Studies of the Class A and Class C Scavenger Receptors

by

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Abstract

Scavenger receptors are cell surface molecules which avidly bind modified lipoproteins and a broad range of other polyanionic molecules and macromolecular complexes. To better understand the structural basis for the ligand specificity of mammalian class A macrophage scavenger receptors (SR-A), I have examined the ability of these receptors to distinguish polynucleotide ligands (poly(I) and poly(G)) from polynucleotides which are not ligands (poly(A) and poly(C)). I found that only those poly- and oligonucleotides which can form four-stranded helical structures are SR-AI ligands. Binding of these molecules is reversibly disrupted by denaturing, and then renaturing, their quadruplex structure. Presumably, this structure generates a negatively charged surface complementary to the positively charged collagenous ligand-binding domain of SR-AI. The *in vivo* functions of the predominantly macrophage-expressed SR-A receptors are largely unknown. Their broad ligand binding specificity, and their ability to bind microbial surface constituents, suggests that these receptors might be pattern recognition receptors involved in the rapid and direct recognition of pathogenic microorganisms during innate immune responses. Since innate immunity is evolutionarily ancient, pattern recognition receptors might also exist in invertebrates such as *Drosophila melanogaster*. Interestingly, *Drosophila* embryos, and a hemocyte-derived *Drosophila* cell line, exhibit an SR-A-like activity. To gain a better understanding of the *in vivo* function(s) of scavenger receptors, a gene was isolated whose product can account for at least some of this scavenger receptor activity. This gene, *dSR-CI,* defines a new class of scavenger receptor which is structurally unrelated to the SR-As. dSR-CI is expressed throughout development, and in embryos is expressed specifically by hemocytes and macrophages. dSR-CI recognizes not only a broad range of polyanionic molecules, but also the microbial ß-glucan laminarin, a potent inducer of the *Drosophila* immune response. dSR-CI might therefore play an important role in *Drosophila* host defense. During work toward isolating *dSR-CI* mutants, two *dSR-CI* - related genes were discovered which are expressed during distinct stages of *Drosophila* development. Future work on the*dSR-CI* gene, and perhaps also on other members of the dSR-C gene family, should provide new insights into the functions of the dSR-CI protein during *Drosophila* development and host defense.

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Chapter One

Scavenger Receptors in Innate Immunity

Introduction

The circulating monocytes and tissue macrophages of mammals, and the hemocytes of invertebrates, mediate many of the innate immune responses to microbial infection (Gordon *et al*, 1988; Hultmark, 1993; Hoffmann, 1995). In some cases, these evolutionarily ancient phagocytic cells recognize and engulf pathogens which have been opsonized by serum proteins such as complement (Gordon *et al*, 1988). However, macrophages and hemocytes can also bind pathogens directly, and this direct recognition plays a crucial role in host defense (Gordon *et al*, 1988; Janeway, 1989, 1992; Hultmark, 1993). Janeway (1989, 1992) has proposed that activation of the innate, or non-adaptive, immune system (and importantly, also of the adaptive immune system; see also Ibrahim *et al*, 1995; Fearon and Locksley, 1996) is initiated when pathogens bind to non-clonally distributed "pattern recognition receptors" on immune cells. These receptors are proposed to exhibit binding specificities for structural patterns typically displayed by the surface molecules of many microorganisms (e.g. lipopolysaccharides (LPS, endotoxin), glucans), patterns which are not normally found on host cells. By virtue of their ability to mediate pathogen binding, pattern recognition receptors should, in principle, be able to participate both in the activation of innate immune cells and in the effector mechanisms (e. g. phagocytosis) these cells employ.

Several soluble and membrane-bound mammalian proteins, including the mannose receptor (Ezekowitz *et al*, 1991; Drickamer and Taylor, 1993), the collectins (Epstein *et al*, 1996), the ß2 integrins CR3 (CD11b/CD18; CR = complement receptor) (Thornton *et al*, 1996) and CR4 (CD11c/CD18) (Ingalls and Golenbock, 1995), and the LPS receptor CD14 (Pugin *et al*, 1994; Kusunoki *et al*, 1996) exhibit the properties of pattern recognition molecules. A common theme among all of these molecules appears to be their ability to recognize non-self carbohydrate and lipid structures including LPS, lipoteichoic acids, mannans and glucans (Janeway, 1989; Fearon and Locksley, 1996; Epstein *et al*, 1996). CR3, which mediates macrophage and polymorphonuclear leukocyte (PMN, neutrophil) engulfment of particles coated with complement component C3, provides a particularly interesting example of the importance of pattern recognition for eliciting an immune response: PMN readily kill iC3b-osponized yeast, but do not lyse iC3b-opsonized erythrocytes or kill iC3b opsonized tumor cells. However, if the PMN are incubated with soluble yeast ß-glucans, which bind directly to CR3 (Thornton *et al*, 1996), the receptor is primed and can subsequently mediate the lysis and killing of iC3b coated erythrocytes and tumor cells (Vetvicka *et al*, 1996). Thus, the CR3 mediated cytotoxic activity of PMN requires the direct recognition by CR3 of a molecular pattern found only on non-self cells. Several invertebrate proteins have also been postulated to be pattern recognition receptors which may participate in hemocyte activation (see Chapter 3), including a crayfish protein with integrin-like characteristics (Duvic and Söderhall, 1992; Söderhall, 1992).

Scavenger receptors (SRs) have been suggested to be attractive candidates for another class of non-self pattern recognition receptors (Freeman *et al*, 1990; Abrams

et al, 1992; Janeway, 1992; Hultmark, 1993; Krieger and Herz, 1994; Hoffmann, 1995; Pearson *et al*, 1995). This suggestion has been based on several findings. First, SRs receptors exhibit broad ligand binding specificity (Table 1), a characteristic feature of pattern recognition receptors. Second, some SRs can bind both microbial surface molecules and intact bacteria. Third, SRs are expressed by macrophages, hemocytes, and other immunologically relevant cells. Fourth, SR activity is evolutionarily ancient (Abrams *et al*, 1992). In addition to their pattern recognition receptor-like properties, some SRs exhibit adhesion molecule-like activities.

Until recently, only two SRs were known, both products of the same gene. In the last three years several additional SR genes have been identified, establishing four independent SR classes (Figure 1, see Acton *et al*, 1994, for definition of classes). Scavenger receptors have historically been defined by their ability to bind modified low density lipoproteins (LDLs), such as oxidized LDL (OxLDL) and acetylated LDL (AcLDL), and they were initially studied primarily for their potential role in atherogenesis, during which lipoprotein-derived cholesterol accumulates in the cells of the arterial wall (Krieger and Herz, 1994). In later chapters, I will discuss my experimental work on the class A and class C scavenger receptors. This chapter, which is an updated and expanded version of a review recently published in *Current Opinion in Immunology* (Pearson, 1996), will review recent developments in the SR field, in particular as they relate to the potential roles of SRs in host defense. The original publication is included in this thesis as Appendix A. A significant amount of the discussion regarding the *Drosophila* class C SR which was included in the originally published review has now been moved to Chapter 3 of this thesis, where it more appropriately belongs. Finally, a note of caution is appropriate here: many findings have "raised the possibility" that SRs may participate, either directly or indirectly, in one or another of a variety of different host defense related processes. Almost all of these possibilities have been based upon 1)*in vitro* observations that a particular molecule or class of molecules can bind to SRs, 2) *in vitro* observations of the effects of one or another treatment (e.g. cytokine, LPS) on SR expression, or 3) observations of the *in vivo* patterns of receptor expression. Until very recently, there has been very little genetic or pharmacologic evidence which would argue for or against any of the suggested SR functions. Until such evidence can be obtained, most of the functions which have been suggested for SRs, and which will be described below, will remain simply suggestions. However, these suggestions provide a valuable source of hypotheses which can be tested as genetic models become available for study.

Scavenger Receptor Classes

The two class A SRs, SR-AI and SR-AII, were the first macrophage SRs to be identified (Kodama *et al*, 1990; Rohrer *et al*, 1990) (see Krieger and Herz, 1994, for a recent review). These receptors comprise six domains, including extracellular α-helical coiled-coil and collagenous regions which oligomerize to form a mature trimeric glycoprotein with a molecular weight of 220 - 240 kD (Figure 1). SR-AI has an

additional C-terminal extracellular scavenger-receptor-cysteine-rich (SRCR) domain, which has been found in more than one dozen other proteins to date (Freeman *et al*, 1990; Resnick *et al*, 1994; Resnick, 1996; Appendix C). In spite of this structural difference, SR-AI and SR-AII exhibit nearly identical binding properties and can specifically bind a broad array of polyanionic ligands with high affinity. Studies with SR-AI have shown that these ligands include surface constituents of both Gram-positive and Gram-negative bacteria, as well as intact bacteria themselves (Table 1) (Krieger and Herz, 1994; Hampton *et al*, 1991; Dunne *et al*, 1994).

SR-AI and/or SR-AII are expressed primarily by monocytes and by peritoneal and most tissue macrophages (Table 2). Some dendritic cells and microglia, which are ontogenetically related to macrophages (Theele and Streit, 1993; Peters *et al*, 1996), certain specialized endothelial cells (EC), and smooth muscle cells in atherosclerotic lesions also appear to express the protein(s). A variety of different stimuli have been found to modulate SR-A/II levels *in vitro* (Table 2), and substantial progress is being made in defining the regulatory elements controlling the macrophage specific expression of the SR-AI/II gene (Horvai *et al*, 1995; Aftring and Freeman, 1995). In a major advance for the SR field, Kodama and colleagues have recently succeeded in disrupting the murine SR-AI/II gene by homologous recombination, and have thereby generated SR-AI/II deficient mice (H Suzuki, Y Kurihara, T Kodama, personal communication).

A third SR-A, termed MARCO, has recently been identified and shown to bind both AcLDL and bacteria (Elomaa *et al*, 1995). MARCO expression has been detected only in peritoneal macrophages and in a limited subset of tissue macrophages residing in the spleen and lymph nodes. All of these macrophages also express SR-AI/II. The C-terminal portion of MARCO is homologous to the collagenous and SRCR domains of SR-AI, although the MARCO collagenous domain is much longer than that of SR-AI/II. MARCO does not share any other domains with SR-AI/II. The primary ligand binding region of all three SR-As is located near the C-terminus. In SR-AI/II, and perhaps also in MARCO, the ligand binding region appears to be a cluster of basic residues located at the C-terminal end of the collagenous domain (Acton *et al*, 1993; Doi *et al*, 1993; Krieger and Herz, 1994; Elomaa *et al*, 1995; Tanaka *et al*, 1996).

The search for additional SRs led to the identification of two class B SRs, CD36 (Endemann *et al*, 1993) and SR-BI (Acton *et al*, 1994). These receptors, together with the lysosomal protein Limp II (Vega *et al*, 1991), the *Drosophila* emp (Hart and Wilcox, 1993) and croquemort (Franc *et al*, 1996) proteins, and a putative *C. elegans* protein (Genbank accession number 728534), define the CD36 family (Greenwalt *et al*, 1992). At the moment, more is known about the biology of the CD36 family proteins than about that of the proteins belonging to any of the other SR classes. These 74 - 88 kD proteins have a single conserved extracytoplasmic domain which shows 25% -- 30% homology between any two family members and is composed of an amino terminal region containing most of the potential N-glycosylation sites, and a carboxy terminal region rich in conserved proline, glycine, and cysteine residues (Figure 1) (Greenwalt *et al*, 1992). Non-conserved putative transmembrane and cytoplasmic domains flank

the extracellular domain. Whether both or only one (C-terminal) of the hydrophobic regions act as transmembrane domains is currently unclear and is a matter of some contention (Vega *et al*, 1991; Pearce *et al*, 1994; Tao *et al*, 1996). CD36 appears to be palmitoylated on two cysteines located near the N-terminus and two located near the C-terminus of the protein (Tao *et al*, 1996). While this would seem to indicate that both termini are cytoplasmic, it has been reported that some CD36 palmitoylation may be extracellular (Jochen and Hays, 1993). In addition, it has been reported that C-terminal deletion mutants expressed in transfected Bowes melanoma cells are secreted into the culture medium (Pearce *et al*, 1994). Interestingly, croquemort lacks a C-terminal hydrophobic region, yet is tightly membrane bound (Franc *et al*, 1996). If it turns out that the N-terminal hydrophobic domain in this family of proteins is not a transmembrane domain, the tight membrane association of croquemort suggests that at least some of the CD36 family proteins may use additional mechanisms for membrane association (Vega *et al*, 1991; Greenwalt *et al*, 1992; Jochen and Hays, 1993).

CD36 and SR-BI bind a wide variety of ligands, at least some of which are polyanionic (Table 1). Interestingly, the class B receptors (and other CD36 family members?) may primarily be lipid binding proteins expressed by tissues and cells involved in host defense and/or lipid metabolism and lipid-dependent processes (e.g. coagulation) (Greenwalt *et al*, 1995; Nicholson *et al*, 1995; Acton *et al*, 1996; Ibrahimi *et al*, 1996; Huh *et al*, 1996; Landschultz *et al*, 1996; Wang *et al*, 1996). These cells include monocytes and monocyte derived macrophages (but apparently not most tissue macrophages), B lymphocytes, capillary endothelial cells, platelets, and adipocytes (Table 2). Similarly, the croquemort gene is expressed in hemocytes and the fat body (Franc *et al*, 1996). In at least some of these cells, CD36 appears to be associated with caveolae (Lisanti *et al*, 1994), which have been implicated in both lipid transport and signal transduction (Parton, 1996). Initial studies of the biosynthesis, processing and intracellular transport of CD36 in macrophage cell lines have recently been reported (Alessio *et al*, 1996), as have studies of the transcriptional regulation of CD36 gene expression (Armesilla and Vega, 1994; König *et al*, 1995).

CD36 and SR-BI have recently been identified as receptors for the anionic phospholipids phosphatidylserine (PS) and phosphatidylinositol (Rigotti *et al*, 1995). Although there are likely to be additional PS receptors (see below), these are currently the only molecularly identified cell surface proteins which can bind PS and PI. Thus, CD36 family proteins may at least partially account for some old results which demonstrated that both mammalian macrophages and insect hemocytes can internalize anionic phospholipid vesicles (Ratner *et al*, 1986). PS is not normally expressed on the outer leaflet of cell membranes, but is externally exposed on activated platelets, damaged tissues, and apoptotic cells (discussed in more detail below). Thus, anionic phospholipids may represent one type of pattern not associated with normal host cells. Indeed, it has been previously suggested that the recognition of anionic phospholipids may be one primitive mechanism by which phagocytes distinguish self from non-self and damaged-self (Ratner *et al*, 1986). Initial reports that SR-AI/II could bind PS vesicles (Nishikawa *et al*, 1990) have since been shown to be

incorrect (Lee *et al*, 1992). The PS binding observed by Nishikawa and coworkers (1990) may have actually been due to one or more of the class B SRs, and/or to other PS receptors expressed on macrophages.

After the striking finding that *Drosophila* embryonic hemocytes express SR activity (Abrams *et al*, 1992), the class C SR gene, *dSR-CI*, was cloned from the hemocyte-like S2 cell line (Pearson *et al*, 1995). The cloning and characterization of this gene are described in more detail in Chapter 3; here I will briefly review the salient features of this receptor. The extracellular portion of the 170 kD dSR-CI protein contains two N-terminal complement control protein (CCP) domains. In mammals, CCP domains mediate the binding of complement receptors and complement regulatory proteins to the central component of the complement cascade, C3 (Reid and Day, 1989). The extracellular region of dSR-CI also contains MAM and somatomedin B domains, and a mucin-like domain which is heavily O-glycosylated. Which of the extracellular domains of dSR-CI mediate ligand binding is currently unknown. It is striking, however, that even though dSR-CI has absolutely no homology to SR-AI/II, it is nonetheless very similar to SR-AI/II in it's ability to bind to a broad array of polyanionic ligands with high affinity (Table 1). Interestingly, dSR-CI also binds the uncharged microbial ß-glucan laminarin, which is a potent inducer of the *Drosophila* immune response (Hultmark, 1993). As discussed in more detail in Chapter 3, these findings suggest that dSR-CI might be able to mediate the direct recognition of microbial pathogens by hemocytes. Indeed, dSR-CI is expressed in the hemocytes of the Drosophila embryo (Pearson *et al*, 1995). Additional *Drosophila* SRs probably exist (see Chapter 3); these could potentially include both SR-A and SR-B homologues.

There are likely to be additional, as yet unidentified, SRs in mammals as well. For example, macrosialin/CD68 has recently been identified as an OxLDL-binding protein present in membrane preparations from elicited peritoneal macrophages and the murine macrophage cell line, RAW 264.7 (Ramprasad *et al*, 1995). Although only membrane blots have so far been used to study the ligand binding specificity of macrosialin/CD68, it appears that this protein may, like SR-AI/II and dSR-CI, have broad polyanionic ligand binding properties. Interestingly, PS vesicles may also be ligands (Ottnad *et al*, 1995; Sambrano and Steinberg, 1995; Ramprasad *et al*, 1995). Macrosialin/CD68 has two extracytoplasmic domains: a mucin-like domain and a domain with homology to lysosomal membrane proteins. Indeed, the protein is expressed predominantly in endosomes, and to a lesser extent in lysosomes; only small amounts are expressed on the cell surface (Holness *et al*, 1993; Ramprasad *et al*, 1995). Thus, the potential roles of macrosialin/CD68 as a SR and as a host defense protein remain uncertain. It is interesting to note, however, that there may be a cell surface isoform which is upregulated in response to inflammatory stimuli (Holness *et al*, 1993). Resolution of these issues is important, as there are clearly mammalian macrophage SR activities distinguishable from both the SR-As and SR-Bs (see, for example Arai *et al*, 1989; Nishikawa *et al*, 1990; Schnitzer and Bravo, 1993; Kuzmenko *et al*, 1994; Nozaki *et al*, 1995; Sambrano and Steinberg, 1995; Lougheed and Steinbrecher, 1996; de Rijke and van Berkel, 1994).

Scavenger Receptor Functions in Innate Immunity

Lipopolysaccharide (LPS) from Gram-negative bacteria is a potent inducer of the immune response in both vertebrates and invertebrates (Hultmark, 1993; Ulevitch and Tobias, 1994; Wright, 1995; Hoffman, 1995). In mammals, serum LPS binding protein (LBP) and the "LPS receptor," CD14, play a critical role in mediating the cellular responses to LPS (Ulevitch and Tobias, 1994; Wright, 1995; Haziot *et al*, 1996). LBP facilitates the formation of LPS-CD14 complexes by transferring LPS from insoluble aggregates (and perhaps also from intact bacteria (Katz *et al*, 1996; Jack *et al*, 1995)) to either of the two CD14 isoforms. The GPI-anchored isoform (mCD14), is expressed primarily by cells of the monocytic lineage, while a soluble isoform (sCD14) is present in serum and can mediate the responses of non-mCD14 expressing cells (particularly endothelial cells) to LPS. Although CD14 enhances the ability of LPS to induce cellular responses by 100 to 10,000 fold, it does not transduce a transmembrane signal itself. Instead, CD14 appears to solubilize LPS and act as a shuttle (Wurfel *et al*, 1995; Wright, 1995) which transports individual LPS molecules through aqueous environments and on the cell surface, most likely to an associated signal transducing receptor (Ulevitch and Tobias, 1994; Wright, 1995; Delude *et al*, 1995; Haziot *et al*, 1996). By their joint action, LBP and CD14 can concentrate LPS molecules at the cell surface, and may thereby amplify the ability of LPS to induce cellular responses (Wright, 1995). This may in fact be a general function of CD14 in host defense, since it appears to act as a pattern recognition receptor which enhances cellular responses to a wide range of microbial surface constituents (Pugin *et al*, 1994; Wright, 1995).

The identification of the mammalian CD14 associated signal transducer(s) is currently the subject of intense research. Recent studies suggest that there are probably several distinct proteins which are important for transducing signals in response to different CD14 bound microbial surface molecules (Pugin *et al*, 1994; Savedra *et al*, 1996). There may even be several different proteins which mediate signal transduction events in response to CD14 bound LPS, since multiple, somewhat independent signal transduction pathways appear to be activated in cells responding to LPS (Delude *et al*, 1994; Arditi *et al*, 1995; Haziot *et al*, 1996). Theoretically, LPS responsive transducer proteins might recognize CD14-bound LPS (Ulevitch and Tobias, 1994), they might recognize LPS directly transferred from CD14 (Wright, 1995; Gegner *et al*, 1995), or they might recognize LPS indirectly transferred from CD14 via a membrane bound protein functionally analogous to LBP. While it is known that CR3 recognizes LPS (Wright *et al*, 1989), and that LPS-mCD14 complexes associate with CR3 (Zarewych *et al*, 1996), this integrin may not be a CD14 associated signal transducing receptor since CD18 deficient and CD18 positive monocytes respond identically to LPS (Wright *et al*, 1990).

The broad ligand binding properties of the SRs suggest that one or more of them might participate in infection induced signal transduction. However, two findings indicate that the mammalian SR-As are probably not critically important for LPS induced, CD14-dependent signal transduction: 1) AcLDL blocks most Lipid IVa (a

bioactive LPS precursor) binding to RAW 264.7 cells, but has no effect on their ability to release TNF -α in response to Lipid-IVa induction (Hampton *et al*, 1991), and 2) CD14 transfected CHO cells respond strongly to LPS (Golenbock *et al*, 1993) yet have only very low levels of endogenous SR activity (Freeman *et al*, 1991).

Whether the SR-B class scavenger receptors can participate in LPS induced signal transduction has not yet been examined. In fact, it is not even known if these proteins can bind LPS. This question should be examined, particularly since it is known that CD36 can bind to both fatty acids and a variety of polyanions and that both CD36 and SR-BI can bind to anionic phospholipids (Table 1). Keeping in mind that the LPS binding capabilities of CD36 and SR-BI are unknown, several findings provide circumstantial evidence that one or more of the CD36 family proteins might be able to participate in CD14 dependent LPS induced signal transduction. First, CD36 family proteins are expressed by most LPS responsive cells (compare Ulevitch to Table II), and are colocalized with CD14 to caveolae (Lisanti *et al*, 1994). Second, the binding of certain CD36 antibodies can initiate signal transduction events in monocytes (Greenwalt *et al*, 1992). Interestingly, CD36 co-immunoprecipitates with several src family tyrosine kinases, including the lyn kinase (Huang *et al*, 1991), which also co-immunoprecipitates with mCD14 and participates in LPS-induced and CD14-dependent signal transduction (Stefanova *et al*, 1993; Arditi *et al*, 1995). However, it is not clear whether the co-immunoprecipitation of lyn with CD36 and CD14 is functionally significant, or simply reflects the colocalization of all three proteins to caveolae (Parolini *et al*, 1996). Third, it has recently been determined that >99% of serum LBP is physically associated with HDL particles, and that purified apo-AI containing lipoproteins can recapitulate the entire process by which whole plasma first enables and then neutralizes the ability of LPS to stimulate cells (Wurfel *et al*, 1994). This process apparently involves the sequential LBP catalyzed transfer of LPS from insoluble aggregates (including bacteria?) to CD14, and then from CD14 to the HDL particle (Wurfel *et al*, 1995). Given the ability of CD36 and SR-BI to bind HDL, it is conceivable that some CD36 family members might localize LBP on the surface of a cell, where it could then fulfill its role in the sequential transfer of LPS from micelles or bacteria to CD14 and HDL. Interestingly, such a role for the CD36 type proteins would not actually require them to bind to LPS. It would be interesting to determine what effects, if any, blocking anti-CD36 or anti-SR-BI antibodies have on LPS induced signal transduction in monocytes, endothelial cells, and other cell types.

In addition to CD14 dependent signal transduction processes, which are sensitive to very low levels of LPS (1 ng or less), there are also CD14 independent signal transduction processes which act at higher LPS concentrations (10 to 100 ng or more). These processes are mediated by low affinity LPS receptors such as CR4 (CD11c/CD18) and may enable macrophage detection of infections in tissues that lack serum proteins and are exposed to high local concentrations of LPS (Ingalls and Golenbock, 1995). Recent data suggests that even monocytes, which express CD14 and are exposed to serum, may predominantly use CD14 independent signal transduction mechanisms at high LPS concentrations (Cohen *et al*, 1995).

Of the various known SRs, only SR-AI/II has thus far been examined to see if it can enable CD14 independent cellular activation. The results of these studies suggest that SR-AI/II probably does not participate in such activation, since CHO cells transfected with rabbit SR-AII do not synthesize arachidonic acid in response to 100 ng/ml LPS or Lipid IVa (Golenbock *et al*, 1993). However, it is important to note that measuring only one indicator of cell activation in a non-macrophage cell line which is exposed to levels of LPS which are below the K_d of this ligand for SR-AI/II, as these researchers did, does not definitively test the involvement of SR-AI/II in LPS induced signal transduction. Since CD14 deficient macrophages can still respond to high levels of LPS (Haziot *et al*, 1996), the involvement of SR-AI/II in LPS induced signal transduction should now be tested in SR-AI/II -CD14 double knockout mice. Such an experiment should finally provide definitive evidence for or against the participation of SR-AI/II in LPS inducted signal transduction events.

Should it be found that CD36 type proteins do bind LPS, it would be interesting to determine if they could participate in CD14 independent signal transduction. This might be done by isolating monocytes and endothelial cells from CD14 deficient mice and testing the effects which blocking antibodies directed against these proteins have on LPS induced cellular activation. As for macrosialin/CD68, there are two findings relevant to its potential participation in CD14 independent LPS induced signal transduction. On the one hand, it has been reported that RAW264.7 membrane fractions contain a 95 kD serum independent lipid IVa binding protein which is strikingly similar to macrosialin/CD68 (B; Ramprasad *et al*, 1995). On the other hand, it has also been reported that lipid A is not able to compete for OxLDL binding to this protein isolated from Kupffer cells (de Rijke and van Berkel, 1994). What is clearly required now is to examine LPS binding to transfected cells expressing macrosialin/CD68.

Rather than regulating cellular activation, SRs may primarily be effector molecules which participate in the phagocytic clearance of microbes and the endocytic clearance of microbial surface constituents, from the body (Hampton *et al*, 1991; Abrams *et al*, 1992; Holness *et al*, 1993; Dunne *et al*, 1994; Bell *et al*, 1994; Krieger and Herz, 1994; Pearson *et al*, 1995). In addition to their broad binding specificities, and their expression in macrophages and liver EC, several recent findings support the involvement of at least some SRs in these processes. First, in clearance studies, the co-injection of SR ligands (poly (I), AcLDL) significantly reduces hepatic uptake of Lipid IVa in mice (Hampton *et al*, 1991). (Similar experiments with alveolar macrophages have demonstrated that SRs may participate in the clearance of anionic environmental particulates from the lung (Resnick *et al*, 1993; Kobzik, 1995)). Second, the addition of AcLDL to alveolar macrophages increases their level of TNF-α production in response to LPS. Since this is evidently not due to priming of the macrophages, the effect of AcLDL may have been to block SR mediated clearance of LPS, thus increasing its availability for CD14 dependent cellular activation (Mendez *et al*, 1995). These results indicate that SRs may play an important protective role in the host by preventing detrimental hyperactivation of the innate immune system by microbial molecules. Third, in support of such a role for SR-AI/II, reports from several

groups now indicate that both peritoneal macrophages (H Suzuki, Y Kurihara, T Kodama, personal communication) and liver EC (van Amersfoort *et al*, 1996) obtained from SR-AI/II knockout mice exhibit a 50% reduction in LPS binding and uptake. Furthermore, these mice appear to be more susceptible to endotoxic shock. Recalling the studies, cited above, which indicated that SR-AI/II probably does not directly participate in LPS induced cellular activation, these more recent studies suggest that the major protective function of SR-AI/II in host defense may indeed be its participation in the clearance of LPS, and perhaps bacteria, from the body. It will be interesting to see if the SR-AI/II knockout mice exhibit an altered susceptibility to infection relative to normal mice. Fourth, LPS can in some circumstances induce the upregulation of SR-AI/II protein expression, presumably enabling efficient endocytic and phagocytic clearance of bacteria (Bell *et al*, 1994). However, this result must be balanced against the finding that LPS can also down-regulate SR-AI/II message levels, and also presumably protein levels (Dufva *et al*, 1995; Hsu *et al*, 1996). The meaning of these conflicting results is not clear. Perhaps they simply demonstrate the long known fact that microenvironmental cues are critically important in regulating macrophage phenotype (Gordon *et al*, 1988; Rutherford *et al*, 1993). Fifth, the association of LPS with HDL suggests that CD36 family proteins, expressed in the liver and elsewhere, may facilitate LPS clearance (Levine *et al*, 1993). Indeed, since only a small fraction (<1%) of the total serum HDL contains LBP under normal circumstances (Wurfel *et al*, 1994; Park and Wright, 1996), the hypothesis presented earlier, that CD36 type proteins participate in LPS induced cellular activation by binding to HDL-LBP, seems somewhat unlikely. In contrast, the hypothesis that CD36 type proteins might play a quantitatively important role in LPS clearance seems more likely since the serum concentration of LBP, and presumably also of LBP-HDL complexes, dramatically increases in response to infection (Gegner *et al*, 1995). As with other hypotheses, this hypothesis could be tested by determining the effects of blocking antibodies directed against the CD36 type proteins on LPS clearance. Although not discussed here, the properties of dSR-CI and croquemort suggest that these receptors could play effector functions in *Drosophila* hemocytes which are analogous to the potential effector functions of the mammalian SRs.

It has been suggested that, as phagocytic and endocytic receptors expressed on macrophages and dendritic cells, some mammalian SRs may also participate in the activation of the adaptive immune system by mediating antigen uptake and/or processing for presentation to B and T cells (Holness *et al*, 1994; Geng and Hansson, 1995). In this regard, it has recently been shown that maleylation of proteins, which converts them to SR ligands, enhances their ability to elicit antibody and T cell responses in the absence of adjuvant *in vivo*, and enhances their presentation to T cells by macrophages *in vitro* (Abraham *et al*, 1995).

One final role for SRs during the immune response has been previously suggested: participation as adhesion proteins in the recruitment of peripheral blood monocytes (PBM) to infected tissues (Holness *et al*, 1994; Hughes *et al*, 1995; Huh *et al*, 1995), a process which requires PBM extravasation through vascular endothelial cell layers (Springer, 1994). Several findings have led to these suggestions: 1) the

monocyte/macrophage growth and differentiation factor M-CSF, which induces monocyte migration (Wang *et al*, 1988) and is locally released by macrophages and TNF α activated EC in response to infection (Hennet *et al*, 1992; Peng *et al*, 1995), also upregulates both SR-AI/II expression by and SR-AI/II mediated adhesion of macrophages (see below) (de Villiers *et al*, 1994a). 2) M-CSF treatment of monocytes upregulates total CD36 levels (Yessner *et al*, 1996), and to a lesser extent surface CD36 levels (Huh *et al*, 1995). It has been suggested that M-CSF might be priming the monocytes for subsequent mobilization of CD36 to the cell surface in response to secondary stimuli (Yessner *et al*, 1996). 3) Interestingly, expression of CD36 on the surface of PBM is upregulated upon their adherence to TNF-α activated EC (Huh *et al* 1995). Thus, PBM adhesion to activated EC may generate one type of secondary stimulus which acts on M-CSF primed monocytes to alter surface levels of CD36 protein. It must be stressed that all of these findings are only consistent with, and far from prove, a role for SRs in the recruitment of PBM to infected tissues.

Scavenger Receptor Functions in Immune Related Processes

Establishment of macrophage, lymphocyte and hemocyte populations

In order to effectively participate as sentinel and effector cells in first line host defense, mammalian monocytes must establish residence throughout the body as permanently differentiated tissue macrophages (Gordon *et al*, 1988; Rutherford *et al*, 1993). It has been suggested that SR-AI/II might be homing and/or retention molecules involved in this process (Hughes *et al*, 1995). Three recent reports support this hypothesis. First, and most importantly, it has been found that the monoclonal antibody 2F8, which recognizes both SR-AI and SR-AII, can inhibit the cation-independent (non-integrin, non-selectin) adhesion of macrophages to tissue culture plastic (Fraser *et al*, 1993) and to frozen splenic, thymic, and lymph node tissue sections (Hughes *et al*, 1995). SR-AI/II mediated adhesion to plastic requires a serum ligand, while adhesion to tissues involves either an endogenously expressed ligand and/or homophilic binding interactions between macrophages and resident SR-AI/II-expressing cells in tissues. Second, as mentioned earlier, M-CSF upregulates both SR-AI/II expression by macrophages and SR-AI/II mediated macrophage adhesion (de Villiers *et al*, 1994a). Indeed, M-CSF may be critical for SR-AI/II expression since such expression is dramatically reduced in M-CSF deficient mice (Gordon *et al*, 1995) Third, CD6, which is composed entirely of SRCR domains, has recently been shown to bind to an Ig domain containing ligand called ALCAM (Bowen *et al*, 1996), thus demonstrating that SRCR domains can mediate binding interactions. It has also recently been suggested that SR-AI/II may participate in the adhesive events regulating lymphocyte recirculation. However, it must be noted that this suggestion is based solely on the observation that SR-AI/II is expressed by lymph node high endothelial venules (Geng and Hansson, 1995). What is now necessary is for these hypotheses to be tested using the SR-AI/II knockout mice.

It is not known whether *Drosophila* hemocytes establish permanent residence in specific tissues. However, they do migrate throughout the body of the developing and adult fly, and in some cases they appear to follow specific guidance cues (Tepass *et al*, 1994; Zhou *et al*, 1995). Since dSR-CI is expressed in hemocytes prior to the onset of migration and contains several potentially adhesive domains, it might be able to participate in the hemocyte migration process (Pearson *et al*, 1995).

Response to wounding

Wounding induces activation of the innate immune system in both mammals and invertebrates (Burnet, 1968; Rizki and Rizki, 1984; Lackie, 1988; Gordon *et al*, 1988; Hultmark, 1993; Baumann and Gauldie, 1994; Mansfield and Suchard, 1994; Matzinger, 1994; Ibrahim *et al*, 1995; Hoffmann, 1995; Harris and Gelfand, 1995;). Dendritic cells, monocytes, macrophages and hemocytes are the central mediators of this innate immune response, playing critical roles in guarding against infection, in clearing wound-associated debris, and in wound healing. It has been suggested that wound recognition by these cells may be somewhat analogous to the recognition of infectious microorganisms (Burnet, 1968; Lackie, 1988; Matzinger, 1994; Mansfield and Suchard, 1994; Hoffman, 1995). Perhaps this process, at least in part, represents another manifestation of pattern recognition. Indeed, as described below, wounding exposes molecular structures and patterns on damaged cells and tissues, and on activated platelets recruited to the wound site, which are not found on normal cells and tissues.

