR domains may participate in ligand binding interactions. Recent studies by Arrufo and colleagues have established such a function for one of the SRCR domains in CD6, a protein expressed by T cells and some B cells (Whitney et al., 1995b).

The SRCR domain in SR-AI, however, does not appear to play a key role in its distinctive broad polyanionic ligand binding (Rohrer et al., 1990; Ashkenas et al., 1993). The SRCR domain in SR-AI presumably confers an as yet undefined binding activity on this endocytic surface receptor. A third structurally related member of the SR-A family has recently been cloned and characterized (Elomaa et al., 1995). This receptor, called MARCO or SR-AIII, has an extended predicted collagenous region and a C-terminal SRCR domain, but no readily apparent coiled-coil domain.

Detailed analysis of the structures of class A scavenger receptors has been limited by the availability of adequate amounts of protein and by technical difficulties inherent in working with water insoluble molecules. To circumvent these problems, we have generated stable CHO cell lines which express substantial amounts of truncated, secreted forms of the human class A scavenger receptors, s-hSR-AI and s-hSR-AII. We have used purified secreted receptors to test predictions made about the structures of the SRCR, coiled-coil, and collagenous domains. We have also directly examined the low resolution structure of these molecules using electron microscopy. Our findings have confirmed the previous predictions and extended our understanding of the structures of the class A macrophage scavenger receptors.

Experimental Procedures

Materials - CHO[DHFR-] cells were obtained from the ATCC. Lysyl endopeptidase from *Achromobacter lyticus* (Lys-C), modified trypsin, and sequencing grade protease V8 (Glu-C) were obtained from Wako Bioproducts, Promega, and Boehringer Mannheim Biochemica, respectively. Uranyl acetate was obtained from Polysciences. Mica sheets (1 x 4 cm), graphite rods (for carbon evaporation), 500-mesh copper grids, and a diffraction grating replica with latex spheres were purchased from Ted Pella, Inc. Poly G beads and other reagents were obtained as previously described (Resnick et al., 1993).

Construction of pRc/CMV-s-hSR-AI and pRc/CMV-s-hSR-AII Expression vectors -- cDNAs encoding soluble forms of the human macrophage scavenger receptor type I and type II were generated by PCR using a 5' oligonucleotide encoding a sequence comprising the myelin-associated glycoprotein (MAG) leader sequence (Arquint et al., 1987) as well as a FLAG epitope tag (Hopp et al., 1988) and overlapping the SR-A cDNAs by 21 bases starting at the lysine at base position 230 (Matsumoto et al., 1990). The 3' oligonucleotides contained the last 21 bases of the type I or type II receptors followed by an Xba I restriction site. The blunt/*Xba* I fragment was ligated to pRc/CMV (Invitrogen) at the *Hind* III(blunt)/*Xba* I sites. The constructs were named pRc/CMV-s-hSR-AI or -AII. After cleavage of the signal sequence, the N-terminal sequence of the expressed proteins is predicted to be D-Y-K-D-D-D-D-K-K^{77}-, where Lys^{77} is the first residue contributed by the scavenger receptor.

Cell culture, transfections, and DHFR amplification - All incubations with intact cells were performed at 37°C in a humidified 5% CO_2 incubator. Stock cultures of CHO [DHFR-] cells were grown in medium A (IMDM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 10^{-4} M hypoxanthine, 10^{-5} M thymidine, and 2 mM glutamine) containing 10% (v/v) fetal bovine serum (FBS) (medium B).

Transfectants expressing the secreted human SR-AI (CHO[s-hSR-AI]) were generated using the Lipofectin method as suggested by the manufacturer (Gibco BRL). On day 0, CHO[DHFR-] cells were plated at 10^6 cells/100 mm dish in medium B. On day 1, the cells were washed twice with Dulbecco's complete phosphate buffered saline (cPBS, Gibco) and refed with a transfection mixture containing 5 ml medium A with 100 µl Lipofectin, 10 µg *Bgl* II linearized pRc/CMV-s-hSR-I, and 200 ng *Bam* HI linearized pSV2-dhfr (Subramani et al., 1981). Following a 5 hr incubation, 5 ml of medium A containing 20% (v/v) FBS were added. On day 2, the cells were refed with medium B, and on day 3, cells were harvested with trypsin and plated at $2x10^5$ - $1x10^6$ cells/100 mm dish in medium C (IMDM supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 0.5 mg/ml Geneticin (Gibco)) containing 7%

(v/v) FBS which was extensively dialyzed against 150 mM NaCl (medium D). On day 4, colonies were switched to medium C containing 10% dialyzed FBS and 50 nM methotrexate (Sigma). Cells expressing increasingly higher levels of s-hSR were selected by progressively increasing the concentration of methotrexate and re-screening for expression using metabolic labeling and a poly G solid-state bead binding assay as previously described (Resnick et al., 1993). One positive clone, designated CHO[s-hSR-AI] (clone 38-2-19) was resistant to 1 μM methotrexate and expressed high levels of s-hSR-AI. It was used for all experiments involving s-hSR-AI.

Transfectants expressing s-hSR-AII (CHO[s-hSR-AII]) were generated using a variation of the Lipofectin method. CHO[DHFR-] cells were grown in Ham's F12 instead of IMDM. Cells were plated at a density of $5x10^6$ cells/ 100 mm dish prior to transfection. Colonies were screened by Western blotting using an anti-peptide antibody raised against a C-terminal peptide of hSR-AII. A highly expressing cell line, CHO[s-hSR-AII]clone-201-16, resistant to 5 μM methotrexate, was obtained by amplifying with increasing concentrations of methotrexate and was used to generate s-hSR-AII.

Expression and purification of s-hSR-AI and s-hSR-AII - Expression: Cells were set in 850 cm^2 roller bottles in Medium E (Ham's F-12 medium containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) at a density of 10^7 cells per bottle. Cells were grown to ~ 50% confluence and were then washed twice with cPBS and refed with 200 ml Medium F (50% Ham's F12, 50% DMEM, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES pH 7.4, and 1 mM sodium butyrate). Four days later the cells were refed and incubated for an additional 4 days. The culture media were then collected, and after addition of sodium azide (final concentration : 0.01% (w/v)), were clarified by centrifugation (4000x*g* for 15 minutes at 4°C), and stored at 4°C or -20°C.

Purification: All steps were carried out at 4°C. Culture media were concentrated using a spiral cartridge ultrafiltration apparatus (Amicon) fitted with an S1Y100 membrane (approximate molecular weight cutoff of 100 kDa). Following concentration of 4-14 liters to approximately 350 ml, the concentrate was diafiltered against 6 liters of 50 mM ammonium bicarbonate, pH 7.5, and further concentrated to a final volume of 125 ml, all in the same device. The sample was then clarified by centrifugation (30 minutes, 4000x*g*). The supernatant was applied at a flow rate of 0.75 ml/min to a 6 by 1.5 cm column containing 10 ml AG-Poly G resin (Sigma) which had been equilibrated in 5% Buffer A (1 M ammonium bicarbonate, pH 7.5)/95% H_2O. The column was washed with 50 ml of 5% A and eluted at 0.5 ml/min with a 95 ml linear gradient containing 5-100% Buffer A. The effluent was monitored at 280 nm, and those fractions containing protein were analyzed by 10% SDS/PAGE and silver staining (Kozarsky et al., 1986; Morrissey, 1981). Fractions containing s-hSR-A

were pooled, diluted 1:1 with H_2O, and applied at 1 ml/min to a Mono Q HR5/5 column (Pharmacia) which had been pre-equilibrated in 7.5% Buffer A/92.5% H_20. The column was then washed with 30 ml 7.5% Buffer A at 2 ml/min, and eluted at 1 ml/min with a 40 ml 7.5-100% linear gradient of Buffer A in H_2O. The eluant was monitored at 280 nm and analyzed by gel electrophoresis as described above; fractions containing s-hSR-A were pooled. Yields were approximately 10.6 mg from 14 liters of s-hSR-AI medium and 10.8 mg from 4 liters of s-hSR-AII medium.

Disulfide mapping of the SRCR domain of s-hSR-AI - Urea (final concentration: 6 M) was added to 200 µg of s-hSR-AI and the sample was submerged in boiling water for 20 minutes and then cooled to room temperature.

LysC digestion: The urea concentration was lowered to 4 M by addition of Tris pH 9.0 (final concentration: 0.1 M) and then Lys-C was added at an enzyme to s-hSR-AI ratio of ~1:45 (w/w). Following incubation at 37° C for 4-13 h, peptides were resolved using HPLC (Hewlett Packard 1090 system). In some cases, the samples were reduced by boiling in the presence of 100 mM DTT for 20 minutes prior to chromatography. Samples were applied at 1 ml/min to an analytical C-18 column (Vydac 218TP54) which was then washed for 5 minutes with H_2O/0.1% trifluoroacetic acid and eluted with a linear gradient containing 0-60% acetonitrile/0.1% trifluoroacetic acid. Peaks containing disulfides were identified by mobility changes upon reduction and by comparison to digests of the type II receptor, which lacks the SRCR domain. The composition of these peaks was determined by both N-terminal sequencing and analysis of the amino acid composition (performed by the Biopolymers Laboratory, M.I.T. Department of Biology). Fractions containing these peptides were isolated and further characterized. Prior to additional proteolytic digestion, the fractions were evaporated to dryness (Speed-vac) and dissolved in 8 M urea by boiling for 20 minutes.

V8 (Glu-C) digestions: HPLC purified fragments of s-hSR-AI were diluted in 25 mM NH_4HCO_3 pH 7.5 to reduce the urea concentration to 1M, and digested with 1 µg enzyme for 10 hours at 37°C. Peptides were resolved on reverse phase HPLC as described above and analyzed by mass spectrometry using MALDI-TOF analysis (HHMI Biopolymers Facility, Harvard Medical School) and either amino acid analysis or N-terminal sequencing.

Trypsin digestions: HPLC purified fragments of s-hSR-AI were diluted in 50 mM Tris/1 mM $CaCl_2$ pH 7.6 to reduce the urea concentration to 1M, and digested with 1 µg enzyme for 10 hr at 37°C. Products were analyzed as described above.

Circular Dichroism - CD spectra were recorded in a 1.0 cm pathlength cell at a sample concentration of 1.32 μM (s-hSR-AI) or 1.39 μM (s-hSR-II) in Buffer B (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 or as indicated). Protein concentration was determined by absorbance at 280 nm in 6 M GuHCl, assuming extinction coefficients of 39680 and 15280 $M^{-1}cm^{-1}$ for the s-hSR-AI and s-hSR-AII, respectively (Edelhoch, 1967). Thermal unfolding studies were also performed in buffer B. An Aviv model 60 DS CD spectrophotometer with a thermo-electric temperature controller was used for all measurements. For calculation of percent α-helicity, θ_{222} = 37,550 degree cm^2 / decimole was assumed to correspond to 100% helicity (Chen et al., 1974). This value was derived by assuming that the length of the α-helical region was 52 residues, which corresponds to the length of the predicted α-helical coiled-coil region before the "skip" in this domain (Kodama et al., 1990; Ashkenas et al., 1993). The second portion of the α-helical coiled-coil domain following the skip may be slightly longer, however the effect of accounting for this on the calculation would be inconsequential. If the α-helical regions were significantly shorter, it would result in a decrease in the value for 100% helicity, and accordingly an increase in the % helicity calculated (Chen et al., 1974). Apparent melting temperature was chosen as the point at which the first derivative of the melting curve was maximal. Melting curves were not fully reversible, therefore, true thermodynamic parameters could not be determined.

Electron Microscopy - Negative-stained samples were prepared using the pleated sheet technique (Smith and Seegan, 1984). A thin layer of carbon was evaporated onto freshly-cleaved mica sheets using a Model DV502A High Vacuum Evaporator (Denton Vacuum). Samples of s-hSR-AI and s-hSR-AII were diluted to 1 μg/ml in PBS and adsorbed to the carbon film using the procedure of Valentine et al. (1968). A small piece (approx. 0.5 x 1.0 cm) of carbon-coated mica was inserted at a 45° angle (carbon-side up) into a small vessel containing 500-600 μl of sample, held there for 5-10 s, and withdrawn. The carbon film partially detaches from the mica sheet, and floats on the aqueous sample, allowing macromolecules to adsorb to the lower surface of the carbon film. Next, the carbon film was washed by inserting the carbon-coated mica into deionized water for 5 s. Finally, the carbon film was floated onto aqueous 1% (w/v) uranyl acetate (pH 4.3), and pleated to approximately 25% of its original length by using the mica sheet to gently compress the carbon film against the edge of the vessel. A 500-mesh copper grid was placed shiny side down on the pleated carbon, and retrieved with a piece of Saran Wrap stretched across the open end of a test tube. Excess stain solution was removed from the filmed grid using pre-moistened Whatman No. 1 filter paper. The filmed grid was removed from the Saran Wrap, and allowed to dry for > 1 hr.

Negative-stained samples were observed in a JEOL JEM 1200EX II transmission electron microscope operating at 80 kV with a 100 μm condenser aperture, a "spot size" of 2 μm, and a 100 μm objective aperture. Photographs were taken at magnifications ranging from 40,000-60,000. Microscope magnification was calibrated using a diffraction grating replica (2160 lines/mm) and latex spheres (0.261 μm).

Measurements of individual molecules were made by projecting the EM negatives onto a Sigma-Scan digitizing board (Jandel Scientific, 22 X magnification). The length of the spacer/coiled-coil domain was taken as the distance from the thick end of the molecule to the junction of the thick fibrous segment with the thin fibrous segment (see Results and Discussion). The length of the collagenous domain was taken as the distance from the junction of the two fibrous segments to the end of the thin fibrous segment. In the case of s-hSR-AI, the end of the thin fibrous segment was taken as the junction between the thin segment and the globular SRCR domains; however, the true end of this segment may have been partially obscured by the SRCR domains (see below). The widths of the two fibrous segments were estimated by measuring a perpendicular bisector of each segment. The "length" of the globular domain (SRCR trimer) was measured along a line which passed through the end of the thin fibrous segment and divided the globular domain into two approximately symmetrical halves. In the majority of the molecules, this line was collinear with the thin fibrous segment. The "width" of the globular domain was then taken as the maximum distance across the domain perpendicular to the "length line".

Results and Discussion

Stable amplified CHO[DHFR-] cell lines expressing high levels of secreted forms of the human type I (CHO-[s-hSR-AI]) and type II (CHO-[s-hSR-AII]) class A macrophage scavenger receptors were generated as described under "Experimental Procedures". The predicted domain structures of the integral membrane forms of the receptor are shown in Fig. 6.1. To generate the secreted forms of the receptor, the cytoplasmic and transmembrane domains were replaced with a myelin associated glycoprotein signal sequence, which was linked to the SR-A protein by a FLAG epitope tag. The signal sequence was presumably removed when the proteins were translated and secreted. The calculated molecular weight of the predicted type I secreted receptor (s-hSR-AI) is 42.3 kDa (383 amino acids), while that of the type II secreted receptor (s-hSR-AII) is 32.1 kDa (290 amino acids). The binding properties of the expressed s-hSR-AI and s-hSR-AII were evaluated using a solid-state bead binding assay previously developed to determine activity of soluble scavenger receptors (Resnick et al., 1993). The secreted receptors exhibited ligand binding activity with the expected polyanion specificity (not shown).

s-hSR-AI and s-hSR-AII were purified from the culture media using ultrafiltration, poly G affinity and Mono Q ion exchange chromatography, as described in "Experimental Procedures". The final material was purified to greater than 95% homogeneity, as evaluated either by HPLC or by SDS/PAGE and silver staining (not shown). Yields were approximately 0.75 mg purified s-hSR-AI per liter of culture medium and 2.7 mg s-hSR-AII per liter. These purified proteins were then used to examine the structures of s-hSR-AI and s-hSR-AII.

Disulfide linkages in the SRCR domain: The pattern of disulfide bonds in the SRCR domain of s-hSR-AI was determined by sequentially cleaving the protein with proteases (Lys-C followed by V8 or trypsin) and determining the composition of the relevant peptide products, using amino acid analysis, mass spectrometry and/or N-terminal sequencing, as described in "Experimental Procedures". Digestion products were separated by HPLC and the fractions containing disulfide-bond linked peptides were identified by both their reduction sensitivity and by comparison to digests from s-hSR-AII (which lacks the SRCR domain). The experimental strategy and the results are summarized in Figure 6.2. Note that the six cysteines in the SRCR domain of SR-AI are numbered 2, 3, 5, 6, 7, 8, as described by Resnick et al. (1994). SRCR domains fall into two groups, those, such as that in SR-AI, without cysteines 1 and 4 and those with them (Resnick et al., 1994). Two products of the Lys-C digestion, designated peptides I and II (Fig. 2), were identified by amino acid analysis and N-terminal sequencing. Unambiguous assignment of disulfide bonds required further proteolytic digestion. Peak I was digested with V8 protease, subjected to reverse phase HPLC, and disulfide containing peaks were again identified. Analysis of these peaks using either mass spectrometry and amino acid analysis (III) or mass spectrometry and N-terminal sequencing (IV) allowed unambiguous assignment of the disulfide bridges as Cys^2-Cys^7 and Cys^3-Cys^8. Digestion of peak II with trypsin followed by reverse phase HPLC yielded fragment V, and both amino acid analysis and mass spectrometry established the presence of the Cys^5-Cys^6 disulfide bond.

This disulfide bonding pattern is consistent with the prediction of a Cys^2-Cys^7 disulfide bond made based on sequence comparisons of many different SRCR family members (Resnick et al., 1994). It seems highly likely that all SRCR domain family members share the disulfide bond pattern established here for the SRCR domain of SR-AI and that cysteines 1 and 4 form a disulfide in those SRCR domains which have all eight cysteines.

Chemical modifications of s-hSR-A characteristic of triple helical collagenous domains: In hSR-AI, the C-terminal SRCR domain (VI) is separated by an 8 amino acid linker from domain V, a 69 amino acid domain which is also found in SR-AII. This domain was predicted to form a collagenous structure because it is composed of 23 Gly-X-Y triplet repeats (Kodama et al., 1990; Matsumoto et al., 1991). One of the hallmarks of collagen

is the presence in the Y position of the triplets of two post-translationally modified amino acids, hydroxylysine and hydroxyproline (Kivirikko and Myllyla, 1985). Hydroxyproline is important for the thermal stability of collagen, while hydroxylysine plays a role in the crosslinking of collagens and also acts as a substrate for glycosylation. Previous electrophoretic studies of SR-A biosynthesis using an inhibitor of hydroxylation, α,α'-dipyridyl, provided indirect evidence for hydroxylation of bovine SR-A (Penman et al., 1991). To measure proline and lysine hydroxylation directly, we performed amino acid analysis of purified s-hSR-AI and -AII. The relative abundances of all of the other amino acids were essentially those predicted from the sequences, assuming that the leader sequence was removed during biosynthesis. Table 6.1 shows that both hydroxylysine and hydroxyproline were present in s-hSR-AI and -AII. Assuming that these modifications occurred only in the Y position of the triplet repeats, they correspond to the hydroxylation of 86% and 107% of the prolines and lysines in the collagenous domain of the type I receptor, and 56% and 106% in the type II receptor. The presence of significant quantities of these modified residues lends further support to the proposal that this domain forms a triple helical collagenous structure (Kodama et al., 1990, Penman et al., 1991).

Circular dichroism analysis of the α-helical contents of s-hSR-AI and -AII: Domain IV is a 121 amino acid segment adjacent to the collagenous domain. The presence of an array of heptad repeats in this domain led to the prediction that it folds into a three stranded α-helical coiled-coil (Kodama et al., 1990). Circular dichroism (CD) spectroscopy of s-hSR-AI and -AII in the 202-250 nm range revealed that these proteins have characteristically α-helical spectra, with minima at 222 and 208 nm (Fig. 6.3 Panels A and C). The α-helicities of s-hSR-AI and -AII calculated from θ_{222} (Chen et al., 1974) are approximately 41% and 40%, respectively. Because domain IV represents 32% and 42% of s-hSR-AI and s-hSR-AII, respectively, and the collagenous domain (V) is not expected to provide a major contribution to θ_{222} (Piez and Sherman, 1970), these results are consistent with the model that domain IV forms an extended α-helical coiled-coil. They also suggest that there is some α-helical character in the SRCR domain. The contributions, if any, of the spacer domain (III) to the α-helical character of these receptors has not been established.