SRs may participate in wound recognition, both as adhesion molecules and also as pattern recognition receptors. For example, it has been suggested that monocyte adhesion to the wound site may be facilitated in part by CD36 mediated binding to thrombospondin (TSP) secreted by activated platelets already attached to the wounded tissue (Silverstein *et al*, 1989). In addition, both collagen and PS are exposed on wounded tissues and cells themselves (Lackie *et al*, 1988; Davie *et al*, 1991; Machin, 1992), and recognition of these damaged-self structures by CD36 may also contribute to direct binding of monocytes to wound surfaces. Finally, since activated platelets also express PS externally (Davie *et al*, 1991; Machin, 1992), CD36 mediated recognition of activated platelets via PS binding might further contribute to monocyte adhesion. After mediating wound detection by one of these mechanisms, CD36 could potentially act as signal transducing receptors which initiate cellular activation, thereby contributing to an inflammatory immune response and enhanced clotting.

SRs could also directly participate in the clotting process. Since both platelets and insect hemocytes express CD36 family members (Greenwalt *et al*, 1992; Franc *et al*, 1996), mechanisms similar to those just described for monocytes may also contribute to the initial recognition of wounds by platelets and hemocytes. In addition, CD36 family members might also participate in the growth and stabilization of platelet and hemocyte clots (Greenwalt *et al*, 1992; but see Saelman *et al*, 1994). This is

suggested by the knowledge that activation of platelets and hemocytes not only induces the secretion of clotting factors, such as TSP in mammals and lipoproteins in invertebrates (Hall *et al*, 1995), but activation also induces changes in the surface characteristics of these cells (Rizki and Rizki, 1984; Lackie, 1988; Davie *et al*, 1991; Machin, 1992; Satta *et al*, 1994). In particular, in addition to exposing PS, activated platelets translocate lysosomal proteins (including LIMPII? Greenwalt *et al*, 1992) and intracellular CD36 stores (Berger *et al*, 1993) to the plasma membrane.

It must be noted, however, that while CD36 can bind TSP, collagen, and PS, these binding events may not be physiologically significant mechanisms for either monocyte or platelet adhesion to wound sites. Indeed, the *in vivo* relevance of CD36 mediated cellular adhesion to collagen and TSP has been repeatedly questioned and is currently a matter of some debate (see Greenwalt *et al*, 1992; McKeown *et al*, 1994). What is clear from several studies (Greenwalt *et al*, 1992; McKeown *et al*, 1994; Saelman *et al*, 1994) is that CD36 is not necessary for adhesion to these proteins. This may be due in part to the existence of several additional monocyte and platelet membrane proteins which can bind to TSP and collagen, which may therefore participate in wound recognition and clotting (McKeown *et al*, 1994). Although CD36 does not appear to be essential for monocyte and platelet binding to TSP and collagen binding, by binding these ligands in cooperation with other receptors, it may enhance cellular adhesion (Greenwalt *et al*, 1992; Saelman *et al*, 1994; Diaz-Ricart *et al*, 1993). It has been found, for example, that CD36 facilitates (but is not essential for) the earliest stages of platelet adhesion to collagen, but subsequently plays no significant role once adhesion has been established (Greenwalt *et al*, 1992; Diaz-Ricart *et al*, 1993).

Finally, it has been suggested that SR-AI/II may participate in the clearance of wound associated debris during the healing process, since SR-AI/II expression is dramatically upregulated in glial cells and recruited macrophages after optic nerve crush (Bell *et al*, 1994). Other SRs may also be involved in the clearance of damaged cells, since macrophage binding of a model damaged cell, the oxidized red blood cell (OxRBC), is completely inhibited by both PS and OxLDL (Sambrano and Steinberg, 1995). The ability of various SRs to bind and perhaps mediate the clearance of advanced glycosylation end-products (SR-AI/II - Araki *et al*, 1995), Type I and Type III procollagen propeptides (Melkko *et al*, 1994), ß-amyloid aggregates (Paresce *et al*, 1996; El Khoury et al, 1996; see also Christie *et al*, 1996), and shed photoreceptor rod outer segments (CD36 - Ryeom *et al*, 1996a) are all consistent with the idea that SRs may generally be involved in the removal of cellular and extracellular debris. The potential for cooperation between SRs and other receptors (e.g. the Receptor for Advanced Glycation End-products (RAGE, Yan et al, 1996) in these processes has not yet been examined.

Recognition and engulfment of apoptotic cells

The induction of apoptosis during an immune response is an important effector mechanism for host defense in higher eukaryotes from plants to animals (Savill *et al*, 1993; Greenberg *et al*, 1994; Shresta *et al*, 1995; Munn *et al*, 1995; Scott *et al*, 1996). In animals, apoptosis also plays an important role in the normal development and turnover of organs, tissues and cells, including those of the immune system (Savill *et al*, 1993; Surh and Sprent, 1994; Steller, 1995; Munn *et al*, 1996). The phagocytic clearance of apoptotic cells, and also of damaged and senescent cells, is a primary function of monocytes, macrophages and hemocytes (Gordon *et al*, 1988; Savill *et al*, 1993; Tepass *et al*, 1994; Sonnenfeld and Jacobs, 1995). These phagocytes may recognize engulf effete cells in part by using pattern recognition mechanisms similar to those used for the recognition and engulfment of infectious microorganisms (Burnet, 1968; Gordon *et al*, 1988; Ibrahim *et al*, 1995). Indeed, apoptotic cells, like damaged cells and tissues, express surface characteristics not usually found on normal self cells (Fadok *et al*, 1992a; Savill *et al*, 1993; Falasca *et al*, 1996). For example, PS is rapidly exposed on the surface of most, if not all, cells undergoing apoptotic cell death (Fadok *et al*, 1992a, 1992b; Martin *et al*, 1995; Verhoven *et al*, 1995).

Multiple receptors and mechanisms have been implicated in the process of apoptotic cell engulfment by macrophages (Savill *et al*, 1993; Flora and Gregory, 1994; Sambrano and Steinberg, 1995; Falasca *et al*, 1996; Ren *et al*, 1996). This is not surprising given the importance of clearing apoptotic cells from the body (Savill *et al*, 1993), and the considerable heterogeneity displayed by macrophages of different origins (defined by differing microenvironments and states of activation). The extent to which these various receptors can replace or compensate for each other (i.e. are functionally redundant) is currently unknown. In addition, with one exception (see below), it is not known whether any of these receptors exhibit either cooperativity or co-dependence during the apoptotic cell engulfment process.

It has repeatedly been suggested that SRs may participate in some of these mechanisms (Abrams *et al*, 1992; Krieger and Herz, 1994; Pearson *et al*, 1995; Hughes *et al*, 1995; Sambrano and Steinberg, 1995; Rigotti *et al*, 1995). Evidence is now accumulating which supports these suggestions, although the precise roles of the SRs, and sometimes their actual identities, have not yet been fully established.

Strong support for the direct involvement of SR-AI/II in apoptotic cell engulfment has been provided by two recent findings: 1) the anti-SR-AI/II monoclonal antibody 2F8 inhibits 50% of apoptotic thymocyte uptake by thymic macrophages and elicited peritoneal macrophages (Platt *et al*, 1995), and 2) thymic macrophages from SR-AI/II knockout mice exhibit a 50% reduction in apoptotic cell uptake (S Gordon, personal communication). The nature of the ligand on apoptotic cells which is recognized by SR-AI/II is not yet known, but can not be PS (Lee *et al*, 1992). Macrophages from SRAI/II deficient mice only exhibit a 50% reduction in apoptotic thymocyte engulfment, demonstrating that additional receptors must also be involved in this process. Since these receptors have not yet been identified, it is difficult to determine the relative

contribution that each makes to apoptotic thymocyte engulfment *in vivo* . Indeed, even for SR-AI/II, it is not yet known if the absence of this receptor has any quantitative or qualitative *in vivo* effects on T cell numbers, T cell development, or thymus function and morphology. It is possible, for example, that by upregulating the expression of other receptors, the thymus may be able to compensate for SR-AI/II deficiency. The identification and targetted disruption of these additional receptors, both alone and in combination with SR-AI/II, may contribute to the resolution of this question.

The results of Platt *et al* (1995) suggest that some of the additional receptors involved in apoptotic thymocyte engulfment may be non-class A SRs. Indeed, it has been found, in other contexts, that some of the SR-Bs can mediate the engulfment of apoptotic cells. For example, both PBM-derived macrophages and bone-marrow derived macrophages can use a combination of CD36, the vitronectin receptor (VnR, $a_v ß_3$ integrin), and thrombospondin to engulf apoptotic leukocytes (Savill *et al*, 1992; Fadok *et al*, 1992b). A direct role for CD36 in this VnR/TSP dependent apoptotic cell uptake mechanism was recently demonstrated by transfecting a CD36 cDNA into Bowes melanoma and COS cells, both of which constitutively express the VnR. The transfected cells, but not the untransfected cells, were able to phagocytose apoptotic cells. In the Bowes cells transfectants, this phagocytosis was inhibitable by blocking antibodies individually directed against CD36, TSP, or the VnR (Ren *et al*, 1995). Importantly (see below), VnR/TSP dependence has not been demonstrated for the engulfment of apoptotic cells by the COS cell CD36 transfectants.

As opposed to PBM and bone marrow derived macrophages, both resident and elicited peritoneal macrophages recognize and/or engulf apoptotic cells and damaged red blood cells by VnR independent mechanisms. At least some of these mechanisms clearly require the involvement of SRs, since OxLDL can partially or completely inhibit the binding and/or engulfment of these cells, depending on the nature of the target (Sambrano and Steinberg, 1995; Ottnad *et al*, 1995). The identity of the relevant SRs is currently unknown, but they may include SR-AI/II, CD36 and/or SR-BI, and macrosialin/CD68, all of which bind OxLDL.

Some of the VnR independent engulfment mechanisms used by elicited and resident peritoneal macrophages are based upon the recognition of PS exposed on the surface of the target cells (Fadok *et al*, 1992a, 1992b). The extent to which the VnR-independent/SR-mediated engulfment mechanisms overlap with the VnR-independent/PS-dependent engulfment mechanisms is currently unknown. Interestingly, OxLDL can partially block the PS dependent uptake of target cells, indicating that both SRs and non-SR PS receptors appear to participate in this process (Fadok *et al*, 1992a, Sambrano and Steinberg, 1995). The ability of macrosialin/CD68 to bind to PS vesicles suggests that it might be one such SR (Sambrano and Steinberg, 1995; but see Ramprasad *et al*, 1995).

Several findings suggest the intriguing possibility that the SR-Bs, and/or other CD36 family members, might also play a role as PS receptors in this process. First, CD36 is expressed by both elicited and resident peritoneal macrophages (Greenwalt

et al, 1992; Endemann *et al*, 1993; Ottnad *et al*, 1995). Second, PS vesicles are SR-B ligands and can cross-compete with OxLDL for receptor binding (Rigotti *et al*, 1995). Third, SR-BI transfected COS cells bind and engulf apoptotic mast cells (Fukasawa *et al*, 1996). Fourth, the same CD36 transfected Bowes cells, described above, which engulf apoptotic cells in a VnR/TSP dependent manner, can also bind and engulf photoreceptor rod outer segments (ROS) using a VnR/TSP independent mechanism (Ryeom *et al*, 1996a). It is now important to determine if the engulfment of apoptotic cells by CD36 and SR-BI transfected COS cells can be inhibited by PS vesicles. There is some reason to believe that it will be, since the engulfment of PS-expressing ROS by CD36-expressing retinal pigment epithelial cells can be inhibited both by an anti-CD36 antibody and by PS vesicles (Ryeom *et al*, 1996a, 1996b). Interestingly, this inhibition is only partial, suggesting the involvement of another receptor in ROS engulfment (Ryeom *et al*, 1996a, 1996b). In further support of this possibility, croquemort-transfected COS cells also recognize and engulf apoptotic thymocytes (Franc *et al*, 1996), and this engulfment is inhibitable by PS vesicles (J.-L. Dimarcq, N. Franc, A. Ezekowitz, and J. Hoffmann, personal communication).

The extent to which CD36 type proteins are physiologically important apoptotic cell receptors is not yet known. However, it appears that CD36 type proteins have the potential to participate in at least two different modes of apoptotic and senescent or damaged cell uptake. This is particularly so for CD36 itself, which can mediate engulfment by both VnR/TSP dependent and PS dependent mechanisms. Interestingly, these two mechanisms so far appear to be mutually exclusive (Fadok *et al*, 1992a; Fadok *et al*, 1993; Ren and Savill, 1995). Is there a way to make some sense out of these results? This question is particularly intriguing because COS cells are not normally phagocytic and because CD36 and SR-BI do not themselves appear to have substantial phagocytic, or even endocytic, capabilities (Greenwalt *et al*, 1995; Ottnad *et al*, 1995; Acton *et al*, 1996). Perhaps the primary role of the CD36 family proteins during apoptotic cell engulfment is not in the actual phagocytic process itself, but rather, is to act as proteins which somehow participate in the activation of macrophage (and COS cell) phagocytic mechanisms by recognizing PS on the surface of apoptotic cells.

In addition to the results already mentioned, several other findings are consistent with, but by no means prove, this hypothesis: 1) external PS appears to be a universal indication that a cell is undergoing apoptosis (Martin *et al*, 1995; Verhoven *et al*, 1995); 2) every macrophage population so far used in studies of apoptotic cell engulfment appears to recognize PS (Nishikawa *et al*, 1990; Fadok *et al*, 1992a, 1992b; Sambrano and Steinberg, 1995; Ottnad *et al*, 1995), as do insect hemocytes (Ratner *et al*, 1986); and 3) as mentioned earlier in regard to LPS induced macrophage activation, CD36 is a signal transducing receptor associated with lyn kinase (and other src family kinases) (Huang *et al*, 1991). This kinase participates in signal transduction events leading to the activation of macrophage and neutrophil phagocytosis in response to a wide range of stimuli (Greenberg, 1995).

Although this hypothesis does not require the involvement of CD36 type proteins in the actual phagocytic process, it does not eliminate the possibility that, after recognizing PS and participating in the activation of macrophage phagocytic mechanisms, they might also participate in phagocytosis. If CD36 type proteins do participate in phagocytosis, it may be important that they do so in cooperation with additional co-receptors. Indeed, this appears to be the case for the VnR/TSP dependent mechanism described above. The choice of co-receptors which might be used for phagocytosis would presumably depend on the particular phenotype of the macrophage, and, in addition to the VnR, may include SR-AI, macrosialin/CD68, other PS receptors (Fadok *et al*, 1992a; Sambrano and Steinberg, 1995, Ottnad *et al*, 1995), and any of several other receptors which have so far been identified solely on the basis of their apoptotic cell engulfment activity (Flora and Gregory, 1994; Falasca *et al*, 1996). In this regard, it is interesting to note the recent report that macrophage uptake of "post-apoptotic" PMN is mediated by a mechanism which is inhibitable by poly(I) and fucoidan, but not by the anti-SRAI/II antibody 2F8, and which also requires the participation of CR3 (Ren *et al*, 1996). It will be interesting to determine whether this polyanion inhibitable activity is a CD36 type protein.

Recent studies have delineated a 28 residue domain of CD36 which may be important for apoptotic cell engulfment (Puento-Navazo *et al*, 1996a), ROS engulfment (Ryeom *et al*, 1996a) and OxLDL binding (Puento-Navazo *et al*, 1996b). Interestingly, this domain is distinct from another region of the protein which has been implicated in TSP binding (Asch *et al*, 1993; Pearce *et al*, 1995). The implications of these findings for CD36 function as an apoptotic cell receptor are currently unclear.

Conclusion

A common characteristic of infectious microorganisms, damaged cells and tissues, and apoptotic and senescent cells is their surface expression of molecular structures and patterns not found on normal host tissues and cells. That the mechanisms used by vertebrate and invertebrate immune cells for the recognition of and response to these diverse targets may be related is an old concept (Burnet, 1968; Gordon *et al*, 1988; Hoffmann, 1995; Ibrahim *et al*, 1995). These mechanisms may include pattern recognition receptor mediated recognition of the target cells, as first proposed by Janeway for the recognition of microbial pathogens (Janeway, 1989, 1992).

Scavenger receptors have often been proposed to be pattern recognition receptors. This chapter has presented the rationale behind these suggestions and has reviewed the current state of knowledge regarding the involvement of the SRs in host defense related processes. While many suggestions have been made over the past several years with regard to the functions of the SRs in these processes, not until very recently has there been any *in vivo* evidence either supporting or refuting these hypotheses. This state of affairs is likely to change as strains of mice and flies are generated which are deficient for one or more of the SRs. Of the numerous potential

functions for SRs which have been proposed, the strongest evidence currently supports a role for the participation SR-AI/II in LPS clearance, and for the participation of both SR-AI/II and CD36 type proteins in apoptotic cell engulfment.

Many potential functions have been proposed, here and by others, for the SR-Bs. Since macrophages must be capable of distinguishing different ligands from each other and responding appropriately, it would seem that for the CD36 family members to participate in even only some of the systems which have been suggested would require either that different family members participate in different functions, or that any given member be coupled to multiple co-receptors which would allow various targets to be distinguished from one another. This may be best illustrated by the fact that, while it is clear that CD36 and SR-BI can participate in the phagocytic engulfment of apoptotic cells, and may do so *in vivo* , there is also substantial evidence indicating that CD36 and SR-BI may participate in normal physiological lipid metabolism (see above). Perhaps a basic function of the CD36 family members is to bind anionic lipids and then deliver them to other proteins which initiate or mediate the various downstream events (lipid/cholesterol transport, adhesion, activation, phagocytosis) induced by these lipids.

Although strains of mice carrying disruptions of these genes have not yet been generated, there are individuals who appear to lack all platelet, monocyte and macrophage CD36 expression. The frequency with which such individuals are found is fairly high (approximately 0.1% in Japan, for example), and as far as anyone knows, these individuals are perfectly healthy (Nozaki *et al*, 1995). This fact is too often ignored when considering the possible functions of CD36. While it seems unlikely that CD36 is a completely vestigial gene, it is clear that, at least in humans, CD36 does not have any functions which are essential for normal health and development.

This raises the more general issue of functional redundancy among the SRs. It is striking that there are multiple SRs which can all recognize some of the same groups of polyanionic ligands. In addition, SRs are not the only cell surface proteins which can bind to some of these ligands (e. g. CD14 and CR3 bind LPS, the VnR and maybe CR3 bind apoptotic cells, GPIa/IIa binds TSP, GPIIb/IIIa binds TSP and collagen, etc.). It is not clear why there should be so many different proteins which have broad and overlapping binding specificities (see also Epstein *et al*, 1996, for a discussion of the collectins). While different temporal and spatial patterns of expression may account for some of this apparent redundancy, many of these proteins appear to have overlapping expression patterns. It is important for host defense, the maintenance of homeostasis, and normal development, that infectious microorganisms and cellular and extracellular debris be eliminated from the body. Perhaps by expressing a variety of different pattern recognition receptors, each having broad and overlapping, but also somewhat distinct, specificities and expression patterns, innate immune cells may be able to effectively distinguish a wide range of non-self and altered self structures from normal self structures anywhere in the body.

Targeted disruption of SR genes should greatly contribute to the elucidation of the roles that SRs play in host defense. SR-AI/II knockout mice have already been generated (H Suzuki, Y Kurihara, T Kodama, personal communication), and SR-BI and CD36 knockouts are under construction. Given the degree of redundancy which may be built into SR and other pattern recognition receptor systems, it may be necessary to construct strains deficient in multiple different SRs in order to see any major effects due to the absence of SR expression. In addition, there are now numerous examples in which multiple receptors either additively or cooperatively participate in the phagocytic engulfment of various targets (Blackwell *et al*, 1985; Gordon *et al*, 1988; Schlessinger *et al*, 1991; Savill *et al*, 1993; Mosser, 1994; Gegner *et al*, 1995; Greenberg, 1995; Petty and Todd,1996;). It seems possible that SRs might also participate in additive or cooperative interactions with other receptors during various immune responses. Therefore, in order to really understand the roles of SRs during immune responses, it might be necessary (and also interesting) to create double mutants lacking both a particular SR gene and a gene encoding another receptor of interest. For example, from *in vitro* and *in vivo* evidence, it seems likely that both CD14 and SRs, including SR-AI/II, may contribute to the clearance of LPS from the body (Hampton *et al*, 1991; Gegner *et al*, 1995; Van Amersfoort *et al*, 1996). Yet, recent reports indicate that any one receptor alone may play only a relatively minor role in this process (Van Amersfoort *et al*, 1996; Haziot *et al*, 1996). It would be interesting to see how LPS clearance is affected when both genes are eliminated. Similarly, multiple knockouts may be required in order to generate noticeable defects in apoptotic cell clearance.

Finally, the similarities between the innate immune systems of mammals and insects, and between the mammalian and *Drosophila* SRs, make it likely that genetic studies in *Drosophila* will also contribute to our understanding of both innate immunity and of SR functions in innate immunity. Thus, in the coming years new studies of SR function in both flies and mammals should provide clear insights into the roles that these receptors play in innate immunity and host defense.

The following chapters describe work I have carried out in the Krieger Lab. In Chapter 2, I discuss experiments designed at elucidating the basis for the polynucleotide ligand specificity of the bovine class A scavenger receptors. Given the similar ligand specificities displayed by all three scavenger receptor classes, these studies may shed some light on the mechanisms by which scavenger receptors recognize their ligands. In Chapter 3, I discuss the identification, cloning, and characterization of the class C scavenger receptor of *Drosophila melanogaster*. As stated above, it is hoped that genetic studies of this receptor will provide insight into the role it plays during *Drosophila* development and host defense. Chapter 4 describes the first steps that have been taken toward identifying and isolating flies lacking dSR-CI function. This work has resulted in the identification of a dSR-C gene family. The initial characterization of this family is reported, and ongoing experiments are described which should yield information on the functions of these genes and how these functions are fulfilled.

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Figure 1-1. Structures of the Class A, Class B, Class C and Class D Scavenger Receptors

There are currently seven defined scavenger receptors, belonging to four independent classes, A, B, C and D. The class A scavenger receptors SR-AI and SR-AII are trimeric glycoproteins generated by alternative splicing of mRNA transcripts from the same gene. They comprise, from amino to carboxy terminus, cytoplasmic (50 amino acids), transmembrane, spacer, α-helical coiled-coil (black), and collagenous (gray) domains. The collagenous domain mediate ligand binding. SR-AI in addition contains a C-terminal scavenger receptor cysteine rich (SRCR) domain. MARCO is a new SR-A protein comprised of N-terminal cytoplasmic (49 amino acids), transmembrane and spacer domains distinct from those of SR-AI/II. These are followed by a long collagenous domain with C-terminal homology to that of SR-AI/II, and then a C-terminal SRCR domain. The somewhat tentative model presented for the Class B receptors SR-BI and CD36 is based on current reports regarding CD36 structure. Note, however, that this issue is a matter of some controversy (see text for discussion). CD36 has a very short C-terminal cytoplasmic domain (< 10 residues; 45 residues in SR-BI), a single transmembrane domain (in this representation), and a single large extracellular domain comprising an N-terminal region containing most of the N-glycosylation sites and a C-terminal region rich in proline, glycine, and cysteine residues (the cysteines are indicated). The *Drosophila* class C scavenger receptor, dSR-CI, comprises several domains. From N-terminus to C-terminus, they are a signal sequence which is likely cleaved from the mature protein, two complement control protein (CCP) domains (gray), a large MAM domain (thick stripes), a small spacer domain, a somatomedin B domain (thin stripes), a serine/threonine rich mucin like domain which may be heavily glycosylated (black), another spacer domain, a transmembrane domain, and finally a small cytoplasmic domain (cross-hatched, 64 amino acids). Macrosialin/CD68 may represent a fourth class of cell surface scavenger receptors (see text). This protein comprises two extracellular domains separated by a proline-rich hinge: an amino terminal mucin-like domain which is heavily O-glycosylated (sticks with open circles), and a compact, membrane-proximal domain probably containing two disulfide bonds which is homologous to domains found in lysosomal membrane proteins. Both domains are N-glycosylated (sticks with closed circles). This protein has a very short (2-10 residue, depending on species) cytoplasmic domain. Figure adapted from M Krieger (unpublished), with permission.

Four Classes of Scavenger Receptors

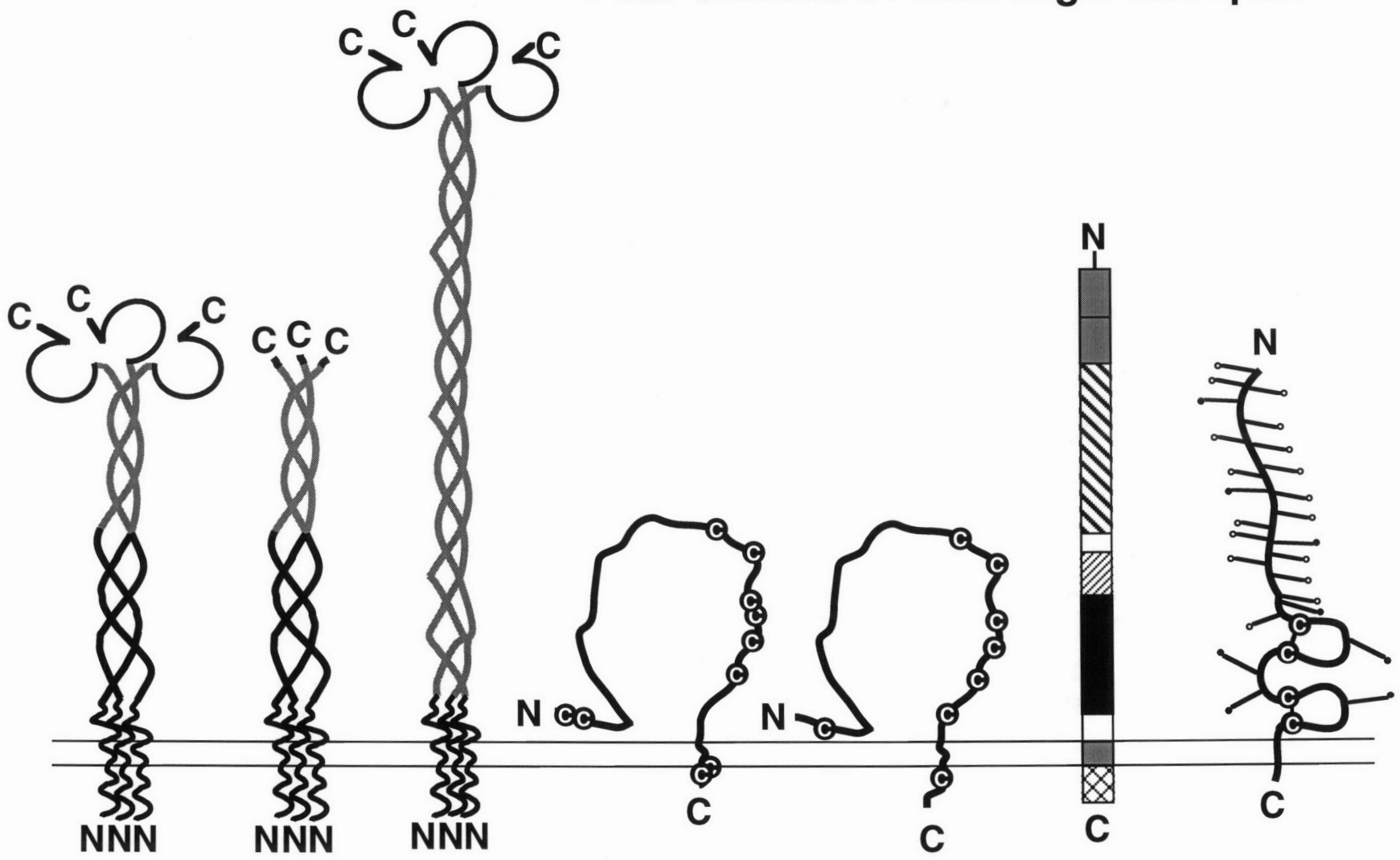

Table 1-1. Ligand Specificities of the Scavenger Receptors

Scavenger receptor ligand specificities reported in the literature have been determined both by directly testing the ability of a given compound to bind to a particular scavenger receptor (SR), and by testing the ability of a given compound to inhibit the binding of a known ligand to SR transfected cells. It is important to note that scavenger receptors exhibit some unusual binding properties, such as species specific differences in ligand binding, and non-reciprocal cross competition between two ligands (Krieger and Herz, 1994; Lougheed and Steinbrecher, 1996). Therefore, the inability of compound A to inhibit the binding of compound B does not necessarily mean that compound A is not a ligand. Caution must be exercised in interpreting negative data unless direct binding studies have been performed.

Notes:

[a] Abbreviations: LDL, low density lipoprotein; AcLDL and OxLDL, acetylated and oxidized LDL; HDL, high density lipoprotein; BSA, bovine serum albumin; M-BSA, maleylated BSA; ReLPS, reduced form of lipopolysaccharide (LPS); LTA, lipoteichoic acid; RBC, red blood cell; TSP, thrombospondin; PMN, polymorphonuclear leukocyte

[b] The binding of AcLDL to CD36 is controversial (Endemann *et al*, 1993; Acton *et al*, 1994; Nicholson *et al*, 1995; Nozaki *et al*, 1995). Since AcLDL is used as an inhibitor to evaluate the contribution of SRs to various macrophage functions, resolution of this issue is important.

[c] nd, not determined

[d] There appear to be species specific differences in the ligand specificity of CD36 (Huang *et al*, 1996).

References:

SR-AI/II: Araki *et al*, 1995; Dunne *et al*, 1994; Hampton *et al*, 1991; Kodama *et al*, 1990; Krieger and Herz, 1994; Melkko *et al*, 1994; Paresce *et al*, 1996; Pearson *et al*, 1993; Platt *et al*, 1995; Resnick *et al*, 1993; Rohrer *et al*, 1990; El Khoury et al, 1996; Chapter Two, this thesis.

MARCO/SR-AIII: Elomaa *et al*, 1995

SR-BI: Acton *et al*, 1994; Acton *et al*, 1996; Fukusawa *et al*, 1996; Huang *et al*, 1996; Rigotti *et al*, 1995.

CD36: Abumrad *et al*, 1993; Endemann *et al*, 1993; Greenwalt *et al*, 1992; Huang *et al*, 1996; Nicholson *et al*, 1995; Nozaki *et al*, 1995; Ren *et al*, 1995; Rigotti *et al*, 1995; Paresce *et al*, 1996.

dSR-CI: Pearson *et al*, 1995; A Pearson, S Xu, M Krieger, N Franc, A Ezekowitz, unpublished data.

Macrosialin/CD68: Ramprasad *et al*, 1995; Sambrano and Steinberg, 1995.

Ligand Specificities of the Scavenger Receptors

	SR-AI/II	MARCO	SR-BI	CD36	dSR-CI	macrosialin/CD68
Lipoproteins/proteins						
AcLDL[a]	+	+	+	±[b]	+	+
OxLDL	+	nd[c]	+	+	nd	+
M-BSA	+	nd	+	+	+	nd
LDL	-	nd	+	-	-	-
HDL	-	nd	+	+	nd	nd
BSA	-	nd	-	-	-	nd
Poly- and oligonucleotides						
polyI /polyG/oligo dG	+	nd	-	±[d]	+	+?
polyC/oligo dA	-	nd	-	-	-	nd
Anionic Polysaccharides						
fucoidan	+	nd	-	±[d]	+	+?
dextran sulfate	+	nd	-	nd	+	nd
chondroitan sulfate	-	nd	-	nd	nd	nd
Phospholipids						
phosphatidylserine	-	nd	+	+	-	+
phosphatidylcholine	-	nd	-	-	-	nd
Poly D-glutamic acid	-	nd	nd	nd	+	nd
Lipid IVa/ReLPS	+	nd	nd	nd	nd	nd

other	apoptotic cells		apoptotic cells	apoptotic cells	apoptotic cells	apoptotic cells ? oxidized RBC?
	LTA, *S. aureus*, other Gram- positive bacteria	*S. aureus* *E. coli*		*P. falciparum* - infected RBC	laminarin (ß-glucan)	
	ß-amyloid aggregates AGE-proteins			ß-amyloid aggregates collagen, TSP		
	PINP/PIIINP			oleate, palmitate		
	phosphorothioate oligonucleotides crocidolite- asbestos					

Table 1-2. Expression Patterns and Regulation of the Scavenger Receptors

Notes:
[a] The superscripts r and n indicate molecular weight determination under reducing and non-reducing conditions, respectively.

[b] Those tissues indicated in bold type have been shown to be major locations for receptor expression

References:
SR-AI/II: Aftring and Freeman, 1995; Ashkenas *et al*, 1993; Bell *et al*, 1994; Bickel and Freeman, 1992; Christie *et al*, 1996; de Villiers *et al*, 1994a; deVilliers *et al*, 1994b; de Rijke and van Berkel, 1994; Dufva *et al*, 1995; Geng and Hansson, 1995; Geng *et al*, 1994; Horvai *et al*, 1995; Hsu *et al*, 1996; Hughes *et al*, 1995; Li *et al*, 1995; Mato *et al*, 1996; Matsumoto *et al*, 1990; Naito *et al* 1991; Shackelford *et al*, 1995; van der Kooij *et al*, 1996.

SR-AIII/MARCO: Elomaa *et al*, 1995.

SR-BI: Acton *et al*, 1994; Acton *et al*, 1996; Landschultz *et al*, 1996; Wang *et al*, 1996.

CD36: Abumrad *et al*, 1993; Alessio *et al*, 1996; Greenwalt *et al*, 1992; Greenwalt *et al*, 1995; Huh *et al*, 1995; Huh *et al*, 1996; König *et al*, 1995; Ottnad *et al*, 1995; Ren and Savill, 1995; Ryeom *et al*, 1996a, 1996b; Swerlick *et al*, 1992; Yesner *et al*, 1996.

dSR-CI: Pearson *et al*, 1995; A. Pearson, D. Trigatti and M. Krieger unpublished.

Macrosialin/CD68: de Rijke and van Berkel, 1994; deVilliers *et al*, 1994; Geng and Hansson, 1995; Holness *et al*, 1993; Ramprasad *et al*, 1995.

Receptor / MW[a] (kD)	Expression Pattern[b]	Regulation
SRAI/II monomer: 76/65[r] trimer: 240/220[n]	Nearly all tissue macrophages, including: Spleen: **marginal zone**, red pulp, metallophilic Lymph node: **subcapsular sinus, medullary cord** germinal center, paracortex Thymus: **medulla**, cortex Lung: **Alveolar macrophages** Liver: Kupffer cells Peritoneal macrophages Other tissues and cells: Lymph node: **postcapillary HEV** follicular dendritic cells - B cell zone Blood: monocytes Brain: macrophages, perivascular microglia Liver: sinusoidal endothelial cells Smooth muscle cells of atherosclerotic plaques No expression observed in: hepatocytes, Langerhans cells, neurons, arterial endothelial cells, normal smooth muscle cells	monocytes < macrophages M-CSF upregulates during differentiation, perhaps via PU.1, AP1/ets, GATA transcription factors monocyte derived macrophages: IFN-γ decreases mRNA /activity levels LPS, GM-CSF decrease mRNA and maybe protein levels M-CSF increases total and surface protein levels IFN-γ, LPS may act via PU.1 elicited peritoneal macrophages: M-CSF increases mRNA, surface protein levels brain: LPS increases protein levels in microglia increased expression in Alzheimer's disease SMC: PDGF-BB, PDGF-AA, TNF-α, and phorbol esters increase mRNA IFN-γ, TGF-ß - conflicting reports of up/down-regulation
SRAIII/MARCO monomer: 80[r] trimer: 210[n]	Selected tissue macrophages: Spleen: marginal zone Lymph node: medulla Peritoneal macrophages No expression observed in spleen metallophilic macrophages Lung or Liver macrophages	Unknown
SR-BI 82[r]	mRNA: **adrenal** and **ovarysteroidogenic cells** moderate: intestine, epididymal fat, mammary low: lung, testis, liver	Adrenal gland: ACTH, estrogen, Apo-AI deficiency and hepatic lipase deficiency, all increase protein/mRNA

	protein: **adrenal** and **ovary steroidogenic cells** moderate: liver, mammary gland low: testis, intestine, epididymal fat	Liver: estrogen decreases protein and mRNA Testis: HCG increases protein and mRNA
CD36 78-88[r,n]	adipocytes, mammary epithelium liver sinusoidal endothelial cells, most capillary endothelial cells	pre-adipocytes < adipocytes capillary endothelial cells: IFN-gamma transiently increases surface expression hi fat diet increases surface expression phorbol esters decreases surface expression TNF-α, IL-1, TGF-ß no effect on surface expression
	monocytes peritoneal macrophages	monocytes < macrophages: mRNA , total and surface protein levels transiently increase, then decrease during maturation
	B cells platelets sickled reticulocytes	monocyte derived macrophages: M-CSF increases mRNA, total and surface protein levels adherence to TNF-α activated EC increases surface protein level GM-CSF, IFN-γ, IL-1ß, TNF-α, TGF-ß no effect
	No expression observed in: Most tissue macrophages Large artery endothelial cells	monocytes: IL-4, IL-6, M-CSF, PMA increase mRNA M-CSF, PMA increase total and surface protein levels dexamethasone, LPS decrease mRNA, total, surface protein
dSR-CI 170[r]	mRNA: embryonic hemocytes Protein: S2 and mbn-2 hemocyte-like cell lines	Largely unknown LPS, laminarin increase protein levels in mbn-2 cells
macrosialin/CD68 87-116[n] usually about 95	Most tissue macrophages, including: Lymph gland: T cell zone, not B cell zone Liver: Kupffer cells Peritoneal macrophages Elicited/activated macrophages Dendritic cells/Langerhans cells Primarily expressed in endosomes, low levels on surface	unstimulated < stimulated (thioglycollate, BCG) macrophages stimulation results in glycoform changes M-CSF decreases levels in elicited macrophages

Chapter Two

Polynucleotide Binding to Macrophage Scavenger Receptors Depends on the Formation of Base-quartet-stabilized Four-stranded Helices

As discussed in the Chapter 1, the class A scavenger receptors exhibit an unusually broad, but circumscribed, polyanionic ligand-binding specificity. These ligands include modified proteins such as Ac-LDL and M-BSA, certain polynucleotides, certain polysaccharides, and a variety of other molecules. Class A scavenger receptors do not bind all polyanions, however. For example, the polyribonucleotides poly(I) and poly(G) are ligands but poly(A) and poly(C) are not. The experiments presented in this chapter were aimed at elucidating the nature of this polynucleotide ligand specificity. The results of these studies indicated that, in order to bind to the class A scavenger receptors, oligo- and polynucleotides must adopt a four-stranded, or quadruplex, helical structure analogous to that formed by telomeric DNA sequences. All of the experiments described in this chapter were performed by me. This work was originally published in February 1993 in *The Journal of Biological Chemistry* (Pearson *et al*, 1993). The following modifications have been made to the original text: 1) the figures have been re-numbered with the prefix "2"; 2) footnotes describing papers submitted for publication have been changed to references since these papers have now been published; 3) the references have been merged with those of other chapters in the "references" section of this thesis.

Since the publication of this work, there have been several developments of note. First, it is now apparent (see Chapter 1), that there are at least three distinct classes of scavenger receptors. The experiments in this chapter were described as having been performed using CHO cells transfected with the bovine type I scavenger receptor (thus the cells were called CHO[bSR-I] cells). This receptor is now known as the class A type I receptor, or bSR-AI. Second, for at least one of the molecules used in these studies, dG_{12}, there has been independent confirmation that the oligonucleotide adopts a parallel stranded quadruplex structure (Miura and Thomas, 1995). Third, I have some unpublished data concerning the ability of phosphorothioate oligonucleotides to bind to bSR-AI. These data are presented in the addendum to this chapter. Fourth, there have been some reports which may shed some light on the possible functional significance of oligo- and polynucleotide binding to scavenger receptors. These are also reviewed in the addendum.

It was originally concluded that the spatial distribution of negatively charged phosphates in the quadruplex structure may form a charged surface which is complementary to the positively charged surface of the collagenous ligand-binding domain of the class A scavenger receptor. The addendum to this chapter further explores this idea. As opposed to most known ligand-receptor systems, in which ligand binding requires very specific interactions between ligand and receptor that are susceptible to minor alterations in the structure of either the ligand or the receptor (Creighton, 1984), the interactions between the SR-As and their ligands may not be strictly specified. Receptor-ligand complementarity in this system may be of a somewhat flexible and dynamic nature.

Polynucleotide Binding to Macrophage Scavenger Receptors Depends on the Formation of Base-Quartet-Stabilized, Four-Stranded Helices*

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Abbreviations - The following abbreviations were used: BSA, bovine serum albumin; CHO, Chinese Hamster Ovary; DEPC, diethyl pyrocarbonate; FBS, fetal bovine serum; FPLC, fast protein liquid chromatography; LDL, low density lipoprotein

As published in *The Journal of Biological Chemistry*

Summary

Macrophage scavenger receptors exhibit unusually broad, but circumscribed, polyanionic ligand-binding specificity. For example, the polyribonucleotides poly I and poly G are ligands but poly A and poly C are not. To further investigate the molecular basis of this polynucleotide-binding specificity, we tested the capacity of various oligodeoxyribonucleic acids to inhibit the scavenger receptor-mediated degradation of ^{125}I-AcLDL by Chinese hamster ovary cells expressing the type I bovine scavenger receptor. A series of short oligodeoxyriboguanines (dG_n, where $5 \leq n \leq 37$) were effective inhibitors. The dG_6, dG_{12} and dA_5G_{37} members of this series were shown by circular dichroism and UV spectroscopy to be assembled into four-stranded helices stabilized by G-quartets. [^{32}P]dA_5G_{37} bound directly to scavenger receptors. Partial or complete denaturation of the quadruplex structures of these oligonucleotides by boiling destroyed their inhibitory activity. Receptor activity was also inhibited by $d(T_4G_4)_4$, a telomere - like oligonucleotide which forms an intramolecular quadruplex. In addition, conversion of the four-stranded potassium salt of poly I to the single-stranded lithium salt dramatically reduced its inhibitory activity. Addition of KCl to the Li^+ salt resulted in the reformation of poly I's quadruplex structure and restoration of its inhibitory activity. A variety of single-stranded and double-stranded oligo- and polydeoxyribonucleotides (e.g., dA_{37}, HaeIII restriction fragments of ΦX174) exhibited very little or no inhibitory activity. Thus, a base-quartet-stabilized four-stranded helix appears to be a necessary structural determinant for polynucleotide binding to and inhibition of scavenger receptors. This conformational requirement accounts for the previously unexplained polyribonucleotide-binding specificity of scavenger receptors. The spatial distribution of the negatively charged phosphates in polynucleotide quadruplexes may form a charged surface which is complementary to the positively charged surface of the collagenous, ligand-binding domain of the scavenger receptor.

Introduction

Macrophage scavenger receptors are trimeric integral membrane glycoproteins which have been implicated in the deposition of lipoprotein cholesterol in artery walls during the formation of atherosclerotic plaques (Brown and Goldstein, 1983; Steinberg *et al.* , 1989) and in pathogen recognition for host defense (Krieger, 1992) . Two classes of macrophage scavenger receptors have been cloned from bovine, murine, human and rabbit cDNA libraries (Kodama *et al.*, 1990; Rohrer *et al*, 1990; Freeman *et al*, 1990; Matsumota *et al*, 1990; Bickel and Freeman, 1992; Ashkenas *et al*, 1993) . The sequence of the type I bovine scavenger receptor cDNA predicts a 453 amino acid protein with the following domains (Kodama *et al*, 1990; Ashkenas *et al*, 1993): I. N-terminal cytoplasmic (amino-acid residues 1-50), II. transmembrane (51-76), III. spacer (77-150), IV. α-helical coiled-coil (151-271), V. collagenous (272-343), and VI. C-terminal (344-453), which includes a 102 residue SRCR (scavenger receptor cysteine-rich) domain. The SRCR domain helped to define a previously unrecognized family of remarkably well-conserved cysteine-rich protein domains (Freeman *et al*, 1990; Aruffo *et al* 1991). The type II scavenger receptor is identical to the type I receptor, except that the 110-amino-acid C-terminus is replaced by a six-amino acid C-terminus (Rohrer *et al*, 1990). Despite its truncated C-terminus, the type II scavenger receptor mediates the binding and endocytosis of ligands with affinities and specificity similar to those of the type I receptor (Rohrer *et al*, 1990; Freeman *et al* 1991; Hampton *et al*, 1991). The collagenous domain appears to play a critical role in ligand binding (Kodama *et al*, 1991; Krieger, 1992; Acton *et al*, 1993; Doi *et al*, 1993).

Unlike most cell surface receptors, macrophage scavenger receptors exhibit unusually broad ligand specificity (Brown and Goldstein, 1983; Krieger, 1992; Goldstein *et al*, 1979; Brown *et al*, 1980; Kodama *et al*, 1988). Their high affinity ligands are polyanions, including certain chemically modified proteins such as acetylated LDL (AcLDL) and maleylated BSA (M-BSA) and certain polysaccharides, phospholipids (Hampton *et al*, 1991; Nishikawa *et al*, 1990), and polyribonucleotides (see below). Bacterial endotoxin (lipopolysaccharide) is also a ligand for scavenger receptors, which appear to play an important role in the rapid clearance of endotoxin from the circulation by the liver (Hampton *et al*, 1991).

To learn more about the mechanism underlying the scavenger receptors' unusual broad binding specificity, we have examined the structural basis of the type I bovine scavenger receptor's specificity for polyribonucleic acids. Poly I and poly G are ligands and competitive inhibitors of the scavenger receptor whereas poly C, poly U and poly A are not (Brown and Goldstein, 1983; Brown *et al*, 1980; Resnick *et al*, 1993). It is striking that the polyribonucleotides which are ligands form four-stranded helices under normal physiological conditions while the polyribonucleotides which are not ligands do not form such quadruplexes (Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975; Howard *et al*, 1977; Roy *et al*, 1979; reviewed in Gushlbauer *et al*, 1990).

A model for a four-stranded deoxyguanosine quadruplex is shown in Figure 2-1. This model is based on the 2.5 Å resolution X-ray crystal structure of the quadruplex formed by a dimer of the telomere-like oligodeoxyribonucleotide $d(G_4T_4G_4)$ (Kang *et al*, 1992). Figure 2-1A shows the hydrogen bonding between the four guanine bases of adjacent strands in this structure. The bases in this "G-quartet" are roughly planar (shaded region). In the quadruplex, the G-quartets are held together by the sugar-phosphate backbones and stack with a right-handed twist, resulting in the formation of a four-stranded helix. Figure 2-1B shows a hypothetical model of a portion of a quadruplex which is based on the $d(G_4T_4G_4)$ crystal structure and the proposed quadruplex structure of poly I and poly G (Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975). Detailed features of quadruplex structures vary depending on the oligo- and polynucleotide sequences and ionic conditions (Gushlbauer *et al*, 1990; Kang *et al*, 1992; Howard and Miles, 1982a; Williamson *et al*, 1989; Sundquist and Klug, 1989; Sen and Gilbert, 1990; Sen and Gilbert, 1992; Hardin *et al*, 1991; Smith and Feigon, 1992; Guo *et al*, 1992a; Lu *et al* 1992; Chen, 1992; Guo *et al*, 1992b; Balagurumoorthy *et al*, 1992). For example, quadruplexes can be composed either of extended oligo- or polynucleotide strands stabilized by intermolecular base-quartets, or of strands which fold back on themselves to form intramolecular or mixed intra- and intermolecular base-quartets. In all cases, the four-stranded helices have very high negative charge densities due to the close proximity of the phosphate groups.

In the current study we have tested and confirmed the hypothesis that assembly of polynucleotide ligands into four-stranded helices is a requirement for their binding to and consequent inhibition of scavenger receptors. We have also demonstrated that other quadruplex forming molecules not previously known to inhibit scavenger receptor activity, including telomeric oligodeoxyribonucleotides, are scavenger receptor ligands.

Experimental Procedures

Polynucleotides and oligodeoxyribonucleotides: Two independent preparations of the lyophilized K^+ salt of poly I were purchased from Pharmacia LKB ($s_{20,w}$ values 6.2 and 5.1; molecular masses, estimated by the manufacturer, 111 kD and 72.5 kD, respectively). These are known to be four stranded in a K^+ solution (Howard and Miles, 1982a). Stock solutions of the K^+ salt of poly I (5 mg/ml) in deionized water (with or without DEPC treatment) were stored at 4°C. Two double stranded DNA preparations were used: HaeIII digested fragments of ΦX174 (New England Biolabs) and pRc/CMV-s-bSR-I (19), a plasmid derived from pRc/CMV (InVitrogen). Most oligonucleotides were synthesized on an Applied Biosystems Model 380B automated DNA synthesizer and deprotected in ammonia at 55°C by the Biopolymers Laboratory at MIT. Deprotected oligonucleotides were dissolved in deionized water and de-salted over NAP10 or NAP25 columns (Pharmacia), or over Sephadex G-10 (Pharmacia) columns for oligonucleotides 15 residues or shorter. The de-salting step is not expected to remove counterions, such as Na^+ and K^+, which are tightly bound to the dG_n oligonucleotides (Hardin *et al*, 1991). The fractions containing oligonucleotides were concentrated by evaporation, resuspended at 0.4 - 4 mg/ml in deionized water, and stored at -20°C. The concentrations of the oligonucleotides were determined from their OD_{260} values (conversion factor: 30 μg/ml for OD_{260} =1.0 (1 cm path length) (Sambrook *et al*, 1989). Due to the hypochromic shift accompanying structure formation, this method underestimates the concentrations of oligonucleotides which fold into four-stranded helices (Saenger, 1984). The oligonucleotide R101 was a gift from Mr. Jonathan Wallach (MIT), and the oligonucleotide oSP24 was provided by Mr. Steven Podos (MIT). Preparations of $d(T_4G_4)_4$ were independently provided by Drs. Q. Guo and N. Kallenbach (New York University) and Dr. J. Williamson (MIT). They were synthesized and isolated in the quadruplex forms as described elsewhere (Williamson *et al* 1989; Guo *et al*, 1992a). The dA_5G_{37} was 5' end labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase (IBI) by standard procedures (Sambrook *et al*, 1989)

Preparation of the Li^+ and Li^+/K^+ salts of poly I - The Li^+ and Li^+/K^+ salts of poly I were prepared following the protocol of Howard and Miles (1982a; 1982b). Briefly, stock solutions of the K^+ salt of poly I (0.5 ml) were converted to the Li^+ salt by successive dialysis against 4 liters of the following solutions: 0.5 M LiCl, 1 mM EDTA (3 hours, room temperature); 0.5 M LiCl (2 hours, 4°C); 3 changes of 0.1 M LiCl (19 to 23 hours total, 4°C); and 4 changes of deionized water (74 to 95 hours total, 4°C). Any poly I molecules significantly smaller than the molecular weight cut-off (MWCO) of the dialysis tubing (6 - 8 kD) were probably lost during dialysis. In some cases, the Li^+ salt of poly I was converted to the Li^+/K^+ salt by addition of KCl (final concentration: 150 or 200 mM). These preparations were stored at 4°C for at least four days before use in ^{125}I-AcLDL degradation assays. The poly I concentration of each sample was determined spectrophotometrically (conversion factor: 40 μg/ml for OD_{260} =1.0 (1 cm

path length) (Sambrook *et al*, 1989)). In several experiments, the concentrations of the poly I isoforms were also determined after digesting the specimens to monomers with Nuclease P1 (Pharmacia; 0.2 units enzyme in 20 µl reaction volume, 4.5 hours at 50°C) and using an extinction coefficient for 5'-IMP of 12.2 ml/µmol-cm at 249 nm (Fasman, 1975) (not shown). This method for correcting errors due to the hypochromic shift did not significantly alter the results.

Other reagents (and sources) include: $Na^{125}I$ (Amersham), and [γ-^{32}P]dATP (New England Nuclear); LDL (Krieger, 1983; Goldstein *et al*, 1983), AcLDL (Goldstein *et al*, 1979), M-BSA (Goldstein *et al*, 1979), and ^{125}I-AcLDL (Goldstein *et al*, 1979; Krieger, 1983; Goldstein *et al*, 1983) were prepared as previously described. Dialysis tubing was obtained from either Spectrum (MWCO 6-8 kD) or Union Carbide (MWCO 12-14 kD). Care was taken to avoid contaminating the tubing with RNAses during preparation and storage.

Gel Filtration Chromatography - Oligonucleotide samples (20 to 50 µg/ml) in Buffer A (10 mM Tris-Cl, pH 7.6, 146 mM NaCl, 3 mM KCl, 0.6 mM $MgCl_2$, 0.3 mM $CaCl_2$; salt composition similar to that of Hams F-12 medium (Ham, 1965) were filtered through a 0.45 µm Millex HA filter (Millipore). The samples were analyzed at 4°C using a Pharmacia LKB 500 FPLC system with a Superose 6 HR 10/30 column (pre-equilibrated in Buffer A) at a flow rate of 0.5 ml Buffer A/min. The absorbance at 254 nm of the eluted material was continuously recorded using a UV-M II optical unit with a 2 mm path length. There was some variability from preparation to preparation in the heterogeneity of the size distributions observed (see Results). Poly I isoforms (50 µg/ml) in Buffer B (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$) were subjected to similar FPLC analysis after clarification by centrifugation (13,000 x g, 15 sec. 4°C).

Spectroscopic (UV) Melting Curves - Melting curves in Buffer C (1 mM Na_2HPO_4, 130 mM NaCl, 3 mM KCl, 0.6 mM $MgCl_2$, 0.3 mM $CaCl_2$, 3 µM $FeSO_4$, 3 µM $ZnSO_4$, 10 nM $CuSO_4$, 10 mM glucose, pH 7.4; formulated to closely match Hams F-12 medium (Ham, 1965)) of the untreated K^+ salt of poly I, its Li^+ salt, and its Li^+/K^+ salt (all at 25 µg/ml) were obtained at 247 nm using an Aviv Model 118 DS spectrophotometer. Absorbance was recorded from 10°C to 90°C in 2°C steps, with an equilibration time of 1.5 minutes, and an acquisition time of 45 seconds. The absorbance values presented represent the differences between the values obtained for poly I in Buffer C and for Buffer C alone. Similar results were obtained for poly I in Hams F-12 medium (data not shown).

Circular Dichroism (CD) - CD spectra of dG_n oligonucleotides dissolved in water at either 35 µg/ml for dG_{12} and dA_5G_{37} (106 µM in nucleotide residues) or 15.3 µg/ml for dG_6 (46 µM) were collected at 37°C on an Aviv Model 60DS equipped with a thermoelectric controller (1 cm cell, dynode voltage remained below 400 mV). Spectral scans of untreated samples were taken in 1 nm steps with an acquisition time of 5 seconds/datum. Each sample was then placed in boiling water for 15 minutes,

cooled at room temperature for 30 minutes, and re-scanned. The ellipticity values presented represent the differences between the values obtained for the oligonucleotide solutions and for a water blank and are corrected for specimen concentrations as previously described (Cantor and Schimmel, 1980). UV spectra were recorded concurrently.

Cell Culture: All incubations with cells were performed at 37°C in a humidified 5% CO_2/95% air incubator. Stock cultures of wild-type CHO cells were maintained in medium A (Ham's F-12 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 5% (v/v) FBS). The isolation of transfectants expressing the type I bovine scavenger receptor (CHO[bSR-I], clone I-B2) and the type II bovine scavenger receptor (CHO[bSR-II], clone II-5) was described previously (Freeman *et al*, 1991). Transfectants were maintained in MAC3 medium (medium A containing 3% new born calf lipoprotein deficient serum in place of the FBS, and supplemented with 0.5 mg/ml geneticin (G418, Gibco), 250 µM mevalonate, 40 µM compactin and 3 µg protein/ml of AcLDL), which provides selective pressure for the expression of scavenger receptor activity as previously described (Freeman *et al*, 1991). Media components, including sera, were obtained or prepared as previously described (Freeman *et al*, 1991; Krieger, 1983).

Assays: Scavenger receptor activity at 37°C was assessed by measuring cellular degradation of ^{125}I-AcLDL in 24 well culture dishes as previously described (Goldstein *et al*, 1979; Krieger, 1983; Goldstein *et al*, 1983). Unless otherwise noted, CHO[bSR-I] cells were seeded at a concentration of 60,000 cells/well in medium B (medium A supplemented with 0.5 mg/ml G418) on day 0. On day 2, 5 µg protein/ml of ^{125}I-AcLDL (153-630 cpm/ng protein) were added to the wells in 0.5 ml of culture medium in the absence (triplicate determinations) or presence (duplicate determinations) of the indicated additions. The culture medium used for these assays was either medium A or medium C (medium A containing 10 units/ml of E. Coli RNAse inhibitor (Calbiochem) and 1% (v/v) ITS+ (an insulin, transferrin, selinium and linoleic acid/albumin supplement from Collaborative Research) in place of FBS). After a 5 or 5.5 hour incubation at 37°C, the amounts of ^{125}I-AcLDL degradation products released into the media were measured and are presented as ng of ^{125}I-AcLDL protein degraded per 5 (5.5) hours per mg of cell protein. Cellular binding and uptake of [^{32}P]dA_5G_{37} after an incubation for 5 hours at 37°C was measured by washing the monolayers 6 times at 4°C as previously described (Goldstein *et al*, 1983), solubilizing each monolayer with 500 µl of 0.1 N NaOH for 15 min at room temperature, neutralizing each sample with 50 µl of 1 N HCl, and counting 110 µl aliquots in 5 ml of Ultrafluor scintillation fluid (National Diagnostics). The binding plus uptake values are given as cpm bound plus internalized per 5 hours per mg of cell protein. Protein concentrations were determined by the method of Lowry et. al. (Lowry *et al*, 1951).

Results

To test the hypothesis that polynucleotide ligands bind to and inhibit scavenger receptors when the ligands are assembled into four-stranded helices, we synthesized and analyzed the properties of an oligodeoxyribonucleotide, dA_5G_{37}. Guanine-rich oligo- and polynucleotides have been shown to adopt four-stranded, or quadruplex, structures via the formation of inter- and/or intramolecular G-quartets (Zimmerman *et al*, 1975; Balagurumoorthy *et al*, 1992; Gray and Bollum, 1974; Panyutin *et al*, 1990; Bonazzi *et al*, 1991). The five adenosine residues at the 5' end were included to facilitate ^{32}P-end-labeling with polynucleotide kinase (see below). Figure 2-2 shows that this oligonucleotide inhibited scavenger receptor activity, as measured by the degradation of ^{125}I-AcLDL by CHO cells expressing the bovine type I scavenger receptor (CHO[bSR-I] cells). The 50% inhibitory dose (ID_{50}) was 1.1 ± 0.5 μg/ml (average ± standard deviation from seven experiments) and virtually all of the scavenger receptor activity was inhibited at an oligonucleotide concentration of 25 μg/ml. The control molecule dA_{37}, which is not expected to form quadruplex structures, did not significantly inhibit ^{125}I-AcLDL degradation at this concentration (95 ± 11% of control activity, from four experiments).

These inhibition data suggested that dA_5G_{37} might bind directly to the scavenger receptor and competitively inhibit ^{125}I-AcLDL degradation. This conclusion was supported by experiments which showed that dA_5G_{37}, but not dA_{37}, could also block the specific binding of a soluble form of the type I bovine scavenger receptor to M-BSA beads (Resnick *et al*, 1993; and data not shown). Thus, it is unlikely that the dA_5G_{37} was interfering with ^{125}I-AcLDL degradation because of binding to ^{125}I-AcLDL rather than to the receptor.

To more directly examine oligonucleotide binding to the receptor, we end-labeled dA_5G_{37} with [^{32}P]phosphate and measured it's binding and uptake by CHO[bSR-I] cells. Figure 2-3A shows that specific [^{32}P]dA_5G_{37} binding and uptake (open circles) at low concentrations (<10 μg/ml) was saturable and was characterized by a dissociation constant of approximately 1 μg/ml. This K_d was similar to the ID_{50} determined by inhibition of ^{125}I-AcLDL degradation. The specific binding plus uptake shown in Figure 2-3A was calculated by subtracting the "nonspecific" cell associated [^{32}P]dA_5G_{37} measured in the presence of a large excess of the scavenger receptor inhibitor M-BSA (400 μg/ml, solid squares, Figure 2-3B) from the total cell associated [^{32}P]dA_5G_{37} (Figure 2-3B, solid circles). The nonspecific binding plus uptake was relatively high and increased linearly with increasing [^{32}P]dA_5G_{37} concentrations. The relationship of this non-specific binding plus uptake to the previously reported cellular uptake of oligonucleotides is not clear (Loke *et al*, 1989; Yakubov *et al*, 1989). To verify that the specific cell association of [^{32}P]dA_5G_{37} (Figure 2-3A) was due to

scavenger receptor expression, we measured the binding plus uptake of [^{32}P]dA_5G_{37} by untransfected CHO cells, which express essentially no scavenger receptor activity (Freeman *et al*, 1991; Hampton *et al*, 1991; Goldstein *et al*, 1979). The untransfected CHO cells exhibited little or no M-BSA competable [^{32}P]dA_5G_{37} binding and uptake over the concentration range shown in Figure 2-3 (data not shown). Figure 2-3C shows the transfection-dependent binding plus uptake of [^{32}P]dA_5G_{37} (open triangles), which was calculated by subtracting the values for the total binding plus uptake for untransfected cells (panel D, solid triangles) from those for the cells expressing the type I bovine scavenger receptor (panel D, solid circles). The shapes of the curves for the transfection-dependent data (panel C) and the M-BSA-competable ("specific") data (panel A) were remarkably similar. Taken together, these data clearly establish that [^{32}P]dA_5G_{37} bound to scavenger receptors in a saturable manner with high affinity.

We next examined the effect of varying the length of dG_n oligonucleotides on their inhibition of ^{125}I-AcLDL degradation by CHO[bSR-I] cells. The monomers 5'dGMP and 3'dGMP and the dimer dGpG at concentrations of up to 100 μg/ml failed to inhibit receptor activity (data not shown). However, all dG_n oligonucleotides tested with $n \geq 5$ were inhibitors (Figure 2-4A, dG_3 and dG_4 were not examined). Essentially complete inhibition was observed at 53 μg/ml for $n \geq 12$. The level of maximal inhibition decreased as the oligonucleotide length decreased from n=10 to n=5. However, the apparent affinity for the receptor for at least one of these shorter oligonucleotides, dG_6, did not appear to be substantially less than that for dG_{12} or dA_5G_{37} (Figure 2-4B). The molecular basis for this length dependence of maximal inhibitory activity has not yet been determined. We have recently observed that a form of bacterial endotoxin, ReLPS (lipopolysaccharide), can only partially inhibit scavenger receptor mediated degradation of ^{125}I-AcLDL by transfected CHO cells expressing the bovine type II or murine type I scavenger receptors (Ashkenas *et al*, 1993). The relationship, if any, between the partial inhibition of activity by the short dG_n's and the partial inhibition of activity by ReLPS has not yet been established.

The relationships of the structures of several dG_n compounds to their inhibitory activities were assessed using CD spectroscopy at 37°C and denaturation by boiling in deionized water. Figure 2-5 shows the CD spectra of dG_6, dG_{12} and dA_5G_{37}, before and after boiling. All three spectra of the untreated materials show maxima centered near 261 nm and 209 nm, minima near 241 nm, and distinctive shoulders near 230 nm (Figure 2-5A). The spectra of these untreated oligo dG_n's were similar to previously reported spectra of quadruplex polynucleotides, including dG_5 (Gray and Bollum, 1974), dG_6 (Bonazzi *et al*, 1991), and dG_{12} (Balagurumoorthy *et al*, 1992), poly G (Howard *et al*, 1977), poly dG (Gray and Bollum, 1974), and telomeric (Guo *et al*, 1992a; Lu *et al*, 1992; Chen, 1992; Lu *et al*, 1993; Guo *et al*, 1995) and model telomeric sequences (Hardin *et al*, 1991; Hardin *et al*, 1992). Furthermore, the location of the maxima near 261 nm suggests that these oligo dG_n quadruplexes were

composed of parallel rather than antiparallel oligonucleotide chains, presumably with all *anti*- glycosidic bond conformations (see Discussion). Boiling in water for 15 minutes reduced the size of the peaks, shifted the minima near 241 nm and the maxima near 261 nm to shorter wavelengths by 2 to 5 nm, and eliminated the shoulder near 230 nm (Figure 5B). These effects, which were most pronounced for dG_6, have previously been seen in similar molecules as a result of heating, and have been ascribed to the melting of G-quartet stabilized four-stranded structures into single stranded oligonucleotides (Gray and Bollum, 1974; Hardin *et al*, 1991; Guo *et al*, 1992a; Lu *et al*, 1992). Concurrent UV scans showed that the absorbances were significantly greater at 290-310 nm for the untreated specimens than for the boiled samples, and that there were significantly more pronounced shoulders near 275 nm in the spectra of the boiled samples than those near 280 nm in the spectra of the unboiled samples (data not shown). Similar results have been reported for dG_5 (Gray and Bollum, 1974), poly G (Pochon and Michelson, 1965) and for telomeric four-stranded G-quartet structures (Zahler *et al*, 1991). Therefore, these CD and UV spectroscopic results provide strong evidence that dG_6, dG_{12}, dA_5G_{37}, and presumably the other dG_n compounds, formed G-quartet-stabilized parallel stranded quadruplexes, and that boiling for 15 minutes in deionized water, followed by re-equilibration for 30 minutes at room temperature, resulted in the partial or complete denaturation of the quadruplexes.

Further characterization of these oligo dG_n's by FPLC analysis supported these conclusions. Superose 6 chromatography at 4°C (Figure 2-6) showed that the untreated dG_6, dG_{12} and dA_5G_{37} preparations exhibited extremely heterogeneous size distributions and included material with very large stokes radii (small elution volumes near the void volume of the column). Similar FPLC profiles were seen at 37°C (data not shown). In every case, boiling in deionized water substantially reduced the apparent sizes of these oligonucleotide complexes (data not shown). Therefore, it is likely that intermolecular G-quartets non-covalently crosslinked the oligonucleotides into large and heterogeneous aggregates. These results are consistent with the previously reported G-quartet-mediated formation of aggregates by guanine rich oligonucleotides (Ralph *et al* 1962; Gray and Bollum, 1974; Frank-Kamenetskii, 1989; Chung and Muller, 1991; however see Bonazzi *et al*, 1991)

Denaturation of the four-stranded dG_6, dG_{12} and dA_5G_{37} complexes by boiling dramatically reduced their capacity to inhibit scavenger receptor activity (Table 2-I). We assume, but have not shown, that the denatured oligonucleotides did not renature in the culture medium during the assay of receptor activity. Taken together, these spectroscopic, chromatographic and inhibition data, along with structural data from other laboratories, provide strong support for the proposal that the formation of G-quartet-stabilized four-stranded structures was responsible for dG_n binding to and inhibition of the scavenger receptor.

Additional evidence for the importance of four-stranded structures in mediating the binding to and inhibition of scavenger receptors by polynucleotide ligands was

obtained in studies of the structure and activity of the polyribonucleotide poly I. X-ray fiber diffraction and other methods have shown that, under normal physiologic conditions, poly I chains form parallel four-stranded helices held together by hydrogen-bonded inosine-quartets (Rich, 1958; Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975). Inosine bases lack the N2 group of guanine; therefore, unlike G-quartets which are stabilized by 2 hydrogen bonds between each pair of bases (Figure 2-1A), inosine-quartets contain only one hydrogen bond between each pair of bases. Formation of poly I quadruplexes has a striking dependence on the type of monovalent cation available. Na^+ and K^+ promote the formation of and stabilize the four-stranded helical structure by forming coordination complexes with the bases. In contrast, the Li^+ salt of poly I in the absence of such stabilizing cations is primarily single stranded (Miles and Frazier, 1978; Howard and Miles, 1982a). Miles and co-workers have shown that preparations of poly I can be cycled between the "denatured" single-stranded form (prepared by extensive dialysis with buffers containing Li^+ as the only monovalent cation) and the four-stranded form (by subsequent addition of Na^+ and/or K^+) (Howard and Miles, 1982a).

The untreated K^+ salt of poly I exhibited a heterogeneous size distribution and included material with very large stokes radii as determined by FPLC (Figure 2-7A). Extensive dialysis against LiCl in water converted the poly I to a homogeneous preparation of presumably single-stranded molecules with substantially smaller apparent mass (smaller stokes radius) than the K^+ salt form (Figure 2-7B). Based on the elution volume, the single-stranded Li^+ salt of poly I was approximately 19 nucleotides in length. Therefore, it appears that the large apparent mass and the heterogeneity of the untreated K^+ salt was due to the formation of inosine-quartet-stabilized aggregates, as was the case for the G-quartet-stabilized oligo dG_n molecules described above. After addition of 200 mM KCl to the Li^+ salt of poly I, and subsequent incubation at 4°C for several days, the heterogeneous mixture of large aggregates was reformed (Figure 2-7C), although the average apparent size was somewhat less than that of the initial K^+-form prior to the salt exchange (Figure 2-7A).