Like many endocytic receptors, the class A scavenger receptors release their ligands at low pH (Naito et al., 1991; Resnick et al., 1993), presumably to allow recycling of the receptor to the cell surface after deposition of its ligand in an acidified endosome. We previously suggested that the presence of histidine residues in two of the normally aliphatic "a" and "d" positions of heptad repeats in the α-helical coiled-coil domain (IV) might be important for pH dependent ligand binding (Kodama et al., 1990). Titration of these imidazoles might disrupt at least portions of the coiled-coil and the adjacent ligand binding collagenous domains. Doi, Kodama and co-workers (1994) provided support for this

suggestion when they showed that mutation of His^{260} to leucine abolished the ability of the receptor to release its ligands in a pH dependent manner. To determine if low pH dramatically altered the secondary structure of hSR-A, we compared the CD spectra of s-hSR-AI and -AII at pH 7.4 and 5.6 (Figure 6.3 panels A and C). The CD spectra at pH 5.6 were only very slightly perturbed relative to those at pH 7.4, suggesting that there were no substantial differences in the secondary structures at these pHs. However, we did observe a substantial pH-dependence in the thermal stabilities of the α-helices in s-hSR-AI (Fig 6.3 Panel B) and -AII (Panel D). At pH 7.4, s-hSR-AI and s-hSR-AII exhibited sharp thermal transitions, "melting" at ~64 °C, whereas at pH 5.6, this transition occurred at ~74 °C (Figure 6.3, Panels B and D).

The higher melting temperatures and apparently increased stability of the coiled-coil at pH 5.6, as well as the unaltered CD spectra, suggest that substantial unwinding of the coiled-coil is unlikely to account for decreased ligand binding at low pH. These results, however, do not preclude the possibility that small pH dependent perturbations in the structure of the coiled-coil domain and associated alterations in the adjacent collagenous domain may play a role in ligand release. Furthermore, it is not yet clear if His^{260} is included within the coiled-coil as was originally predicted (Kodama et al., 1990), because it is difficult to predict precisely where the coiled-coil segment ends based solely on the amino acid sequence (Ashkenas et al., 1993). Additional studies will be required to define precisely the mechanism by which His^{260} influences ligand binding.

Studies by Anachi, Brodsky, and co-workers using a peptide model of the collagenous ligand binding domain have recently demonstrated that this region is massively destabilized at low pH (Anachi et al., 1995). As they observed this effect only below pH 4.5, the relationship of their results to pH-dependent ligand binding is unclear. It is possible that in the context of the whole receptor, or in a receptor-ligand complex, this structural transition could occur at a higher pH.

Electron Microscopy of s-hSR-AI and -AII: Negative-stained images of s-hSR-AI (Fig. 6.4A and B) and s-hSR-AII (Fig. 6.4C and D) confirmed that these molecules have elongated structures, and are not assembled into higher order oligomers (Resnick et al., 1993). By negative staining, both SR-A isoforms appeared to be composed of two fibrous segments which differed noticeably in thickness (see also footnote 1). The principle difference between the two isoforms was the presence in s-hSR-AI, but never in s-hSR-AII, of a globular domain. This globular domain, which sometimes appeared to be resolved into two or, less frequently, three small subdomains, was observed at the end of the thin segment but never at the end of the thick segment. This globular domain almost certainly represents three SRCR domains. Therefore, we propose that the thin segment is the collagenous domain and the thick segment is the α-helical coiled-coil domain. The thick segment may also include a contribution from the spacer domain (Figure 6.1 and see below).

Length, width, and angle measurements of the individual s-hSR-AI/II domains were performed on the negatively stained images as described in "Experimental Procedures". The results are summarized in Table 6.2 and Figure 6.5. The average length of the thin segment of the type II receptor, which consists of 23 Gly-X-Y repeats and a 15 residue C-terminal domain, was 210 ± 29 Å, a value similar to the predicted length of the collagenous domain, 197 Å (69 res. x 2.86 Å/res., Bella et al., 1994). The average thin segment length for the type I isoform, 170 ± 27 Å, was significantly shorter, raising the possibility that the SRCR domains may partially obscure the C-terminus of the collagenous domain in these images. It seems unlikely that the SRCR domains would normally block access to the C-terminus of the collagenous domain in solution, because this portion of the collagenous domain is required for ligand binding (Acton et al, 1993; Doi et al, 1993) and most of the binding properties of the type I and type II isoforms are similar (Ashkenas et al, 1993). The average lengths of the thick segments in both the type I and type II isoforms, 210 ± 32 Å and 230 ± 28 Å respectively, were somewhat longer than the predicted length of the α-helical coiled-coil domain, 181 Å (121 x 1.5 Å, Harbury et al., 1994). The quaternary structure of the 75-residue spacer domain at the N-terminus of the α-helical coiled-coil domain, and its contribution to the length of the thick segment, are unknown.

The average widths of the thick and thin segments were not as well defined as their lengths (Table 6.2) and were almost 2-fold greater than the widths predicted from crystal structures of a three-stranded α-helical coiled-coil (24 Å, Harbury et al., 1994) or a collagenous triple helix (10 Å, Bella et al., 1994). Absolute width measurements are particularly susceptible to small errors in measurement caused by distortion of the molecules during EM specimen preparation. Furthermore, it is difficult to assess the contributions of the many N-linked oligosaccharides in the α-helical coiled-coil domains (Kodama et al, 1990, Resnick et al., 1993; Matsumoto et al., 1990) to the observed widths. However, in both s-hSR-AI and s-hSR-AII, the thick segments were approximately 2-fold wider than the thin segments, a ratio similar to the predicted ratio of 2.4. Thus, the relative widths of the segments are consistent with the proposal that the thick segment represents the α-helical coiled-coil domain with some contribution made by the spacer domain and that the thin segment is the collagenous domain.

The globular structure observed at the end of the thin segments in s-hSR-AI is presumably composed of three individual SRCR domains. Its average dimensions were 58 ± 12 Å x 76 ± 18 Å. In a small number of the negatively stained images, these structures could be resolved into two or three discrete subdomains whose average dimensions were 54 ± 10 Å x 35 ± 9 Å (n=23).

Many of the negatively stained molecules appeared to be bent near the junction of the two fibrous segments. The angle between these segments varied

from 20° to 180°, with 80% of the molecules having angles greater than 100°. Thus, there appears to be a flexible hinge linking the α-helical coiled-coil and collagenous domains, with some preference for molecules with a more extended conformation. Segmental flexibility might contribute to the ability of these receptors to bind a wide variety of ligands, e.g., relatively small molecules (Pearson et al., 1992), large macromolecules and macromolecular complexes (Krieger and Herz, 1994) and even intact microorganisms (Dunne et al., 1994).

The extended nature of the scavenger receptors (overall length ~ 440 Å) could serve to project their collagenous ligand binding domains away from the cell surface and thus make them more accessible to very large ligands. Interestingly, the other member of the SR-A family, MARCO, does not have an α-helical coiled-coil domain (Elomaa et al., 1995). However, it does contain a much longer collagenous domain (87 Gly-X-Y repeats), resulting in a predicted fibrous length of about 750 Å. Antibody blocking experiments suggest that, as is the case with SR-AI and SR-AII, MARCO's ligand binding domain is at the C-terminal end of the receptor (Elomaa et al., 1995). Strikingly, the Gly-X-Y repeats in MARCO are interrupted by a skip. Similar disruptions in short collagenous domains have been reported to introduce a flexible hinge which separates two fibrous segments (e.g., C1q, Schumaker et al., 1981, Thiel and Reid, 1989). The skip in MARCO may be functionally equivalent to the flexible hinge in SR-AI and -AII. Thus, all of the class A scavenger receptors appear to project their ligand binding domains away from the cell surface via a hinged fibrous structure.

A somewhat different perspective on the structures of SR-A is provided by rotary metal shadowing techniques. Rotary metal-shadowed images of s-hSR-AI and s-hSR-AII at pH 7.4 (not shown) indicated that both molecules are elongated rods, with an average length of approximately 310 Å. Both s-hSR-AI and -AII appeared relatively uniform in thickness along their length. However, in the s-hSR-AI images, there was also an approximately 100 Å diameter globular region at one end of each rod, presumably formed by the three SRCR domains of the trimer. This structure was absent in all of the s-hSR-AII images. Stained images obtained after dialysis of the type I receptor into pH 4.3 buffer looked similar to those described above. It thus appears that at neutral pH these molecules are "snapped-back" on themselves, giving an angle of 0° between the segments.

The reason behind the different results with the different methods is unclear. While the negative stained images were nominally obtained at pH 4.3, the molecules were adhered to the carbon film at pH 7.4. It is not known whether structural rearrangement would occur once the molecules are on the carbon. The CD studies presented above suggest that if a major structural change occurs between pH 7.4 and 5.7, it does not significantly affect secondary structure. It may well be that the putative ligand binding region of the collagenous interacts with acidic residues in the spacer domain, folding the

molecule in half at neutral pH. Whatever the cause of the apparent snap-back, it may not apply to the receptor when it is anchored in a membrane. Hydrodynamic studies are in progress to evaluate whether this pH dependant conformational change occurs in solution as well. If this is a real effect, it could in some way relate to the mechanism for pH dependant ligand release.

The studies of the structures of s-hSR-AI and s-hSR-AII described here have provided significant support for the fibrous structural model originally proposed for the class A scavenger receptors (Figure 6.1). They also have provided a disulfide map of the SRCR domain and, by low resolution electron microscopic imaging, have improved our understanding of the overall structure of the extracellular portion of the receptor.. Future higher resolution studies of these molecules will further our understanding of the structure and unusual binding properties of these multiligand/multifunction receptors.

Acknowledgments:

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Footnotes

[1] The majority of the negatively stained molecules (77%) had this overall structure. We also observed a number of shorter molecules in every sample. The average dimensions of these molecules (230 x 49 Å) were similar to those of the thick fibrous segments. Presumably, the thin fibrous segment of SR-A was present, suggesting that the thin fibrous segment was not resolved in these molecules.

Figure Legends

Figure 6.1. Predicted quaternary structures of the type I and type II class A macrophage scavenger receptors.

The domain structures of the type I and type II receptors were deduced from their cDNA sequences. These domains include a cytoplasmic domain, a single transmembrane domain (TM), a spacer domain, a region predicted to form an α-helical coiled-coil, and a putatively collagenous domain containing 23 Gly-X-Y repeats. The type I receptor has an 110 amino acid COOH terminal region comprising an 8 residue hinge and a 102 amino acid scavenger receptor cysteine rich (SRCR) domain, while the type II receptor has a 17 amino acid C-terminus. In the secreted forms used in this paper, the cytoplasmic and transmembrane domains were replaced with a cleavable signal sequence followed by a FLAG epitope tag.

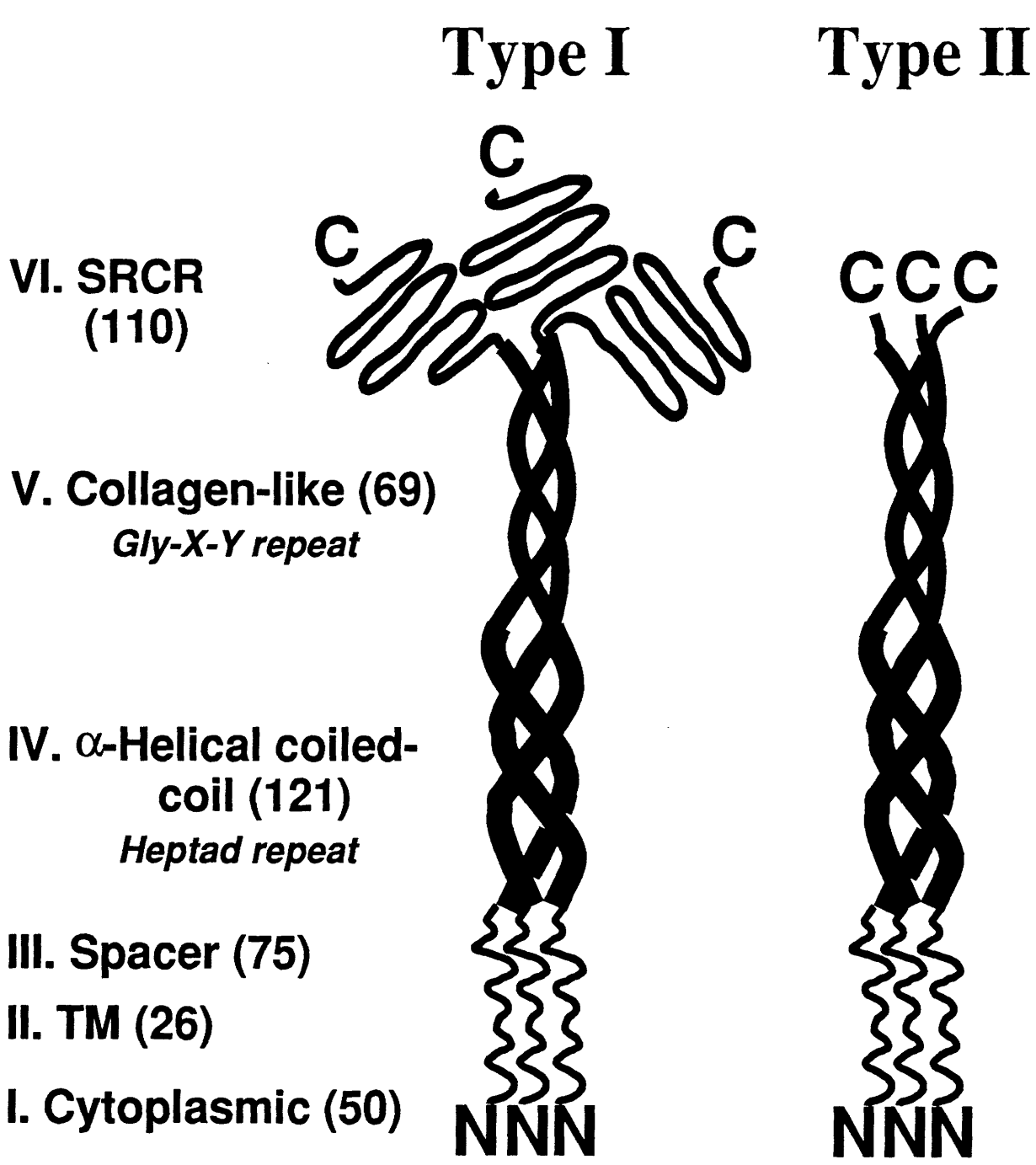

Type I
Type II
VI. SRCR (110)
V. Collagen-like (69)
Gly-X-Y repeat
IV. α-Helical coiled-coil (121)
Heptad repeat
III. Spacer (75)
II. TM (26)
I. Cytoplasmic (50)
C
C
C
C
C C C
N N N
N N N

Figure 6.2. Identification of disulfide bonds in the SRCR domain.

Purified s-hSR-AI was subjected to proteolytic digestion with LysC as described in materials and methods. Shown at the top are this enzyme's target sites in the SRCR sequence, with amino acids numbered as in the full length protein. Following cleavage with LysC, fragments I and II were identified as reduction sensitive peaks by reversed phase HPLC and further characterized using both N-terminal sequencing and amino acid analysis. Peak I was digested with V8 protease and the resulting peptides were resolved by HPLC yielding fragments III and IV. Peak II was digested with trypsin, yielding peak V. The identities of peaks III, IV, and V were determined using mass spectrometry and either amino acid analysis or N-terminal sequencing.

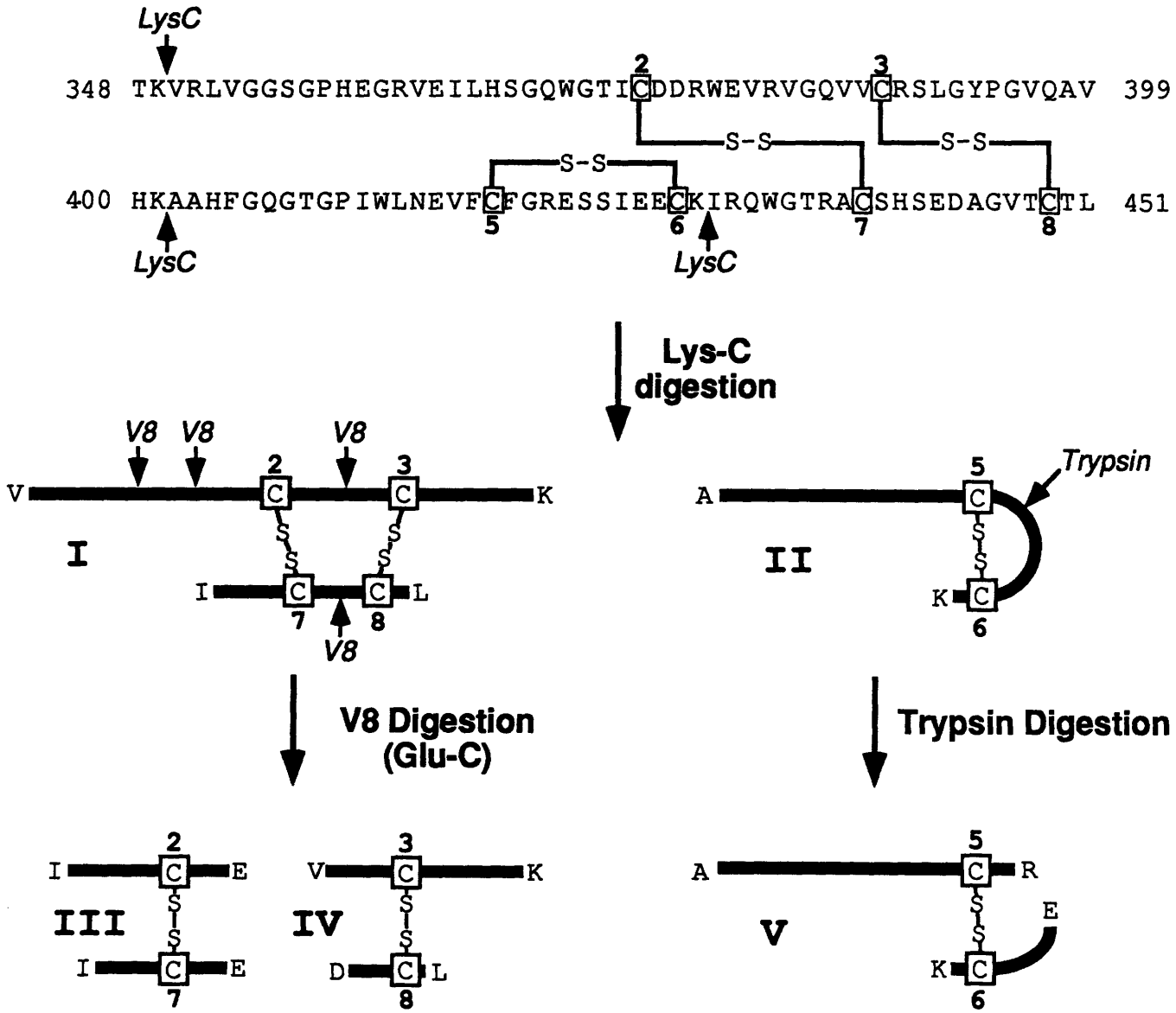
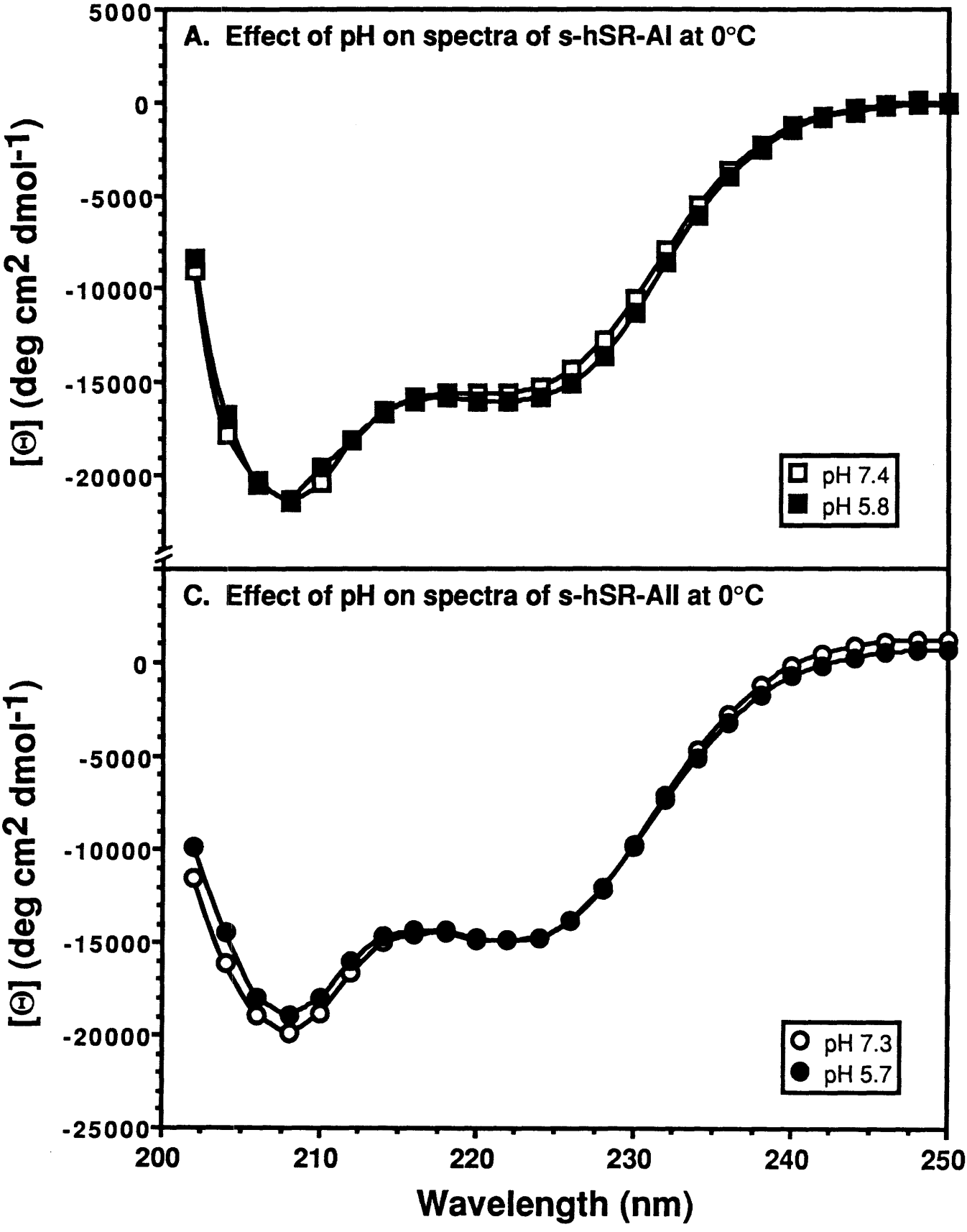