To confirm that the structures of these poly I preparations were undergoing the expected cation-dependent quadruplex/monomer transitions, we examined the temperature-dependence of their UV absorbances at 247 nm (Howard and Miles, 1982a, 1982b) As shown in Figure 2-8, both untreated poly I (solid circles) and the Li^+/K^+ salt (open diamonds) displayed cooperative melting transitions between 30°-45°C in a buffer containing a variety of salts at physiologic concentrations (see Experimental Procedures). In contrast, the Li^+ salt of poly I (open circles) did not exhibit a similar temperature-dependent shift in absorbance at 247 nm between 10°C and 75°C; indeed, it's absorbance at all temperatures was similar to those of the high temperature (melted) forms of the untreated and Li^+/K^+ salts of poly I. Together with the FPLC data described above, these results indicate that our preparation of the Li^+

salt of poly I consisted predominantly of denatured, single-stranded molecules, while the untreated and Li^+/K^+ preparations were predominantly aggregated quadruplexes.

Figure 2-9 shows a comparison of the abilities of the quadruplex and predominantly single stranded poly I isoforms to inhibit ^{125}I-AcLDL degradation by CHO[bSR-I] cells. The untreated K^+ salt of poly I (open squares) inhibited scavenger receptor activity with an ID_{50} of ~1.0 μg/ml, and the inhibition was essentially complete at 4 μg/ml. In contrast, the Li^+ salt of poly I (open triangles) was a much less efficient inhibitor; there was 50% inhibition at ~7 μg/ml and the activity was not fully inhibited even at the highest concentrations of poly I tested (18% of control activity at 71 μg/ml). Although we were unable to detect, using FPLC, the formation of poly I aggregates during the course of this experiment (data not shown), the relatively low residual activity of the Li^+ salt may have been due in part to the presence of small amounts of residual quadruplex structure after dialysis and addition to the assay medium (Miles and Frazier, 1978; Howard and Miles, 1982a). The addition of 200 mM KCl to the Li^+ salt of poly I (solid squares) restored it's inhibitory activity: the ID_{50} was ~1.2 μg/ml, and inhibition was essentially complete at 6 μg/ml. Three other independent Li^+-dialysis/KCl-addition experiments, each using either one or two different commercial preparations of poly I, gave similar results. The inhibitory activity of the Li^+/K^+ salt of poly I at 37°C was somewhat greater than might be expected based on the melting curve in Figure 2-8. This may be due to differences in the experimental conditions of the spectroscopic and receptor-activity assays which might alter the stability of the four-stranded form of poly I. For example, the Na^+ concentration in the degradation assay was approximately 146 mM, while it was 132 mM in the spectroscopic assay. Small differences in ion concentrations can have a significant effect on poly I stability (Howard and Miles, 1982a). Also, BSA and other proteins were present in the Ham's F12 medium used in the receptor activity assays but not in the UV melting experiments (see Experimental Procedures).

In the experiment shown in Figure 2-9, an RNAse inhibitor from *E. Coli* was used to protect all three poly I isoforms and poly C (used as a negative control, not shown) from extensive RNAse degradation during the 5 hour incubation with cells. We have found that the inclusion of an RNAse inhibitor to the cell culture assay media was essential to prevent the virtually complete hydrolysis of poly C to mono and dinucleotides. The quadruplex polyribonucleotides appeared to be resistant to this degradation, as assessed by FPLC analysis (data not shown). The enzymatic and chemical lability of polyribonucleotides such as poly C should be considered when using these molecules as negative controls in scavenger receptor studies.

All of the quadruplex-forming oligonucleotide and polyribonucleotide molecules described above aggregated into heterogeneous, higher order structures as determined by FPLC analysis (for example, see Figures 2-6 and 2-7). To determine if oligonucleotides which form small, homogeneous, quadruplex structures could also inhibit scavenger receptor activity, we tested the inhibitory activities of several

telomere-like oligonucleotides. Unlike the heterogeneous four-stranded dG_n and poly I molecules described above, many model telomeres fold into well-defined intramolecular G-quartet-stabilized monomeric structures (Williamson *et al*, 1989; Sundquist and Klug, 1989; Kang *et al*, 1992; Smith and Feigon, 1992; Guo *et al*, 1992a). Two preparations of $d(T_4G_4)_4$ were obtained in the monomeric, intramolecularly folded four-stranded form from two independent sources (Williamson *et al*, 1989; Guo *et al*, 1992a) Both of these oligonucleotides exhibited moderate scavenger receptor-inhibitory activity (the average receptor activity in the presence of 25 μg oligonucleotide/ml was 43% of control), which was similar to those of dG_5 and dG_6 (Figure 3 and data not shown). We synthesized the related oligonucleotide $d(G_4T_4)_5$ and found using FPLC (not shown) that it was composed of a mixture of two discrete species with Stokes radii approximately equivalent to those expected for dA_{22} and dA_{67}. The $d(G_4T_4)_5$ was an effective inhibitor of scavenger receptor activity (22 ± 9% of control activity at 25 μg/ml, average from three experiments), with activity approaching that of dG_{12}. We therefore conclude that at least some oligonucleotides with model telomeric sequences can inhibit scavenger receptors, presumably due to their binding directly to the receptors. Thus, short oligonucleotides with well-defined G-quartet structures, as well as longer polynucleotides and the heterogeneous dG_n complexes described above, can be effective inhibitors of scavenger receptors.

We have examined the capacity of several other oligo- and polynucleotides to inhibit scavenger receptors. Table 2-2 shows that those molecules not expected to form quadruplexes, including double stranded DNA, were very poor inhibitors of scavenger receptor activity, while those expected or previously shown to form base-quartet stabilized four-stranded molecules were effective inhibitors. In combination with previous reports of the polynucleotide specificity of scavenger receptors (Brown *et al*, 1980; Brown and Goldstein, 1983), the current studies provide strong support for the proposal that the formation of quadruplex structures is an important determinant in the binding to and inhibition of scavenger receptors by oligo- and polynucleotides.

Discussion

The broad ligand binding specificity of macrophage scavenger receptors has been well-defined but is poorly understood. A large number of polyanions inhibit scavenger receptor-mediated binding, uptake and degradation of AcLDL (Goldstein *et al*, 1979; Brown *et al*, 1980; Brown and Goldstein, 1983; Kodama *et al*, 1988; Nishikawa *et al*, 1990; Hampton *et al*, 1991 Krieger, 1992). These inhibitors include certain modified proteins, carbohydrates, lipids and polyribonucleic acids. In many cases, these inhibitors have been shown to be ligands for the receptor and thus competitive inhibitors. However, there are many other polyanionic proteins, carbohydrates, and polyribonucleic acids that do not inhibit AcLDL degradation. For example, poly I and poly G are good inhibitors but poly A, Poly C, and poly U are not. After determining the primary structures of the type I and type II bovine macrophage scavenger receptors, we suggested that their common extracellular collagenous domain, which is highly cationic and well conserved among mammalian scavenger receptors (Ashkenas *et al*, 1993), was a likely site of polyanionic ligand binding (Kodama *et al*, 1990; Rohrer *et al*, 1990). Recent experiments have confirmed this proposal (Acton *et al*, 1993; Doi *et al*, 1993; Kodama *et al*, 1991). It seems likely that the spatial distribution of the positively and negatively charged side chains in this domain plays a critical role in distinguishing between polyanionic ligands and polyanions that do not bind (Krieger *et al*, 1993).

In the current study, we have extended the list of scavenger receptor inhibitors to include specific types of oligodeoxyribonucleic acids, and we have determined the structural basis of the type I bovine scavenger receptor's specificity for particular polyribonucleic and oligodeoxyribonucleic acids. Short dG_n oligonucleotides, where $5 \leq n \leq 37$, and certain telomere-like oligonucleotides (e.g., $d(G_4T_4)_5$) effectively inhibited type I bovine scavenger receptor activity. Experiments using transfected CHO cells expressing the type II bovine scavenger receptor gave similar results, although oligonucleotide inhibition of the type II receptors was generally not as potent as was inhibition of the type I receptors (A. Pearson and M. Krieger, unpublished data). Cell binding studies with $[^{32}P]dA_5G_{37}$ established that this oligonucleotide, and presumably the other dG_n 's, bound directly to the type I scavenger receptor and suggest that these molecules functioned as competitive inhibitors. These oligonucleotides and all of the previously described polyribonucleotide inhibitors can form four-stranded helices stabilized by either inter- or intramolecular hydrogen-bonded base-quartets (e. g., "G-quartets" see Figure 2-1) (Brown *et al*, 1980; Brown and Goldstein, 1983; Rich, 1958; Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975; Howard *et al* 1977; Roy *et al*, 1979; Williamson *et al* 1989; Panyutin *et al*, 1990; Bonazzi *et al*, 1991; Guo *et al*, 1992a)

The inhibitory activities of these molecules were dramatically reduced when their four-stranded helical structures were disrupted, either by boiling in deionized water in the case of the dG_n's or by Li^+ denaturation in the case of poly I. The coordinate denaturation and loss of activity of poly I were reversible; its quadruplex

structure and its inhibitory activity were restored by the addition of KCl to the Li^+ salt. A set of control oligo- and polynucleotides which do not form, or are not expected to form, four-stranded helices (e.g., dA_{37}, ΦX DNA) did not inhibit scavenger receptor activity. Thus, the formation of a base-quartet-stabilized four-stranded helix appears to be a necessary structural determinant for the activity of oligo- and polynucleotide inhibitors of scavenger receptors.

It has recently been reported that dG_{10} aggregates, which were presumably four-stranded, but not dC_{10}, dA_{10} or boiled dG_{10}, can bind to topoisomerase II and inhibit its activity (Chung and Muller, 1991). A comparison of the bovine scavenger receptor amino acid sequence with that of the human (Tsai-Pflugfelder *et al*, 1988) or yeast (Giaever *et al*, 1986) topoisomerases showed no significant homology (unpublished observations). The topoisomerase II sequences do contain many clusters of basic residues which might interact with the phosphates in aggregated dG_{10}.

Three other proteins have been reported to bind to guanine-rich oligonucleotides. The Oxytricha telomere-binding protein (Raghuraman and Cech, 1990), the Oxytricha telomerase (Zahler *et al*, 1991), and an avian telomere-binding protein (Gualberto *et al*, 1992) bind to oligonucleotides with telomeric sequences (e. g. $d(T_4G_4)_4$). The two invertebrate proteins appear to differ from the scavenger receptor since they bind to the unfolded form of this telomeric oligonucleotide, rather than to the folded quadruplex form (Raghuraman and Cech, 1990; Zahler *et al*, 1991). It has been suggested that the vertebrate binding protein recognizes G - G base pairs in telomere-like oligonucleotides (Gualberto *et al*, 1992). The scavenger receptor may provide an additional tool for the analysis of telomeric structures in natural nucleic acids. For example, the scavenger receptor may be able to detect quadruplex conformations in chromosomes.

It is important to note that the detailed three dimensional structures of quadruplex oligonucleotides and polynucleotides can vary. The quadruplexes can be stabilized by intermolecular base-quartets between either parallel (Rich, 1958; Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975; Gushlbauer *et al*, 1990; Sen and Gilbert, 1988; Gupta *et al*, 1993) or antiparallel (Gushlbauer *et al*, 1990; Jin *et al*, 1990; Hardin *et al*, 1991; Wang *et al*, 1992) extended polynucleotide strands. Alternatively, the strands can fold back on themselves to form quadruplexes stabilized by intramolecular or mixed intra- and intermolecular base-quartets (Williamson *et al*, 1989; Sundquist and Klug, 1989; Gushlbauer *et al*, 1990; Panyutin *et al*, 1990; Kang *et al*, 1992; Smith and Feigon, 1992). The orientations of the bases with respect to the sugar-phosphate-backbones can be all *anti* in the case of parallel strands (Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975; Gupta *et al*, 1993), or they can alternate between *syn* and *anti* when the strands fold back on themselves and/or are antiparallel (Henderson *et al*, 1987; Gushlbauer *et al*, 1990; Kang *et al*, 1992; Smith and Feigon, 1992; Wang *et al*, 1992). The four polynucleotide chains are equidistant from each other when the strands are parallel. However, when the chains fold back on

themselves, some of the sugar-phosphate chains are closer together and others are further apart, as in the $d(G_4T_4G_4)$ dimer (Kang *et al*, 1992) (Figure 1A), so that the helical grooves are alternately wide or narrow. Quadruplex structures can also vary depending on the ionic environment, the method of sample preparation, and sample concentration and history (compare Howard and Miles, 1982a; Sen and Gilbert, 1988, 1990, 1992; Sundquist and Klug, 1989; Williamson *et al*, 1989; Gushlbauer *et al*, 1990; Panyutin *et al*, 1990; Jin *et al*, 1990; Hardin *et al*, 1991; Zahler *et al*, 1991; Kang *et al*, 1992; Smith and Feigon, 1992; Guo *et al*, 1992a; Lu *et al*, 1992; Chen, 1992; Guo *et al* 1992b; Balagurumoorthy *et al*, 1992; Wang *et al*, 1992; Gupta *et al*, 1993; Lu *et al*, 1993; Guo *et al*, 1995). For example, the telomeric oligonucleotide $d(G_4T_4G_4)$ has been reported to form a quadruplex with Na^+ counterions whose structure differs in a number of ways from that of the quadruplex formed with K^+ counterions (Kang *et al*, 1992; Smith and Feigon, 1992).

The detailed structures of the quadruplexes used in this study varied considerably. Some consisted of large aggregates of non-covalently associated oligo- and polynucleotides, while others were clearly small monomeric structures. The poly I quadruplex comprises four individual and parallel polyribonucleotide strands (Rich, 1958; Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975). It is likely that the individual strands are not in register. As a consequence, the ends of the quadruplexes might be free to fold-back to form intramolecular, antiparallel structures. The telomere-like oligonucleotide $d(T_4G_4)_4$ repeatedly folds back on itself to form an intramolecular quadruplex containing G-quartets and T loops (Williamson *et al*, 1989; Guo *et al*, 1992a). Adjacent guanine segments are therefore oriented in an antiparallel fashion in this quadruplex. The telomere-like oligonucleotide $d(G_4T_4)_5$ apparently forms at least two different quadruplex species whose detailed structures have not been determined.

The dG_n oligonucleotides used in this study could have formed either parallel quadruplexes, like poly I and poly G, or folded back antiparallel quadruplexes, like the telomeric oligonucleotides, or perhaps a mixture of both. Recent studies of telomere-like oligonucleotides have demonstrated that circular dichroism provides a sensitive technique for distinguishing between parallel and antiparallel quadruplex conformations (Chen, 1992; Balagurumoorthy *et al*, 1992; Lu *et al*, 1993; Gupta *et al*, 1993). A strong positive band centered near 260-265 nm in the CD spectrum correlates with a parallel quadruplex conformation (Hardin *et al*, 1991, 1992; Guo *et al*, 1992a; Lu *et al*, 1992; Chen, 1992; Balagurumoorthy *et al*, 1992; Gupta *et al*, 1993; Guo *et al*, 1995). On the other hand, a strong positive band centered near 295 nm accompanied by a weak negative band near 265 nm correlates with an antiparallel conformation (Williamson *et al*, 1989; Jin *et al*, 1990; Hardin *et al*, 1991; Kang *et al*, 1992; Smith and Feigon, 1992; Guo *et al*, 1992a; Chen, 1992; Balagurumoorthy *et al*, 1992; Wang *et al*, 1992; Lu *et al*, 1993; Guo *et al*, 1995). These recent results are supported by CD studies of dG_5 and poly G (Howard *et al*, 1977; Gray and Bollum, 1974), as well as more recent studies of several oligo dG_n's (Bonazzi *et al*, 1991).

Thus, our CD results indicate that the oligo dG_n molecules used here were predominantly parallel-stranded quadruplex structures.

We have not yet determined whether all or only a subset of the many possible quadruplex conformations can inhibit scavenger receptor activity. Therefore, even though a four-stranded structure appears to be necessary for nucleic acid inhibition of the scavenger receptor, it may not be sufficient. There may be quadruplex-forming oligonucleotides which cannot inhibit scavenger receptor activity.

The results of this study suggest the possibility that the spatial distribution of negatively charged phosphates in oligo- and polynucleotide quadruplexes can form a charged surface which is complementary to the positively charged surface of the collagenous domain of the scavenger receptor. This could account for the previously unexplained, striking polynucleotide binding specificity of this receptor (Brown *et al*, 1980; Brown and Goldstein, 1983) and our recent finding that the collagenous domain of complement factor C1q exhibits polyanionic ligand binding whose specificity is similar, but not identical, to that of scavenger receptors (Acton *et al*, 1993; Raepple *et al*, 1976). These results suggest that further analysis of the known structures of collagen and quadruplexes will provide additional insights into the binding properties of scavenger receptors.

Acknowledgments: We thank Richard Cook and the staff of the MIT Biopolymer Laboratory, Jonathan Wallach, Steve Podos, and Nanda Sinha for oligonucleotides, and Drs. Qiu Guo and Jamie Williamson for the gifts of four-stranded $d(T_4G_4)_4$ samples. We also thank Dr. Peter Kim and Brenda Schulman for very generously providing access to and instruction on the use of the scanning spectrophotometers. We are grateful to Drs. Guo, Williamson, H. Miles, S. Acton, J. Ashkenas, D. Hudson, and M. Ross, and to D. Resnick, for helpful discussions and suggestions. We are also grateful to Drs. Guo, M. Lu, A. Garcia, G. Gupta, and N. Kallenbach for sharing and permitting us to cite their results prior to publication, and to Dr. Vernon Ingram who first pointed out that some scavenger receptor ligands could adopt four-stranded structures. We also thank Julia Khorana for help with preparation of the figures, David Resnick for help and advice with M-BSA binding assays, and Marsha Penman and Alison Lux for excellent technical assistance.

Figure 2-1. Structure of a G-Quartet (A) and model of a segment of an extended base-quartet stabilized four-stranded helix (B).

Panel A shows a molecular model of a G-quartet, adapted from the x-ray crystal structure of a d($G_4T_4G_4$) dimer (Kang *et al*, 1992). The oxygen, nitrogen, and phosphorus atoms are shown as open, solid, and hatched circles, respectively. Hydrogen bonds are indicated by dashed lines. In the structure of the d($G_4T_4G_4$) dimer the oligonucleotide chains fold back on themselves to form an antiparallel quadruplex (not shown). As a consequence, the base conformations around the G-quart*et* a*l*ternate between *syn* and *anti* and the quadruplex has wide and narrow helical grooves (see Discussion). Panel B shows a hypothetical model of an extended parallel, four-stranded helix, based in part on the X-ray fiber structures of poly I and poly G (Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975). The shaded planes represent the base-quartets (see text).

Figure 1.

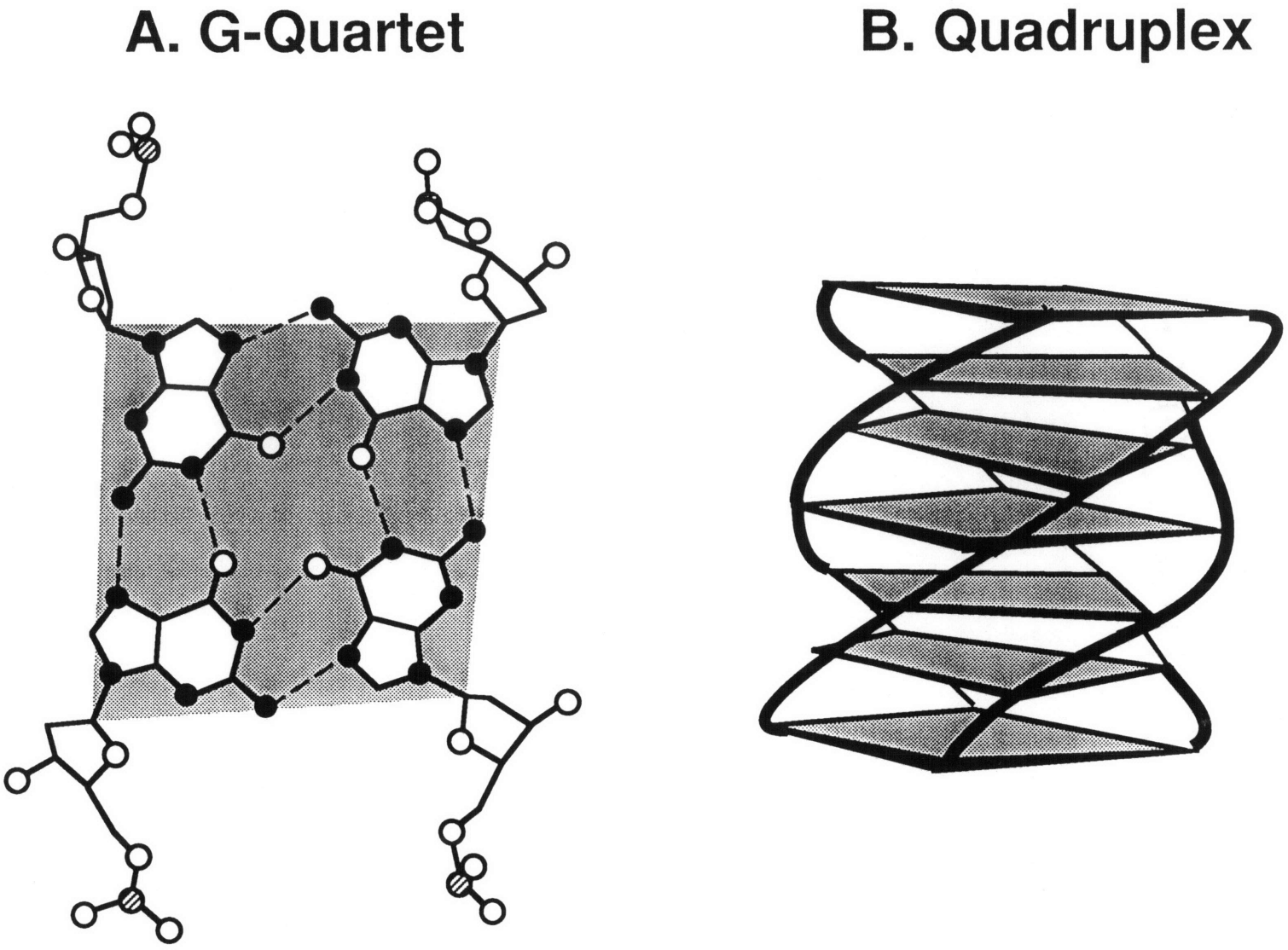


Figure 2-2. Inhibition of cellular ^{125}I-AcLDL degradation by dA_5G_{37}.

CHO[bSR-I] cells, which express bovine type I scavenger receptors, were plated on day 0 in medium B in 24-well dishes (80,000 cells/well). On day 2, the cells were incubated for 5 hours at 37°C in medium A containing 5 μg protein/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated amounts of dA_5G_{37}. The degradation values represent the results from a single experiment and were measured as described in experimental procedures.

Effect of dA_5G_{37} on ^{125}I-AcLDL Degradation by CHO[bSR-I] Cells

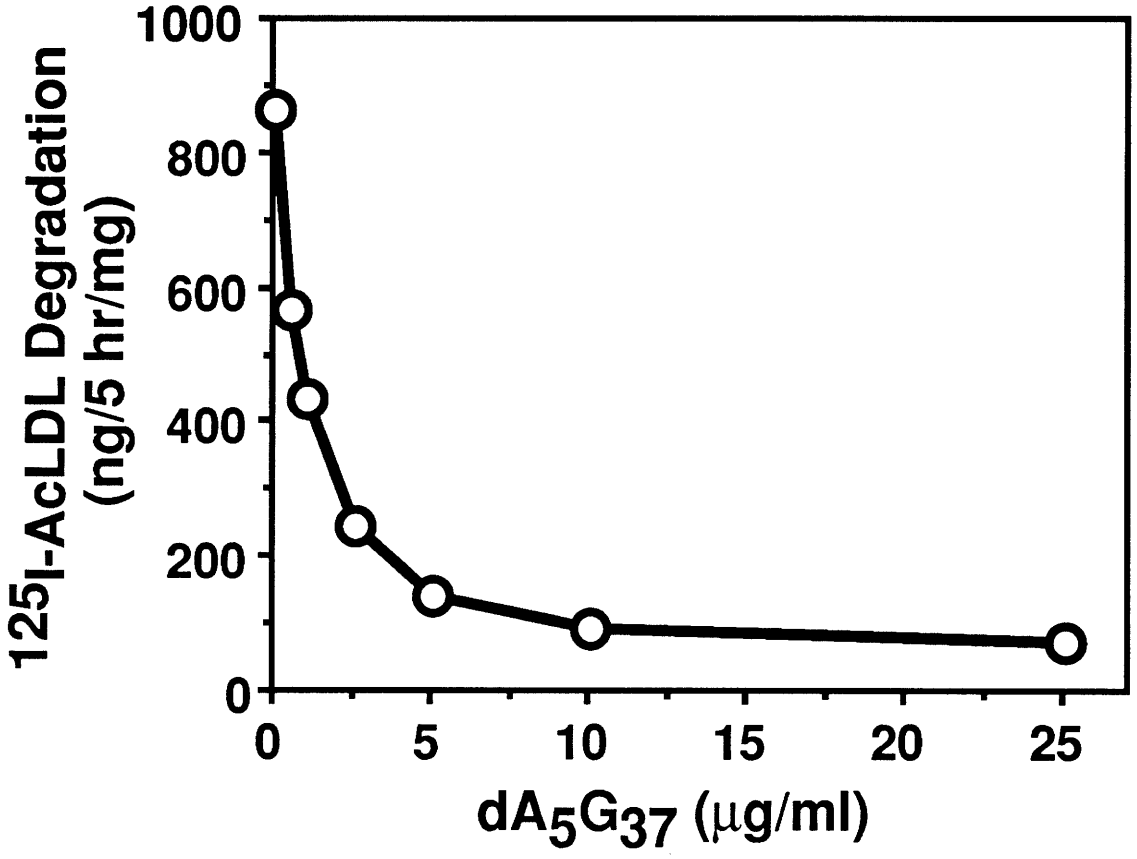
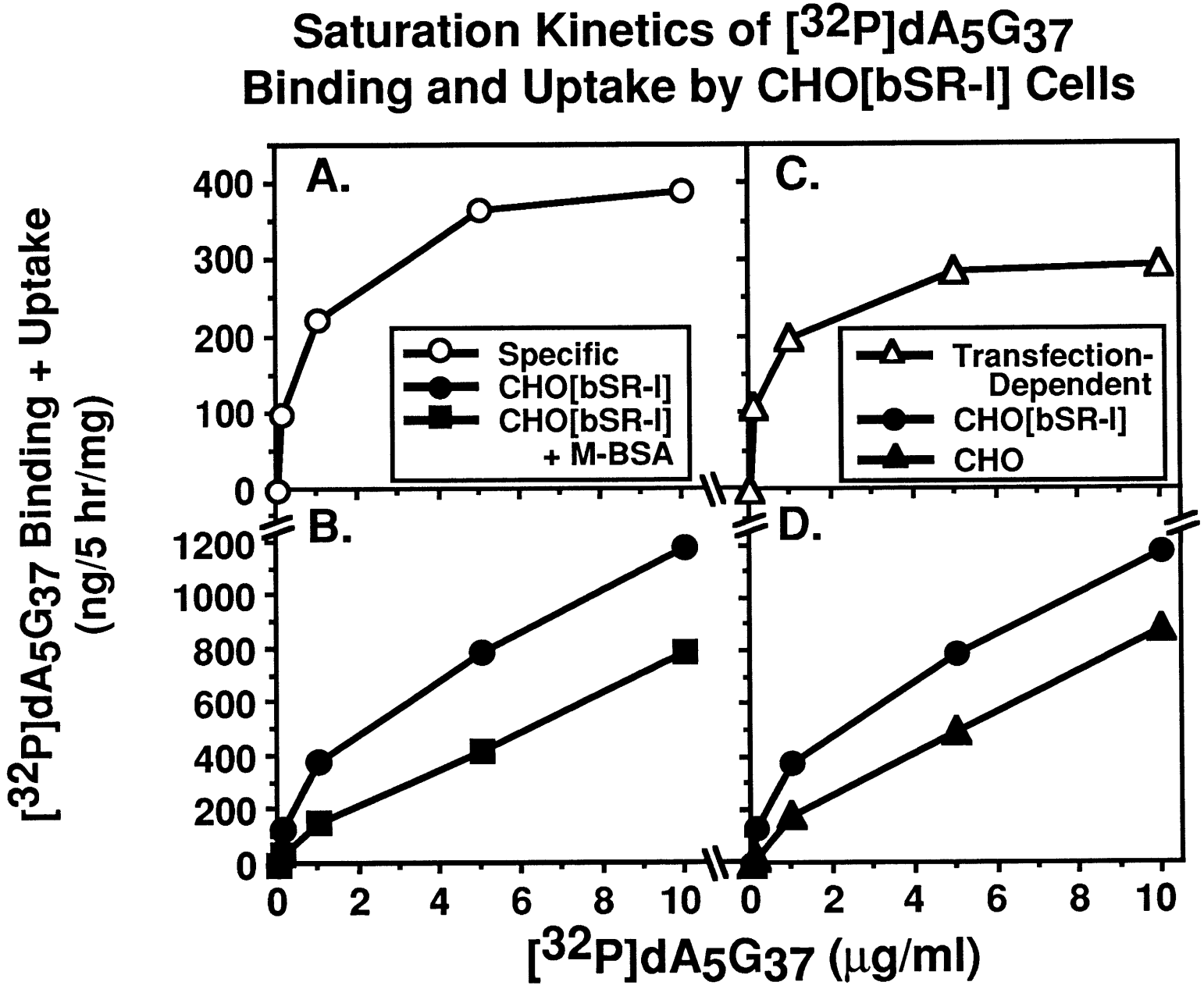
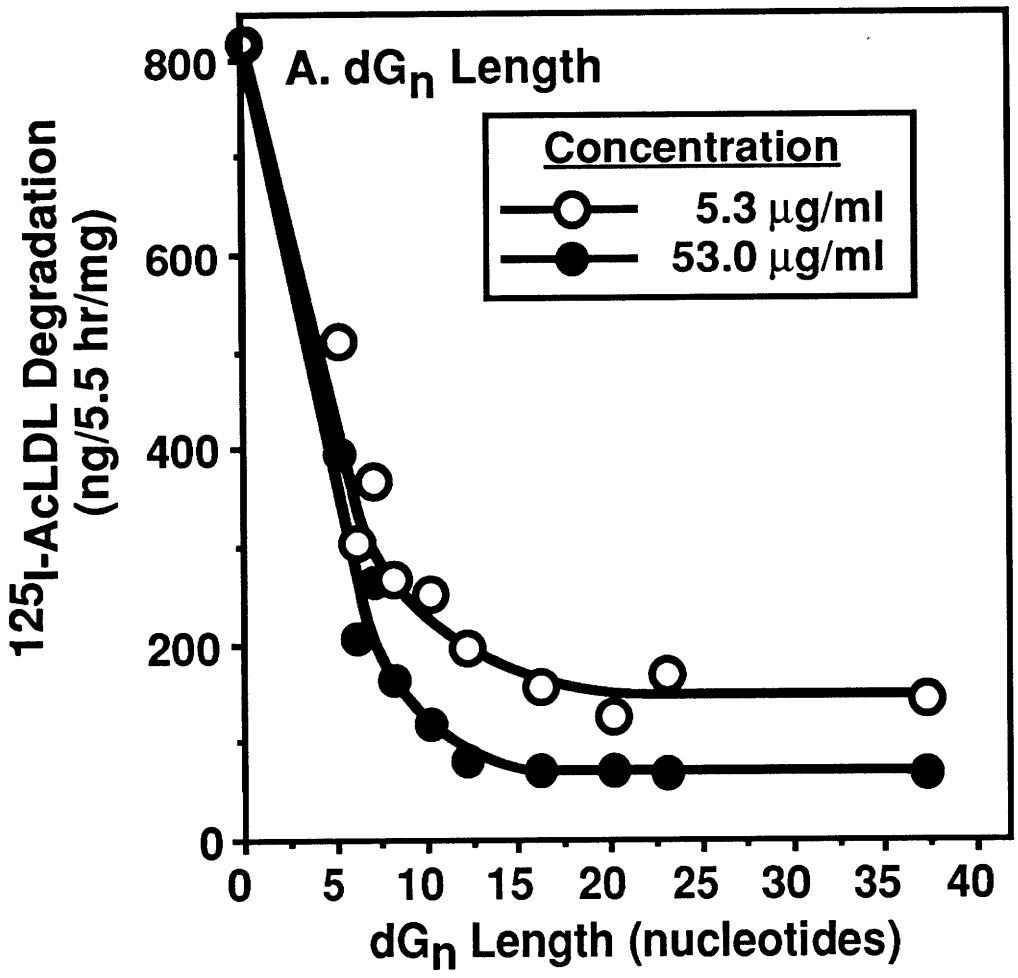


Figure 2-3. Binding plus uptake of [^{32}P]dA$_5$G$_{37}$ by CHO[bSR-I] cells.

Untransfected CHO and CHO[bSR-I] cells were plated on day 0 in medium B in 24-well dishes (60,000 cells/well). On day 2, the cells were incubated for 5 hours at 37°C in medium A containing the indicated concentrations of [^{32}P]dA$_5$G$_{37}$ (4580 cpm/ng) in the absence (duplicate incubations) or presence (single incubations) of 400 μg/ml of M-BSA. Binding and uptake were measured as described in experimental procedures. The values for the specific binding plus uptake of [^{32}P]dA$_5$G$_{37}$ (panel A, open circles) represent the differences between total binding plus uptake (panel B, solid circles) and nonspecific binding plus uptake determined in the presence of M-BSA (panel B, solid squares). The values for the transfection-dependent binding plus uptake (panel C, open triangles) represent the differences between the total binding plus uptake values for the transfected CHO[bSR-I] cells (panel D, solid circles) and for the untransfected CHO cells (panel D, solid triangles).


Saturation Kinetics of [32P]dA5G37
Binding and Uptake by CHO[bSR-I] Cells
[32P]dA5G37 Binding + Uptake
(ng/5 hr/mg)
A.
B.
C.
D.
Specific
CHO[bSR-I]
CHO[bSR-I]
+ M-BSA
Transfection-
Dependent
CHO[bSR-I]
CHO
400
300
200
100
0
1200
1000
800
600
400
200
0
0
2
4
6
8
10
[32P]dA5G37 (μg/ml)


A. dG$_n$ Length
Concentration
5.3 μg/ml
53.0 μg/ml
^{125}I-AcLDL Degradation (ng/5.5 hr/mg)
800
600
400
200
0
0
5
10
15
20
25
30
35
40
dG$_n$ Length (nucleotides)

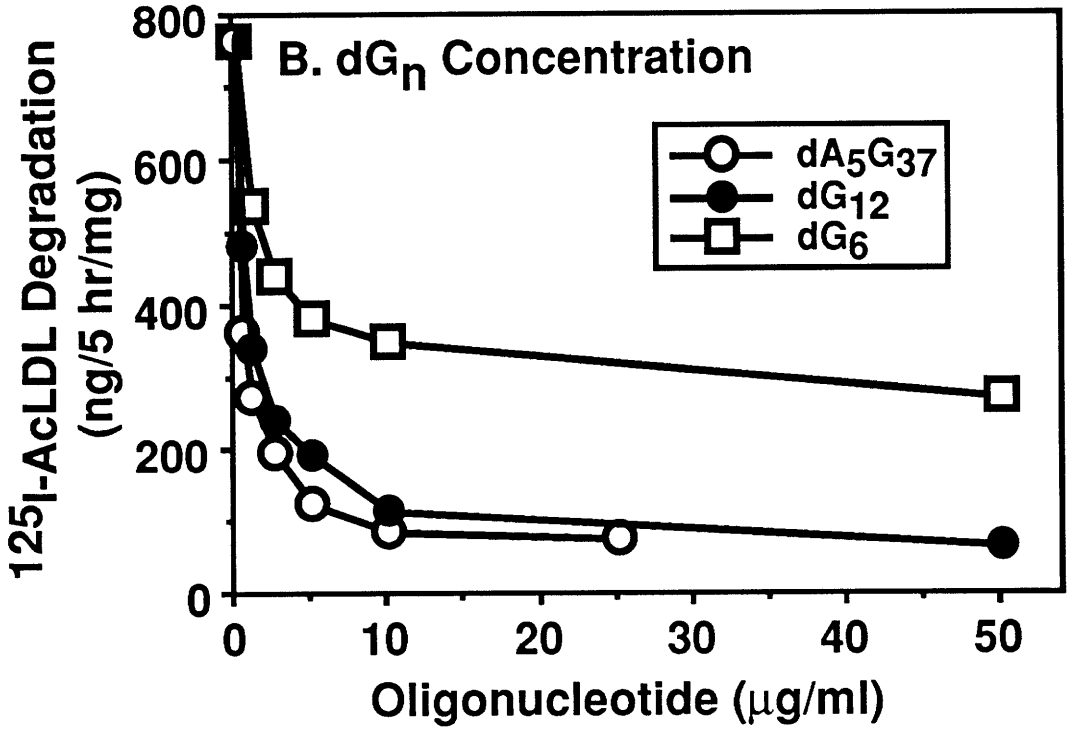

B. dG$_n$ Concentration
dA$_5$G$_{37}$
dG$_{12}$
dG$_6$
^{125}I-AcLDL Degradation (ng/5 hr/mg)
800
600
400
200
0
0
10
20
30
40
50
Oligonucleotide (μg/ml)

Figure 2-4. Effect of dG_n length on the inhibition of ^{125}I-AcLDL degradation by CHO[bSR-I] cells.

Panel A: CHO[bSR-I] cells were plated on day 0 (30,000 cells/well). On day 3, the cells were incubated at 37°C for 5.5 hours in medium A containing 5 μg protein/ml of ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated dG_n oligonucleotides at concentrations of either 5.3 μg/ml (open circles) or 53.0 μg/ml (solid circles). Degradation was measured as described in experimental procedures. For n=37, dA_5G_{37} was used in place of dG_{37}. Panel B: Cells were plated on day 0 at 60,000 cells/well. On day 2, the cells were incubated at 37°C for 5 hours in medium A containing 5 μg protein/ml of ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated concentrations of oligo dG_6, dG_{12} or dA_5G_{37}. Degradation was measured as described in experimental procedures.

Figure 2-5. CD spectra of untreated and boiled dG_n oligonucleotides.

The indicated oligonucleotides were dissolved in 3 ml of deionized water at 15.3 to 35 μg/ml (46 to 106 μM in nucleotide residues). CD spectra for each oligonucleotide were recorded at 37°C before (panel A) and after (panel B) boiling as described in experimental procedures.

CD Spectra of Untreated and Boiled dG_n's

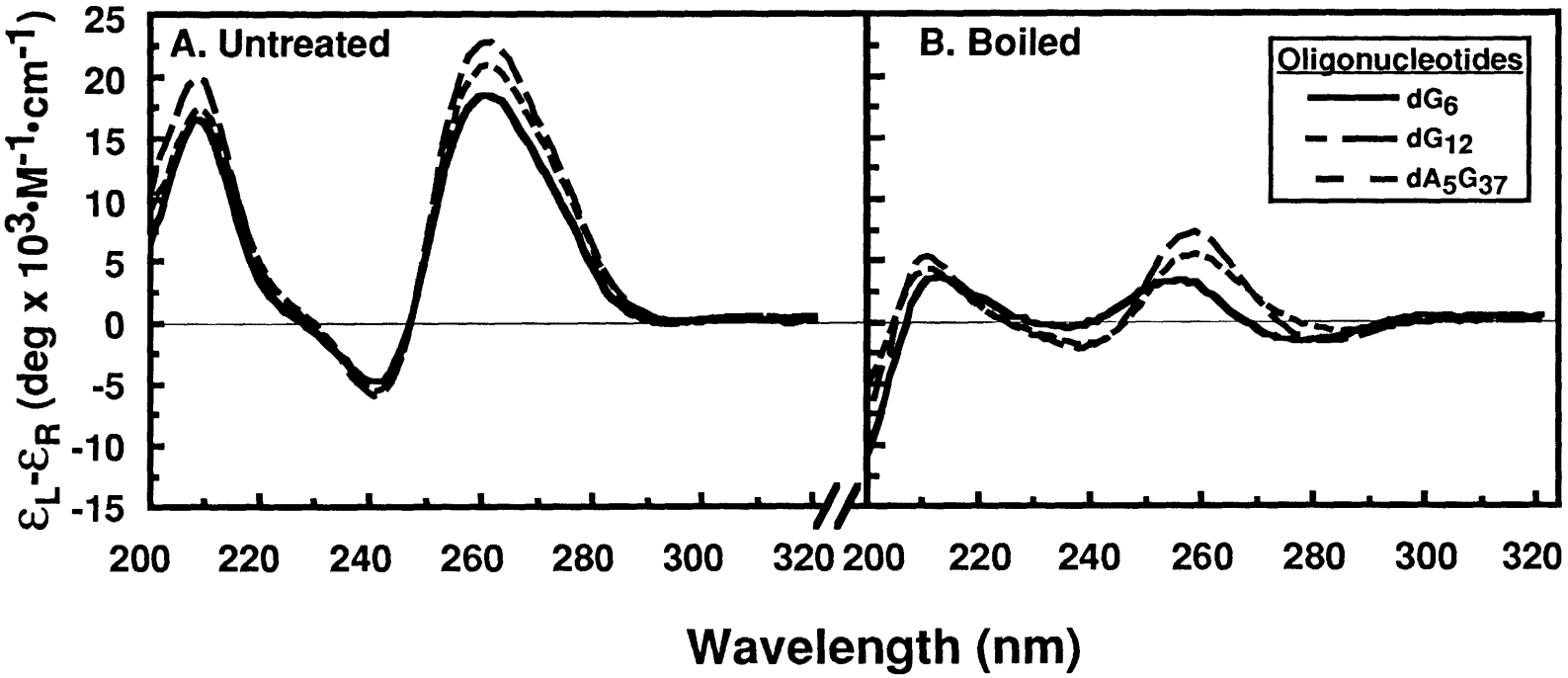
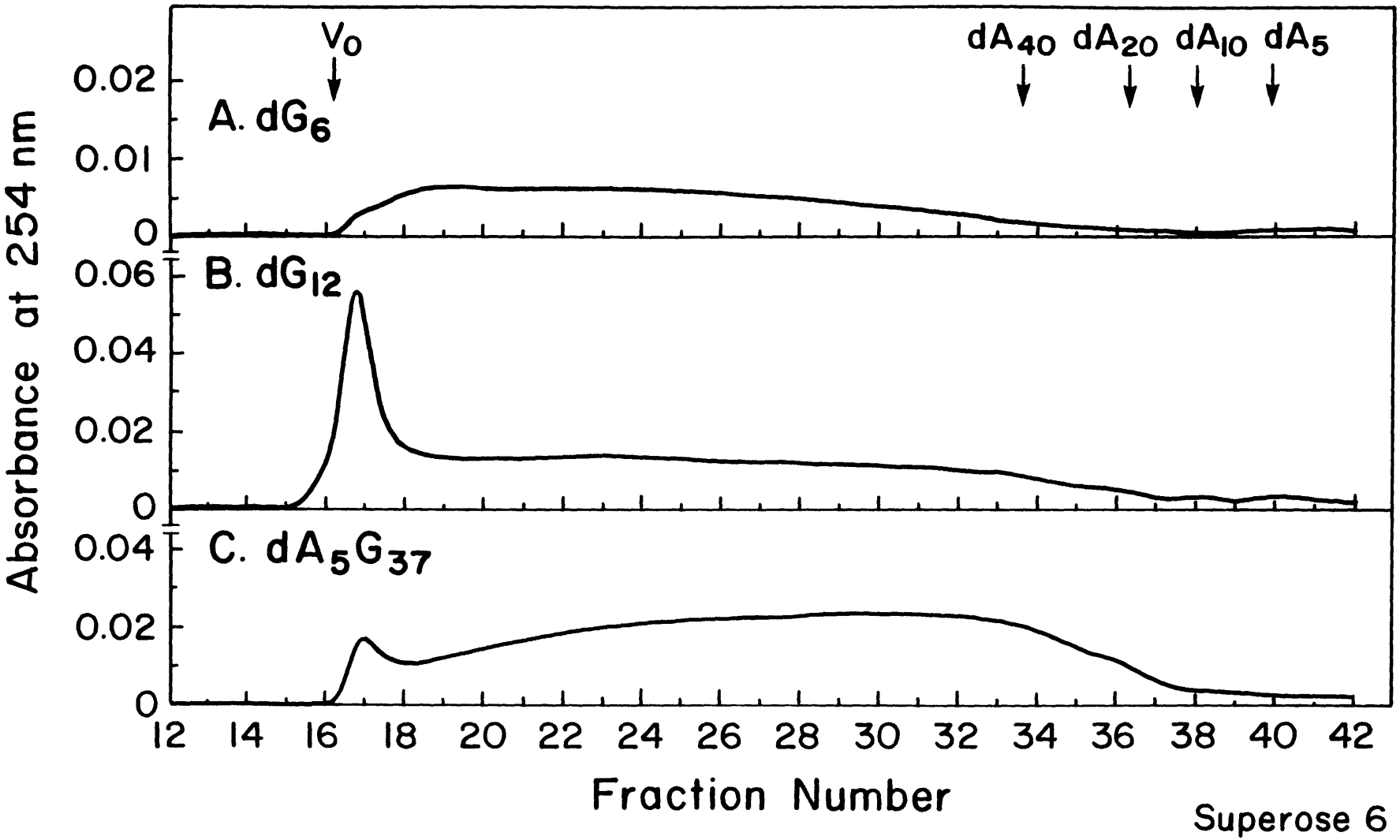


Figure 2-6. FPLC analysis of dG_6, dG_{12} and dA_5G_{37}.

The dG_6 (panel A), dG_{12} (panel B) and dA_5G_{37}(panel C) oligonucleotides (20 to 50 µg/ml in Buffer A, 200 µl/sample) were subjected to Superose 6 column chromatography as described in experimental procedures. The locations of the void volume (globular proteins with masses $\geq 5x10^6$ are excluded) and dA_n standards (50 µg/ml; n = 5, 10, 20, 40) are shown. For the dA_n standards, the peak-widths at half-height were approximately 1.5-2 fractions.

FPLC Analysis of Oligo dG's



Superose 6

Table 2-1: Effect of Boiling on the Ability of Oligo dG_n's to Inhibit ^{125}I-AcLDL Degradation in CHO[bSR-I] Cells

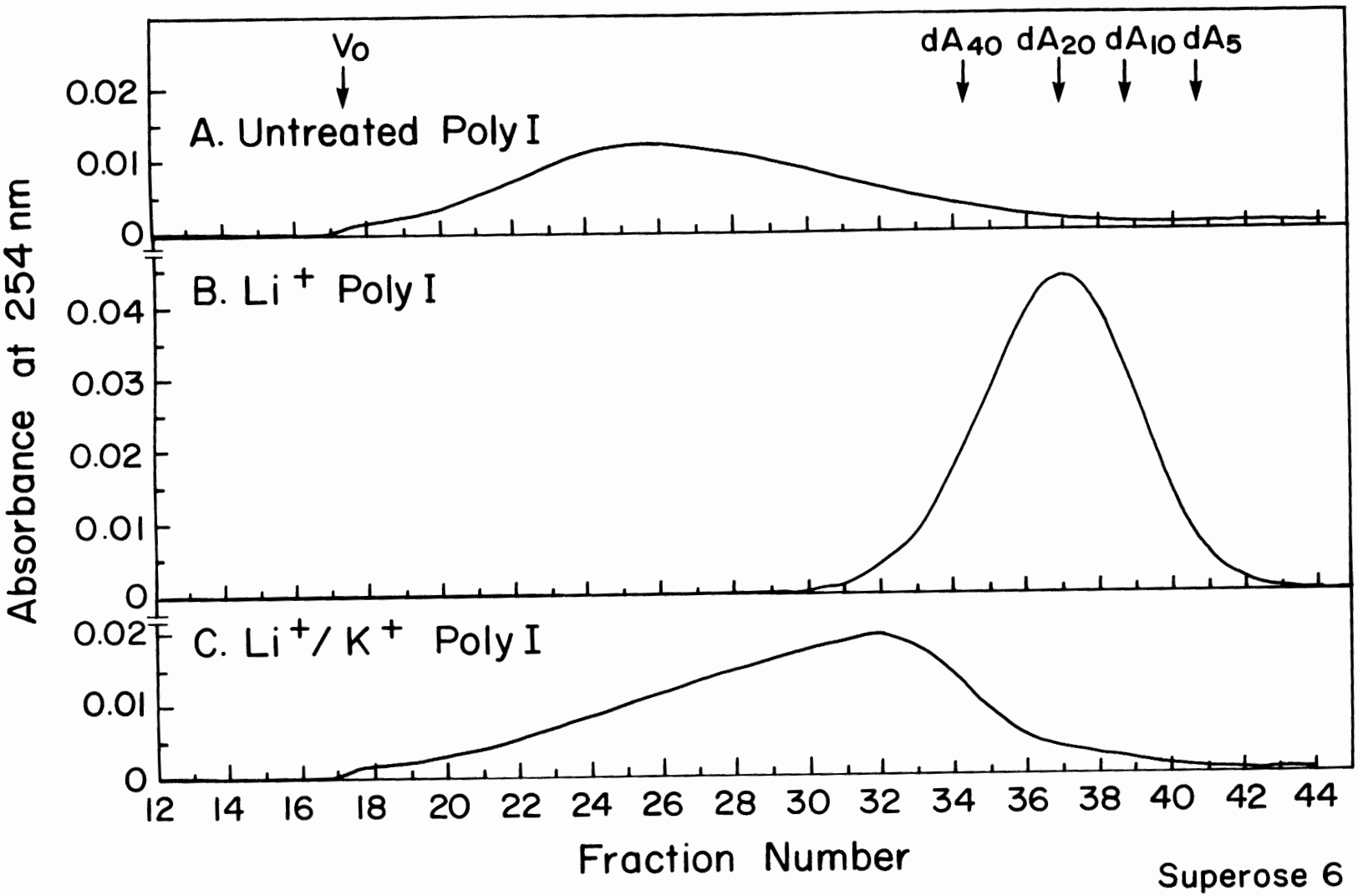
Oligonucleotide	Concentration	Scavenger Receptor Activity*	
		Untreated	Boiled
	(μg/ml)	(% of control)	
none	-	100%	100%
dG_6	5.0	53 ± 5%	92 ± 9%
dG_{12}	5.0	29 ± 6%	85 ± 10%
$d(A_5G_{37})$	5.0	23 ± 6%	82 ± 13%
	25.0	12 ± 3%	85 ± 9%

* Cells were plated on day 0 (20,000-60,000 cells/well) and scavenger receptor activity (^{125}I-AcLDL degradation) was assayed on day 2 or 3 as described in experimental procedures. The 100% control values measured in the absence of added oligonucleotides ranged from 565 - 2640 ng /5 hr/mg protein. For each oligonucleotide, the values are the averages ± standard deviations determined in 5 - 7 experiments, using 2 or 3 independently synthesized preparations of each oligonucleotide.

Figure 2-7. FPLC analysis of poly I isoforms.

The untreated (panel A), the Li^+ salt (panel B) or the Li^+/K^+ salt (panel C) of poly I (50 µg/ml in Buffer B, 200 µl/sample) were subjected to Superose 6 column chromatography as described in experimental procedures. The locations of the void volume and dA_n standards (25 µg/ml; n = 5, 10, 20, 40) are shown.

Effects of Li^+ and K^+ on the Structure of Poly I: FPLC Analysis



Superose 6

Figure 2-8. Effects of Li^+ denaturation and K^+ renaturation on the temperature-dependent UV absorbance of Poly I.

The untreated K^+ salt (solid circles), the Li^+ salt (open circles) , or the Li^+/K^+ salt (open diamonds) of Poly I were prepared in buffer C and the temperature-dependent UV spectra were recorded as described in experimental procedures.

Effects of Li^+ Denaturation and K^+ Renaturation on the Temperature-Dependent UV Absorbance of Poly I (25 μg/ml)

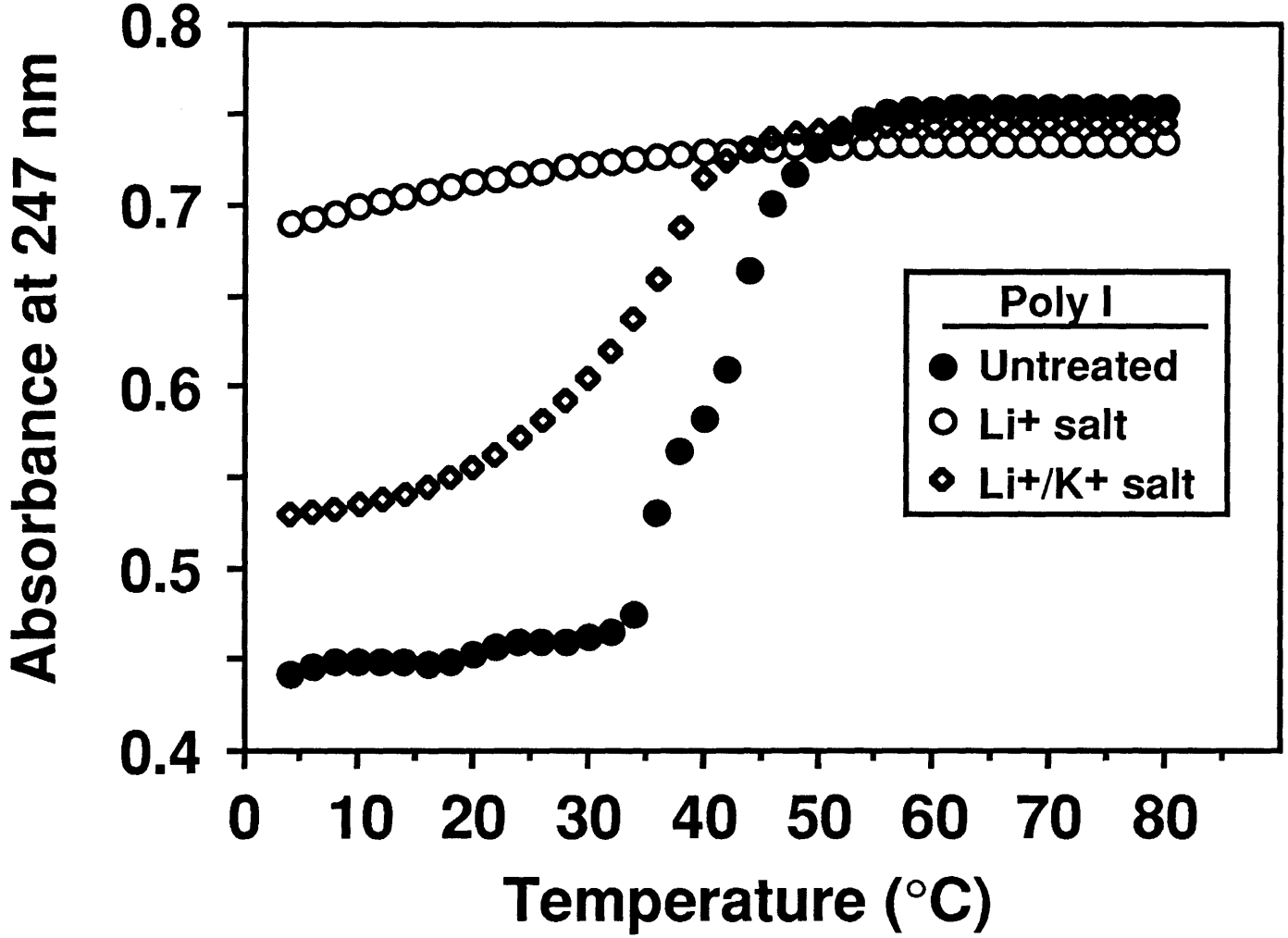


Figure 2-9. Effects of Li^+ denaturation and K^+ renaturation on the inhibition by poly I of ^{125}I-AcLDL degradation by CHO[bSR-1] cells.

On day 2 of culture, degradation of 5 μg protein/ml ^{125}I-AcLDL in medium C was assayed in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated concentrations of the untreated K^+ salt (open squares), the Li^+ salt (open triangles), or the Li^+/K^+ salt (solid squares) of poly I as described in experimental procedures.

Effects of Li^+ Denaturation and K^+ Renaturation on the Inhibition by Poly I of ^{125}I-AcLDL Degradation in CHO[bSR-I] Cells

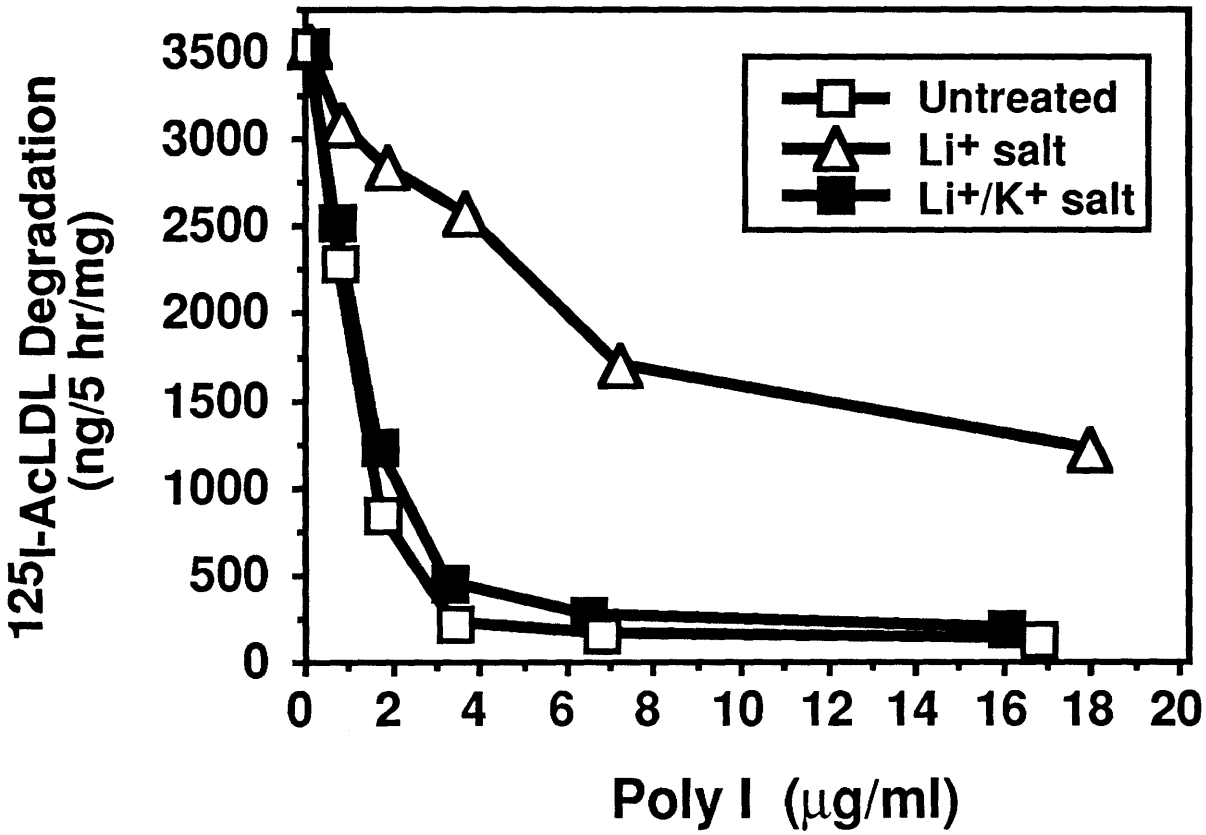


Table 2-2: Effective and Ineffective Oligo- and Polynucleotide Inhibitors of Macrophage Scavenger Receptors[a]

Effective Inhibitors: Four-stranded molecules[b]	Ineffective Inhibitors: one- or two-stranded molecules[c]
Compound	**Compound**
poly I	poly A
poly G	poly C
polyriboxanthinylic acid	poly U
dG_n (n=5-8,10,12,16,20,23,37,60)	GMP, GTP, GpG
dA_5G_n (n=12,37)	dA_{37}
	oSP24[d]
$d(T_4G_4)_4$	dAP1[d]
$d(G_4T_4)_5$	$d(CG)_9C(CG)_9$
	TH1[d]
	R101[d]
	pRc/CMV
	ΦX174 (HaeIII digest)

[a] The inhibition of scavenger receptor activity was measured using cellular ^{125}I-AcLDL degradation assays. All of the compounds listed were examined in this study except for poly A, poly U, poly G, polyriboxanthinylic acid, and GTP, which, along with poly I, poly C , were tested previously (Brown *et al*, 1980; Brown and Goldstein, 1983).

[b] These molecules are expected to be four-stranded under the conditions of this assay, based on experiments reported in this work or in the literature (Rich, 1958; Gray and Bollum, 1974; Arnott *et al*, 1974; Chou *et al*, 1977; Zimmerman *et al*, 1975; Howard *et al*, 1977; Roy *et al*, 1979; Williamson *et al* 1989; Panyutin *et al*, 1990; Bonazzi *et al*, 1991; Guo *et al*, 1992a; Balagurumoorthy *et al*, 1992; Saenger, 1984).

[c] Ineffective inhibitors are defined as molecules which, at 25 μg/ml, decreased ^{125}I-AcLDL degradation by CHO[bSR-I] cells by less than 10% for single stranded molecules or less than 20% for double stranded molecules. These molecules are assumed to be single or double stranded under the conditions of this assay (Saenger, 1984; Sklenar and Feigon, 1990).

[d] Sequences of oligonucleotides: dAP1, 5'ATCCCACTGTGCCAT3'; oSP24, 5'AGAAATGGGCCTGAGTG3'; TH1 (Sklenar and Feigon, 1990), 5'GAGAGAACCCCTTCTCTCTTTCTCTCTT3'; R101, 5'GTCAGTCGACACACTAGTTCAGACGTAGTGTCTGATGAGGCCGAAAGGCCGAA ACGCGATCGGCATAACGCGTGGATCCTAGC3'

Chapter 2 - Addendum

While the studies presented here were in press, Doi *et al* (1993) proposed a model for poly(G) binding to the SR-As in which a single stranded form of the polynucleotide ligand wraps around the collagen triple helix of the receptor such that the phosphodiester backbone lies in a channel formed by the lysine residues of three consecutive Gly-X-Y repeats. The results presented in this chapter clearly negate this model by demonstrating that the quadruplex structure is required for the binding of homopolymeric oligo- and polynucleotides to bSR-AI and bSR-AII. This serves as a good reminder of the fact that, while model building can be quite informative, it must take into account the known properties of the various components of the system being modeled, and it must be backed up by experimental data.

How can the binding specificity of the SR-As for quadruplex nucleic acids be explained? dA_{37} is not significantly degraded during the course of these assays and inhibiting the RNAse mediated degradation of poly(C) and Li^+-poly(I) does not confer scavenger receptor inhibitory activity on these molecules. Thus, the nuclease resistance of quadruplex nucleic acids (unpublished observations) relative to single stranded and double stranded nucleic acids can not account for the observed results. In our original publication, we proposed that "the spatial distribution of the negatively charged phosphates in polynucleotide quadruplexes may form a charged surface which is complementary to the positively charged surface of the collagenous, ligand-binding domain of the scavenger receptor" (Pearson *et al*, 1993). Whatever this structure is, it might not be formed by a linear determinant in the quadruplex, but rather, might be formed by phosphate groups from multiple strands of the quadruplex. This seems reasonable since the phosphate backbone structure of any single strand in the quadruplex is fairly similar to that of any single strand in B-form DNA duplexes (Smith and Feigon, 1992; Kang *et al*, 1992; Cheong and Moore, 1992; Miura and Thomas, 1995) while the relative arrangement of the adjacent backbones differs in these two forms.

The binding of nucleic acids to SR-As may require a rather specific phosphate group conformation which the quadruplex structure can provide. Alternatively, within certain limits, the phosphate groups may be able to adopt a variety of conformations all of which are loosely complementary to the receptor's ligand binding domain. This possibility is suggested by the finding that both parallel and anti-parallel quadruplexes can inhibit bSR-AI activity with approximately equal efficacy, even though their spatial distributions of phosphate groups differ significantly from each other. Thus, the different phosphate group conformations displayed by various quadruplex structures might all fall within a set of negative charge distributions which are adequate for high affinity binding.

The parameters which define this set of spatial distributions might be governed by the length and flexibility of the lysine residues in the three consecutive Gly-X-Lys repeats which form the collagenous ligand binding domain of the SR-As. The extended side chains of these residues project away from the collagen helix and may not be involved in strong interactions within the protein. Thus, they may have a large

amount of free rotation which would allow them to occupy a variety of different spatial positions around the collagen helix. Quadruplex nucleic acid structures may cluster enough phosphate groups close enough together to allow for multiple and specific interactions with these lysines. Since the lysine side chains may be relatively flexible, a variety of different quadruplex structures, all of which bring multiple phosphates into close proximity, would be capable of binding. Duplex structures, on the other hand, may not bring enough phosphate groups close enough together to allow for multiple interactions with the lysine side chains. Indeed, the distances separating adjacent strands of either parallel or anti-parallel quadruplex's are as short, and usually shorter, than the distance which separates adjacent strands across the minor groove of duplex DNA (Kang *et al*, 1992; Smith and Feigon, 1992; Wang and Patel, 1993; Miura and Thomas, 1995; Patel and Wang, 1995). It should be noted that there are conserved negatively charged residues in the SR-As, and that these may in some manner modulate ligand binding specificity (Ashkenas *et al*, 1993).

The model I have suggested is rather different from the conventional view of ligand-receptor interactions, in which ligand binding requires very specific interactions between ligand and receptor that are susceptible to minor alterations in the structure of either the ligand or the receptor (Creighton, 1984). This is not necessarily surprising since Class A scavenger receptors (and for that matter, class B and C SRs as well), with their broad ligand binding specificities, are rather different than most other receptors. The interactions between the SR-As and their ligands may not be strictly specified, and receptor-ligand complementarity in this system may be of a somewhat flexible and dynamic nature. This model could explain the promiscuous binding properties of the SR-As: all of the various SR-A ligands may form structures in which negatively charged moieties are constrained within the set of spatial distributions defined by the positions that the SR-A collagen domain lysine side chains can occupy in space. Indeed, M-BSA, all of the nucleic acid ligands, and at least some of the polysaccharide ligands (Millane *et al*, 1988; Piculell and Rymdén, 1989) form ordered structures which place constraints on the relative distribution of negative charges. LPS aggregates can also form regular ordered structures (Hayter *et al*, 1987; Wang and Hollingsworth, 1996) which might restrict the distances separating individual LPS molecules to within the set of spatial distributions compatible with SR-A binding. This model might also explain the binding promiscuity of quadruplex forming oligonucleotides for a variety of different, structurally unrelated proteins (Stein, 1995), including CD36 and dSR-CI (see Chapter 1). By placing phosphate groups very close together, quadruplex nucleic acids may be able to complement a variety of different positive charge distributions displayed on protein surfaces.

A detailed comparison of the ligand binding capabilities and structures of different quadruplex oligonucleotides would be very useful in defining the set of negative charge spatial distributions which the SR-As require for ligand binding. This model for SR-A ligand binding predicts that a wide range of quadruplex structures could be accommodated within the SR-A ligand binding domain. An interesting test of this model would be to see if spatially different protein - nucleic acid contacts are

observed in the crystal structures of synthetic SR-A collagen domains (Tanaka *et al*, 1996). complexed with a variety of quadruplex ligands.