LysC
348 TKVRLVGGSGPHEGRVEILHSGQWGTICDDRWEVRVGQVVCRSLGYPGVQAV 399
S-S
S-S
S-S
400 HKAAHFGQGTGPIWLNEVFCFGRESSIEECKIRQWGTRACSHSEDAGVTCTL 451
LysC
LysC
Lys-C digestion
V8 V8 V8
I
V8
Trypsin
II
V8 Digestion (Glu-C)
Trypsin Digestion
III
IV
V

Figure 6.3. Circular dichroism (CD) analysis of s-hSR-AI and s-hSR-AII.

CD spectra of s-hSR-AI and -AII were recorded at concentrations of 1.32 and 1.39 μM, respectively, in 10 mM sodium phosphate, 150 mM NaCl, at the indicated pH values. Panels A, C - spectra were recorded for 3 seconds at each wavelength at 0°C. Panels B, D - samples were equilibrated at each temperature for 1.5 minutes prior to measuring θ_{222} for 30 seconds.


A. Effect of pH on spectra of s-hSR-AI at 0°C
pH 7.4
pH 5.8
C. Effect of pH on spectra of s-hSR-AII at 0°C
pH 7.3
pH 5.7
[Θ] (deg cm2 dmol-1)
5000
0
-5000
-10000
-15000
-20000
-25000
200
210
220
230
240
250
Wavelength (nm)

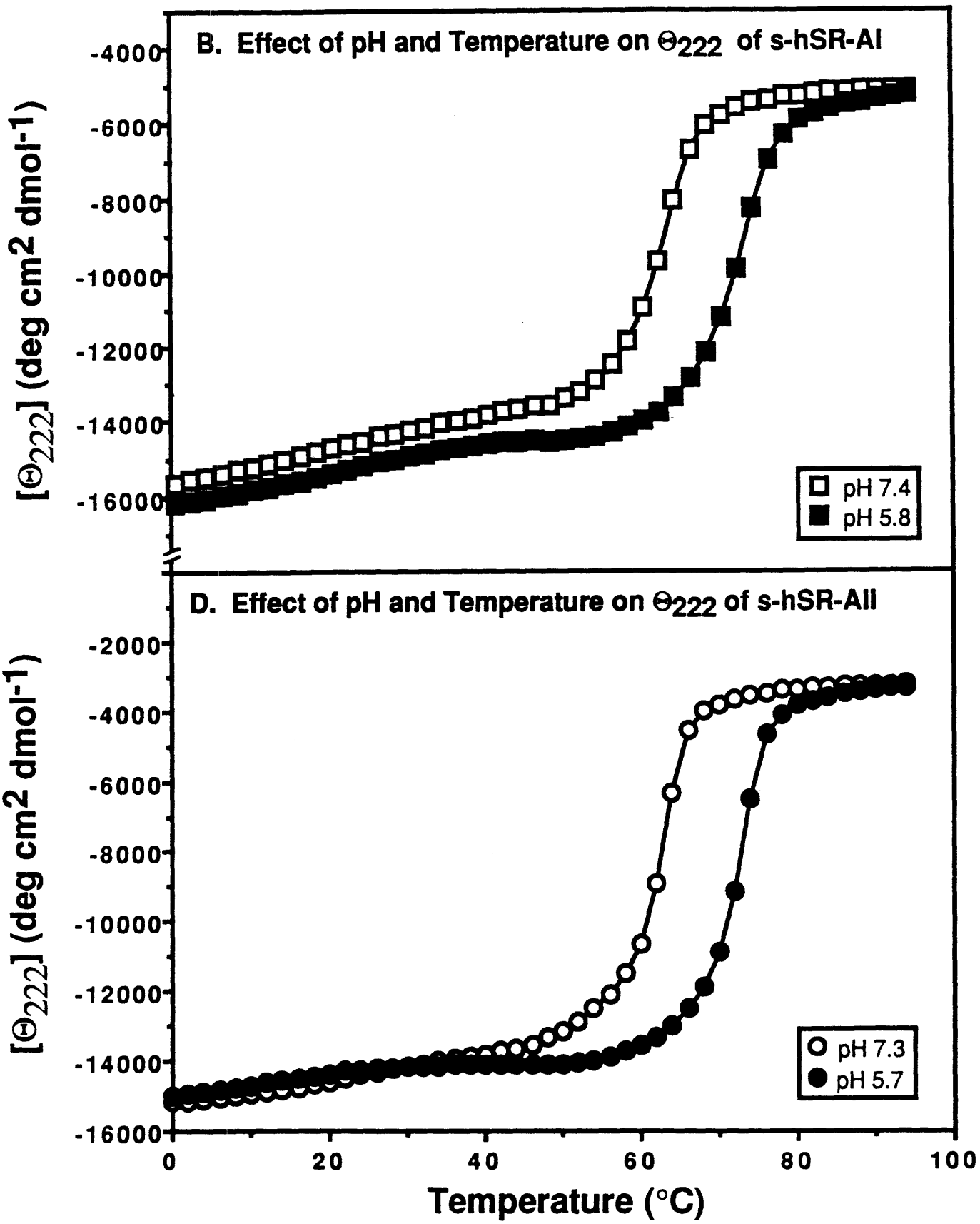

B. Effect of pH and Temperature on Θ222 of s-hSR-AI
-4000
-6000
-8000
-10000
-12000
-14000
-16000
[Θ222] (deg cm2 dmol-1)
pH 7.4
pH 5.8
D. Effect of pH and Temperature on Θ222 of s-hSR-AII
-2000
-4000
-6000
-8000
-10000
-12000
-14000
-16000
[Θ222] (deg cm2 dmol-1)
pH 7.3
pH 5.7
0
20
40
60
80
100
Temperature (°C)

Figure 6.4. Negative stain images of s-hSR-AI and s-hSR-AII.

Samples of purified s-hSR-AI or s-hSR-AII were prepared as described in "Experimental Procedures" and visualized using transmission electron microscopy. Double-tailed arrows indicate coiled-coil/spacer domains (thick fibrous segments), single-tailed arrows indicate collagenous domains (thin fibrous segments), and arrow heads indicate SRCR domains. Panels A and B, s-hSR-AI; Panels C and D, s-hSR-AII. Bars indicate 0.1 μm.

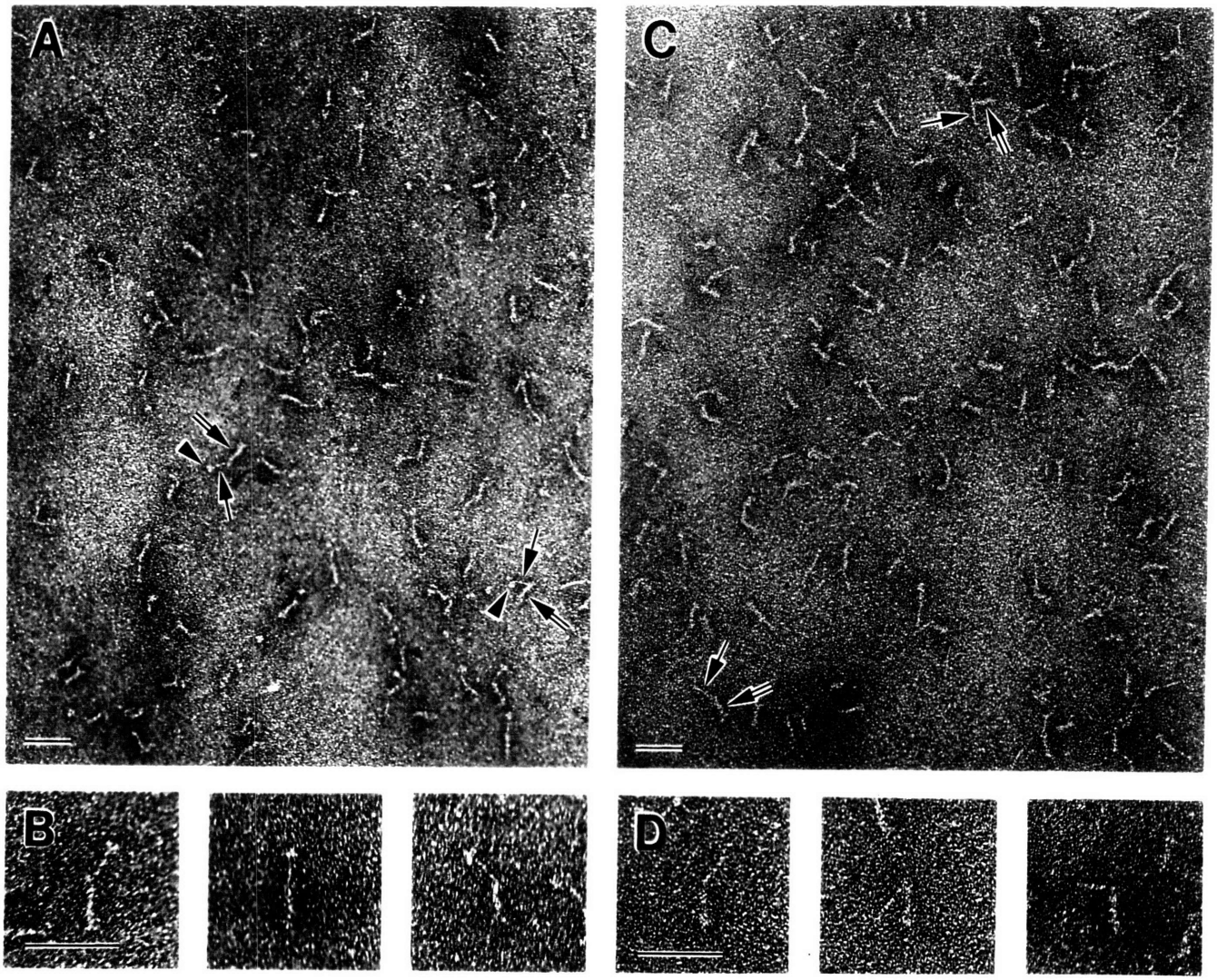

A
C
B
D

Figure 6.5. Dimensions of s-hSR-AI and s-hSR-AII based on negative stain electron microscopy.

Values (mean ± s.d.) are based on the measurements of >100 negatively stained images. The angle between the two fibrous domains was variable, ranging from 20 - 180°, with 80% of the values > 100°.

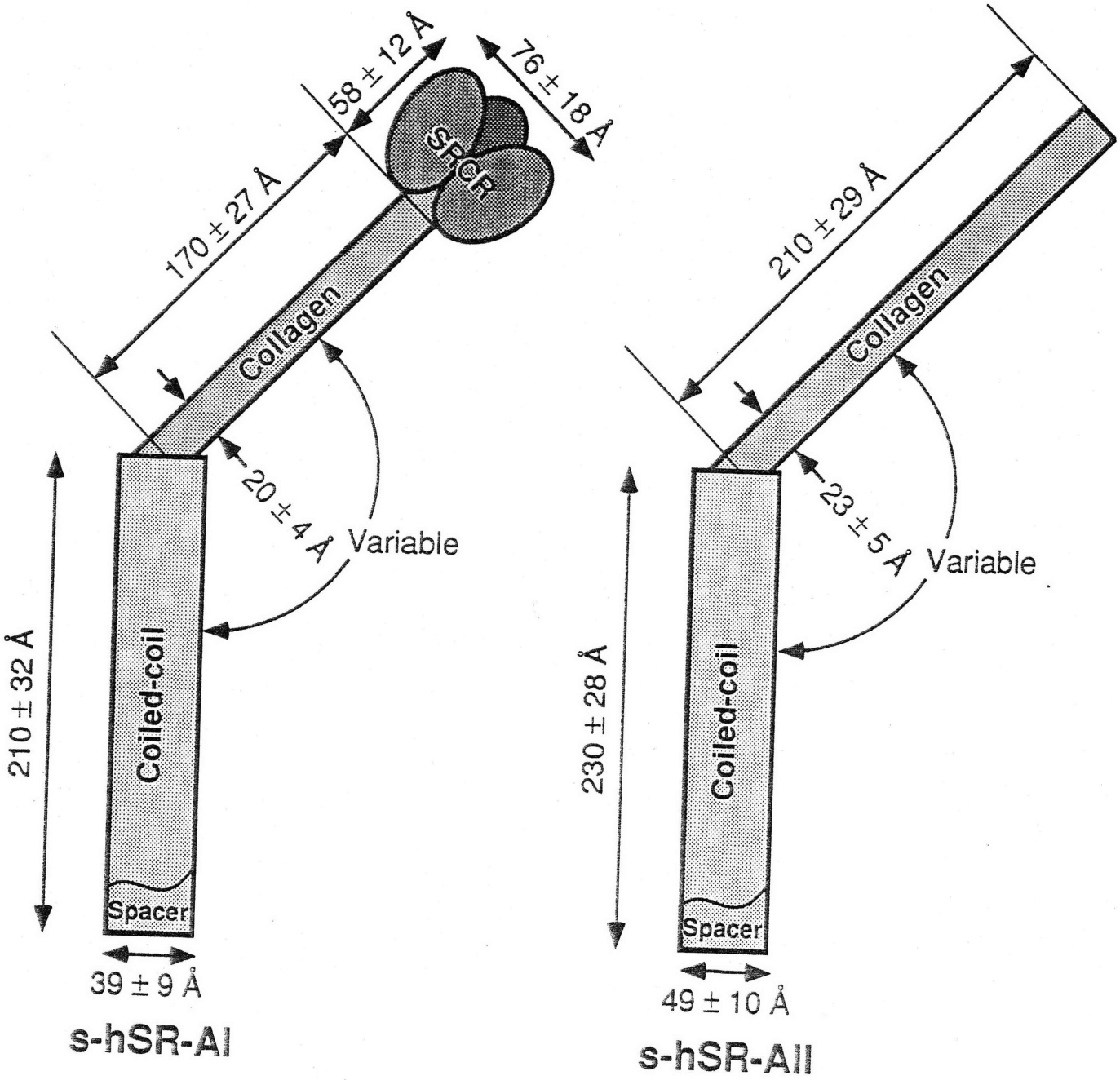

58 ± 12 Å
76 ± 18 Å
SRCR
170 ± 27 Å
Collagen
20 ± 4 Å
Variable
210 ± 32 Å
Coiled-coil
Spacer
39 ± 9 Å
s-hSR-AI
210 ± 29 Å
Collagen
23 ± 5 Å
Variable
230 ± 28 Å
Coiled-coil
Spacer
49 ± 10 Å
s-hSR-AII

Table 6.1. Quantitation of hydroxylated lysine and proline in s-hSR-AI and -AII.

Amino Acid	s-hSR-AI		s-hSR-AII	
	Predicted	Measured	Predicted	Measured
Lysine	22	16.5 ±1.0	19	14.9 ±0.1
Hydroxylysine	-	5.35±0.13	-	5.31±0.09
Proline	18	16.4 ±1.0	16	14.0 ±0.1
Hydroxyproline	-	5.16±0.25	-	3.35±0.17

Values given are the average number of residues per polypeptide± the standard deviation based on two experiments. The average deviation of predicted from the observed values for the other amino acids was 8% and 5.7% for s-hSR-I and s-hSR-II.

Table 6.2. Dimensions of negative-stained EM Images of s-hSR-AI and -AII.

Domain	s-hSR-AI		s-hSR-AII	
	Length (Å)	Width (Å)	Length (Å)	Width (Å)
Coiled-coil/Spacer	210 ± 32	39 ± 9	230 ± 28	49 ± 10
Collagenous	170 ± 27	20 ± 4	210 ± 29	23 ± 5
SRCR (trimer)	58 ± 12	76 ± 18	NA	NA
Overall Length	440 ± 49	NA	440 ± 43	NA

Values given are the means of at least 100 measurements ± the standard deviation. Overall length is defined as the sum of the measured lengths of the coiled-coil/spacer, collagenous, and SRCR domains.

Chapter 7

Perspective - scavenger receptors and the future

The purification, cloning, and sequencing of the type I and type II class A scavenger receptors by Kodama, Krieger, and co-workers (Kodama et al., 1988; Kodama et al., 1990; Rohrer et al., 1990) revealed that these receptors contained features which immediately allowed a plausible quaternary structure to be proposed (presented in Fig. 1.1, introduction). A major goal of this thesis has been to both evaluate these predictions and to extend them, yielding an increasingly accurate picture of receptor structure. An additional aim has been to try and better understand receptor function by screening some candidate SR ligands.

Studies in this thesis have provided structural information about the receptor as a whole as well as about all of the domains individually. These findings are summarized in Figure 7.1. These experiments have provided considerable support for the validity of many of the structural features predicted based on the primary sequence (Kodama et al., 1990; Rohrer et al., 1990), and have extended those predictions. However, there remain some unanswered questions in the realm of receptor structure, in particular with regard to the nature of the ligand binding interaction. Perhaps the most intriguing is the mechanism by which a positively charged rigid rod selectively binds along its surface some, but not all, negatively charged molecules with quite high affinity. More typically, high affinity specific binding occurs in fairly restrictive pockets of a protein, with selectivity enforced by steric considerations and interactions with surrounding residues. An original suggestion that the scavenger could make such a binding pocket by forming higher order oligomers (as is the case for C1q) was shown to be incorrect. A related question pertains to the number of ligand binding sites. The receptor's predicted trilateral symmetry raises the possibility that it could have some multiple of 3 binding sites, although some of the ligands may be large enough to sterically hinder simultaneous occupancy of those sites. Very preliminary studies using affinity co-electrophoresis (not shown) have suggested that there may be multiple binding sites, although the results are not unambiguous. The receptors have been shown to exhibit pH dependent ligand binding; however the mechanism for this remains unclear. Studies in this thesis have demonstrated that one reasonable hypothesis, namely that major disruption of receptor structure in response to protonation of a key histidine in the coiled-coil domain causes ligand release, does not appear to be the mechanism for this process.