Recent findings suggest that binding of quadruplex DNA to scavenger receptors may be biologically significant (Kimura *et al*, 1994; Stacey *et al*, 1996). Bacterial DNA is highly immunogenic (Pisetsky, 1996; Stacey *et al*, 1996;), at least in part due to the common occurrence of unmethylated CpG dinucleotides which are rare in mammalian DNA (Krieg *et al*, 1995; Ballas *et al*, 1996). Macrophages and/or dendritic cells appear to mediate at least some of the immune responses to both bacterial DNA and CpG containing oligonucleotides (Stacey *et al*, 1996; Ballas *et al*, 1996). In studies using splenocyte cultures, it has been found that the immunogenicity of such oligonucleotides can be enhanced by embedding the CpG motifs within quadruplex forming guanine tracts (Kimura *et al*, 1994; Ballas *et al*, 1996). Interestingly, this increased immunogenicity is completely eliminated by the addition of scavenger receptor ligands to the assay medium (Kimura *et al*), suggesting that scavenger receptors may be involved in this process. The role of the quadruplex structure may simply be to enhance the cellular uptake of the oligonucleotides, and that the CpG motifs in these oligonucleotides then stimulate the immune system via scavenger receptor independent mechanisms (Peyman *et al*, 1995). Consistent with this hypothesis, it has recently been demonstrated that maleylation of proteins, which converts them to SR ligands, enhances their ability to elicit antibody and T cell responses in the absence of adjuvant *in vivo*, and enhances their presentation to T cells by macrophages *in vitro* (Abraham *et al*, 1995). Alternatively, it has been suggested that quadruplex DNA itself may be immunogenic or may potentiate the immune response (Pisetsky and Reich, 1993 ;Pisetsky, 1996), although whether this immunogenicity is due to binding to any of the molecularly identified scavenger receptors is unknown.

In the introduction to this chapter, a mention was made of some studies I performed using phosphorothioate oligodeoxyribonucleotides. During attempts to use scavenger receptors to facilitate the uptake of antisense oligonucleotides coupled to quadruplex forming sequences, I chose to use phosphorothioate oligonucleotides because of their greater nuclease resistance relative to phosphodiester oligonucleotides (Stein, 1995). In initial experiments, it was found that the oligonucleotide dBGTAS1 was an excellent inhibitor of bSR-AI activity (Figure 2A-1). This oligonucleotide comprises 15 phosphorothioate-linked residues followed by 27 phosphodiester-lined residues which include a stretch of 12 guanines. Unexpectedly, the control oligonucleotide dBAS1, which comprises only the first 15 phosphorothioate linked residues of dBGTAS1, was also a good inhibitor (Figure 2A-1). However, the oligonucleotide dAP1, identical in sequence to dBAS1 but having phosphodiester linkages, had no inhibitory activity. A second phosphorothioate, dAP2, also inhibited scavenger receptor activity. Phosphorothioate oligonucleotides appear to bind directly to the receptor, and not to the AcLDL ligand, since dBAS1 blocked the specific binding of a soluble form of bSR-AI to M-BSA beads (D. Resnick and M. Krieger, unpublished observations). Unlike normal phosphodiester bonded oligonucleotides, the

phosphorothioates do not require oligomerization in order to bind to bSR-AI, since dBAS1 is single stranded in solution as determined by FPLC analysis (not shown).

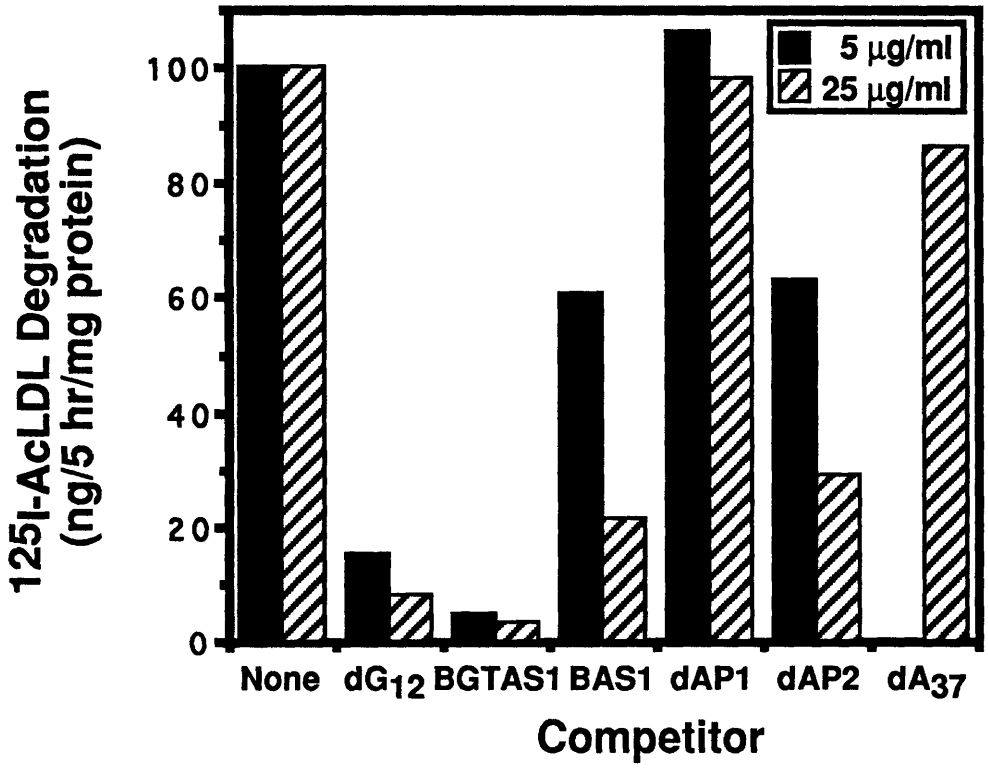
The apparent ability of non-quadruplex-forming phosphorothioate oligonucleotides to bind to bSR-AI is surprising. Interestingly, recent studies have reported that phosphorothioates can bind with high affinity to a large number of structurally unrelated proteins, all of which have regions of high positive charge density (Wyatt *et al*, 1994; Stein, 1995; Guvakova *et al*, 1995; Benimetskaya *et al*, 1995; Beltinger *et al*, 1995). This binding ability is not due to the increased nuclease resistance of the phosphorothioates. Rather the phosphorothioate backbone is mechanistically required for the tight binding of these oligonucleotides to proteins (Wyatt *et al*, 1994). Thus, the phosphorothioate moiety appears to confer some distinctive property on nucleic acids which enables them to bind to a variety of proteins with a significantly higher affinity than their phosphodiester counterparts. Consistent with this conclusion, the phosphorothioate analog of dG12 has greater bSR-AI inhibitory activity than the phosphodiester analog (84.8% inhibition versus 57.1% at 5 μg oligonucleotide/ml, 93.0% versus 75.7% at 25 μg oligonucleotide/ml). The converse result has also been demonstrated: proteins interact more avidly with phosphorothioates which can form quadruplex structures than with phosphorothioates which cannot form such structures (Wyatt *et al*, 1994; Stein, 1995; Guvakova *et al*, 1995).

It is not clear why phosphorothioate oligonucleotides have such a strong affinity for numerous proteins. Two models have been suggested. In one model, the substitution of a sulfur atom for one of the non-bridging oxygen atoms of the backbone results in a more rigid oligonucleotide structure. Since this molecule has already lost a significant degree of entropy, the entropic cost of binding to a protein is not as great and high affinity binding is possible (Benimetskaya *et al*, 1995). The fact that phosphorothioate quadruplexes bind to numerous proteins with a significantly greater affinity than phosphodiester quadruplexes would seem to argue against this model, since quadruplexes are already very rigid structures. In the second model, the phosphorothioate backbone has a looser counterion shell than the phosphodiester backbone. Thus, it is easier to remove the counterions and to make ionic bonds between protein residues and the backbone (Cho *et al*, 1993). This model might account for the higher affinity of phosphorothioate quadruplexes relative to their phosphodiester analogs. Quadruplex structures may enhance the binding of phosphorothioate oligonucleotides because they change the mechanism of binding from one involving primarily non-specific electrostatic interactions between ligand and protein to one in which specific electrostatic interactions between ligand and protein moieties can occur due to the tighter packing of phosphorothioate groups. Regardless of the mechanism by which proteins bind to phosphorothioate oligonucleotides, this sequence-non-specific binding has been problematic for researchers attempting to use anti-sense oligonucleotides to modulate protein expression (Stein). The possibility of interactions with scavenger receptors must now be added to the list of potential causes which have been proposed for the non-sequence-specific effects of anti-sense phosphorothioate oligonucleotides (Stein, 1995).

Figure 2A-1. Phosphorothioate oligonucleotide inhibition of ^{125}I-AcLDL degradation by CHO[bSR-AI] cells.

CHO[bSR-AI] cells were plated on day 0 in medium B in 24-well dishes (60,000 cells/well). On day 2, the cells were incubated for 5 hours at 37°C in medium A containing 5μg protein/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated concentrations of oligonucleotides. Degradation of ^{125}I-AcLDL was measured as described in experimental procedures, and the amount of degradation relative to control (no additions), determined in from 1 to 3 experiments is indicated. The sequences of the oligonucleotides used are: dBGTAS1 (phosphorothioate/phosphodiester), $^{5'}$ATCCCACTGTGCCATGGGGGGGGGGGGTTATTATTATTATATA$^{3'}$; dBAS1 (phosphorothioate),$^{5'}$ATCCCACTGTGCCAT$^{3'}$; dAP1 (phosphodiester), $^{5'}$ATCCCACTGTGCCAT$^{3'}$; dAP2 (phosphorothioate), $^{5'}$CATCGTCATCTAGCC$^{3'}$.

Phosphorothioate Oligonucleotide Inhibition of ^{125}I-AcLDL Degradation by CHO[bSR-I] Cells



Chapter Three

Expression Cloning of dSR-CI, a Macrophage-Specific Scavenger Receptor from *Drosophila melanogaster*

Introduction

It has been over 100 years since Metchnikoff's observations of phagocytosis led him to propose the cellular theory of immunity (Metchnikoff, 1884). Since that time, numerous studies have shown that phagocytes, including circulating monocytes and tissue macrophages, neutrophils, and dendritic cells, play a key role in the adaptive immune system, both as effector cells and as activators and regulators of the immune response (Janeway, 1989, 1992; Matzinger, 1994; Ibrahim *et al*, 1995; Fearon and Locksley, 1996). In addition, monocytes, macrophages and neutrophils are key components of the innate, or non-adaptive, immune system which provides an important and immediate first line of host defense prior to the activation of the adaptive immune system (Gordon *et al*, 1988; Janeway, 1992; Fearon and Locksley, 1996). The ability of monocytes and macrophages to activate and regulate adaptive immune responses may be inseparable from their earlier participation in the innate immune response (Janeway, 1992; Ibrahim *et al*, 1995; Fearon and Locksley, 1996).

As discussed in Chapter One, macrophages and other phagocytic cells can recognize and bind pathogens directly (i.e., independent of pathogen opsonization), and this direct form of self/non-self discrimination may play a crucial role in the activation and regulation of both the innate and adaptive immune responses (Gordon *et al*, 1988; Janeway, 1989, 1992; Ibrahim *et al*, 1995). It has been proposed that the direct recognition of pathogens by phagocytic cells is mediated by non-clonally distributed "pattern recognition receptors" which exhibit broad ligand binding specificity for molecular structures common among microbial pathogens, but absent from the host (Janeway 1989, 1992; Ibrahim *et al*, 1995). In addition to activating the immune response, these pattern recognition receptors may also play a role as effector molecules in the immune response (Krieger *et al*, 1993; Krieger and Herz, 1994; Pearson 1996).

Both innate immunity and the key role of phagocytes in host defense appear to be evolutionarily ancient, predating the development in vertebrates of the adaptive immune system (Janeway, 1989, 1992; Fearon and Locksley, 1996). Indeed, invertebrate immunity, which comprises both humoral and cellular components (Rizki and Rizki, 1984; Lackie, 1988; Hultmark, 1993; Habicht, 1994; Hoffmann, 1995), is strikingly similar to vertebrate innate immunity (Hultmark, 1993; Habicht, 1994; Hoffmann, 1995). One of the most obvious similarities is the crucial role played by macrophages and other hemocytes (blood cells) during the invertebrate response to infection (Rizki and Rizki, 1984; Lackie, 1988; Iwanaga *et al*, 1992; Jomori and Natori, 1992; Söderhall, 1992; Hultmark, 1993; Habicht, 1994).

As with mammalian macrophages, the immune responses of invertebrate macrophages, and other hemocytes, are induced both by contact with intact microorganisms and by exposure to isolated microbial surface constituents, including LPS and the ß-glucan laminarin (Samakovlis *et al*, 1992; Iwanaga *et al*, 1992; Söderhall, 1992; Hultmark, 1993). The mechanisms by which invertebrate macrophages become activated in response to infection is not yet understood.

However, it is clear that in *Drosophila* and other invertebrates, macrophage activation induces the nuclear translocation of transcription factors homologous to the mammalian Rel/NF-κB family of transcription factors which play a crucial role in mediating vertebrate innate immune responses (Sha *et al*, 1995). Furthermore, many of the transcriptionally activated immune response genes contain upstream NF-kB binding sites which are essential for their activation (Sun and Faye, 1992; Engström *et al*, 1993; Kappler *et al*, 1993; reviewed in Hultmark, 1993 and Hoffmann 1995).

It has been proposed that, as in vertebrates, the direct recognition of pathogens and their surface constituents by invertebrate macrophages (Rizki and Rizki, 1984; Lackie, 1986) is mediated by pattern recognition receptors (Hultmark, 1993; Hoffmann 1995). Several invertebrate proteins have been proposed as candidate pattern recognition receptors (Sun *et al*, 1990; Iwanaga *et al*, 1991; Iwanaga *et al*, 1992; Jomori and Natori, 1992; Hultmark, 1993; Cerenius *et al*, 1994; Theopold *et al*, 1996). Included among these are the *Drosophila* protein 18-wheeler (Eldon *et al*, 1994; Eldon and Williams, 1996.), and a *B. mori* protein GNBP (Lee *et al*, 1996). Both 18-wheeler and its homolog, Toll, have leucine-rich repeats reminiscent of CD14, coupled to intracellular domains homologous to the IL-1 receptor which are involved in Rel transcription factor activation (Schneider *et al*, 1991; Rosetto *et al*, 1995). 18-wheeler has been shown to recognize non-self molecules and *18-wheeler* mutants are immune compromised, but a direct role in inducing an immune response has not yet been demonstrated for this protein. Interestingly, Toll has recently been found to play a direct role in the induction of immune responses in *Drosophila* , although it does not appear to act as a pattern recognition receptor in doing so (Lemaitre *et al*, 1996). Two additional Toll/18-w homologs have also recently been identified (Mitcham *et al*, 1996). GNBP is a secreted protein which has pattern recognition receptor like properties (Lee *et al*, 1996). Interestingly, it is also homologous to CD14, may have a GPI-anchored cell surface isoform, and cross-reacts with polyclonal CD14 antibodies. A homolog has also been detected in *Drosophila* (Lee *et al*, 1996). However, the involvement of GNBP in immune activation has not yet been explored.

Among the various mammalian proteins which may be pattern recognition receptors are the macrophage-specific class A scavenger receptors (see Chapter One). These receptors exhibit broad polyanionic ligand binding specificity (Brown and Goldstein, 1983; Krieger and Herz, 1994), and among their high affinity ligands are the bacterial surface constituents lipopolysaccharide (LPS) and lipoteichoic acid (Hampton *et al*, 1991; Krieger *et al*, 1993; Doi *et al*, 1993; Dunne *et al*, 1994;). However, as discussed in Chapter One, the actual non-pathological functions of the SR-As are, to a large extent, still unknown. Given the notion that innate immunity based on pattern recognition receptors might be evolutionarily ancient, it seemed reasonable to suggest that scavenger receptors might also exist in an invertebrate such as *Drosophila*. Indeed, Abrams and colleagues (1992) recently demonstrated that *Drosophila* embryonic macrophages and the macrophage-like *Drosophila* Schneider S2 cell line exhibit a scavenger receptor activity similar to that of the mammalian SR-As (Abrams *et al*, 1992).

To follow up on this observation, I set out to clone a *Drosophila* homolog of the mammalian SR-As. It was hoped that the identification of such a gene, when coupled with the power of *Drosophila* genetics, would yield insight into the normal functions of these SR-As. This chapter first describes my initial demonstration of S2 cell scavenger receptor activity which established the basis for the S2 cell studies published by Abrams and colleagues (1992). This is followed by a description of attempts to clone a scavenger receptor cDNA from S2 cells. Interestingly, rather than isolating a homolog of the mammalian SR-As, a new scavenger receptor gene, *dSR-CI*, was isolated. This chapter describes the characteristics of *dSR-CI*, and discusses the potential functions of this receptor in *Drosophila* host defense and development.

Portions of the work presented here were originally published in April, 1995 in *The Proceedings of the National Academy of Sciences, USA* (Pearson *et al*, 1995). Alison Lux performed the expression screening of cDNA pools in COS cells, and generated the CHO[dSR-CI] cell line. The 4 °C and 37 °C saturation binding experiments shown in Figure 3-4 were performed by Marsha Penman and Shangzhe Xu. Under my supervision, Shangzhe Xu also performed some of the CHO[dSR-CI] competition experiments shown in Figures 3-5 through 3-8, and most of the sequencing of the dSR-CI cDNA clone. All other work presented in this chapter was performed by me. The original publication is included in this thesis as Appendix B.

Experimental Procedures

cDNA Library Construction and Expression Cloning of *dSR-CI*: Poly A+ RNA (30 μg) was prepared from *Drosophila* S2 and Kc_0 cells as described (Libermann *et al*, 1987), except that cells were homogenized and DNA was sheared with a Brinkmann Polytron disrupter (PT10S probe, three times 5 s, setting 4). cDNAs, synthesized from S2 cell mRNA using an oligo(dT) primer, were ligated to phosphorylated BstXI linkers (pCTTT AGAGCACA and pCTCTAAAG (Research Genetics)), size selected into >1.5 kbp and >2.0 kbp fractions, and ligated into the BstXI site of the expression plasmid pcDNAI (InVitrogen) (Aruffo and Seed, 1987). MC1061/p3 cells (InVitrogen) were transformed with this library by electroporation and DNA pools (1600 - 4000 clones/pool) were transfected into COS-M6 cells. The transfected cells were visually screened for uptake of fluorescent DiI-AcLDL (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate labeled acetylated low density lipoprotein), and a single clone (S0o24.11c) was isolated from one positive pool, all as previously described (Lin *et al*, 1991; Acton *et al*, 1994). This clone, designated pdSR-CI, was sequenced on both strands using the Sequenase 2.0 (US Biochemicals) and the Gibco-BRL PCR-based sequencing kits. Sequence comparisons and database surveys were performed using the GCG Sequence Analysis Software Package (versions through 7.3) (Devereux *et al*, 1984), and BLAST from NCBI (Altschul *et al*, 1990).

Southern Screening of S2 cDNA Library: 1 μg of DNA from each cDNA pool analyzed was doubly-digested with HindIII and XbaI, electrophoretically separated on a 1% agarose gel, and transferred to Genescreen membranes (Dupont NEN) following standard protocols. The filters were hybridized to ^{32}P random prime labeled (Sambrook *et al*, 1989) probes at 50 °C overnight in 0.5 M phosphate buffer, 1 mM EDTA, 7% SDS, 1% BSA, 100 μg/ml denatured salmon sperm DNA. The filters were then washed six times at 50 °C - 54 °C in 0.3 M phosphate buffer, 1mM EDTA, 0.5%-1.0% SDS (0.5% BSA included in first two washes). The following probes, generously provided by A. Karson, were used: 1) AK817, generated by PCR amplification of the first 817 coding base pairs of mSR-AI, encoding all but the collagen and SRCR domains of this protein; 2) AKN1A, generated by StyI/XbaI digestion of the mSR-AI cDNA clone, comprising that portion of the cDNA encoding the collagen and SRCR domains, plus a small portion of the mSR-AI 3' UTR (Ashkenas *et al*, 1993).

Northern Analysis: Northern blot analysis of S2 and Kc_0 poly A+ RNA (Sambrook *et al*, 1989) was performed using a random primed labeled PCR amplified, gel purified, full-length dSR-CI cDNA probe. Developmental Northern analysis of dSR-CI expression in strain *Canton S* was performed using a mixture of gel purified, random primed labeled probes 1101p (generated by PCR amplification of the 5' terminal 1101 bps of the dSR-CI cDNA) and 428p (generated by PCR amplification of the 3' terminal 428 bps). An elongation factor 1 α probe was used for normalization of mRNA loading. The developmental Northern blot was generously provided by Patrick McCaw.

In situ Hybridization to *Drosophila* Embryo's: Clone p6-5, containing the 5' terminal 815 bps of the dSR-C1 cDNA, was constructed as follows: pdSR-CI was doubly-

digested with EcoRV and XbaI and blunted with Klenow, and the large vector-containing fragment was purified and re-circularized. Two digoxygenin labeled RNA probes were generated from ApaI (sense strand) and SpeI (antisense strand) digested p6-5 DNA by *in vitro* transcription from the T7 (sense) and SP6 (antisense) promoters using DIG-dUTP (Genius 2.0 DIG RNA labeling kit, Boehringer Mannheim). *Canton S* embryo's (0-16 hr) were collected and processed, *in situ* hybridizations were performed, and embryos were staged, as previously described (Campos-Ortega and Hartenstein, 1985; White *et al*, 1994).

Cell Culture: Cells were maintained at 37 °C in a humidified 5% CO_2/95% air incubator (mammalian cells), or at 25 °C in tightly capped flaskettes (*Drosophila* cells). Wild type CHO cells were grown in medium A (Hams F-12 supplemented with PSG (100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine), and 5% (v/v) FBS (fetal bovine serum)). COS-M6 cells were grown in medium B (DME supplemented with PSG and 10% (v/v) FBS). *Drosophila* S2 cells were grown in medium C (Schneider's media supplemented with PSG and 10% heat-inactivated FBS), and *Drosophila* Kc_0 cells were grown in medium D (D22 medium supplemented with PSG minus glutamine). CHO[mSR-AII] cells, which express murine SR-A type II (Ashkenas *et al*, 1993), were grown in medium E (medium A containing 0.5 mg/ml geneticin (G418, Gibco)). A stable transfectant (clone 2.6a) expressing dSR-CI, designated CHO[dSR-CI], was isolated by transfecting 1 x 10^6 CHO cells with 0.5 µg pSV2neo and 9.5 µg pdSR-CI using the polybrene method (Sambrook *et al*, 1989; Penman *et al*, 1991), selecting transfectants in medium E and cloning a receptor positive (uptake of 1 µg protein/ml DiI-AcLDL) colony by dilution plating (Penman *et al*, 1991)

Assays and reagents: Scavenger receptor activities in mammalian cells at 4 °C (measured in 6-well dishes) and 37 °C (24-well dishes) and in *Drosophila* S2 cells at 25 °C were determined as previously described (Abrams *et al*, 1992; Ashkenas *et al*, 1993; Pearson *et al*, 1993; Chapter 2), with minor modifications for S2 cells. Briefly, after incubation of S2 cells for 5 hours at 25 °C with medium containing ^{125}I-AcLDL, the cells were washed two times rapidly with BSA/Tris-Cl buffer, once slowly (10 minutes) with the same buffer, and then once rapidly with Tris-Cl buffer . Cell surface binding of ^{125}I-AcLDL (2 hours, 4 °C), and its binding plus internalization (5 hours, 37 °C), are expressed as ng cell associated ^{125}I-AcLDL protein per mg cell protein. Degradation activity is expressed as ng ^{125}I-AcLDL protein degraded in 5 hours per mg cell protein. Protein determination was by the method of Lowry (Lowry *et al*, 1951). For competition experiments, cells were incubated with ^{125}I-AcLDL for 5 hours in the absence (triplicate incubations) or presence (duplicates) of competitors during the assay. Stock solutions of competitors (e.g., laminarin (Sigma)) were prepared in Dulbecco's complete phosphate buffered saline (at 4 - 10 mg/ml). These and other reagents (e.g., sodium butyrate from Pfaltz and Bauer) were obtained and/or prepared as indicated or as previously described (Abrams *et al*, 1992; Pearson *et al*, 1993; Acton *et al*, 1994; Rigotti *et al* 1995). Phosphatidylserine vesicles were generously provided by A Rigotti.

Results

Initial experiments reported elsewhere (Abrams *et al*, 1992) indicated that embryos from the Canton S strain of *Drosophila melanogaster* exhibit scavenger receptor like activity. To facilitate the analysis of this activity, the *Drosophila* S2 cell line (Cherbas *et al*, 1994), was examined for scavenger receptor activity. As shown in Figure 3-1, this cell line exhibits specific degradation and binding plus uptake activities for the classical scavenger receptor ligand ^{125}I-AcLDL. These scavenger receptor activities were high affinity, saturable processes, having K_m values of approximately 3.0 and 5.0 μg protein/ml, respectively. Subsequent studies reported elsewhere have confirmed and extended these results, and have demonstrated that the Kc_0 embryonic cell line lacks this scavenger receptor activity (Abrams *et al*, 1992).

Two approaches were taken to identify the gene responsible for the macrophage scavenger receptor-like activity in *Drosophila* S2 cells. In the first approach, a cDNA expression library was prepared from S2 cell poly A+ mRNA, divided into small pools, and screened for SR-AI homologs by low stringency Southern hybridization using probe AK817, as described in Experimental Procedures. In this way, over 40 pools were identified which gave hybridization signals, 19 of which are shown in Figure 3-2. Some of these pools contained cDNAs which gave very strong hybridization signals in this experiment. Ten of these positive pools were chosen for subsequent analysis using the non-overlapping 5' and 3' mSR-AI probes AK817 and AKN1A. The two probes hybridized to similarly sized fragments in four of the pools (data not shown). Although these cDNA pools have not yet been further analyzed, the results obtained thus far suggest that there may indeed be *Drosophila* homologs of the mammalian class A scavenger receptors.

In the second approach, COS-M6 cells were transfected with the cDNA pools and visually screened for endocytosis of fluorescent AcLDL (DiI-AcLDL). A single receptor-positive pool was identified (~350,000 clones screened) and was subdivided repeatedly to obtain a single functional plasmid (designated pdSR-CI for plasmid encoding *Drosophila* scavenger receptor Class C, type I; (for nomenclature, see Acton *et al*, 1994). Northern blot analysis of poly A+ RNA demonstrated that the level of the dSR-CI message (~2.1 kb) was at least 50-fold higher in S2 cells than in Kc_0 cells (Figure 3-3). As the expression cloning strategy yielded a scavenger receptor cDNA, the homology cloning approach was discontinued.

A CHO cell line stably transfected with pdSR-CI was generated. These cells, called CHO[dSR-CI] cells, exhibited high affinity, saturable ^{125}I-AcLDL binding at 4° C (K_d ~ 2 μg protein/ml, Figure 3-4A) and, at 37° (Figure 3-4B), high affinity and saturable binding plus internalization (open circles) and degradation (solid squares, K_d ~5.5 μg protein/ml). The degradation of bound and internalized ^{125}I-AcLDL by CHO[dSR-CI] cells, which was chloroquine-sensitive (not shown) and thus presumably lysosomal, was more efficient than that in S2 cells (Abrams *et al*, 1992). This difference may be

due to differences in the assay conditions (e.g., temperature, media) or in the fundamental properties of the cultured cells. The affinity of dSR-CI for ^{125}I-AcLDL was comparable to the affinities of the receptors in *Drosophila* S2 cells and of mammalian SR-A (Kodama *et al*, 1990; Rohrer *et al*, 1990; Abrams *et al*, 1992;). Thus, CHO[dSR-CI] cells express a scavenger receptor-mediated endocytic pathway which is similar to those of S2 cells and mammalian macrophages (Brown and Goldstein, 1983; Kodama *et al*, 1990; Rohrer *et al*, 1990; Abrams *et al*, 1992; Krieger and Herz, 1994).

A hallmark of SR-A and S2 cell scavenger receptors is their broad polyanion binding specificity, usually assessed by measuring inhibition of ^{125}I-AcLDL binding, and subsequent uptake and degradation (Brown and Goldstein, 1983; Krieger *et al*, 1993). Using such an assay, it was found that numerous SR-A polyanionic ligands, in addition to AcLDL itself, were effective inhibitors competitors of ^{125}I-AcLDL degradation by both CHO[dSR-CI] and S2 cells (Figure 3-5). These included the modified protein M-BSA, the four-stranded polynucleotides poly(I) and dA_5G_{37}, and the polysaccharide dextran sulfate. At concentrations of 400 μg/ml (100 μg/ml for dA_5G_{37}) all of these molecules reproducibly inhibited scavenger receptor activity by > 85% in both cell types. Furthermore, M-BSA, poly (I), dA_5G_{37}, and dextran sulfate were all high affinity competitors for both cell types: their concentrations which gave half-maximal inhibition ranged from ~0.1 to 5 μg/ml (for example, Figure 3-6). As with SR-A, single stranded dA_{37} (100 μg/ml), unmodified LDL and BSA (400 μg/ml) and phosphatidylserine vesicles (500 μg/ml) did not compete (< 15% inhibition). Surprisingly, poly D-glutamic acid inhibited dSR-CI (see below), although it is not an SR-A inhibitor (Brown and Goldstein, 1983; Krieger and Herz, 1994). These results suggest that expression of dSR-CI could account for at least some of the S2 cell-scavenger receptor activity.

While there were many similarities in the scavenger receptor activities of CHO[dSR-CI] and S2 cells, two notable differences were observed. First, the apparent K_i values for poly(I) and dA_5G_{37} were ~10-fold lower for CHO[dSR-CI] than S2 cells (Figure 3-6). Second, poly D-glutamic acid inhibited both cell types with relatively high affinities (apparent K_i's: CHO[dSR-CI] - 7 μg/ml, S2 - 18 μg/ml), but with substantially different maximal levels of inhibition (measured using 400 μg/ml) (Figure 3-7). While poly D-glutamic acid inhibited most of the ^{125}I-AcLDL degradation by CHO[dSR-CI] cells (80%), it inhibited only about 20% of the activity in S2 cells. These disparities between the CHO[dSR-CI] and S2 cells may be due to differences in the assay conditions or in the properties of the receptors expressed in dissimilar cells from different species. Alternatively, they raise the possibility that S2 cells may be like mammalian macrophages (Krieger and Herz, 1994) and express multiple types of scavenger receptors, some of whose specificities may differ from that of dSR-CI (e.g., insensitive to poly D-glutamic acid).

Because of the broad polyanion specificity of CHO[dSR-CI] and S2 cell scavenger receptor-mediated ^{125}I-AcLDL degradation, it was surprising to find that laminarin, an uncharged, ß1-3 glucose polymer, was also a highly effective inhibitor of

both the CHO[dSR-CI] and S2 cell scavenger receptor activities (Figure 3-8, apparent K_i's of ~ 2 and 8 µg/ml, respectively). In contrast, dextran (400 µg/ml), another uncharged glucose polymer, did not inhibit ^{125}I-AcLDL degradation (not shown). Laminarin does not appear to inhibit dSR-CI activity by binding to the AcLDL ligand, since it has virtually no effect on the degradation of ^{125}I-AcLDL by CHO cells expressing bovine SR-AII (data not shown). The precise mechanism of laminarin inhibition of dSR-CI activity and its relationship to laminarin induced immune responses in cultured *Drosophila* cells (Samakovlis *et al*, 1992) have not yet been established.

The 2032 bp dSR-CI cDNA (Figure 3-9; Genbank Accession #U17693) encodes a 629 residue polypeptide (Figure 3-9B). The cDNA has a 39 bp 5' untranslated region (UTR) (Figure 3-9A) containing a sequence (ACCGT) which closely matches the canonical *Drosophila* transcription initiation start site (Cherbas and Cherbas, 1993). The presence of this sequence and the estimated message size of 2.1 kb (Figure 3-3) suggests that the cDNA clone is probably close to full length. The 5' UTR also contains an in frame stop codon 15 bps upstream of the putative initiator methionine codon. The 106 bp 3' UTR (Figure 3-9A) contains a polyadenylation signal 84 bps downstream of the termination codon. The predicted dSR-CI protein is a multidomain type I transmembrane protein (Figure 3-9C) which has no significant homology to the mammalian SR-A or SR-B molecules (Ashkenas *et al*, 1993; Acton *et al*, 1994). Its N-terminal 20 residues represent a putative signal sequence (see also Chapter 4) which is followed by a 609 amino acid (67.6 kD) mosaic protein comprising nine domains with six potential N-linked glycosylation sites (underlined in Figure 3-9B). Domains I (54 residues) and II (53 residues) (Figure 3-9B) are members of the complement control protein (CCP) family of domains (Reid and Day, 1989). Their sequences conform to the overall CCP consensus sequence (69% and 65% identities, respectively) about as well as other randomly selected CCP sequences (not shown). Over 160 CCP domains have been found in more than 30 proteins, including many complement proteins (e.g., C1r, C2, DAF, CRI), and other proteins in vertebrates (e.g., clotting factors, selectins (Bevilacqua and Nelson, 1993), proteoglycans (Yamada *et al*, 1994)) and invertebrates (the *Drosophila hikaru genki* gene product (Hoshino *et al*, 1993) and *Limulus* Coagulation Factor C (Iwanaga *et al*, 1992)). In many cases, these ~60 residue domains participate directly in binding interactions with other proteins.

Domain III (185 residues) is the first known invertebrate member of the MAM (for Meprin, A5 antigen and receptor protein tyrosine phosphatase Mu) family of extracellular domains (Beckmanm and Bork, 1993). This domain appears to modulate homophilic binding interactions between receptor protein tyrosine phosphatases (Zondag *et al*, 1995). A 25 residue spacer (domain IV) separates the MAM domain from domain V, which is a 48 amino acid somatomedin B-like domain (Baughman *et al*, 1992). This motif was first described as a fragment of the extracellular matrix molecule vitronectin, in which it acts as the binding site for plasminogen activator inhibitor-1 (Deng *et al*, 1996).

Domain VI is a 129 residue Ser/Thr-rich domain. Threonine and serine comprise 55% and 12%, respectively, of all amino acids in this domain. Within an 87 residue sub-region (positions 381 - 467), they account for 79% of all residues, while Lys, Arg, and Pro compose all but one of the other 18 residues. This domain is thus reminiscent of both vertebrate and *Drosophila* mucins (Martin *et al*, 1988; Toribara *et al*, 1993). However, unlike the mucins, domain VI contains no identifiable internal repeat units at the DNA or protein sequence levels. By analogy with the mucins and other cell surface proteins, such as the LDL receptor, this domain is presumably heavily O-glycosylated and highly extended (Jentoft, 1990). Indeed, Western analysis indicates that, in contrast to its predicted unmodified molecular weight of 68 kD, in S2 cells this protein has a molecular weight of approximately 170 kD in S2 cells (D. Trigatti, S. Xu, A. Pearson and M. Krieger, unpublished observations). Furthermore, the protein is sensitive to both N-glycanase and O-glycanase treatments, indicating that it contains N-linked and O-linked oligosaccharides. In fact, the protein is heavily O-glycosylated, since O-glycanase treatment alone results in a substantial decrease in its mass to 95 kD (D. Trigatti, S. Xu, A. Pearson and M. Krieger, unpublished observations).

The remainder of the protein is composed of a 29 residue spacer segment (domain VII), a 22 residue putative transmembrane domain (domain VIII), and a 64 residue cytoplasmic domain (IX), none of which show significant sequence similarity to other proteins. The cytoplasmic domain contains several potential sites for phosphorylation by various kinases (Figure 3-9B) (Feramisco *et al*, 1980; Glass and Smith, 1983; Woodgett *et al*, 1986; Pinna, 1990).

To begin to explore the physiological functions of *dSR-CI*, *in situ* hybridization was used to examine its expression during *Drosophila* embryonic development (Figure 3-10). Throughout the stages of development examined, the expression pattern of dSR-CI was essentially identical to the distribution of hemocyte/macrophages (Nelson *et al*, 1994; Tepass *et al*, 1994). For example, dSR-CI expression was first seen during developmental stage 10 (4-5 hours after egg laying (ael)) in the procephalic mesoderm (panel A), which gives rise to all of the embryonic hemocytes (Tepass *et al*, 1994). During stage 11 (5-7 hours ael, panel B), the stained cells appeared to migrate posteriorly into the gnathal buds and into the tail end of the germ band (which is adjacent to the head due to germ band elongation), and anteriorly into the head and clypeolabrum. During stage 12 (7-9 hours ael), germ band retraction carried the tail region cells to the posterior end of the embryo (panel C). In addition, dSR-CI expressing cells appeared to migrate both posteriorly and anteriorly along the ventral and dorsal surfaces of the ventral nerve cord (not shown), so that by late stage 12 (panel C), punctate single cell staining could be seen in the grooves of the ventral nerve cord. By stages 13/14 (10-11 hours ael, panel D), stained cells appeared to have migrated not only throughout the head and tail regions of the embryo, but also around the gut. By stage 11, some dSR-CI positive cells were found in cavities (panel E), where hemocyte/macrophages accumulate (Abrams *et al*, 1993; Tepass *et al*, 1994). In later stages, stained cells, rather than being integrated into defined tissues, were found scattered throughout the hemocoel. Here, macrophages

both deposit extracellular matrix (Nelson *et al*, 1994) and phagocytose the apoptotic cells which begin to appear during stage 11 (Campos-Ortega and Hartenstein, 1985; Abrams *et al*, 1993; Tepass *et al*, 1994). Expression of dSR-CI precedes the onset of apoptosis in stage 11 (Tepass *et al*, 1994). Finally, it was observed that dSR-CI was expressed in multi-vesicular macrophage-like cells, which presumably contained apoptotic corpses (panel F and Abrams *et al*, 1993; Tepass *et al*, 1994). These results demonstrate that dSR-CI expression in embryos is primarily, if not exclusively, restricted to hemocyte/macrophages. Occasionally unstained cells with a macrophage-like morphology were observed. It is not clear whether this was due to low sensitivity of the staining assay or to the presence of a distinct population of dSR-CI-negative macrophages.

Developmental Northern analysis was undertaken in order to determine the pattern of dSR-CI expression during *Drosophila* development (Figure 3-11A). The results of this analysis demonstrated that the dSR-CI message is first expressed in 2-5 hour embryo's, in agreement with the *in situ* results above. dSR-CI mRNA expression is subsequently maintained throughout development, with a significant increase in expression during the early pupal stage (Figure 3-11B). The dSR-CI message is also expressed in adults, although at lower levels. Interestingly, however, there is a very high level of expression in adult heads. It is not yet known which cell types in the late embryonic and post-embryonic stages express the dSR-CI message.

Discussion

Previous work clearly demonstrated the existence of receptors in *Drosophila* having properties which are strikingly similar to the mammalian class A scavenger receptors (Abrams *et al*, 1992). Scavenger receptor activity was found both in *Drosophila* embryos and in the S2 cell line (Figure 3-1; Abrams *et al*, 1992). Therefore, a cDNA expression library was constructed from S2 mRNA in order to clone the scavenger receptor gene responsible for this activity. Two cloning strategies were followed: 1) functional cloning was used to isolate a cDNA which would confer scavenger receptor activity on an otherwise receptor negative mammalian cell line, and 2) homology cloning was used to isolate SR-AI homologs. Expression cloning yielded a scavenger receptor cDNA first, and so the homology cloning strategy was discontinued. While a scavenger receptor gene has been successfully cloned, it is not yet known whether it is responsible for any or all of the scavenger receptor activity which has previously been observed. Resolution of this issue awaits the generation of flies lacking a functional dSR-CI gene product.

The newly identified *Drosophila* scavenger receptor exhibits a broad ligand binding specificity for polyanionic ligands (Figures 3-4 and 3-5). In addition, it appears to recognize at least one microbial surface constituent, laminarin (Figure 3-8). The dSR-CI message is expressed specifically in embryonic hemocytes until at least 11 hours of development (stage 13/14) (Figure 3-10). The dSR-CI message is expressed throughout all subsequent stages of development, and is highly expressed in the adult head (Figure 3-11). Although the cell types expressing dSR-CI in the head remain to be determined, they may be glial cells since: 1) in mammals, certain microglia share a common origin with macrophages (Theele and Streit, 1993), and 2) in *Drosophila* embryos, certain microglia (the subperineurial glia) are derived from the mesoderm rather than from the neuroectoderm, as was previously believed (Edwards *et al*, 1993; Ito *et al*, 1995). These particular glial cells are not yet present in the embryos examined for dSR-CI expression.

In both its ligand binding properties and its expression pattern, the newly identified *Drosophila* scavenger receptor is very similar to the mammalian class A scavenger receptors. However, the *Drosophila* receptor defines a new class of scavenger receptors, the class C receptors, which share absolutely no homology with the SR-As. While this may be striking case of convergent functional evolution, it is also possible that class A and class C receptors exist in both mammals and invertebrates. At least for the class B scavenger receptors, homologs have been identified in organisms as divergent as mammals, *Drosophila* and *C. elegans* (Hart and Wilcox, 1993; Endemann *et al*, 1993; Acton *et al*, 1994; Franc *et al*, 1996; Genbank accession number 728534). The inability of poly D-glutamic acid to fully inhibit the S2 cell scavenger receptor activity (Figure 3-7) suggests that dSR-CI may not be the only scavenger receptor expressed by these cells. Given the preliminary results from the homology screens of the S2 cell cDNA library, the putative additional receptors may include an SR-A homolog.

The *dSR-CI* gene encodes a multi-domain putative transmembrane protein (Figure 3-9). Disregarding those regions of the protein which seem to be spacers or transmembrane segments, only the cytoplasmic domain of dSR-CI is nondescript. All of the other domains have homologs in other mammalian and invertebrate proteins. Interestingly, three of these domains (CCP, MAM, and mucin) are adhesive domains in mammalian proteins (Springer, 1994; Li *et al*, 1994; Zondag *et al*, 1995). The serine/threonine-rich mucin domain VI appears to be heavily glycosylated and, based on analogy with the vertebrate mucins (Jentoft, 1990), may extend more than 320 Å from the cell surface. This would significantly project the N-terminal domains (I-V) out into the extracellular space, potentially facilitating their interactions with ligands.

The function(s) of dSR-CI remain to be determined by genetic, physiological, and biochemical studies. However, based on what is already known about the dSR-CI and about *Drosophila* biology, several hypotheses can be formulated.

As with the mammalian scavenger receptors, the broad polyanionic binding specificity of dSR-CI, coupled with its mosaic structure and hemocyte/macrophage-specific expression, make it an attractive candidate for a pattern recognition receptor which can help confer the polyspecificity and self/nonself discrimination required for innate immunity in *Drosophila* (Janeway, 1992; Abrams *et al*, 1992; Hultmark, 1993; Krieger *et al*, 1993; Hoffman, 1995; see also Chapter One). In support of this hypothesis is the finding that dSR-CI recognizes the ß-glucan laminarin (Figure 3-8), which is a potent stimulator of the Drosophila immune response (Samakovlis *et al*, 1992). Furthermore, dSR-CI protein expression appears to be upregulated in the *Drosophila* mbn-2 hemocyte cell line upon LPS and laminarin treatment (D. Trigatti, A. Pearson and M. Krieger, unpublished observations). It is conceivable, but still wholly untested, that dSR-CI might cooperate with other putative pattern recognition receptors, such as 18-wheeler and GNBP (see above), during the immune response.

The presence of CCP domains in dSR-CI places this receptor in a superfamily of proteins, many of which are involved in both vertebrate and invertebrate host defense. In mammals, CCP domains serve to mediate interactions between C3, the central component of complement, and many C3 receptors and complement regulatory proteins (Reid and Day, 1989). This suggests that, in addition to its potential capability to recognize pathogens directly, dSR-CI might also be able to recognize pathogens which have been opsonized by invertebrate α-2-macroglobulin, a protease inhibitor which is both structurally and functionally ancestral to C3 (Sottrup-Jensen *et al*, 1985; Armstrong *et al*, 1992; Melchior *et al*, 1995). Likewise, dSR-CI might recognize pathogens opsonized by products of the prophenoloxidase melanization cascade, a complement-like serine protease cascade important to insect and crustacean immunity (Ashida, 1990; Söderhall, 1992). Indeed, studies of the *Manduca sexta* and *Drosophila* prophenoloxidase genes have demonstrated structural and functional similarities between these proteins and C3 (Hall *et al*, 1995b; Fujimoto *et al*, 1995; Beck *et al*, 1996).

Subsequent to recognizing pathogens by one or more of these mechanisms, dSR-CI may participate as a major effector molecule in the immune response. Several effector functions for dSR-CI can be envisioned. Most obviously, by binding to pathogens, dSR-CI may facilitate their phagocytic clearance from the host. Additionally, dSR-CI might participate in clot formation when the infectious load is too great for efficient phagocytic clearance (Rizki and Rizki, 1984; Lackie, 1986; Iwanaga *et al*, 1992; Söderhall, 1992; Hultmark, 1993; Hoffmann, 1995) by recognizing some of the secreted adhesion molecules and clotting factors which are known to bind to both pathogens and hemocyte membrane receptors (Söderhall, 1992; Iwanaga *et al*, 1992). For example, as described earlier, dSR-CI might recognize invertebrate C3-like clotting factors whose existence is suggested by the existence of both α-2 macroglobulin and the presence of CCP domains in *Limulus* coagulation Factor C (Iwanaga *et al*, 1992; Armstrong *et al*, 1992) . Further, since invertebrate lipoproteins bind to microbial surface constituents and are hemolymph clotting factors (Lackie, 1988; Hall *et al*, 1995a), dSR-CI could participate in clot formation by acting as a lipoprotein receptor.

dSR-CI mediated pathogen binding might also be important for the initial stages in the formation of cellular capsules around very large pathogens such as the eggs of parasitic wasps (Rizki and Rizki, 1984; Lackie, 1988). Alternatively, or perhaps additionally, dSR-CI could conceivably play a role in the later stages of this process, when well-ordered and multi-layered capsules are formed via extensive hemocyte-hemocyte adhesive interactions, and in which the outer layers of cells never contact the pathogen (Rizki and Rizki, 1984; Lackie, 1988). Since dSR-CI contains a MAM domain and several other potentially adhesive domains (Springer, 1994; Li *et al*, 1994; Zondag *et al*, 1995), it could be an adhesion molecule which contributes to the growth and strengthening of the cellular capsules by binding to proteins either expressed on or adherent to the surfaces of already adherent hemocytes (Rizki and Rizki, 1984; Söderhall, 1992). It is important to note, however, that even though MAM domains can modulate homophilic binding interactions in mammalian proteins, the MAM domain of dSR-CI does not appear to do so in normal, resting, S2 cells, since these cells are not aggregated in tissue culture.

In all of the responses discussed so far, dSR-CI plays an effector function. However, the components of the clotting and prophenoloxidase cascades must be released from intracellular stores in hemocyte/macrophages before they can perform their functions (Rizki and Rizki, 1984; Lackie, 1988; Söderhall, 1992; Iwanaga *et al*, 1992; Hultmark, 1993; Hoffmann, 1995). *Drosophila* hemocytes also release a battery of antibacterial peptides (such as the cecropins) in response to infection and may send signals to the fat body to do the same (Hultmark, 1993; Hoffmann, 1995). Thus, a very early event in the immune response must be the activation of hemocyte/macrophages in response to pathogen recognition. Rel domain transcription factors homologous to NF-kB are probably crucial for this initial activation event (Hultmark, 1993; Hoffmann, 1995). However, little is currently known about the mechanisms by which the activities of these and other transcription factors are themselves regulated in response to infection. Given its ligand binding properties, dSR-CI clearly has the potential to be

involved in the initial detection of pathogens and the subsequent activation of hemocyte/macrophages. Again, dSR-CI could mediate cellular activation following either direct detection of microbes and/or indirect detection of microbes which have been opsonized by endogenous serum components such as α-2 macroglobulin. While the cytoplasmic domain of dSR-CI contains several potential serine/threonine phosphorylation sites, its lack of homology with other proteins provides no insight into how dSR-CI might activate hemocyte/macrophages.

It is important to note that the *Drosophila* fat body is the major site of antibacterial peptide synthesis in response to infection (Hultmark, 1993; Hoffmann, 1995). As in the hemocytes, rel domain transcription factors, and other transcription factors, are probably required for this response. However, again as in the hemocytes, little is currently known about the mechanism by which the fat body is activated. As mentioned above, the process may depend on signals generated by previously activated hemocytes. Alternatively, the fat body may contain receptors which themselves recognize soluble microbial surface constituents (Hultmark, 1993; Hoffmann, 1995). Since terminally differentiated fat body cells have not yet appeared at the stages of embryonic development examined for dSR-CI mRNA expression, it is not yet known whether dSR-CI has the potential to contribute to fat body activation.

The expression, ligand binding properties, and domain structure of dSR-CI also suggest functions for this protein in processes distinct from host defense. For example, *Drosophila* hemocyte/macrophages migrate throughout the tissues and interstitial spaces of the developing and adult fly (Rizki and Rizki, 1984; Nelson *et al*, 1994; Tepass *et al*, 1994). Migration along the embryonic ventral nerve cord appears to be dependent upon tissue-specific guidance cues, since the migration does not occur in *single-minded* mutant embryos lacking CNS midline and ventral epidermal cells (Zhou *et al*, 1995). Since dSR-CI is expressed prior to the onset of hemocyte/macrophage migration, and since it contains several potentially adhesive domains, it may play a role in these migratory processes.

Apoptotic cells are efficiently recognized and engulfed by macrophages during both mammalian and *Drosophila* development (Abrams *et al*, 1993; Savill *et al*, 1993). Both the class A and class B scavenger receptors may participate in apoptotic cell engulfment in mammals (see Chapter 1), and it has been proposed that the *Drosophila* scavenger receptors may participate in apoptotic cell engulfment during embryonic development (Abrams *et al*, 1992; Abrams *et al*, 1993). The broad binding specificity of dSR-CI suggests that it might recognize apoptotic cells, since these cells express molecular patterns on their surface which are not found on normal cells (Savill *et al*, 1993). Indeed, apoptotic cells can be considered to be "altered self" cells somewhat analogous to non-self microbial pathogens (see Chapter 1). While these findings have all been made studying mammalian apoptotic cells, given the conservation of the apoptotic cell death program across the animal kingdom (Steller, 1995), it seems likely that they will hold true for *Drosophila* as well. Several findings are consistent with the possibility that dSR-CI might participate in apoptotic cell engulfment during *Drosophila* development: 1) dSR-CI expression in the embryo

precedes the onset of apoptosis (Abrams *et al*, 1992); 2) dSR-CI expression is upregulated during the early stages of pupal metamorphosis, a time of extensive cell death; and 3) COS cell transfectants expressing dSR-CI (but not untransfected cells) are able to bind and engulf apoptotic thymocytes *in vitro* (N. Franc and A. Ezekowitz, personal communication). Importantly, PS vesicle do not appear to be dSR-CI ligands (Figure 3-5). It seems possible that the CD36 homolog croquemort might recognize PS on apoptotic cells, and the dSR-CI might act as a phagocytic receptor subsequent to this initial recognition event. Interestingly, however, croquemort is expressed coincidentally with the onset of apoptosis (Franc *et al*, 1996). This suggests that an alternative role for dSR-CI might be to mediate the initial recognition of apoptotic cells by hemocyte/macrophages, which would then lead to the upregulation of croquemort and the subsequent engulfment of the dying cells. Finally, the potential expression of dSR-CI in subperineurial glia, if confirmed, is consistent with this receptor playing a role in the reported participation of these cells in the engulfment of apoptotic cells arising in the central nervous system (Sonnenfeld and Jacobs, 1995). For the moment, the hypothesis that dSR-CI participates in apoptotic cell engulfment remains speculative. However, this possibility seems great enough to warrant experimental exploration of this issue in the future.

Many different functions for dSR-CI have been suggested here. It will be interesting to determine the actual function of dSR-CI. This is particularly interesting since, to date, dSR-CI and 18-wheeler are the only molecularly identified invertebrate membrane receptors which directly recognize microbial surface molecules. The application of genetic techniques, along with additional molecular and physiologic studies, should soon begin to provide information on the roles of dSR-CI in *Drosophila* development and host defense.

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Figure 3-1. Concentration dependence of the ^{125}I-AcLDL interaction with S2 cells.

1.5×10^6 S2 cells were incubated in medium C containing the indicated amounts of ^{125}I-AcLDL for 5 hours at 25 °C, and cell association (binding plus internalization, solid circles) and degradation (solid squares) were determined as described in Experimental Procedures. The high affinity values shown represent the specific binding plus uptake and degradation of the ligand, determined as the difference between measurements made in the absence (duplicate incubations) and presence (single incubations) of excess unlabeled AcLDL (400 μg protein/ml).

Concentration Dependence of the ^{125}I-AcLDL Interaction With S2 Cells

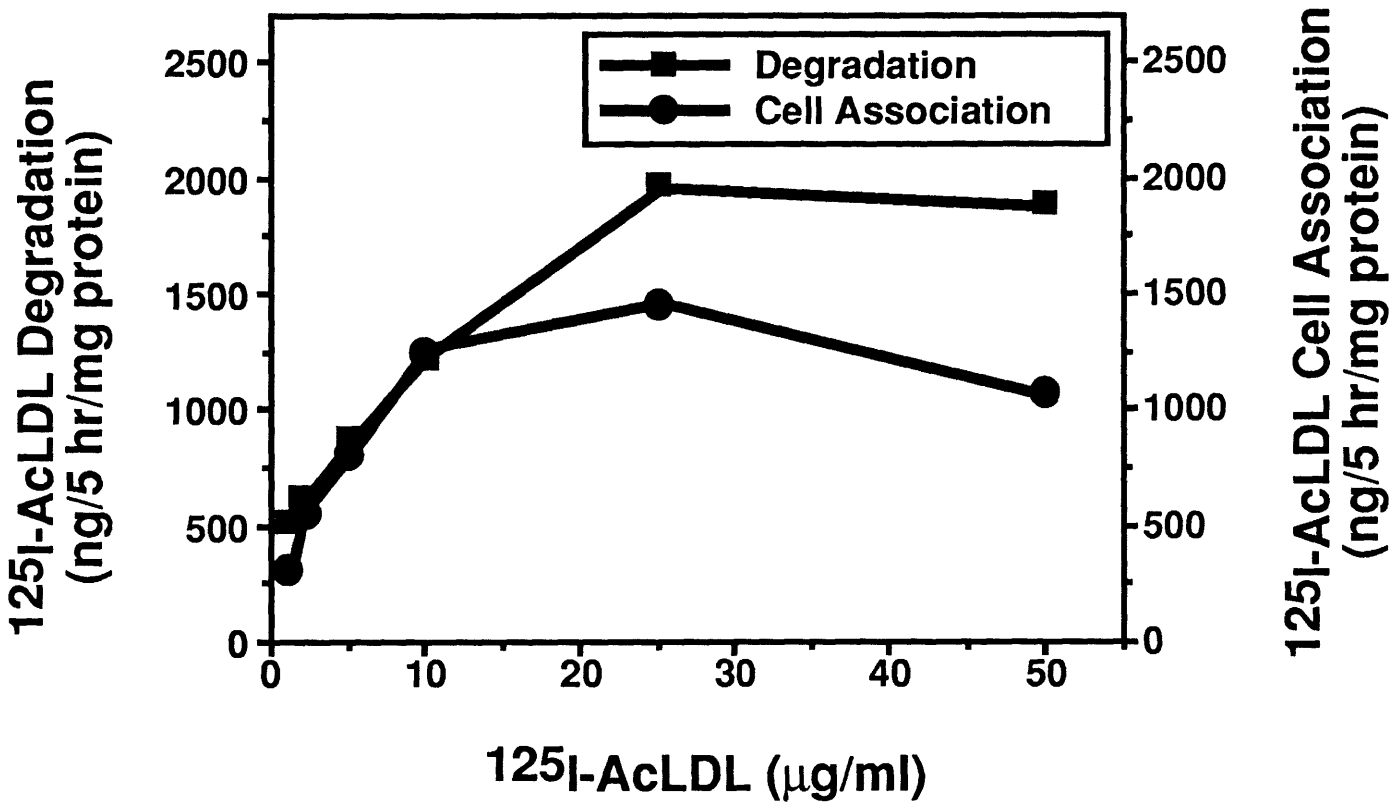


Figure 3-2. Southern hybridization screening of S2 cDNA pools for SR-AI homologs.

1 μg of DNA from each of 19 S2 cDNA pools was screened by Southern hybridization for cDNA clones containing sequences homologous to mSR-AI as described in Experimental Procedures. There was incomplete digestion of the DNA in these cDNA pools (data not shown), which may account for some of the fragments greater than 4 kbp in size which were detected. DNA size standards, in kbp, are indicated (λBstEII digest, New England Biolabs).

Hybridization Screening of S2 cDNA Pools with an mSR-AI Probe

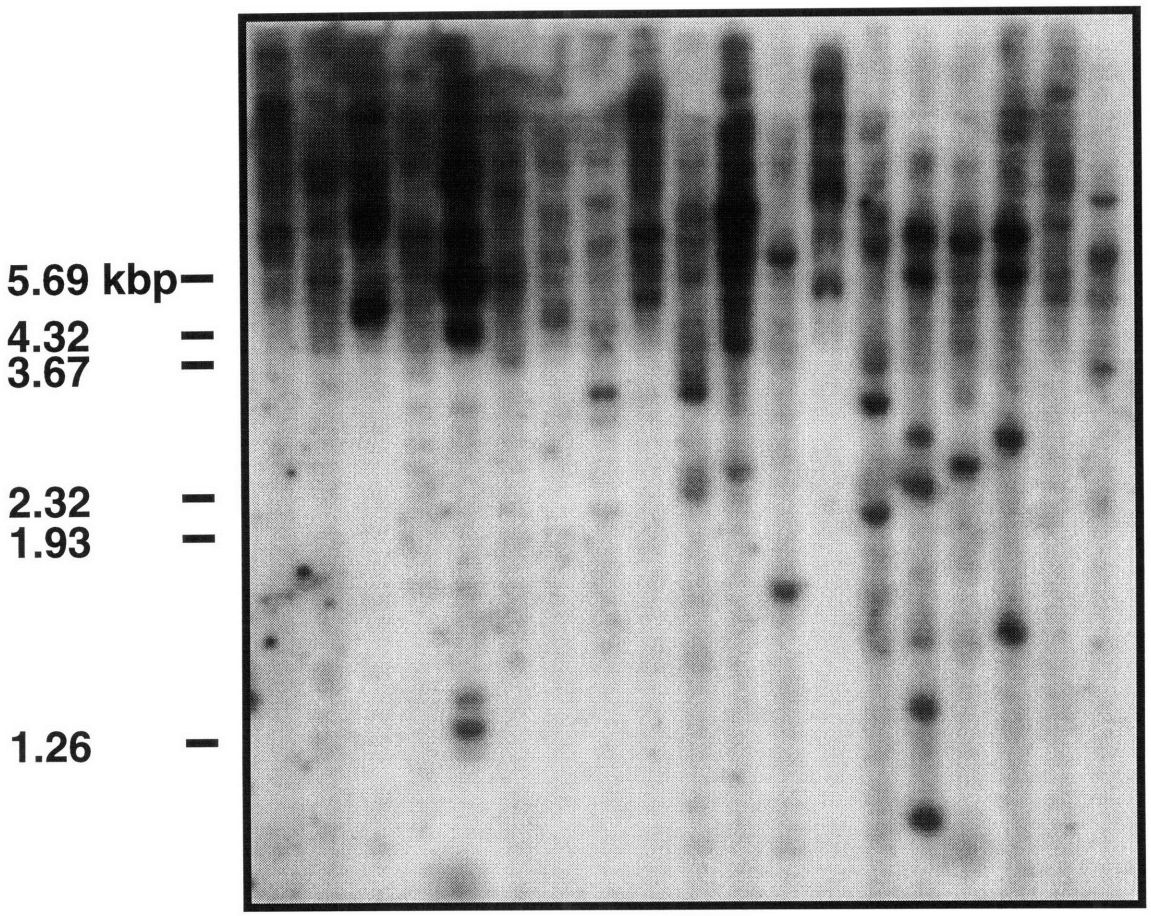


Figure 3-3. Northern hybridization analysis of dSR-CI expression in S2 and Kc_0 cells.

Approximately 4 μg of polyA+ mRNA from S2 and Kc_0 cells was analyzed using a PCR generated dSR-CI probe and an elongation factor 1 α probe as described in Experimental Procedures. A single band of approximately 2.1 kb was detected in the S2 lane. Only a very weak signal can be detected from Kc_0 cells.

Northern Analysis of dSR-CI Expression

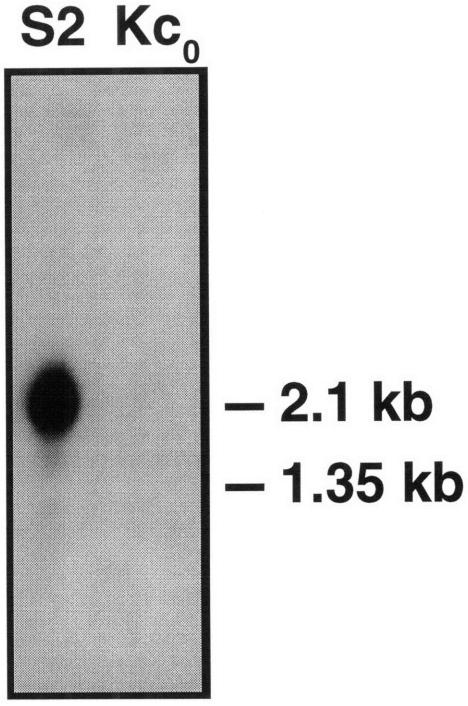


Figure 3-4. Concentration dependence of the ^{125}I-AcLDL interaction with CHO[dSR-CI] cells at 4°C and 37°C.

On day 1, CHO[dSR-CI] cells were plated in medium E into either 6-well dishes (125,000 cells/well, panel A) or 24-well dishes (60,000 cells/well, panel B). On day 3, the indicated amounts of ^{125}I-AcLDL in medium A were added and binding for 2 hours at 4° C (panel A, solid circles) or binding plus internalization (open circles) and degradation (solid squares) for 5 hours at 37° C (panel B) were determined as described in Experimental Procedures. The high affinity values shown represent the differences between measurements made in the absence (duplicate incubations) and presence (single incubations) of excess unlabeled AcLDL (400 μg protein/ml). Untransfected CHO cells exhibit virtually no scavenger receptor activity (Freeman *et al*, 1991).

Concentration Dependence of 125I-AcLDL Interaction with CHO[dSR-CI] Cells

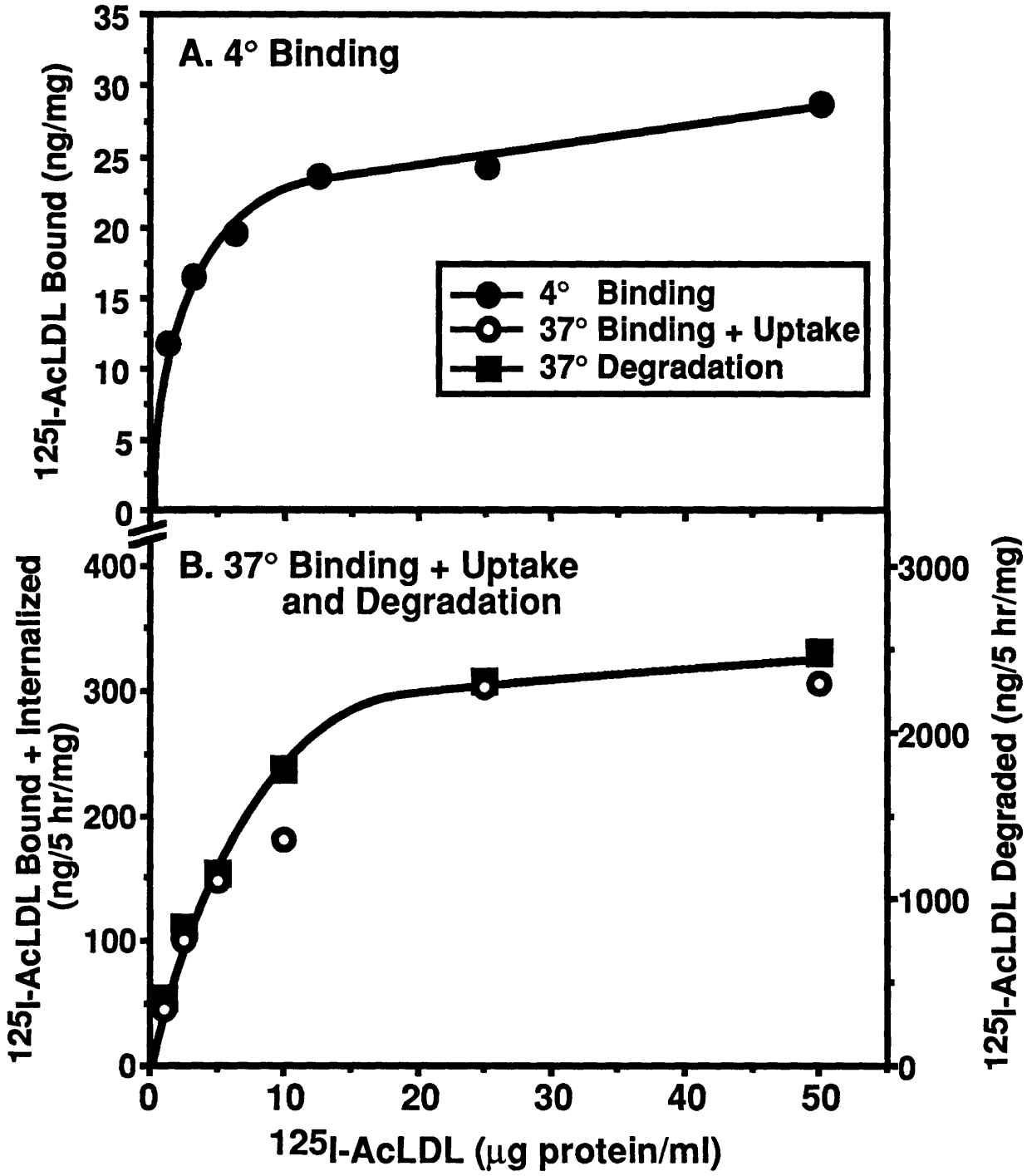


Figure 3-5. Specificity of the CHO[dSR-CI] and Schneider S2 scavenger receptors.

CHO[dSR-CI] cells were plated on day 0 in medium E in 24-well dishes (60,000 or 30,000 cells/well). On days 2 or 3, respectively, the cells were incubated for 5 hours at 37 °C in medium E lacking G418 and containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of 400 μg/ml of the indicated competitors, except that dA_{37} and dA_5G_{37} were used at 100 μg/ml and phosphatidylserine vesicles were used at 500 μg/ml. S2 cells (750,000 cells/tube) were incubated for 5 hours at 25 °C in medium C containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the same concentrations of the same competitors. The degradation values, expressed as a percent of the control degradation levels observed in the absence of added competitor, were measured as described in Experimental Procedures and represent the results from a single experiment. Similar results have been observed in multiple experiments, except for dA_{37} and phosphatidylserine vesicles, which were only tested once. The control degradation values in these experiments ranged from 1163 - 1571 ng/5 hours/mg protein for CHO[dSR-CI] cells, and 2965 - 4313 ng/5 hours/mg protein for S2 cells. Abbreviations: MBSA - maleylated BSA; pI - poly(I); DSO_4 - dextran sulfate; CSO_4 - chondroitan sulfate; pD-E - poly D-glutamic acid; PS - phosphatidylserine vesicles (1:1 phosphatidlyserine:phosphatidlycholine).