Studies are underway to generate higher resolution structural information about the receptor which may provide an answer to some of these questions. Currently, efforts are focused on two different approaches. One line of investigation involves attempts to crystallize the entire secreted type I and II class A human receptors and to determine the receptor structure by X-ray diffraction techniques. Small crystals of SR-AI have been grown, and further experiments are in progress. These experiments are presented in Appendix D of this thesis. An alternative approach for the determination of receptor

structure involves the crystallization of receptor fragments individually. It is possible that this would make the problem somewhat more tractable. Efforts to independently crystallize or obtain via NMR the structure of the SRCR domain are described in appendix C. Such a structure would be highly useful, although it would not show how the domains interact. Similarly, structures could also perhaps be obtained for the collagenous and coiled-coil domains, either from proteolytic products from the full receptor, or perhaps by independent expression of these domains.

Recently, both small collagens and three stranded α-helical coiled-coils formed from synthetic peptides have been crystallized, and their structures have been determined at high resolution (Harbury et al., 1994; Bella et al., 1994). This raises the possibility that several interesting structural questions could be addressed using peptide models of putatively important regions of the analogous scavenger receptor domains. In the case of the coiled-coil domain, the question of pH dependent release of ligands could be investigated by determination of the structure of a peptide which encompasses the critical C-terminal histidine residue (Doi et al., 1994). Perhaps more significantly, structures of peptide models of the collagenous ligand binding domain could help us understand how this domain recognizes ligands. Anachi, Brodsky, and colleagues have demonstrated that maintenance of the structure of a peptide model which includes the putative ligand binding region of the collagenous domain is pH dependent, with significant destabilization at low pH (Anachi et al., 1995). They do not report that their model trimer is capable of mediating ligand binding activity; however perhaps theirs or a similar model could be used for such structural studies. This assumes that the ligand binding activity could in fact be reconstituted in a peptide model and does not require any of the other structural elements present in the scavenger receptor. Given our current understanding of SR structure, higher resolution studies such as those presented above seem like the best way to address the many remaining structural issues.

While structural studies have slowly revealed the physical features of the class A scavenger receptors, the actual *in vivo* function of these receptors remains shrouded in mystery. The vast majority of studies investigating SR function have used *in vitro* competition and binding studies to identify plausible ligands and have "raised the possibility" that scavenger receptors could play a role in many (or even most?) macrophage-receptor mediated processes. Examples of this sort of study are found in chapters 2 and 3 of this thesis, where I show that crocidolite asbestos and lipoteichoic acid are SR-A ligands. Asbestos and non-opsonized Gram-positive bacteria (which have lipoteichoic acid on their surfaces) both are bound by macrophages, which of course express SR-A on their surfaces. These are, therefore, potentially physiologically relevant ligands. However, this type of approach is ultimately unsatisfying, and even well conceived and designed experiments fall short of the mark in convincingly defining the SR-A role. The relatively few *in vivo*

studies that have been carried out have been illuminating (e.g. Hampton et al., 1991; Kobzik, 1995). However these studies take place in a complex physiological background containing many scavenger receptors. Even if these and similar sorts of experiments conclusively demonstrate that a scavenger receptor is involved in the process being studied, which particular scavenger receptor or receptors are acting often remains an open question.

Recently, Kodama and co-workers have reported the targeted disruption of the class A scavenger receptor in mice (unpublished, Pearson, 1996). This is a critical development for the field, as it will finally allow some definitive experiments on SR-A function to be carried out. Preliminary results have indicated that these mice are healthy, and accordingly that the SR-A does not appear to be an essential gene. While there is no readily apparent phenotype, more detailed investigations applying some of the numerous findings obtained from *in vitro* studies have produced some interesting observations. Binding of OxLDL by peritoneal macrophages is reduced by 50%, providing more support for this receptors involvement in atherogenesis (Kodama, unpublished). Crossing these SR-A deficient mice with strains with atherogenic propensities such as apoE deficient mice (Breslow, 1993) will help to still better define the pathophysiological role of this receptor in atherogenesis. Studies by Gordon and colleagues (unpublished, Pearson, 1996) have revealed that the ability of thymic macrophage to engulf apoptoic thymocytes is decreased by 50% in these SR-A deficient animals, supporting their previous findings which had been based on antibody inhibition of this process (Platt and Gordon, 1995). This suggests that the SR-A plays a role in the clearance of apoptotic cells, at least in this limited case. Obviously the SR is not the principle mediator of the clearance of apoptotic cells, as the animals develop normally. Experiments investigating the potential role of the SR-A in host defense have yet to be carried out. Studies with these mice and macrophage cell lines derived from them will ultimately provide the answers to this and other questions concerning the *in vivo* function of the scavenger receptor.

An essentially untouched aspect of SR function lies in the SRCR domain. Its presence for the most part has little effect on the polyanionic ligand binding properties associated with the scavenger receptors, although in at least one case it does modulate them somewhat (Ashkenas et al., 1993). Given its location at the end of a long, rigid rod, and that some of the other SRCR domain superfamily members have been shown to be involved in ligand binding, it seems highly likely that it has an as yet unknown soluble, cell surface, or cell matrix ligand. Use of recombinant SRCR domain to search for such ligands using an approach similar to that used to identify a ligand for the lymphocyte homing receptor in high endothelial venules could be successful (Watson et al., 1990), assuming that the ligand is expressed on the surface of tissues or cells with which macrophages interact. One such potential location would be the vascular endothelium, given its role in recruitment of circulating monocytes into tissues. Differential expression of SR-AI and SR-AII has not yet been well

studied. Possibly knowledge of the differences in expression patterns would suggest likely functions for the SRCR domain.

This thesis has answered many structural questions about the class A scavenger receptors. As has been discussed above, many structural and functional questions remain unanswered. With the reagents currently available, in particular the availability of high levels of the protein for crystallization and other structural studies as well as the SR-A deficient mice, the tools are now in place to begin to answer these questions.

Figure 7.1. Summary of structural findings about class A scavenger receptors.

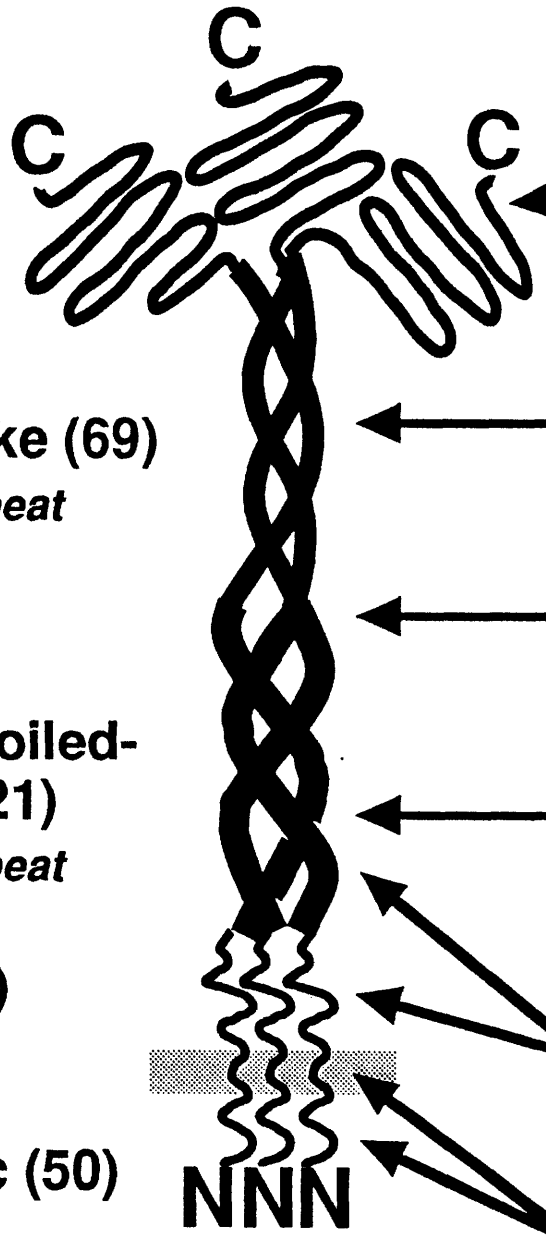
Elements of receptor structure discovered or confirmed in the course of this thesis are indicated in boxes adjacent to the relevant domain(s). Number of residues in each domain in the human receptor are as indicated.
Abbreviations: SRCR, scavenger receptor cysteine-rich, TM, transmembrane.

Summary of the Structural Findings in this Thesis about the Class A Scavenger Receptors

Findings about overall SR-A structure

- Receptor is trimeric, and does not appear to form higher order oligmers
- Hydrodynamic studies reveal that receptor is highly elongated, a finding supported by electron microscopy (EM) studies (length ~ 440 Å)

Type I



VI. SRCR (110)

V. Collagen-like (69)
Gly-X-Y repeat

IV. α-Helical coiled-coil (121)
Heptad repeat

III. Spacer (75)

II. TM (26)

I. Cytoplasmic (50)

Findings about individual domains

- Disulfide bond pattern identifed as Cys 2-7, 3-8, 4-5.
- Globular structure and approximate dimensions observed by EM (54 x 35 Å each).

- Identifed as ligand binding domain.
- Presence of hydroxylated lys/pro supports proposed structure.
- EM comfirms highly elongated structure.

- EM reveals that hinge between domains IV/V is flexible, with angles ranging from 20-180°.

- Quantity of α-helix in protein consistent with pred. structure.
- EM confirms highly elongated structure.
- Protonation of His^{261} doesn't cause major structural change.

- Degree of N-linked glycosylation in domains III and IV determined (6 out of 7 potential sites are glycosylated in bovine receptor).

- Receptor trimerizes and binds ligands without these domains, but is not membrane associated in their absense.

Appendix A

The Type I and Type II bovine scavenger receptors expressed in Chinese hamster ovary cells are trimeric proteins with collagenous triple helical domains comprising noncovalently associated monomers and Cys^{83}-disulfide linked dimers

This appendix contains studies designed to examines in detail issues related to the biosynthesis and processing of the class A scavenger receptors. It addresses the following questions: 1) What are the kinetics of receptor glycosylation, oligomerization and degradation; 2) what is the oligomeric state of the receptor, and what role does a cysteine found in the spacer domain (Cys^{83}) play in this oligomerization; 3) is the receptor hydroxylated (as predicted for the collagenous domain); 4) is the receptor expressed on the cell surface. This work was published in 1991 in *The Journal of Biological Chemistry* **266**, 23985-23993.

My contribution to these studies was limited to the identification and preliminary characterization of a reagent which could successfully crosslink scavenger receptors in intact cells both on the cell surface and internally. Figure 10 was based on this result, though the actual experiment shown was performed by Marsha Penman, as were most of the others in this work. The paper was written primarily by Dr. Monty Krieger, and I and others contributed to its editing and revision.

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The Type I and Type II Bovine Scavenger Receptors Expressed in Chinese Hamster Ovary Cells Are Trimeric Proteins with Collagenous Triple Helical Domains Comprising Noncovalently Associated Monomers and Cys^{83}-Disulfide-linked Dimers*

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Scavenger receptors have been implicated in the development of atherosclerosis and other macrophage-associated functions. The structures and processing of type I and type II bovine macrophage scavenger receptors were examined using polyclonal anti-receptor antibodies. Pulse/chase metabolic labeling experiments showed that both types of scavenger receptors expressed in Chinese hamster ovary (CHO) cells behaved as typical cell surface membrane glycoproteins. They were synthesized as endoglycosidase H-sensitive precursors which were converted to endoglycosidase H-resistant mature forms expressed on the cell surface. The reduced precursor and mature forms were doublets on sodium dodecyl sulfate-gel electrophoresis, primarily because of heterogeneous *N*-glycosylation. The approximate molecular sizes were: type I precursor, 65/63 kDa; type I mature, 82/76 kDa; type II precursor, 57/53 kDa; and type II mature, 72/65 kDa. During post-translational processing, the cysteine-rich C terminus (SRCR domain) of some of the type I receptors was proteolytically removed to form a relatively stable, approximately 69-kDa degradation product. Type II receptors differ from type I receptors in that they do not have SRCR domains and an analogous proteolytic cleavage was not observed. Several experiments provided strong evidence that the Gly-*X*-*Y*-repeat domains in the scavenger receptors oligomerize into collagenous triple helices. For example, α,α'-dipyridyl, an inhibitor of the collagen-modifying enzymes prolyl and lysyl hydroxylases, interfered with both the kinetics and nature of post-translational receptor processing, and both precursor and mature forms of the receptors in intact cells could be cross-linked with difluorodinitrobenzene into reduction-resistant trimers. In intact cells, precursor receptor trimers (type I, 198 kDa; type II, 176 kDa) were assembled in the endoplasmic reticulum by the noncovalent association of monomers and Cys^{83}-disulfide-linked dimers (type I, 129 kDa; type II, 119 kDa). When cells were lysed in the absence of the sulfhydryl trapping agent iodoacetamide, oxidation of the side chain of Cys^{17} in the cytoplasmic domain leads to the artifactual formation of reduction-sensitive covalently linked trimers. The approximate masses of the mature dimer and trimer forms were 162 and 237 kDa for type I receptors and 147 and 219 kDa for type II receptors. Cys^{83}-disulfide-linked dimer formation was not required for function because mutant receptors ($Cys^{83} \rightarrow Gly^{83}$) assembled into trimers of noncovalently associated monomers and exhibited normal receptor activity. Treatment of cells with difluorodinitrobenzene cross-linked some of the receptors into complexes larger than trimers, raising the possibility that the trimers may assemble into higher order oligomers.

The macrophage scavenger receptors are integral membrane glycoproteins which exhibit unusually broad ligand specificity (Goldstein *et al.*, 1979; Brown and Goldstein, 1983; Kodama *et al.*, 1988, 1990; Rohrer *et al.*, 1990; Freeman *et al.*, 1990). They bind a wide variety of polyanions, including chemically modified LDL,[1] acidic phospholipids, and poly(I). Numerous studies have suggested that the scavenger receptors may be critically involved in cholesterol deposition in artery walls during atherogenesis (Brown and Goldstein, 1983; Steinberg *et al.*, 1989; Jurgens *et al.*, 1987, Steinbrecher *et al.*, 1990). The broad binding specificity of the scavenger receptors and their expression in macrophages (Brown and Goldstein, 1983; Pitas *et al.*, 1985; Nagelkerke *et al.*, 1983; Kodama *et al.*, 1990) suggest that they may also play a role in other physiologic and pathophysiologic systems, such as macrophage-associated immune responses and inflammation (Kodama *et al.*, 1990; Freeman *et al.*, 1990). For example, bacterial endotoxin (lipopolysaccharide) is a ligand for scavenger receptors, and scavenger receptors play a quantitatively important role in the clearance of endotoxin from the circulation by the liver (Hampton *et al.*, 1991; Raetz *et al.*, 1988).

Purified bovine scavenger receptor(s) are comprised of roughly equal amounts of reduction-sensitive trimers (220 kDa) and dimers (150 kDa) (Kodama *et al.*, 1988). On reduction, these oligomeric forms collapse into monomers of about

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[1] The abbreviations used are: LDL, low density lipoproteins; SRCR, scavenger receptor cysteine-rich; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DFDNB, difluorodinitrobenzene; SDS, sodium dodecyl sulfate.

77 kDa, approximately 15–20 kDa of which are due to *N*-linked glycosylation. The trimeric form, but not the dimeric or monomeric forms, can bind the ligand acetyl-LDL (AcLDL). We used the partial sequence of the purified bovine receptor to clone two classes of scavenger receptor cDNA, type I and type II, from bovine lung (Kodama *et al.*, 1990; Rohrer *et al.*, 1990) and subsequently to clone their homologues from cultured murine macrophages (Freeman *et al.*, 1990). The homologous human cDNAs have also been cloned (Matsumoto *et al.*, 1990). When incorporated into mammalian expression vectors, the cloned cDNAs confer scavenger receptor activity on transiently transfected COS cells (Kodama *et al.*, 1990; Rohrer *et al.*, 1990) and stably transfected CHO cells (Freeman *et al.*, 1991; Matsumoto *et al.*, 1990).

The sequence of the type I bovine scavenger receptor cDNA predicts a 453 amino acid protein with the following domains (Kodama *et al.*, 1990): I, N-terminal cytoplasmic (amino acid residues 1–50); II, transmembrane (51–76); III, spacer (77–108); IV, α-helical coiled-coil (109–271); V, collagenous (272–343); and VI, C-terminal cysteine-rich (344–453), designated SRCR (scavenger receptor cysteine-rich). The SRCR domain helped to define a previously unrecognized family of remarkably well conserved cysteine-rich protein domains (Freeman *et al.*, 1990). The type I receptor has 7 potential *N*-linked glycosylation sites in the spacer and α-helical coiled-coil domains and 8 cysteines, 6 of which are in the SRCR domain. The type II scavenger receptor is identical to the type I receptor, except that the 110-amino acid SRCR domain is replaced by a 6-amino acid C terminus (Rohrer *et al.*, 1990). Despite its truncated C terminus, the type II scavenger receptor mediates the endocytosis of modified LDL with essentially the same affinity and broad specificity as the type I receptor (Rohrer *et al.*, 1990; Freeman *et al.*, 1991). In the current study, we have generated rabbit polyclonal anti-peptide antibodies to the bovine scavenger receptor and used them to examine the synthesis, post-translational processing, and structure of bovine scavenger receptors expressed in stable CHO cell transfectants.

MATERIALS AND METHODS

Cell Culture—All incubations with intact cells were performed at 37 °C in a humidified 5% CO_2, 95% air incubator unless specified otherwise. As described previously (Krieger, 1983), stock cultures of wild-type CHO cells were grown in medium A (Ham's F-12 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) containing 5% (v/v) fetal bovine serum (medium B), and transfectants were grown in medium C (medium A containing 3% (v/v) newborn calf lipoprotein-deficient serum, 0.5 mg/ml geneticin (G418, Gibco Laboratories), 250 μM mevalonate, 40 μM compactin, and 3 μg of protein/ml of AcLDL). The isolation of transfectants expressing the type I bovine scavenger receptor (CHO[bSR-I], clone IB2) and the type II bovine scavenger receptor (CHO[bSR-II], colony II-5 or a subclone, IIB3) was described previously (Freeman *et al.*, 1991). Transfectants expressing the Cys^{83} to Gly^{83} mutant forms of the type I (CHO[bSR-I(Gly^{83})], colony 1–27) and type II (CHO[bSR-II(Gly^{83})], colony 3–12) receptors were generated using the Polybrene method (Sambrook *et al.*, 1989) as follows. On day 0, wild-type CHO cells were plated at 5×10^5 cells/100-mm dish in medium B. On day 1, the monolayers were refed with 3 ml of medium B containing 10 μg/ml Polybrene (Aldrich), 0.5 μg/dish pSV2dhfr* (Stuhlmann *et al.*, 1989), 0.5 μg/dish pRc/CMV (Invitrogen), which contains a G418 resistance marker, and 10 μg/dish of either the expression vector pCMV4bSRI(Gly^{83}) (type I) or pCMV4bSRII(Gly^{83}) (type II) (see below). After a 6-h incubation at 37 °C, the polybrene-containing media were removed, and the cells were incubated with medium B containing 30% (v/v) dimethyl sulfoxide for 4 min at room temperature. The monolayers were then washed with medium A and refed with medium B. On day 3, the cells were reset at 10^5 cells/100-mm dish in medium E (medium B supplemented with 0.5 mg/ml geneticin). On days 13–14, the surviving colonies were screened for the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled AcLDL (3 μg of protein/ml in medium E, overnight incubation followed by fluorescence microscopy (Kingsley and Krieger, 1984)), and receptor-positive colonies were isolated and used in the indicated experiments. Native LDL and AcLDL and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled AcLDL were prepared as previously described (Krieger, 1983, Kodama *et al.*, 1988).

Preparation of Expression Vectors for Cys^{83} to Gly^{83} Mutant cDNAs—The expression vectors pCMV4bSRI and pCMV4bSRII were constructed as follows: the cDNAs for the type I and type II scavenger receptors were excised from pXSR7 (Kodama *et al.*, 1990) and pXSR3 (Rohrer *et al.*, 1990) by *Hin*dIII with Klenow blunting and then *Bam*HI, and these cDNAs were inserted between the *Bgl*II and *Sma*I sites of pCMV4 (gift of David Russell, Andersson *et al.*, 1989). A single-stranded phagemid was generated by M13K07 infection of CJ236 *Escherichia coli* cells (Bio-Rad) transformed with pCMV4bSRI, and this phagemid served as the template for single primer oligonucleotide-mediated mutagenesis by the Kunkel method (Sambrook *et al.*, 1989). The complementary mutagenic oligonucleotide CCAACCGTGCCATTCTTCGT was used to create a Cys^{83} to Gly^{83} point mutation (UGC → GGC) at nucleotides 247–249. The sequence of the 0.7-kilobase *Sma*I/*Xba*I fragment of the mutant cDNA was confirmed by dideoxy sequencing, and this fragment was then subcloned into the pCMV4bSRI and pCMV4bSRII vectors to generate the mutant expression vectors pCMV4bSRI(Gly^{83}) and pCMV4bSRII(Gly^{83}). The receptor activities expressed by COS M6 cells (Kodama *et al.*, 1990; Rohrer *et al.*, 1990) transiently transfected with these mutant vectors were equivalent to or slightly greater than those of cells transfected with the corresponding wild-type expression vectors (data not shown).

Antibodies—Oligopeptides were coupled via their terminal cysteine residues to hemocyanin as previously described (Kozarsky *et al.*, 1986; Beisiegel *et al.*, 1981; Russell *et al.*, 1984). The peptides were the: 1) 17 N-terminal residues (MAQWDDFPDQQEDTDSC), 2) 16 amino acids from the spacer domain plus an additional cysteine not found in the receptor (DISPSPEGKGNGSEDEC), and 3) 15 amino acids from the scavenger receptor cysteine-rich (SRCR) domain (CRSLGYKGVQSVHKR). Antibodies were raised in New Zealand White rabbits (Kosarsky *et al.*, 1986) and purified from the serum prior to use by Protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) chromatography (Sambrook *et al.*, 1989). The protein concentrations of the purified antibody preparations were: 4 mg/ml, anti-N terminus; 7 mg/ml, anti-spacer: and 5 mg/ml, anti-SRCR.

Cell Labeling—Cells were plated on day 0 in 3 ml of medium D (medium A containing 3% newborn calf lipoprotein deficient serum) or medium E in six-well dishes (150,000 cells/well).

Metabolic Labeling with [^{35}S]Methionine—On day 2, monolayers were washed two times with PBS and then incubated with 0.5 ml of pulse-labeling medium F (medium D prepared with methionine-free Ham's F-12 (Hazelton)) containing 300 μCi/ml [^{35}S]methionine (>800 Ci/mmol, Du Pont-New England Nuclear). After the indicated times of pulse-labeling, cells were either washed once with Hepes-buffered saline and harvested by lysis or washed once with medium A, refed with 3 ml/well medium B or medium D supplemented with 1 mM unlabeled methionine (chase media), and harvested at the indicated times. Cells were lysed. and the extracts were prepared for immunoprecipitation as previously described (Kozarsky *et al.*, 1986) using lysis buffer A (calcium- and magnesium-free PBS with 1% (v/v) Triton X-100, 1 mM methionine, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin) without or with 10 mM iodoacetamide as indicated. In some experiments, 2 μg/ml tunicamycin were included in a 3-h pretreatment in medium D or E and in the subsequent labeling and chase media. In others, α,α'-dipyridyl (100 μM, Sigma) was included in a 1-h pretreatment in medium D and in the subsequent labeling and chase media. To chemically cross-link receptors in intact [^{35}S]methionine-labeled cells, the labeled cells were washed twice with ice-cold PBS and incubated for 1 h at 4 °C in PBS with or without 350 μM 1,5-difluoro-2,4-dinitrobenzene (DFDNB, Pierce). The cells were then washed three times with PBS containing 5 mM glycine to inactivate unreacted cross-linker (Bischoff *et al.*, 1988) and harvested in lysis buffer A containing 5 mM glycine with or without 10 mM iodoacetamide.

Cell Surface Labeling with ^{125}I—On day 2, monolayers were placed on ice, washed three times with ice-cold PBS, overlayed with an Enzymobead (Bio-Rad) labeling mixture (see below) on ice for 30 min with rocking every 5 min, washed four times with ice-cold PBS containing 20 mM Kl, and harvested with a lysis buffer as described above. Each vial of beads was prepared by hydration with 500 μl of

PBS overnight at 4 °C according to the manufacturer's instructions. Immediately prior to use, 50 μl of 2% glucose (w/v) in water were added to a vial of hydrated beads, and labeling mixtures for each well of cells were prepared in polypropylene tubes as follows: aliquots of beads (62.5–67 μl) were diluted to 375–500 μl with PBS containing 20 mM glucose, and 1.7–2.5 mCi of $Na^{125}I$ (Amersham, 13.5 mCi/μg of iodine) were added.

Immunoprecipitation, Electrophoresis, and Autoradiography—Immunoprecipitation of LDL receptors with anti-C antibody and sample preparation for electrophoresis were performed as previously described (Kozarsky *et al.*, 1986). The same protocol was used for immunoprecipitations with anti-scavenger receptor antibodies except that the detergent mixture was prepared without SDS and contained 1–2 million solubilized, unlabeled, wild-type CHO cells per ml. These cells were included to reduce nonspecific background. Additionally, 5 μl of 20% (w/v) SDS in water were included in each precipitation. The amounts of anti-peptide antibody used for precipitations varied as indicated. SDS-polyacrylamide gel electrophoresis, for which the specimens were boiled with or without β-mercaptoethanol (5%), gel fixation, staining, and autoradiography were carried out as previously described (Kozarsky *et al.*, 1986) using 8%, 10%, or 3–10% gradients of acrylamide as indicated. The following reduced ^{14}C-methylated standards (Amersham) were used to estimate molecular masses: myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). The estimated molecular mass values presented are averages from multiple experiments. Additional unlabeled standards (Pharmacia) included α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamate dehydrogenase (53 kDa).

Enzymatic Digestions—Pronase (20 μg/ml, Sigma) treatment of labeled monolayers to identify cell surface receptors was carried out as previously described (Kozarsky *et al.*, 1986; Tolleshaug *et al.*, 1983). For endoglycosidase H (Endo H, Genzyme) digestions, washed immunoprecipitates were resuspended in 20 μl of 50 mM sodium phosphate, pH 5.5, 0.5% SDS, and 1 mM phenylmethylsulfonyl fluoride, with or without 2% (v/v) β-mercaptoethanol. The samples were boiled for 5 min and cooled to room temperature, sodium azide was added to a final concentration of 0.1% (w/v), and 2.5 μl of 50 mM sodium phosphate, pH 5.5, with or without 0.3 μg/ml Endo H were added. Samples were incubated at 37 °C overnight prior to electrophoresis. For *N*-glycanase digestions, washed immunoprecipitates were resuspended in 11.1 μl of 0.5% SDS with or without 0.7% β-mercaptoethanol and boiled for 3 min. Subsequently, the following additions were made: 15.3 μl of 0.44 M sodium phosphate, pH 8.6, 2 mM phenylmethylsulfonyl fluoride, 5.6 μl of 7.5% (v/v) Nonidet P-40 in water, and 1.5–2.5 μl of *N*-glycanase (250 units/ml, Genzyme). Samples were incubated at 37 °C overnight prior to electrophoresis.

Assays—Specific, high affinity scavenger receptor activity at 37 °C was assessed as previously described (Krieger, 1983; Freeman *et al.*, 1991) by measuring cellular degradation of ^{125}I-AcLDL (1–50 μg of protein/ml) in 24-well culture dishes using 400 μg/ml poly(I) to correct for nonspecific degradation. Protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

To permit further analysis of the structures of the type I and type II bovine scavenger receptors, we prepared three polyclonal rabbit anti-peptide antibodies which recognize peptide sequences from the cytoplasmic N terminus, the extracellular spacer domain, or the extracellular SRCR (scavenger receptor cysteine-rich) domain. Fig. 1 shows the ability of these antibodies to immunoprecipitate bovine scavenger receptors from CHO transfectants expressing either the type I receptor (CHO[bSR-I] cells) or the type II receptor (CHO[bSR-II] cells) and from control untransfected CHO cells. The cells were pulse-labeled for 30 min with [^{35}S] methionine and then immediately processed (0 chase) or incubated for an additional 3 h in medium supplemented with 1 mM unlabeled methionine prior to immunoprecipitation, SDS-gel electrophoresis, and autoradiography. After pulse-labeling, the anti-N-terminal peptide antibody (anti-N-term) precipitated doublet precursor (p) forms of both types of scavenger receptor with apparent molecular sizes of 65 kDa

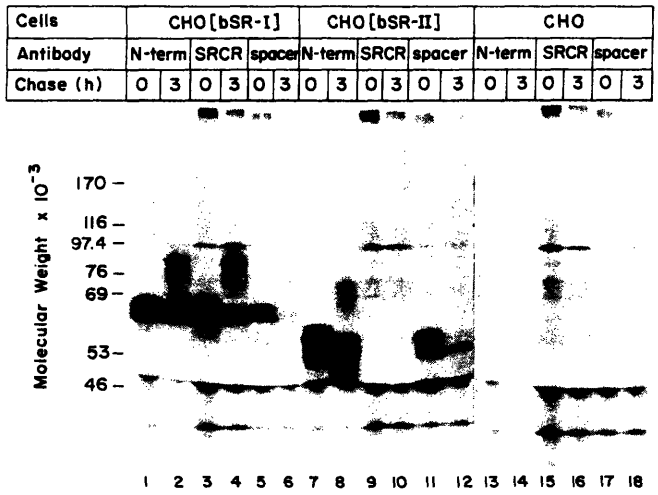

FIG. 1. **Immunoprecipitation of bovine scavenger receptors expressed in transfected CHO cells with polyclonal anti-peptide antibodies.** The type I (CHO[bSR-I]) and type II (CHO[bSR-II]) scavenger receptor-expressing cells and wild-type untransfected cells were plated on day 0 in 3 ml of medium D in 6-well dishes (150,000 cells/well). On day 2, the cells were pulse-labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for 0 or 3 h, and lysed with buffer A containing 10 mM iodoacetamide, and the extracts were subjected to immunoprecipitation as described under "Materials and Methods" with one of the following antibodies: anti-N-term (6 μl/tube), anti-SRCR (10 μl/tube), or anti-spacer (7 μl/tube). Metabolically labeled proteins in the immunoprecipitates were reduced with β-mercaptoethanol, separated by SDS-polyacrylamide (8%) gel electrophoresis, and visualized by autoradiography as described under "Materials and Methods."

and 63 kDa for the type I receptor (*lane 1*) and 57 kDa and 53 kDa for the type II receptor (*lane 7*). After the 3-h chase, the precursors were converted into less well resolved doublet mature (m) forms with apparent molecular masses of 82 kDa and 76 kDa for the type I receptor (*lane 2*) and 72 kDa and 65 kDa for the type II receptor (*lane 8*). The results with the anti-SRCR peptide antibody (anti-SRCR) were similar to those with the anti-N-term antibody for the type I receptor (*lanes 3* and *4*), although in other experiments, the anti-SRCR antibody was not as quantitatively effective as the anti-N-term antibody in precipitating type I receptors. As expected, the anti-SRCR antibody did not recognize the type II receptor (*lanes 9* and *10*) which does not have an SRCR domain (Rohrer *et al.*, 1990). The anti-spacer domain antibody recognized the precursor forms of both types of receptor, although not as well as did the other antibodies; however, it did not precipitate the mature forms, suggesting that the anti-spacer domain antibody may recognize a conformation/maturation-sensitive epitope(s). The radioactive bands seen in both the untransfected CHO lanes (*13–18*) as well as in the lanes from the transfected cell (*e.g.* the high molecular weight bands in *lanes 3–5, 9–11,* and *15–17*) are presumed to be background precipitation artifacts. Scavenger receptor bands of the expected size were not detected in the untransfected CHO cells using these antibodies (*lanes 13–18*).

The relationships between the "p" and "m" forms of the receptors were examined in greater detail in the pulse/chase experiment shown in Fig. 2. Cells were pulse-labeled for 10 min, and immunoprecipitates from each of the indicated chase times were treated with or without endoglycosidase H (Endo H). Conversion from an endoglycosidase H-sensitive to a resistant form indicates that a protein contains *N*-linked oligosaccharides which are processed from a high mannose, Endo H-sensitive precursor form to a complex, Endo H-resistant mature form in the *medial* Golgi compartment

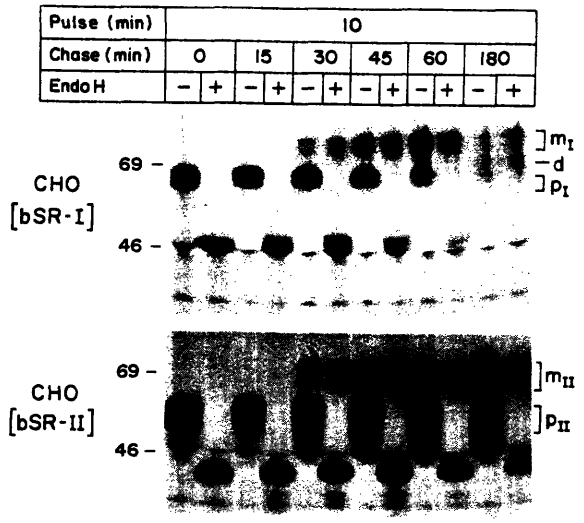

FIG. 2. **Kinetics of scavenger receptor biosynthesis: endoglycosidase H sensitivity.** Type I and type II scavenger receptor-expressing cells were plated in medium D and subsequently pulse-labeled with 300 μCi/ml [^{35}S]methionine for 10 min, chased for the indicated times, and lysed with buffer A; the extracts were subjected to immunoprecipitation (anti-N-term antibody, 6 μl/tube). The precipitates were treated with endoglycosidase H (Endo H) or subjected to control incubations without the enzyme as indicated and were reduced with β-mercaptoethanol before 10% gel electrophoresis and autoradiography as described under "Materials and Methods." The precursor and mature forms of the type I and type II receptors are indicated by p_I, m_I, p_{II}, and m_{II}, respectively. A degraded form of the type I receptor is indicated by *d*. Molecular mass markers are 69 kDa and 46 kDa.

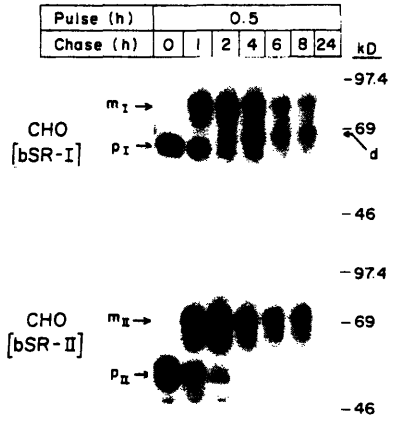

FIG. 3. **Processing and stability of scavenger receptors in transfected CHO cells.** Type I and type II scavenger receptor-expressing cells were plated in medium E and subsequently pulse-labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for the indicated times, and lysed with buffer A with 10 mM iodoacetamide, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 1 μl/tube), reduction with β-mercaptoethanol, 8% gel electrophoresis, and autoradiography as described under "Materials and Methods."

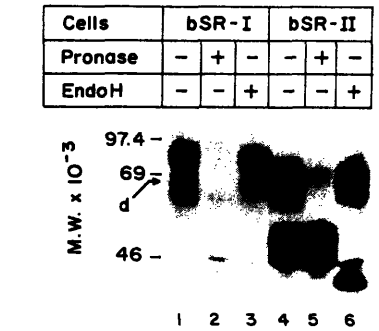

FIG. 4. **Pronase and endoglycosidase H sensitivity of scavenger receptors.** Type I and type II scavenger receptor-expressing cells were plated in medium D and subsequently pulse-labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for 2 h, and treated with (*lanes 2* and *5*) or without (*lanes 1, 3, 4,* and *6*) pronase (20 μg/ml, 20 min, 37 °C) before harvesting with buffer A, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 6 μl/tube). Some of the immunoprecipitates (*lanes 3* and *6*) were treated with endoglycosidase H (Endo H), and then all samples were subjected to reduction with β-mercaptoethanol, 8% gel electrophoresis, and autoradiography as described under "Materials and Methods."

(Kornfeld and Kornfeld, 1980). Fig. 2 shows that the precursor forms of both types of scavenger receptor were Endo H-sensitive while the mature forms were Endo H-resistant. Thus, the scavenger receptors expressed in CHO cells are *N*-glycosylated proteins, and the Endo H-sensitive *N*-linked sugars contribute approximately 15–16 kDa to the apparent mass of the precursor forms of the receptors. The apparent masses of the type I and type II receptors without *N*-linked sugars, 50 kDa and 41 kDa, respectively, are consistent with those (50 kDa and 38 kDa) predicted from the primary sequence of the receptors derived by cDNA cloning (Kodama *et al.*, 1990; Rohrer *et al.*, 1990). There was an apparent precursor/product relationship between the precursor and mature forms, and the maturation for both types of receptor was slow: no mature form was seen after 15 min of chase, and only a small amount was seen after 30 min of chase. In the case of the type I receptor, a second, Endo H-resistant form of the receptor (*d* in Fig. 2) with an apparent molecular mass of about 69 kDa was seen after 180 min of chase.

Fig. 3 shows the results of a more extended pulse/chase experiment. Significant amounts of the mature forms of the type I and II receptors as well as the d form of the type I receptor remained stable for up to 8 h of chase, while virtually no receptor protein was detectable after 24 h of chase. The kinetics of the appearance of the d form of the type I receptor and its reduced size relative to the mature form m_I suggested that it might be a degraded product derived from the proteolytic removal of part of the extracellular C terminus from the mature form of the type I receptor. This was confirmed using the anti-SRCR antibody, because this antibody could not immunoprecipitate the d form of the type I receptor (not shown). Removal of the *N*-linked oligosaccharides with *N*-glycanase demonstrated that the differences in the m_I and d forms cannot be attributed to alterations in the *N*-linked sugars (data not shown). The estimated molecular masses of the *N*-glycanase-treated type I and type II receptors were 53 kDa and 44 kDa, respectively. These values are similar to those for the Endo H-treated precursors and suggest that most of the differences in the electrophoretic mobilities of the precursor and mature forms were due to post-translational processing of *N*-linked oligosaccharides. We and others have previously described a partially degraded form of the native LDL receptor in CHO cells (Kozarsky *et al.*, 1986) and human fibroblasts (Lehrman *et al.*, 1985) which is analogous to the d form of the type I scavenger receptor. For LDL and scavenger receptors, the degraded forms arise as a consequence of the proteolytic removal of extracellular cysteine-rich terminal domains.

To determine the location (surface or internal) of the various forms of the receptors in intact cells, we used extracellular pronase sensitivity (Kosarsky *et al.*, 1986; Tolleshaug *et al.*, 1983) and cell surface iodination assays. Fig. 4 shows the effects of pronase on receptors from cells pulse-labeled with [^{35}S]methionine for 0.5 h and chased for 2 h in medium

supplemented with 1 mM unlabeled methionine. Under the conditions of this experiment, the bulk of the labeled type I receptor had been processed into the mature and degraded forms, whereas there were roughly equal amounts of precursor and mature forms of the type II receptor (compare *lanes 1* and *4*). Essentially all of the mature and degraded Endo H-resistant forms were removed by extracellular pronase treatment, while the low level of type I precursor and the substantial level of type II precursor were pronase-resistant (*lanes 2* and *5*). These results were confirmed by the analysis of steady state cell surface receptor expression using surface iodination. Fig. 5 shows that mature, *N*-glycanase-sensitive forms of both type I and type II receptors were observed after immunoprecipitation, electrophoresis, and autoradiography of ^{125}I-labeled surface receptors.

These experiments indicated that both types of scavenger receptor expressed in CHO cells behaved as typical cell surface membrane proteins which were co-translationally modified with high mannose *N*-linked sugars in the endoplasmic reticulum and transported through the Golgi apparatus, where the *N*-linked chains were processed to the complex form, en route to the cell surface. The bovine scavenger receptors differ from other previously described integral membrane receptors in that their primary sequences contain a domain with 24 Gly-*X*-*Y* repeats which, we proposed, folds into a collagen-like triple helix (Kodama *et al.*, 1990; Rohrer *et al.*, 1990). A distinctive characteristic of Gly-*X*-*Y* repeat collagenous triple helical domains is that some of the prolines and lysines are hydroxylated by α,α'-dipyridyl-sensitive hydroxylases prior to triple helix formation (Hurych and Chvapil, 1965; Kivirikko and Myllyea, 1986; Drickamer *et al.*, 1986; Colley and Baenziger, 1987a, 1987b; Jimenez *et al.*, 1973; Muller *et al.*, 1978; O'Reilly *et al.*, 1988; Persson *et al.*, 1988). Proline hydroxylation stabilizes the triple helical structure and is thought to occur in the endoplasmic reticulum (Kivirikko and Myllyea, 1986).

Fig. 6 shows the independent and combined effects of α,α'-dipyridyl and tunicamycin (an inhibitor of *N*-glycosylation) on the structures of type I and type II scavenger receptors in

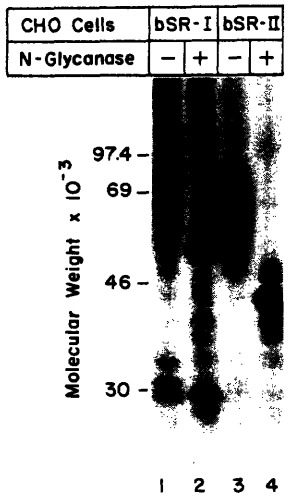

FIG. 5. **Surface iodination of transfected CHO cells.** Type I and type II scavenger receptor-expressing cells were plated in medium D and subsequently labeled with ^{125}I as described under "Materials and Methods." The cells were then lysed with buffer A; the extracts were subjected to immunoprecipitation (anti-N-term antibody, 6 μl/tube), treatment with (*lanes 2* and *4*) or without (*lanes 1* and *3*) *N*-glycanase; and then all samples were subjected to reduction with β-mercaptoethanol, 10% gel electrophoresis and autoradiography as described under "Materials and Methods."

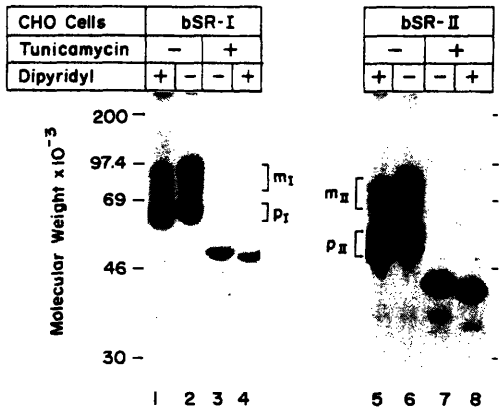

FIG. 6. **Effects of α,α'-dipyridyl and tunicamycin on the synthesis and processing of scavenger receptors.** Type I and type II scavenger receptor-expressing cells were plated in medium E and subsequently treated with either 100 μM α,α'-dipyridyl, 2 μg/ml tunicamycin, or both as described under "Materials and Methods," pulse-labeled with 300 μCi/ml [^{35}S]methionine for 2 h in the presence or absence of the same drugs, and lysed with buffer A, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 0.5 μl/tube), reduction with β-mercaptoethanol, 10% gel electrophoresis, and autoradiography as described under "Materials and Methods."

cells labeled for 2 h with [^{35}S]methionine. Under the conditions of this experiment, both precursor and mature forms of the receptors were observed without drug treatment (*lanes 2* and *6*). Treatment of the cells with α,α'-dipyridyl alone (*lanes 1* and *5*) led to somewhat increased electrophoretic mobilities of the precursors which were also observed in 30-min pulse-labeled cells (not shown) and a reduction in the amount of precursor converted to mature form. This interference of α,α'-dipyridyl with normal precursor-to-mature processing was confirmed in pulse/chase experiments (not shown). Inhibition of the post-translational processing and intracellular transport of collagen domain-containing proteins by α,α'-dipyridyl has been reported previously (Colley and Baenziger 1987b; Jimenez *et al.*, 1973; Muller *et al.*, 1978; O'Reilly *et al.*, 1988; Persson *et al.*, 1988). Treatment of the cells with tunicamycin alone (Fig. 6, *lanes 3* and *7*) resulted in the collapse of the doublets into more rapidly migrating singlet forms of both type I and type II scavenger receptors with apparent molecular masses of 49 kDa and 40 kDa, respectively. These values are similar to those obtained after *N*-glycanase or Endo H treatments (see above). Both forms of the receptor synthesized in the presence of tunicamycin exhibited α,α'-dipyridyl-sensitive electrophoretic mobilities (*lanes 4* and *8*). Thus, the effects of α,α'-dipyridyl on scavenger receptor electrophoretic mobility were not dependent on the addition of *N*-linked sugars to the receptor. These data indicate that both forms of the scavenger receptor were subject to prolyl and/or lysyl hydroxylation, that the collagenous domains probably fold into collagen triple helices, and that the hydroxylation, and thus the folding, facilitates intracellular transport.

The presence of triple helical collagenous domains in the scavenger receptors appears to explain, at least in part, our previous finding that about half of the scavenger receptors purified from bovine lung membranes was isolated as reduction-sensitive trimers (Kodama *et al.*, 1988). However, most of the rest of the purified receptor was isolated as reduction-sensitive dimers. The receptors' ligand AcLDL bound only to the trimeric form. To examine the disulfide-dependent covalent structure of the bovine receptors synthesized in the CHO cells, we determined the electrophoretic mobilities of reduced and unreduced receptors after [^{35}S]methionine labeling for

either 20 min or 2 h (Fig. 7). After a 20-min labeling, the unreduced precursor forms of the type I and type II receptors (*lanes 2* and *6*) migrated as three distinct bands, designated p-1^0, p-2^0, and p-3^0 for the monomeric, dimeric, and trimeric forms. The apparent masses for the type I receptor were 65/63 kDa (doublet), 129 kDa, and 198 kDa, and for the type II receptor were 57/53, 119, and 176 kDa. On reduction (*lanes 1* and *5*), these bands collapsed into the precursor forms described above. After labeling for 120 min, the unreduced receptors migrated as sets of monomeric, dimeric, and trimeric precursor and mature forms (*lanes 4* and *8*). The apparent masses of the mature forms were 82/76, 162, and 237 kDa and 72/65, 147, and 219 kDa for the type I and type II receptors, respectively. The monomeric unreduced precursor and mature forms of the type I receptor (*lanes 2* and *4*, "$1^{0\prime\prime}$") exhibited slightly increased electrophoretic mobilities (smaller apparent masses) relative to their monomeric reduced counterparts (*lanes 1* and *3*). The increased mobilities of the unreduced forms may be a consequence of intramolecular disulfide bonding as has previously been observed for the LDL receptor (Daniel *et al.*, 1983). These results establish that both type I and type II bovine scavenger receptors synthesized in CHO cells can be isolated as disulfide-linked dimers and trimers, that this oligomerization can be detected in the precursor, Endo H-sensitive, forms, and, because the results were similar for type I and type II receptors, that the cysteine residues in the SRCR domain are probably not involved in the interchain disulfide bonds.

The presence of reduction-sensitive trimers in the type II receptor was unexpected. This form of the molecule has only 2 cysteines per chain (Cys^{83} and Cys^{17}), only 1 of which is extracellular (Cys^{83}) and, thus, available to participate in a disulfide bond. The reducing environment in the cytoplasm should prevent the side chain of Cys^{17} from forming disulfide bonds in intact cells. Therefore, we tested the possibility that the reduction-sensitive trimeric forms of the receptors seen in Fig. 7 were experimental artifacts due to oxidation of intracellular cysteines during or after cell lysis. Fig. 8 shows the effects of including the sulfhydryl trapping agent iodoacetamide in the cell lysis buffer. The use of 10 mM iodoacetamide (compare *lanes 1* and *5* with *lanes 2* and *6*) eliminated

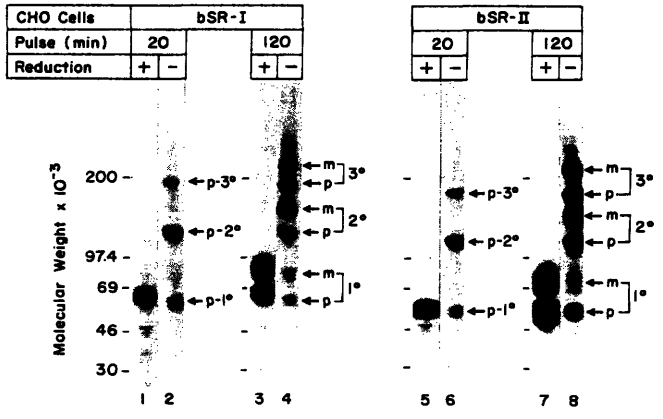

FIG. 7. **Comparison of the electrophoresis of reduced and unreduced scavenger receptors.** Type I and type II scavenger receptor-expressing cells were plated in medium E and subsequently pulse-labeled with 300 μCi/ml [^{35}S]methionine for either 20 min or 120 min and lysed with buffer A, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 1 μl/tube), reduction with β-mercaptoethanol as indicated, 3–10% gradient gel electrophoresis, and autoradiography as described under "Materials and Methods." The monomeric, dimeric, and trimeric precursors are indicated by p-1^0, p-2^0, and p-3^0, respectively. The monomeric, dimeric, and trimeric mature forms are indicated by m-1^0, m-2^0, and m-3^0.

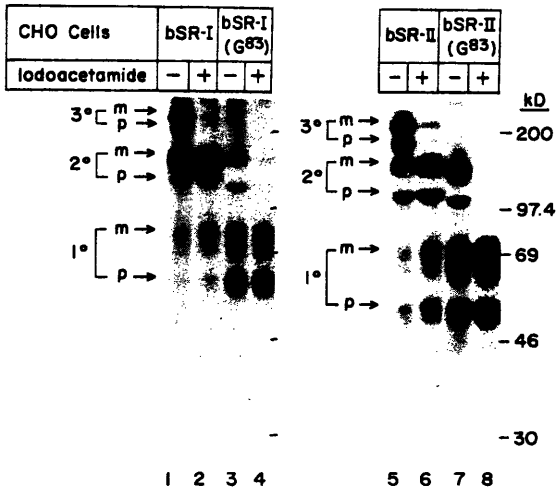

FIG. 8. **Effects of iodoacetamide and mutation of Cys^{83} to Gly^{83} on scavenger receptor oligomerization.** Transfected cells expressing the wild-type (Cys^{83}) forms of the type I and type II scavenger receptors and cells expressing the Gly^{83} mutant forms were plated in medium E and subsequently pulse-labeled with approximately 300 μCi/ml [^{35}S]methionine for 20 min, chased for 2 h, and lysed with buffer A with or without 10 mM iodoacetamide as indicated, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 1 μl/tube), 8% gel electrophoresis without sample reduction, and autoradiography as described under "Materials and Methods." Abbreviations: *bSR-I(G^{83})* and *bSR-II(G^{83})*, cells expressing the mutant type I and type II receptors.

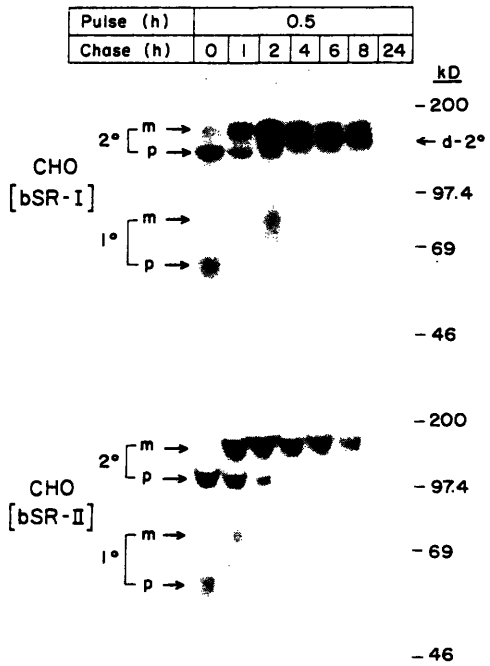

FIG. 9. **Kinetics of processing of unreduced scavenger receptors in transfected CHO cells.** Type I and type II scavenger receptor-expressing cells were plated in medium E and subsequently pulse-labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for the indicated times, and lysed with buffer A containing 10 mM iodoacetamide, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 1 μl/tube), 8% gel electrophoresis without sample reduction, and autoradiography as described under "Materials and Methods."

most of the trimeric, but not the dimeric or monomeric forms of both the type I and type II receptors. Thus, the reduction-sensitive trimers were apparently artifacts of cell lysis.

Fig. 9 shows the kinetics of type I and type II scavenger receptor processing examined using iodoacetamide-containing

lysis buffer and nonreducing electrophoresis. The maturation of the precursor forms was fairly slow, and the mature forms were stable for the first 8 h of chase and were degraded by 24 h of chase. The dimeric degraded form of the type I receptor, d-2^0, was detected by 2 h of chase, and its stability was comparable to that of the full length mature form. The d-2^0 form was not recognized by the anti-SRCR domain antibody (not shown), suggesting that both of the chains in this form of the dimer had been proteolytically cleaved.

The results with iodoacetamide suggested that the extracellular cysteine, Cys^{83}, is probably the only cysteine in either the type I or type II receptors which normally participates in the interchain disulfide bond responsible for covalent dimer formation. To directly test this, we generated expression vectors for mutant type I and II receptors (Cys^{83} to Gly^{83}) and isolated stable CHO transfectants, CHO[bSR-I(Gly^{83})] and CHO[bSR-II(Gly^{83})], which express the mutant receptors. Analysis of the saturation kinetics for the degradation of ^{125}I-AcLDL by cells expressing the wild-type (Cys^{83}) and mutant (Gly^{83}) type I and type II receptors showed that the mutations did not alter the receptors' activities (Freeman *et al.*, 1991, and data not shown). The affinities of the wild-type and mutant receptors for ^{125}I-AcLDL were virtually identical. Fig. 8 shows the effects of the mutations and iodoacetamide on the covalent oligomerization of the scavenger receptors. The cells were pulse-labeled with [^{35}S]methionine for 20 min and chased for 2 h prior to cell lysis, immunoprecipitation, electrophoresis, and autoradiography. All of the samples were unreduced. For the Gly^{83} mutants in the absence of iodoacetamide (*lanes 3* and *7*), monomeric and dimeric, but no trimeric, forms were observed. In the presence of iodoacetamide, only monomeric Gly^{83} mutant receptors were observed (*lanes 4* and *8*). Thus, in intact cells, wild-type receptors were comprised of monomers and covalent dimers linked by Cys^{83} disulfides. The covalent, reduction-sensitive trimers observed in the transfected CHO cells after lysis without iodoacetamide (*lanes 1* and *5*), and presumably the trimers that we isolated from bovine tissue (Kodama *et al.*, 1988), were artifacts of oxidation during cell disruption and probably arise because of disulfides formed by Cys^{17}.

Although these experiments show that the scavenger receptors do not normally form covalent trimers, the presence of the collagenous domain, the receptors' α,α'-dipyridyl-sensitive post-translational processing, and the efficient formation of covalent trimers when the cells were disrupted without a sulfhydryl trap all indicate that the receptors are normally noncovalent trimers. To independently confirm this, we pulse-labeled CHO transfectants expressing the wild-type receptors for either 20 or 120 min with [^{35}S]methionine and examined the effects of treating the labeled, intact cells with the lipid-soluble chemical cross-linker DFDNB. The cells were subsequently lysed in the presence of iodoacetamide, and the oligomerization state of immunoprecipitated receptors was analyzed by electrophoresis (Fig. 10). After a 20-min pulse, the precursor forms of the type I and type II receptors were cross-linked into reduction-resistant dimers and trimers (*lanes 1, 2, 5,* and *6*). Without reduction (*lanes 3, 4, 7,* and *8*), cross-linking significantly increased the amounts of trimeric forms. The electrophoretic mobilities of the reduction-resistant cross-linked dimers and trimers were virtually identical with the non-cross-linked reduction-sensitive dimers and trimers (*lanes 3* and *7,* and results using iodoacetamide-free lysis buffer, not shown). In the unreduced, cross-linked specimens (*lanes 4* and *8*), the minor bands migrating with apparent masses greater than those of the trimeric forms (*e.g.* those highlighted by *dots*) have not been identified, but might

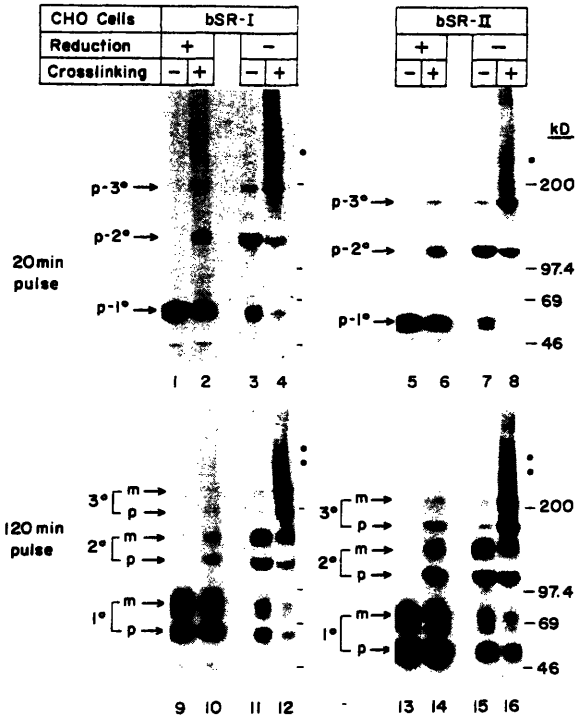

FIG. 10. **Effects of treating transfected cells with the cross-linker difluorodinitrobenzene on wild-type scavenger receptor electrophoresis.** Cells expressing wild-type (Cys^{83}) type I and type II scavenger receptors were plated in medium E, subsequently pulse-labeled with 300 μCi/ml [^{35}S]methionine for either 20 min or 120 min, and treated with or without 350 μM DFDNB as described under "Materials and Methods." The cells were then lysed with buffer A containing 10 mM iodoacetamide and 5 mM glycine, and the extracts were subjected to immunoprecipitation (anti-N-term antibody, 1 μl/tube), 3–10% gradient gel electrophoresis with or without sample reduction, and autoradiography as described under "Materials and Methods." The *solid dots* above the molecular mass markers indicate the locations of high molecular weight bands in the cross-linked material.

represent higher order receptor oligomers. The results of cross-linking after a 120-min pulse showed that the oligomerization of the mature forms of the receptors (*lanes 9–16*) was similar to that of the precursors. Similar cross-linking results were observed for the Gly^{83} mutants (data not shown). Thus, both wild-type and mutant type I and type II receptors form noncovalently associated trimers.

DISCUSSION

We recently cloned type I and type II scavenger receptor cDNAs from both bovine lung (Kodama *et al.*, 1990; Rohrer *et al.*, 1990) and murine macrophages (Freeman *et al.*, 1990). The homologous human cDNAs have also been cloned (Matsumoto *et al.*, 1990). Both types of scavenger receptor sequences were predicted to encode transmembrane proteins with multiple extracellular domains, including an α-helical coiled-coil domain and a 72-amino acid domain comprising 24 Gly-*X*-*Y* triplets. Because of these triplet repeats and our demonstration that a trimeric, but not dimeric or monomeric form of the purified bovine receptor can bind ligands (Kodama *et al.*, 1988), we proposed that this domain oligomerizes into a collagenous triple helix (Kodama *et al.*, 1990). The type I and II receptors differ only by the presence in the type I receptor of an extracellular cysteine-rich C-terminal domain, called the SRCR domain (Rohrer *et al.*, 1990; Freeman *et al.*, 1990). In the current work, we generated polyclonal anti-peptide antibodies which recognize bovine scavenger receptors expressed in stable CHO cell transfectants (Freeman *et al.*,

1991) and used these antibodies to study the processing and structures of the receptors.

Both type I and type II scavenger receptors behaved as typical cell surface membrane proteins. Precursor forms were co-translationally modified with high mannose *N*-linked sugars and oligomerized (see below) in the endoplasmic reticulum and were transported to the Golgi apparatus, where the *N*-linked chains were converted into Endo H-resistant complex-type oligosaccharides. The mature forms of the receptors were then expressed on the cell surface. The 15–17-kDa increases in the apparent masses of the receptors on conversion from the precursor to the mature forms were primarily due to maturation of the *N*-linked oligosaccharides. The apparent masses of the deglycosylated receptors were similar to those predicted from the deduced amino acid sequences (50 kDa and 38 kDa for the type I and type II receptors). Proteolytic cleavage of the extracellular C terminus of the mature form of the type I receptor lead to the formation of a relatively stable truncated form of the molecule. Similar proteolytic processing of the native LDL receptor has also been observed (Kozarsky *et al.*, 1986; Lehrman *et al.*, 1985). The functional significance of this proteolysis of the scavenger receptor remains to be determined.

The efficiency of the post-translational processing and intracellular transport of the type I and type II scavenger receptors, as well as their electrophoretic mobilities, were altered by treatment of the transfectants with α,α'-dipyridyl, an inhibitor of the collagen-modifying enzymes prolyl hydroxylase and lysyl hydroxylase (Hurych and Chvapil, 1965; Kivirikko and Myllyea, 1986). The effects of α,α'-dipyridyl were similar to those previously observed with other proteins containing collagenous domains, *e.g.* serum mannan binding protein (Colley and Baenziger, 1987a, 1987b) and the lung surfactant apoprotein (O'Reilly *et al.*, 1988), and therefore provide strong experimental support for the proposal that the Gly-*X*-*Y* repeat domain folds into a collagenous triple helix. The relatively slow transport of the receptors from the endoplasmic reticulum to the Golgi (30–60 min) may, in part, reflect the time required for the extensive post-translational steps in collagen triple helix assembly (prolyl and lysyl hydroxylation, prolyl *cis-trans* isomerization, alignment, and folding of the monomers into trimers, see Kivirikko and Myllyla, 1986) in addition to the time required for the folding and intermolecular association of the other portions of the receptor (*e.g.*, the α-helical coiled-coil domain).

The covalent and noncovalent association of the receptors' polypeptide chains into oligomers was examined using the sulfhydryl trapping agent iodoacetamide, the chemical cross-linker DFDNB, and site-specific mutagenesis. Both type I and type II scavenger receptors have cysteine residues at positions 17 (cytoplasmic domain) and 83 (extracellular spacer domain). The precursor and mature forms of both type I and type II receptors can be found as monomers and Cys^{83}-dependent, reduction-sensitive dimers. Disulfide-linked trimers are not present in intact cells, but are formed when the cells are lysed in the absence of a sulfhydryl trapping agent because of oxidation of the side chain of Cys^{17}. The reducing environment of the cytoplasm apparently prevents the participation of Cys^{17} in disulfide bonding in intact cells. The type I receptor has 6 cysteines in the SRCR domain which are not present in the type II receptor. The similarity of the oligomeric structures of the type I and type II receptors and the evolutionary conservation of the cysteines in SRCR domains (Freeman *et al.*, 1990) strongly suggests that the cysteines in the SRCR domain are probably all involved in intramolecular disulfide bonds. We previously reported that the scavenger receptors purified from bovine lung membranes were composed of reduction-sensitive dimers and trimers (Kodama *et al.*, 1988). Furthermore, ligand blotting experiments indicated that only the trimeric form could bind the ligand AcLDL. The current studies using transfected CHO cells suggest that the disulfide-linked trimeric form of the purified bovine lung receptor was also an oxidation artifact.

Although disulfide-linked trimers are experimental artifacts, our findings strongly suggest that both precursor and mature type I and type II receptor chains normally associate into *noncovalent* trimers. Reduction-resistant dimers and trimers were observed after cross-linking receptor chains with DFDNB in intact cells. The facile formation of disulfide-linked trimers when the cells are lysed as well as the presence of the collagenous domain and α,α'-dipyridyl sensitivity support the conclusion that the receptor chains associate as trimers. Thus, the type I and type II scavenger receptors are noncovalently associated trimers comprising disulfide-linked dimers and monomers. While it seems likely that the collagenous domain contributes significantly to the trimerization of receptor subunits, other domains, particularly the α-helical coiled coil domain, may also play an important role in trimerization. Clearly, the disulfide bond at Cys^{83} is not required for trimerization. Assessment of the relative contributions of the different domains to receptor trimerization will require additional experiments.

The stoichiometry of the dimers and monomers in the functional receptors has not yet been established. In the simplest trimeric model, each dimer would associate with a single monomer to form a functional "unit" trimer. An attractive, alternative model for the structure of the scavenger receptors is a higher order oligomerization model based on the structures of complement factor C1q (Schumaker *et al.*, 1981; Reid *et al.*, 1982), lung surfactant apoprotein (Voss *et al.*, 1988; Hass *et al.*, 1991), and the serum mannose binding protein (Thiel and Reid, 1989). These secreted proteins, which also contain short collagenous domains, assemble into higher order oligomers containing multiple collagenous triple helices. They have a distinctive ultrastructure which resembles a bouquet of tulips (collagenous stems with C-terminal globular bulbs). In addition to the higher order oligomerization of proteins containing collagenous domains, proteins with large α-helical coiled-coil domains are also known to assemble into higher order oligomers (Bourne, 1991). Our observation of chemically cross-linked species with apparent masses greater than those of the trimeric forms is consistent with possible higher order oligomeric structures. Additional studies will be required to characterize more precisely the oligomerization states and structures of the functional type I and type II scavenger receptors.

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Appendix B

The SRCR domains: an update

This appendix provides an update to the review of the scavenger receptor cysteine rich (SRCR) domain superfamily presented in chapter 5. In particular, it presents information about the 5 new proteins which have joined the superfamily since the publication of the review, and correctly aligns CD6 domain 1, which was improperly aligned in the published figure. It also reviews the recent discovery of a ligand for CD6.

Two new members of the group A SRCR domains have been identified, MARCO and Enterokinase. The distinguishing property of the group A SRCR domains is the presence of 6 cysteines, and in particular the absence of Cys^1 and Cys^4 (see chapter 5). MARCO, or SR-AIII, is a glycoprotein which has an extended collagenous region in addition to its single SRCR domain and is expressed on the surface of a subset of macrophages. (Elomaa et al., 1995). It has been suggested that MARCO's SRCR domain is involved in the interaction of this receptor with bacteria; however this is highly questionable. Alignment of the MARCO SRCR domain with the group A consensus sequence (Figure C.1) reveals that it matches the consensus quite well - of the residues in the consensus sequence, 78% are matched by MARCO.

Enterokinase is an intestinal membrane-associated protease that cleaves and activates trypsinogen, and has been found to contain a single SRCR domain (Kitamoto et al., 1994). It is a modular protein, and also includes domains homologous to the LDL-receptor, meprin, C1r/s, as well as a serine protease domain. Alignment of the SRCR domain of the bovine sequence with the group A consensus reveals that it is a relatively poor fit to the consensus (Figure C.1). While the N-terminal half of the domain fits the consensus fairly well (55 % identity), the C-terminal portion is extremely difficult to align. One of the cysteines (probably Cys^5) is apparently missing. In the alignment reported by Kitamoto and colleagues, it was proposed that Cys^7 and Cys^8 are missing, and that the cysteine that I have aligned as 8 is not part of this domain. In any case, it may well be that this domain is the remnant of an SRCR domain, and no longer has the SRCR fold. On the other hand, perhaps it is stable without formation of the canonical disulfide bonds.

Three additional members of the group B SRCR domain have been at least partially cloned and sequenced. The major distinguishing feature of group B is the presence of 8 characteristic cysteine residues (see chapter 5). The new group B members include Ebnerin, PEMA, and a gall-bladder mucin, and they contain a total of at least 11 additional SRCR domains. One additional group B domain, human CD6 domain 1, has been added to the alignment (Figure C.1), as it was improperly aligned in the published work (chapter 5). The reason for this misalignment is fairly clear - there is a very large sequence inserted between cysteines 5 and 6.

The first of the new proteins, Ebnerin, is a multidomain glycoprotein which contains 4 SRCR domains, 3 bone morphogenic protein-I domains, and a region homologous to the zona pelucida domain (Li and Snyder, 1995). It is expressed in the von Ebner's glands, which are salivary glands involved in taste perception. The 4 SRCR domains from the rat protein are obviously a fairly recent result of tandem duplication within the gene, as the domains are all virtually identical to each other (Fig C.1). The domains all match the consensus sequence extraordinarily well: for example domain 2 has 98% identity with the consensus. In the published sequence, domain 1 is incomplete, starting well within the SRCR domain. It seems quite likely that the authors failed to clone the N-terminal sequence of the protein, a conclusion supported by the lack of a signal peptide and by their own finding that the protein expressed in 293 cells has a substantially greater mobility on SDS-PAGE gels than the native Ebnerin.

PEMA (*Petromyzon marinus* SRCR containing protein), a protein consisting of 2 SRCR domains and 5 EGF-like repeats has recently been identified (Mayer and Tichy, 1995). PEMA is a sea lamprey protein, and was cloned from a larval intestinal tissue. It is in the Genbank database, but no information concerning its function or expression has been published. PEMA's two SRCR domains are fairly similar to each other, and match the consensus sequence quite well (Figure C.1).

The gall-bladder mucin is a protein secreted by gall-bladder epithelial cells which presumably plays a role in the protection of those cells. Partial cDNA clones of the bovine gall-bladder mucin which contains 5 SRCR domains in addition to multiple presumably O-glycosylated serine/threonine rich regions have been identified (Nunes et al., 1995). One of these clones, pGBM7-1, contains 4 SRCR domains, which are termed Ga711-Ga714 in Figure C.1. These 4 SRCR domains are virtually identical to each other, and are extraordinarily similar to those found in Ebnerin -- Ebnerin domain 2 and mucin domain 1 have 80% sequence identity. The 4 mucin sequences are an excellent match for the consensus sequence, as is the SRCR domain from the other cDNA clone pGBM31-1 (called Ga311 in Figure C.1)

The use of the SRCR domains in such a broad variety of proteins argues that it is a "tinker-toy" type module, able to function in numerous different contexts. It seems likely that our observation in the review (Chapter 5) that the mammalian SRCR domains are mainly associated with proteins which are potentially involved in host defense is not particularly significant -- it seems unlikely that the mucin or enterokinase has a role in host defense, although it is a possibility. The functions of these newer SRCR domains are in no case known. However since the publication of the review, studies of CD6 been illuminating in this regard. Bowen, Aruffo and colleagues have cloned the Activated Leukocyte-Cell Adhesion Molecule (ALCAM), a Ig domain containing protein found on activated leukocytes which binds to CD6 (Bowen et al., 1995). Studies with individual segments of CD6 demonstrated that SRCR domain 3

expressed as a soluble independent module is capable of binding ALCAM, proving that the SRCR domain is responsible for this interaction (Whitney et al., 1995b). The discovery of other ligands for the SRCR domains should help further our understanding of this domain.

Figure B.1. Comparison of the sequences of 13 new SRCR domains to previously derived consensus sequences.

The sequences were manually aligned. Abbreviations used are as follows: ConsA/B, consensus sequences for the group A and B SRCR domains (see chapter 5); Enter, bovine enterokinase; MARCO, mouse MARCO; PEMA, sea lamprey PEMA; Ga71 and Ga311, bovine gall-bladder mucin clones pGBM7-1 and pGBM31-1. Murine CD6 domain-1 is also presented, as it was incorrectly aligned in the review presented in chapter 5. Where multiple domains exist within the same protein, they are numbered starting at the amino terminus. Consensus sites are indicated in the top row for both domain types as described in chapter 5. These consensus residues have not been adjusted to take the new sequences into account, and are denoted by a single amino acid (capital letter) or a single class of residue (a, aliphatic (A, I, L, V); π, aromatic (F, W, Y); h, hydrophobic (a, π, M); +, positively charged (H, K, R); -, negatively charged (D, E); ±, charged (-, +); o, S or T).

```
ConsA  aRLa....G....EG.VEah....WG.aC.-..W...-A.VVC+.aGπ..GA.a.....FG....Phha..a.C.G..............E..L.-C.h.......C.....DAGV.C.
MARCO  VRIMG...GTNR..GRAEVYYNNEWGTIC.DDDWDNNDATVFCRMLGYSRGRALSSYGGGSGNIWLDN...VNCRG..............TENSLWDCSKNSWGNHNC.VHNEDAGVECS
Enter  VRLFN...GTTDSSGLVQFRIQSIWHVAC.AENWTTQISDDVCQLLGLGTGNSSVPTFSTGGGPYVNLNTAPNGSL..............ILTPSQQCLEDSLILLQC......NYKSCG

ConsB  aRL......G..C.G.VEa...G.WGoaC.±..π.a..A.VaC+QLGCG.Aa.h...a.h......aWh..h.C.G..............E..aW.C.........C......A.VhCS
Ebne1  ........................MC.DDSWDINDANVVCRQLGCGWALSAPGSAQFGQGLGPIVLDDVACRGH..............EAYLWSCSHRGWLSHNC.GHQEDAGVICS
Ebne2  VRLVN...GGDRCRGRVEILYQGSWGTVC.DDSWDINDANVVCRQLGCGWALSAPGSAQFGQGSGSIVLDDVACRGH..............EAYLWSCSHRGWLSHNC.GHQEDAGVICS
Ebne3  VRLVN...GGDRCQGRVEILYQGSWGTVC.DDSWDTKDANVVCRQLVCGWALSAPGSAHFGQGSGSIVLDDVACTGH..............EAYLWSCSHRGWLSHNC.GHHEDAGVICS
Ebne4  LRLVN...GTNRCEGRVEILYRGSW.VPCADDSWDINDANVVCRQLGCGSALSAPGNAWFGQGSGLIVLDDVSCSGY..............ESHLWNCRHPGWLVHNC.RHVEDAGVICS
Pema1  VRLVQ...GGS.CSGRVEILYGGRWGTVC.DDNWGIEDATVVCRQLGCGYAVYAPTHARFGMGSGPIWMDEVYCSGS..............ESYLSHCLFNGWNSHDC.SHGEDASVICS
Pema2  VRLVG...GGW.CQGRVEVYYAGSWGTVC.DDSWDRQDAEVVCRQLSCGYAVSAPSNAHFGVGSGQIWLDDVNCYGQ..............ESSLARCNHSGWGRHNC.GHNEDASVICS
Ga711  LRLVN...GSDRCQGRVEVLYGGSWGTVC.DDSWDTNDANVVCRQLGCGWAISAPGDARFGQGSGPIVLDDVGCSGY..............ETYLWSCSHSPWNTHNC.GHSEDASVICS
Ga712  LRLVN...GGDRCQGRVEVLYRGSWGTVC.DDSWDTNDSNVVCRQLGCGWANSAPGNARFGQGSGPIVLDDVGCSGY..............ETYLWSCSHNPWNTHNC.GHSEDASVICS
Ga713  LRLVN...GSDRCQGRVEVLYRGSWGTVC.DDSWDTNDANVVCRQLGCGWGISAPGDARFGQGSGPIVLDDVGCSGY..............ETYLWSCSHNPTNTHNC.GHSEDASVICS
Ga714  LRLVN...GSDRCQGRVEVLYGGSWGTVC.DDSWDTNDANVVCRQLGCGSGISAPGDARFGQGSGPILLDDVGCSGY..............DTYLWSCSHSPWNSHNC.GHSKDASVICS
Ga311  LRLVNWNSSHPTCAGRVEIYHGGQWGTVC.DDNWDVQDAQVVCRQLGCGYAVSAPGNAYFGSGSGPITLDDVVCSGA..............ESNLWQCRNRGWFYHNC.GHHEDAGVICS
hCD61  VRLTN...GSSSCSGTVEVRLEASWEPAC.GALWDSRAAEAVCRALGCGGAEAASQLAPPTPELPPPPAAGNTSVAANATLAGAPALLCSGAE..WRLCEVVEHA...CRSDGRRARVTCA
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Appendix C

Progress in the determination of the high resolution structure of an SRCR domain

Introduction

Generation of a high resolution structure of an SRCR domain would provide a significant resource. Presumably all of the SRCR domains have the same overall fold. Accordingly, structural information about a single SRCR domain should provide data about all the members of this increasingly widespread superfamily. There are two basic approaches one can take to determine the structure of a domain this size (102 amino acids). The classical approach used to determine such a structure is X-ray crystallography. X-ray structure determination can be quite difficult, as conditions which promote the nucleation and growth of crystals which diffract adequately are not always readily discovered. X-ray crystallography typically requires significant quantities of protein. Determination of a structure of a domain this large by means of nuclear magnetic resonance (NMR) is also feasible (Wuthrich, 1990). In order to facilitate this process, substitution of ^{15}N-labeled amino acids is highly desirable. Thus, expression in bacterial systems or perhaps yeast is the most practical way to generate material for this approach. This appendix describes efforts to express the SRCR domain of the type I bovine scavenger receptor in both *Pichia pastoris* and *E. coli*. It also summarizes the progress which has been made on the expression and refolding of the membrane proximal domain of CD6 in *E. coli.*

I carried out most of the experiments described in this appendix. Gus Leotta, an undergraduate (UROP) working with me, generated and sequenced the *Pichia* pHIL-S1[b-SRCR] construct, and made the *Pichia* His^-Mut^+ transformants. Michael Bowen and Alejandro Aruffo at the Bristol-Myers Squibb Pharmaceutical Research Institute have generously provided reagents and advice for the work on CD6 SRCR domain 3.

Experimental procedures

Materials - pHIL-S1 and pPIC-9 were obtained from Invitrogen, Ni-NTA agarose was from Qiagen, pET15b and *E. coli* BL21(DE3)pLysS were from Novagen. All basic molecular biological manipulations were carried out as described in Sambrook et al., (1989).

Generation of Pichia pastoris *bSRCR expression constructs* - To attempt to express the SRCR portion of the bovine SR-AI in *Pichia pastoris*, three different expression constructs were generated. The first of these was generated using PCR and two oligonucleotides, primer 1 (5' GCCGCCCTCGAGCTCACCACCA TCACCATCATCACCACAGCGGCCGCAGAGCTAGCGTTTTAATTAAGACTAGT ATGCAAAGACAATCTAAT 3') and primer 2 (5' - GCCGCCGGATCCGCGGAATT CGAATGAAAATATGATGCA 3'). These oligonucleotides were used with plasmid pXSR7 (Kodama et al., 1990) to generate a PCR product that contained the entire SRCR domain. The PCR product was digested with *Xho* I and *Bam* HI, and the digested product was ligated with pHIL-S1 which had been digested with the same enzymes. The construct was then sequenced on both strands using the dideoxy method (Sequenase, US Biochemical). The expressed construct, pHIL-S1[His-bSRCR] would consist of an N-terminal PHO1 signal, 8 His residues for affinity purification, an in-frame *Not* I sequence (for insertion of an epitope tag if desired), and a heart muscle kinase sequence for ^{32}P labeling. The final N-terminal amino acid sequence after signal sequence cleavage would be predicted to be HHHHHHHHSGRRASVLIKTSM^{345}-, where M^{345} corresponds to the beginning of homology with the bovine SRCR domain. M^{345} is the only methionine in the SRCR domain, and thus the N-terminal "utility peptide" could be cleaved off with cyanogen bromide after use for purification. The SRCR domain was then transferred into the pPIC9 vector, which has an α-factor leader sequence instead of the PHO1 sequence found in pHIL-S1. In order to do this, the insert was excised from the pHIL-S1 construct with *Xho* I and *Eco*R-I, and then linkered with two oligonucleotides (5'-CAGGGCCCATG-3' and 5'-CATGGGCCCTGAGCT-3') to blunt the *Xho* I site. This insert was then ligated to pPIC9 digested with *Sna* BI and *Eco* RI, and the finished construct, pPIC9[His-bSRCR] was sequenced as described above. It is possible that the leader sequence could interfere with expression, so a construct without it was also made. The leader was excised by digestion of pHIL-S1[His-bSRCR] with *Xho* I, partial blunting with Klenow and dGTP, dTTP, and dCTP, digestion with *Spe* I, removal of 5' overhangs with mung bean nuclease, and ligation. The excised region was sequenced as described above. The product of this, pHIL-S1[b-SRCR], should produce protein product which, after signal sequence cleavage, would begin with the sequence RSM^{345}.

Transfection and expression in Pichia pastoris - The three expression constructs were linearized with *Bgl* II and transfected into the histidine auxotrophic *Pichia pastoris* strain GS115 using the spheroplast method as described in the manufacturer's instructions (Invitrogen, *Pichia* expression kit).

Transfection was evaluated by ability of transfectants to grow in the absence of histidine, and disruption of the AOX1 locus was verified by determination of the loss of ability to grow on methanol as a sole carbon source (i.e. the yeast should change from His^-Mut^+ to His^+Mut^-). Alternatively, the vectors were digested with *Sal* I prior to transfection. This should result in integration of the vector at the His4 locus, resulting in a His^+Mut^+ transfectant. Presence of the insert in the transfected yeast was verified by PCR using primers flanking the insert (AOX5' and AOX3', Invitrogen). Expression of the transfected gene was carried out as described (see Invitrogen manual for all solutions/details). Briefly, transfected yeast were inoculated into BMGY (which contains glycerol as a carbon source) in baffled flasks and grown in baffled flasks on a rotary shaker at 30°C until they reach an OD_{600} of ~1.0. They were then pelleted at 1500x*g*, and induced by resuspending at an OD_{600} of 1.0 (His^+Mut^+) or 10.0 (His^+Mut^-) in BMMY (which contains methanol as a carbon source). Methanol was added to 0.5% every day to compensate for evaporation. After 5 days, the yeast were harvested by centrifugation at 1500x*g* and the supernatant and the pellet were frozen at -20°C. Secreted proteins were visualized by SDS-PAGE and Coomassie or silver staining (Kozarsky et al., 1986; Morrissey, 1981).

Generation of bSRCR DNA constructs for expression in E. coli - The bacterial expression construct was generated as follows. The bSRCR insert was excised from pHIL-S1[His-bSRCR] by digestion with *Spe* I, blunting with Klenow, and *Bam* HI digestion. The gel purified insert was then ligated with pET-15b which had been digested with *Nde* I, blunted with Klenow, and digested with *Bam* HI. The resulting vector, pET-15b[bSRCR], was sequenced at the ligation joins. Expression of this construct in bacteria should give a product with the N-terminal sequence $MGSSHHHHHHSSGLVPRGSHTSM^{345}$, which includes a His tag for purification, and both a thrombin site and a unique methionine to allow cleavage of this leader region from the SRCR domain. This plasmid was then transfected into the expression host *E. coli* BL21(DE3)pLysS.

Expression and purification of bSRCR from E. coli - Transfected bacteria were inoculated into LB containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol, and grown at 37°C until reaching an OD_{600} of ~0.6. Expression was then induced by addition of isopropyl β-D-thiogalactopyranoside to 1 mM. Three hours after induction, the bacteria were harvested by centrifugation for 15 minutes at 6000x*g*, and the resulting pellets were frozen at 20°C. Proteins were solubilized by sonicating the pellets for 1 min. on ice (setting 7, Model 185 Branson Sonifier) in 40 ml Buffer A (6 M guanidine•HCl, 100 mM sodium phosphate, 100 mM Tris pH 8.0). The resulting mixture was stirred for 1 hr at room temperature, and sonicated twice for 1 min. The sample was then clarified by centrifugation for 2x15 min. at 9000x*g*. The supernatant was mixed with an equal volume of a 1: 1 suspension of Ni-NTA agarose in Buffer A, and incubated at room temperature on a rotator for 1 hr. The mixture was poured into an column (1.5 cm internal diameter). The matrix

was washed with 100 ml buffer A, and 50 ml Buffer B (Buffer A, pH 6.3). It was then eluted with buffer C (Buffer A, pH 4.5). The eluate was monitored by OD_{280}. Fractions containing protein were pooled, and evaluated by SDS-PAGE and Coomassie Blue staining as described above.

Refolding and analysis of bSRCR - Many refolding protocols were attempted, however the most generally useful one was as follows. Purified bSRCR (10-20 mg/ml) was reduced by addition of dithiothreitol to 100 mM and immersion in a boiling water bath for 20 minutes. The sample was then diluted to 1 mg/ml with 8 M GuHCl. Refolding reactions were typically done at a final protein concentration of ~25µg/ml in 200 mM Tris pH 8.5 containing 1 mM EDTA. Additives and variations attempted included the addition of 10% glycerol, 1-2 M urea, 1-2.5 M guanidine HCl, 0.5 M arginine, Ham's F12 media, and complete Dulbecco's phosphate buffered saline (Gibco). Redox reagents added were typically 3 mM reduced glutathione and 0.3 mM oxidized glutathione, although different ratios and quantities were attempted. Refolding reactions were tried at 4°C, 25°C, and 37°C for 1 to 10 days at varying pH values. Refolding reactions were analyzed using reversed phase HPLC. The reaction mixture was injected over a Vydac C18 analytical column (218TP54). The column was eluted with the following gradient (buffer D = acetonitrile/0.1% trifluoroacetic acid (TFA); buffer E, H2O/0.1% TFA): 5 minutes 0-23% buffer D (balance was buffer E), 20 minutes 23-43% D, and 2 minutes 43-100% D. Initial purified material (which was oxidized) eluted at 20.4 min, while fully reduced material eluted at 23.7 min. Refolded material which looked promising was further analyzed by proteolytic digestion. Refolded material (200 µg) was boiled in 8 M Urea for 20 minutes, cooled to room temperature, diluted with 50 mM Tris/1 mM $CaCl_2$, pH 7.6 to a final urea concentration of 1 M, and digested with 4 µg modified trypsin (Promega) for 16 hr at 37°C. Digests were evaluated by reversed phase HPLC as described above using a slightly different gradient (0-10 min, 0% D, 10-50 min, 0-40% D, 50-51 min, 40-100% D). Peaks were analyzed by amino acid analysis (MIT Biopolymers Facility, Dept. of Biology).

Generation of murine CD6 domain 3 DNA expression constructs - Two different mCD6-3 constructs were made. These were SmCD6, which contains residues 260-361, and LmCD6, which includes residues 260-394 (Whitney et al., 1995a). The constructs were generated by PCR, using murine CD6-pCDM8 (provided by Michael Bowen, Brystol-Myers Squibb) vector as a template, and two oligonucleotides, Primer 1 (5'-GGGAATTCCATATGGAGCACCAGTCCTGGCG C-3') and either Primer 2 (SmCD6-3) (5'-CGTTGAAGGGATCCTCAGCCTGAGC AGACAACCCT-3') or Primer 3 (LmCD6-3) (5'-CGTTGAAGGGATCCTCATGAGT CCTTATCCTTCAC-3'). The resulting PCR products were digested with *Nde* I and *Bam* HI, and ligated with pET-15b which had been cut with the same enzymes. The resulting constructs were then sequenced. SmCD6-3 had a base change that resulted in the conversion of Arg^{324} to a Ser, and will be reconstructed at a future time. LmCD6-3 had a single conservative base

change. These constructs when expressed will encode proteins with the N-terminal sequence MGSSHHHHHHSSGLVPRGSHME^{260}, which includes a His tag and a thrombin cleavage site. The short construct encodes a protein product that ends at residue 361, the long at residue 394. These plasmids were transfected into the expression host *E. coli* BL21(DE3)pLysS.

Expression, purification, and refolding of mCD6-3 from E. coli - SmCD6-3 and LmCD6-3 were expressed, purified, refolded, and subjected to HPLC analysis exactly as described for the bSRCR protein product. ELISA analysis was carried out using monoclonal antibody #38 (a conformation specific rat-anti-mCD6 domain 3 antibody), and using COS cell expressed mCD6-3 as a positive control (the antibody and mCD6-3 were generous gifts of Michael Bowen). Briefly, samples to be analyzed were diluted in coating buffer (50 mM sodium bicarbonate, pH 9.6), and incubated overnight in Immobilon-1 96 well dishes (Fisher). Wells were washed 3 times with PBS containing 0.05% Tween-20, and blocked for 1 hour with 100 µl EIA specimen diluent (Genetic Systems). The wells were then washed twice with PBS/Tween-20, and mAB#38 diluted to 1 µg/ml in specimen diluent was added. After a 1.5 hr incubation at room temperature, the wells were washed 4 times with PBS/Tween-20, and horse-radish peroxidase-anti-rat-IgG (Biosource International) was added at a dilution of 1:5000 in specimen diluent. Following a 1 hour incubation, the wells were washed 4 times with PBS/Tween-20, and developed with EIA chromagen RG (Genetic Systems). The reaction was stopped by addition of 1N H_2SO_4, and wells were read at 450 nm.

Results and discussion

As an initial approach to the high level expression of a SRCR domain, expression of the bovine SR-AI's SRCR domain (bSRCR) in the methylotropic yeast *Pichia pastoris* was chosen. This system was selected for several reasons. It has been reported that *Pichia* is capable of producing correctly folded secreted proteins with yields exceeding 1 gram per liter (Tschopp et al., 1987). As *Pichia* is eukarytoic, it may be expected to correctly arrange disulfide bonds in the proteins it secretes. *Pichia* can grow in defined media, and the very high levels of expression make isotopic labeling for NMR feasible.

Constructs to express and secrete bSRCR with and without a His tag leader sequence were generated and transfected into *Pichia* as described in materials and methods. Secretion of the product into the media was evaluated by SDS-PAGE and silver staining or Coomassie Blue staining, or by ^{32}P labeling of the heart muscle kinase site (His containing constructs only) followed by SDS-PAGE and autoradiography. Despite several variations of constructs, integration sites, and expression conditions, protein expression was never detected. The different constructs used contained either a PHO1 or an α-factor leader sequence. In the case of PHO1, constructs with and without the N-terminal His tag and heart muscle kinase sites were tried. The *Pichia* constructs were integrated into the *Pichia* genome at both the AOX1 site and the His 4 locus. Apparently, approximately 50% of the proteins that are attempted to be expressed in *Pichia* express poorly or not at all (Jim Cregg, personal communication). It would appear that the bovine SRCR domain is among this 50%.

An alternative approach to expression is to use *E. coli*. *E. coli* has several advantage as an expression host. It can express many proteins at moderately high levels (typically ~50 mg/l), and isotopic substitution for NMR is simple. However there are drawbacks. Disulfide containing molecules will typically not be produced in the correctly folded form. Thus, the expressed protein must be subjected to *in vitro* refolding reactions. The conditions which enable a protein to fold successfully are highly variable, and may require considerable effort to discover. This problem is exacerbated in the case of the bSRCR domain, for which there are no known ligands. Thus, the only way to assess whether a refolding reaction has been successful is to determine the disulfide bond arrangement and compare the result to that found in the whole receptor expressed in CHO cells (see chapter 6).

The bovine SR-AI SRCR domain was inserted into a vector placing the cDNA under the control of a T7 promoter. Addition of IPTG to the growth media resulted in massive induction of SRCR domains, which were found upon lysis of the bacteria to be completely insoluble unless denaturants were added, presumably in inclusion bodies. Yields of Ni^{2+}-affinity purified protein were

around 50 mg/l. The purified protein was assessed by SDS-PAGE and reversed phase HPLC, and found to be fairly pure.

Efforts to refold the bSRCR domain were ultimately unsuccessful. The material purified from the bacteria was oxidized. Upon reduction, its mobility by reversed phase HPLC was substantially different. Many refolding conditions caused the domain to refold to the initial fully oxidized form. Proteolytic digestion and amino acid analysis of this form revealed that the cysteines were forming nearest neighbor disulfides, i.e. Cys^2-Cys^3, Cys^5-Cys^6, and by inference Cys^7-Cys^8, instead of the correct Cys^2-Cys^7, Cys^3-Cys^8, Cys^5-Cys^6 arrangement. Occasionally different forms were observed, however they were not particularly reproducible. The difficult of the assay as well as the strong propensity of the protein to form vicinal disulfides was the eventual downfall of this approach.

Current efforts are focused on the refolding of bacterially expressed murine CD6 SRCR domain #3 (mCD6-3). The issues and problems facing this task are the same as those facing bSRCR, with one important difference: mCD6-3 has a known ligand, ALCAM (Whitney et al., 1995b; see Appendix B for details). This enables the refolding reaction to be monitored by means of a simple ELISA assay, allowing numerous refolding conditions to be readily screened in parallel. Furthermore, conformation specific monoclonal antibodies have been isolated, providing an alternative assay.

Two expression constructs were produced. The first of these, SmCD6-3, includes only the third SRCR domain. The second, LmCD6-3, encodes this SRCR domain and an additional 34 amino acid stretch between this domain and the membrane spanning region of the protein. This extension apparently enhances ligand binding to some degree (Whitney et al., 1995b). Following induction and Ni^{2+}-affinity purification, yields were approximately 26 mg SmCD6-3 and 43 mg LmCD6-3 per liter. HPLC analysis of the purified material revealed that it was oxidized, with substantially different mobility than the reduced material. Refolding reactions yielded products with still different mobility. Analysis of these products is in progress. Conformation specific monoclonal antibodies are capable of recognized several different forms of refolded material, however no refolded form is as yet able to bind ALCAM. Assuming that this material can be correctly refolded, NMR studies and X-ray analysis should prove highly illuminating.

Appendix D

Progress in the crystallization of the secreted type I class A human scavenger receptor

Introduction

Many of the predictions made concerning the structure of the type I and type II class A scavenger receptors have been verified and extended in the course of the work reported in this thesis. Support was provided for the receptor being trimeric, and for the existence of the collagen and α-helical coiled-coil domains, as well as the determination of the disulfide pattern of the SRCR domain. Low resolution images of the receptor were obtained via electron microscopy. Expression of the receptor at high levels (Chapter 6) allowed for the possibility of obtaining higher resolution information via X-ray crystallography. Possession of a high resolution structure of the receptor would help us investigate the puzzling questions remaining concerning scavenger receptor structure/function. Crystallization of a long, fibrous glycoprotein may prove difficult, however initial results appear promising.

The crystallization trials described in this chapter were initiated by Gus Leotta, am undergraduate (UROP) working with me. He did the initial factorial screen. I've done the subsequent refinement of conditions and characterization of crystals. Dr. Larry Stern has provided much advice and assistance in all aspects of this project.

Materials and methods

Expression and purification of s-hSR-AI - Secreted human type I receptor (s-hSR-AI) was expressed and purified as described in chapter 6. The purified material was buffer exchanged into 10 mM Tris, pH 7.4 using a Centricon 100 (Amicon) and subsequently concentrated to 10-20 mg/ml (Lowry et al., 1951).

Crystallization conditions - Crystallization trials were carried out using the hanging drop method as previously described (Ducruix and Giege, 1992). Briefly, 1 µl purified s-hSR-AI was mixed with 1 µl precipitant on a cover slip. This cover slip was rapidly inverted and placed over a grease rimmed well of a 24 well dish (Linbro) containing 500 µl of precipitant. The sealed drops are incubated at room temperature to allow crystals to form. Initial selection of precipitants was guided by a standard factorial screen of conditions which had been successfully used to crystallize other proteins (Jancarik and Kim, 1991). Conditions which led to precipitation were further investigated to find precipitant concentrations in which crystals could nucleate and grow to larger sizes. The composition which works best is currently 15% PEG-4K containing 100 mM sodium cacadolite at pH 6.5.

X-ray diffraction studies - Crystals were harvested in 17% PEG-4K containing 100 mM sodium cacadolite at pH 6.5 and mounted in 0.5 mm Lindeman tubes as previously described (Ducruix and Giege, 1992). Crystals were initially characterized with 10 min still shots at 0° and 90° angles, as well as 6 hr shots with 1° oscillation at the same angles. Studies were carried out using a Rigaku RU200 X-ray set with a rotating copper anode and a 0.3 x 3 mm filament run at 50,000 volts, 100 mA. K_α radiation was selected with a graphite monochrometer, and diffraction data were collected using a R-axis-2 image plate area detector.

Results and Discussion

Initial attempts to crystallize s-hSR-AI gave promising results. The factorial screen gave numerous conditions which precipitated the receptor. Refinement of these conditions led to the identification of 15% polyethylene glycol (4000 Da molecular weight) and pH 6.5 as conditions which could promote crystal growth. These conditions resulted in the growth of crystals of several different morphologies. The most prevalent form is apparently star shaped (diagrammed in Figure D.1). These stars are actually composites of diamond shape plates. The two forms drawn to the right of the star are frequently observed. Mechanical disruption of the stars causes them to separate into single diamond shaped plates. The dimensions of these diamonds are typically 100 μm long, 50 μm wide, and about 5 μm thick. Under the same conditions, two other structures are occasionally observed. Needles of approximately 160 μm by 20 μm have been seen, as have amorphous crystals with dimensions of 80 mm by 20 μm. It has not been established that these crystals are in fact s-hSR-AI. The other drop constitutents, namely PEG, sodium cacadolyte, and Tris, are highly unlikely to crystallize at these concentrations. It is possible that protein or other contaminants in the preparation could be crystallizing. Accordingly, an essential control experiment at this point is to isolate several crystals and establish via SDS-PAGE that they are in fact composed of scavenger receptor.

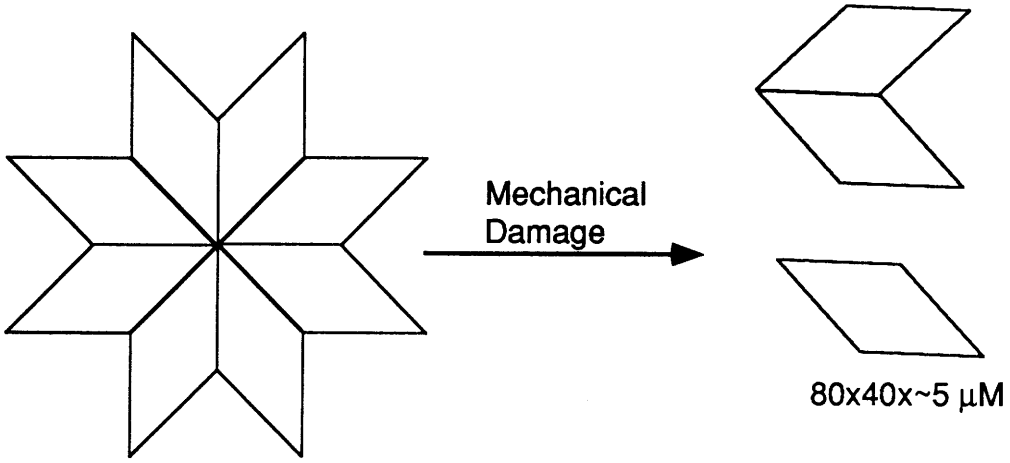
Initial attempts to characterize these crystals using X-ray diffraction have been unsuccessful. Analysis of single diamond shaped and needle-like crystals gave either no diffraction or very weak diffraction. There are several possible reasons for this. Electron microscopy of single molecules has revealed that the receptor is roughly 400 Å long (see chapter 6). Assuming that the long axis of the receptor lies in the short dimension of the crystal, this means that a 5 μm thick crystal would have only ~125 molecules in this dimension. This may well not be enough to diffract adequately. It may also be that the crystals are simply disordered. Attempts to grow larger crystals are in progress, as are attempts to crystallize the type II receptor. The most promising prospect is to use the small crystal forms to seed different conditions which do not inherently support nucleation, but might support further crystal growth. Efforts are currently being hindered by batch-to-batch variation of crystallization conditions, however this problem appears to have been overcome. Further studies will be required to iron out this and other technical difficulties.

Figure D.1 Crystal forms obtained using s-hSR-AI

Morphologies and sizes of observed crystals are shown. Crystals were observed at 40x magnification with a dissecting microscope, and measurements were estimated using an optical micrometer.

Crystal forms obtained using s-hSR-AI

Stars



Needles

Amorphous

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