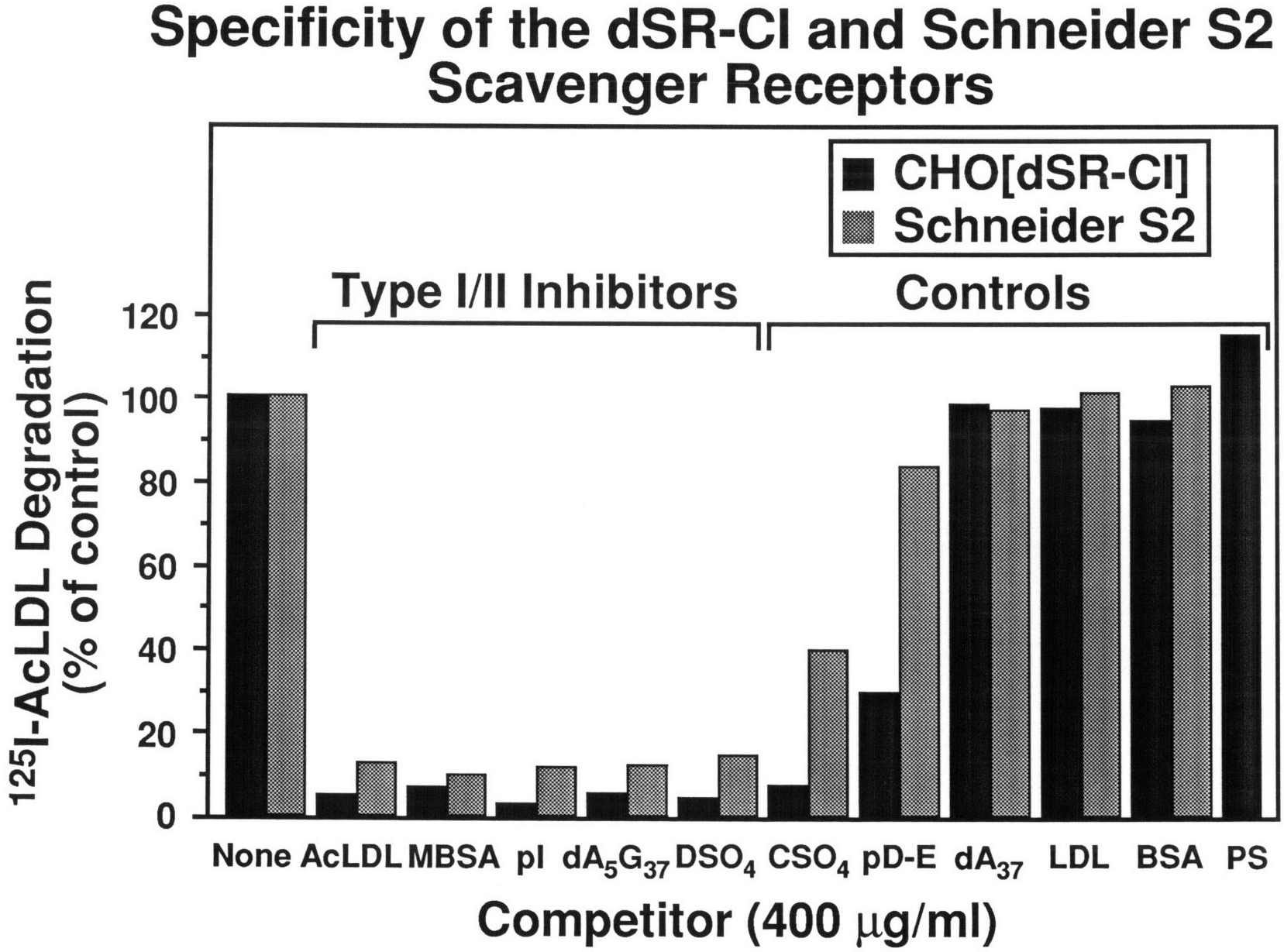

Specificity of the dSR-CI and Schneider S2 Scavenger Receptors
CHO[dSR-CI]
Schneider S2
Type I/II Inhibitors
Controls
125I-AcLDL Degradation (% of control)
120
100
80
60
40
20
0
None
AcLDL
MBSA
pI
dA5G37
DSO4
CSO4
pD-E
dA37
LDL
BSA
PS
Competitor (400 μg/ml)

Figure 3-6. Inhibition of ^{125}I-AcLDL degradation in CHO[dSR-CI] and S2 cells by dA_5G_{37} and poly(I).

A. CHO[dSR-CI] cells (solid circles) were plated on day 0 in medium E in 24-well dishes (60,000 or 30,000 cells/well). On days 2 or 3, respectively, the cells were incubated for 5 hours at 37 °C in medium E lacking G418 and containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated concentrations of either dA_5G_{37} (part A) or poly(I) (part B). S2 cells (solid squares, 750,000 cells/tube) were incubated for 5 hours at 25 °C in medium C containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the same concentrations of these competitors. The degradation values, expressed as a percent of the control degradation levels observed in the absence of added competitor, were measured as described in Experimental Procedures and represent the averages of the results from one to three experiments per concentration. The control degradation values in these experiments ranged from 586 - 1032 ng/5 hours/mg protein for CHO[dSR-CI] cells, and 510 - 1335 ng/5 hours/mg protein for S2 cells.

Inhibition of ^{125}I-AcLDL Degradation in CHO[dSR-CI] and S2 Cells by dA_5G_{37} and poly(I)

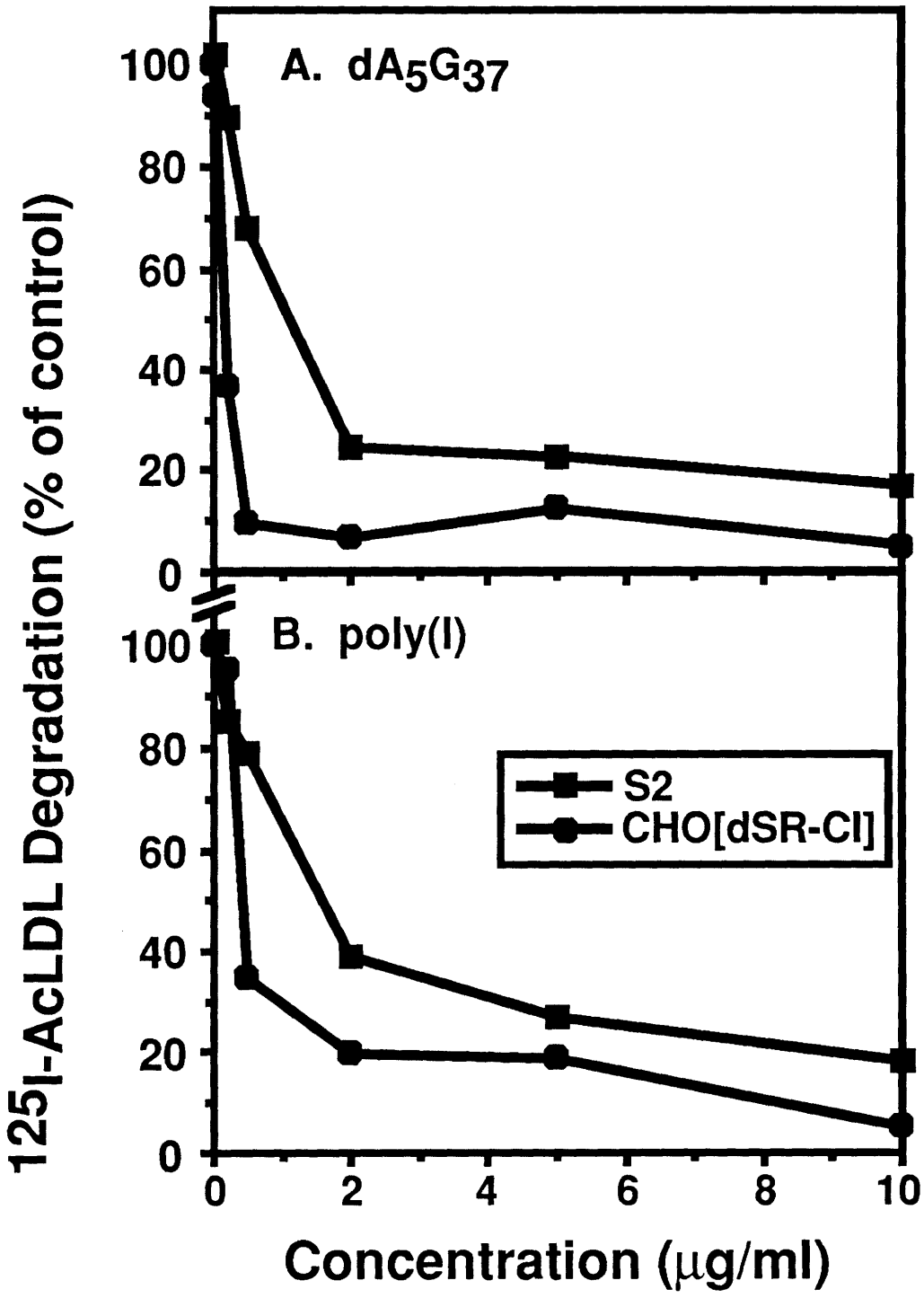


Figure 3-7. Inhibition of ^{125}I-AcLDL degradation in CHO[dSR-CI] and S2 cells by poly D-glutamic acid.

CHO[dSR-CI] cells were plated on day 0 in medium E in 24-well dishes (30,000 cells/well). On day 3, the cells were incubated for 5 hours at 37 °C in medium E lacking G418 and containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated concentrations of poly D-glutamic acid. S2 cells (750,000 cells/tube) were incubated for 5 hours at 25 °C in medium C containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the same concentrations of poly D-glutamic acid. The degradation values, expressed as a percent of the control degradation levels observed in the absence of added competitor, were measured as described in Experimental Procedures. The control degradation values in this experiment were 1163 ng/5 hours/mg protein for CHO[dSR-CI] cells, and 4313 ng/5 hours/mg protein for S2 cells.

Inhibition of 125I-AcLDL Degradation in CHO[dSR-CI] and S2 Cells by poly D-glutamic acid

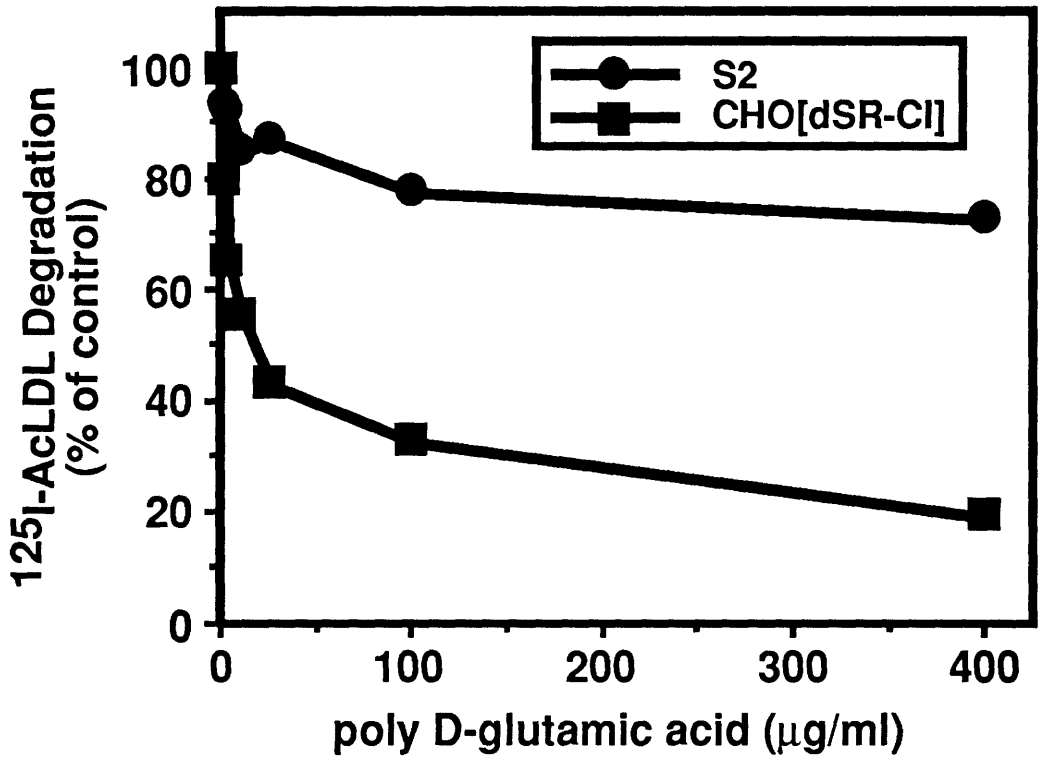


Figure 3-8. Inhibition of ^{125}I-AcLDL degradation in CHO[dSR-CI] and S2 cells by laminarin.

CHO[dSR-CI] cells were plated on day 0 in medium E in 24-well dishes (60,000 or 30,000 cells/well). On days 2 or 3, respectively, the cells were incubated for 5 hours at 37 °C in medium E lacking G418 and containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the indicated concentrations of laminarin. S2 cells (750,000 cells/tube) were incubated for 5 hours at 25 °C in medium C containing 5 μg/ml ^{125}I-AcLDL in the absence (triplicate incubations) or presence (duplicate incubations) of the same concentrations of laminarin. The degradation values, expressed as a percent of the control degradation levels observed in the absence of added competitor, were measured as described in Experimental Procedures and represent the averages of the results from one to three experiments per laminarin concentration point. The control degradation values in these experiments ranged 582 - 727 ng/5 hours/mg protein for CHO[dSR-CI] cells, and 510 - 1335 ng/5 hours/mg protein for S2 cells.

Inhibition of ^{125}I-AcLDL Degradation in CHO[dSR-CI] and S2 Cells by Laminarin

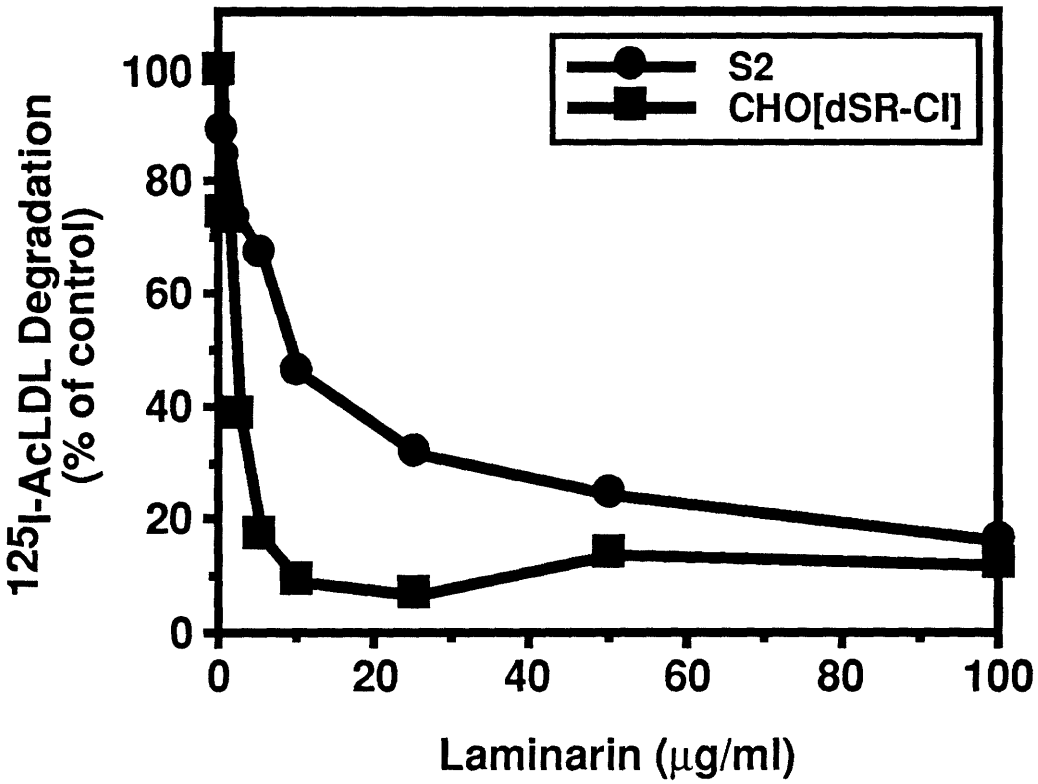


Figure 3-9. Predicted protein sequence and domain organization of the *Drosophila* scavenger receptor type CI (dSR-CI).

Panel A: The sequences of the 5' and 3' untranslated regions (UTR) of the dSR-CI cDNA. The putative transcription start site (underline) and an in frame stop codon (***) in the 5' UTR, and the putative polyadenylation signal (^^^^^^) in the 3' UTR, are indicated.

Panel B: The cDNA for dSR-CI was cloned and sequenced as described in Experimental Procedures. The predicted protein sequence is numbered from -20 for the first in-frame Met in the putative signal sequence; the first residue (Arg) after the predicted cleavage site (von Heijne, 1986) is designated +1. Cysteines are boxed and potential N-linked glycosylation sites are underlined. Potential phosphorylation sites in the cytoplasmic domain are indicated (* casein kinase II (Pinna, 1990), † protein kinase C (Woodgett *et al*, 1986), ^ cAMP/cGMP dependent protein kinase (Feramisco *et al*, 1980; Glass and Smith, 1983)). The protein is divided into nine domains, some belong to previously described motif families (see text). Consensus sequences for those motifs are indicated below the corresponding sequences in dSR-CI. The CCP consensus sequence is from Perkins, *et al.* (1988). The MAM and somatomedin B consensus sequences were generated from 13 MAM sequences (7 independent sequences and 6 sequences of homologs from different species), and 15 somatomedin B sequences (8 independent sequences from 6 proteins, and 7 sequences of homologues). MAM consensus criteria: single amino acids or combinations with aromatic (π=F,W,Y), hydroxyl (o=S,T), or positive or negative (+=H,K,R; -=D,E) side chains must be present in $\geq$ 5 independent sequences, aliphatic (a=A,V,L,I) or charged (c=+,-) residues in $\geq$ 6, and hydrophobic residues (h=a,π,M) in all 7. At positions in which only two amino acids occur in at least 6 of the sequences, both are shown. Somatomedin B consensus residues occur $\geq$ 5.67 of the 8 independent sequences. For these calculations, residues were assigned an appropriate fractional occupancy weight if they occurred in a sequence represented by several species homologues. The MAM consensus sequence differs somewhat from that assembled by Beckmann and Bork (1993) when fewer cloned sequences were available.

Panel C: Schematic diagram of the domain structure of dSR-CI. The signal sequence (Sig Seq), complement control protein (CCP), MAM, somatomedin B (Som B), spacer (Sp), ser/thr rich putative O-glycosylated (Ser/Thr), transmembrane (TM), and cytoplasmic (Cyto) domains and the potential N-linked glycosylation sites (ball and stick symbols) are indicated. The domains are numbered as in part A.

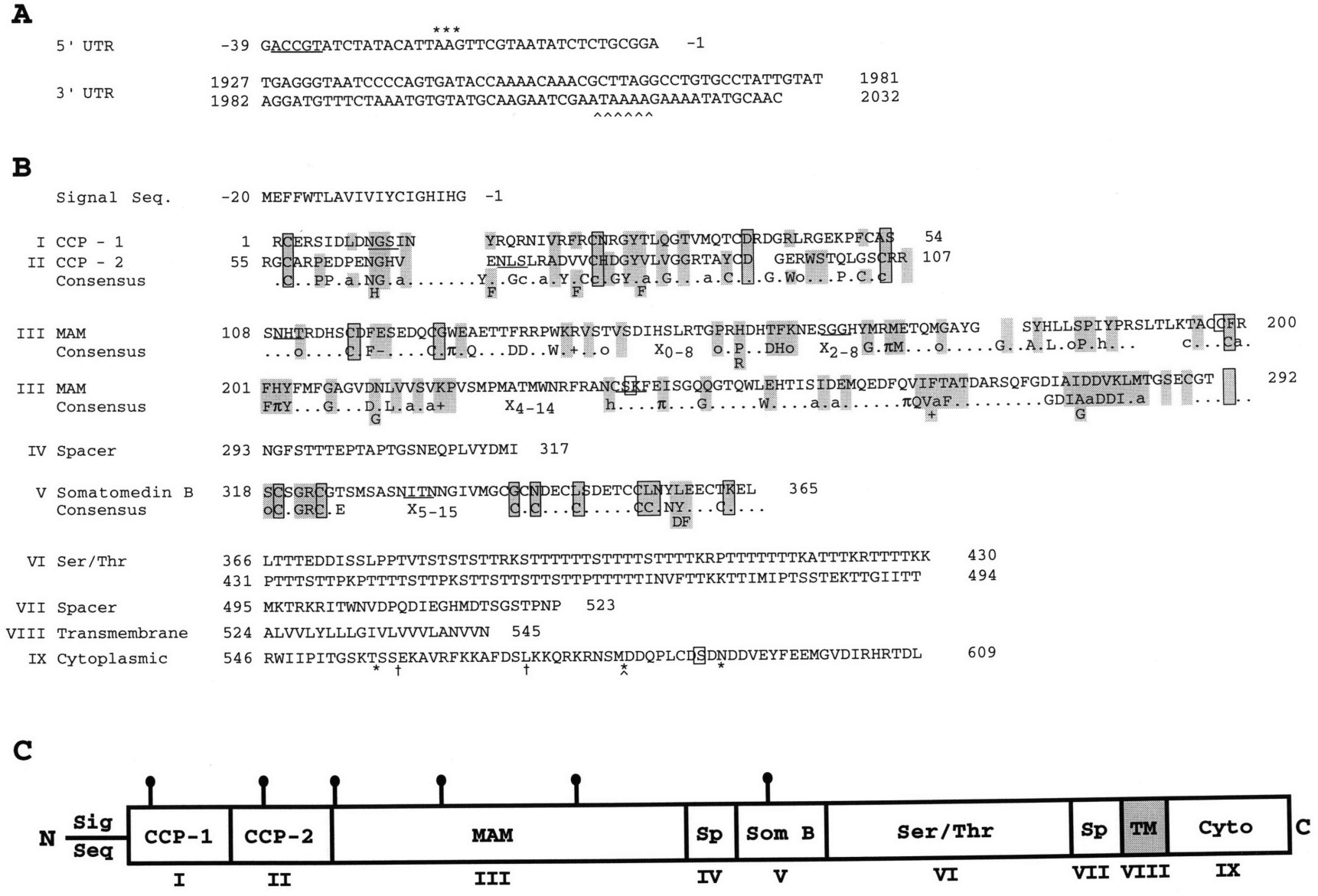

A
5' UTR
-39 GACCGTATCTATACATTAAGTTCGTAATATCTCTGCGGA -1
3' UTR
1927 TGAGGGTAATCCCCAGTGATACCAAAACAAACGCTTAGGCCTGTGCCTATTGTAT 1981
1982 AGGATGTTTCTAAATGTGTATGCAAGAATCGAATAAAAGAAAATATGCAAC 2032
B
Signal Seq. -20 MEFFWTLAVIVIYCIGHIHG -1
I CCP - 1 1 54
II CCP - 2 55 107
Consensus
III MAM 108 200
Consensus
III MAM 201 292
Consensus
IV Spacer 293 NGFSTTTEPTAPTGSNEQPLVYDMI 317
V Somatomedin B 318 365
Consensus
VI Ser/Thr 366 430
431 494
VII Spacer 495 MKTRKRITWNVDPQDIEGHMDTSGSTPNP 523
VIII Transmembrane 524 ALVVLYLLLGIVLVVVLANVVN 545
IX Cytoplasmic 546 609
C
N
Sig Seq
CCP-1
CCP-2
MAM
Sp
Som B
Ser/Thr
Sp
TM
Cyto
C
I
II
III
IV
V
VI
VII
VIII
IX

Figure 3-10. Expression of dSR-CI mRNA in *Drosophila* embryos.

In situ hybridization in embryos was performed using a digoxygenin labeled dSR-CI antisense RNA probe as described in Experimental Procedures and was visualized using Nomarski optics. The stage ("St.") of embryonic development is indicated and the bars in panels E and F represent 10 μm. Panel E is a high magnification view of a portion of the ventral nerve cord of a stage 11 embryo. Panel F is a high magnification view of a portion of the posterior region of a stage 12 embryo. Abbreviations: cl, clypeolabrum; gb, germ band; pm, procephalic mesoderm; vnc, ventral nerve cord.

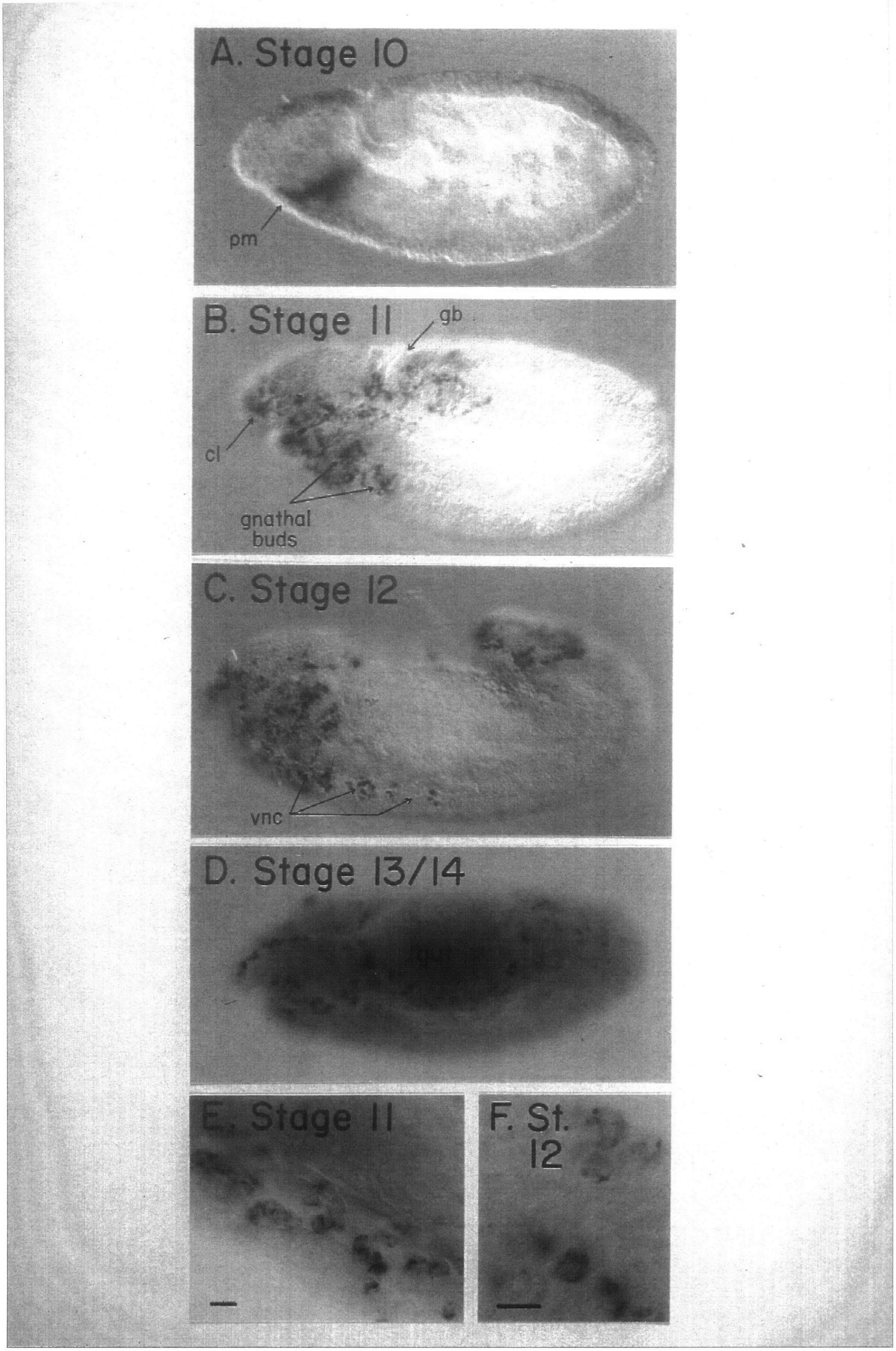

A. Stage 10
pm
B. Stage 11
gb
cl
gnathal buds
C. Stage 12
vnc
D. Stage 13/14
E. Stage 11
F. St. 12

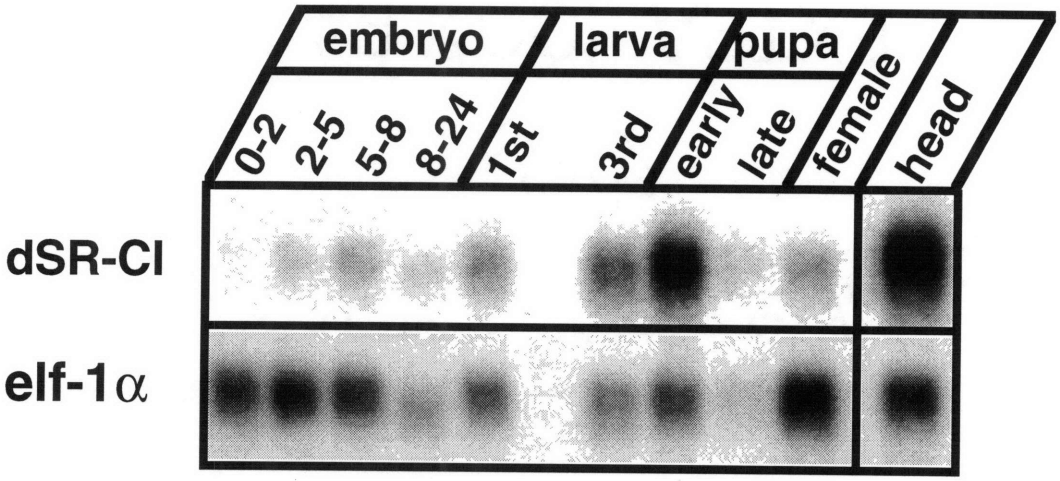
Figure 3-11. Developmental Northern analysis of dSR-CI mRNA expression.

A. A developmental Northern blot was analyzed using the dSR-CI probe 1101p and an elongation factor 1α probe as described in Experimental Procedures. The lack of a dSR-CI signal in late pupa is due to insufficient loading of these lanes.

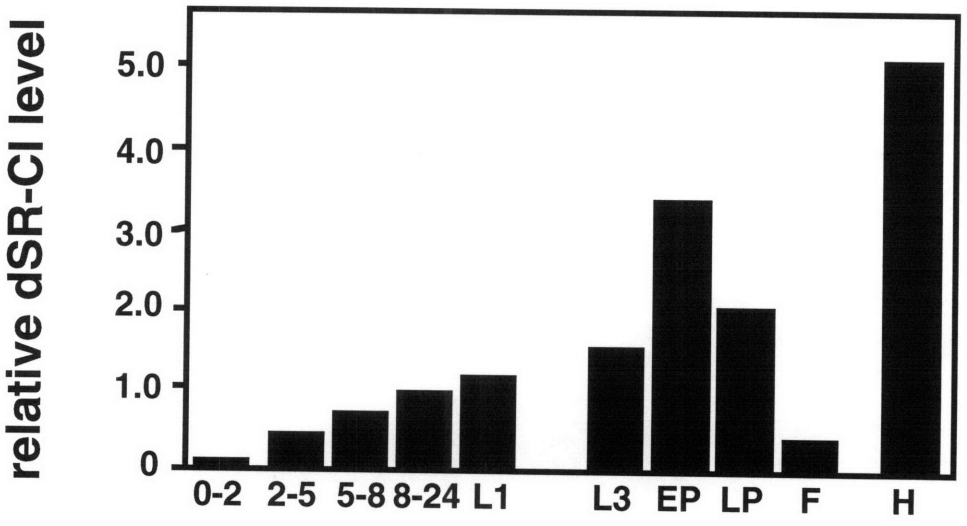
B. The level of dSR-CI expression was determined at every stage of development relative to the level observed in 8 - 24 hour embryos. dSR-CI message levels were normalized to each other by correcting for loading differences using the elongation factor 1α signal. Note that elongation factor 1α is known to be expressed at higher levels in embryos than in subsequent developmental stages (Walldorf *et al*, 1985).

Developmental Northern Analysis of *dSR-CI* Expression

A.



B.



Chapter Four

Identification and Analysis of a Family of dSR-CI - Related Genes in *Drosophila melanogaster*

Introduction

In Chapter 3, the cloning and characterization of the *Drosophila dSR-CI* gene was described. The initial goal of the cloning project was to identify a gene responsible for the scavenger receptor activity which had been previously observed in *Drosophila* embryos and S2 cells. While it was hoped that this gene would be an SR-A homolog, the expression cloning approach taken was not biased toward the isolation of one particular class of SR, and indeed, the gene which was identified which defined a completely new and distinct SR class. In retrospect, this is not necessarily surprising since, as discussed in Chapter one, there are now at least three distinctly different classes of proteins which have scavenger receptor activity and pattern recognition receptor like broad binding specificity, and there are numerous other proteins which also exhibit pattern recognition receptor characteristics.

The expression pattern of the dSR-CI message, and the sequence and ligand binding properties of the dSR-CI protein, make this gene interesting in its own right. In the preceding chapter, a number of potential developmental and host defense functions for *dSR-CI* were discussed. The next step toward determining the actual functions of this gene *in vivo* was to identify and/or generate mutants lacking dSR-CI function. Therefore, *in situ* hybridization to polytene chromosomes was used to localize the gene within the *Drosophila* genome. Following this localization, it was decided that genomic clones containing the *dSR-CI* gene should be isolated. These genomic clones were desired for several reasons. First, analysis of the region containing the *dSR-CI* gene indicated that the best way to obtain mutants might be to use P element mediated mutagenesis (Zhang and Spradling, 1993). To do so would require a source of genomic DNA which could be used for analysis of P element insertions into or near the *dSR-CI* gene. Second, genomic clones could be used for breakpoint analysis of the various chromosome aberrations affecting the *dSR-CI* region. Third, genomic clones containing *dSR-CI* with all of its regulatory elements could be used to generate P element rescue constructs. Successful rescue of a mutant phenotype with cloned DNA is the best way to demonstrate that the phenotype is due to a mutation in the gene of interest. Fourth, the upstream regulatory sequences of the *dSR-CI* gene could be used to express altered forms of the dSR-CI protein in a tissue-specific manner for functional studies. Finally, these clones would also be useful for studying the regulated expression of the *dSR-CI* gene.

In the process of isolating genomic clones, a new family of *Drosophila* genes homologous to *dSR-CI* was discovered. This chapter describes the identification and characterization of these new *dSR-CI* homologs, as well as the current state of knowledge regarding the location and organization of these genes within the *Drosophila* genome. Most of the work described herein was performed by me. Some of the genetic analysis of the deficiency and single mutant strains shown in Figure 4-2 was performed by Y. Yu under my direction and supervision. C. Bae gave some assistance in the PCR analysis of various deficiency strains (Figure 4- 12), and D. Trigatti subcloned a piece of one of the *dSR-CII* genomic lambda clones for sequence analysis (Figure 4-9).

Experimental Procedures

Reagents and sources. The standard molecular biology techniques used throughout this work can be found in Sambrook *et al* (1989). Labeling of probes with ^{32}P-nucleotides, obtained from DuPont NEN, was sometimes performed using the Amersham Megaprime labeling kit.. Agarose (SeaKem GTG, and LE grades) was from FMC Bioproducts. The following filters were used: Genescreen (DuPont NEN) and Zetabind (CUNO Inc.) for Northern and Southern blotting, and Nitrocellulose HA (Millipore) for genomic and cDNA library screening.

In situ hybridization to polytene chromosomes. Salivary glands were dissected from wandering third instar larva in 45% acetic acid, and their chromosomes were spread and squashed between a glass slide and a siliconized glass coverslip in 3:2:1 acetic acid:deionized water:lactic acid. After incubation at 4 °C overnight, the slides were dipped into liquid nitrogen, the coverslips were removed, and the slides were washed twice in 100% ethanol and allowed to dry. Slides were stored at RT. Just before use, the slides were acetylated for 10 minutes in 0.1M triethanolamine containing 355 µl acetic anhydride/300 ml, and, after washing and drying in ethanol, the chromosomes were denatured for 3 minutes in 0.07 N sodium hydroxide. A biotinylated dSR-CI probe was prepared by nick translation of 450 ng of clone pdSR-CI (Chapter 3, Pearson *et al*, 1995) using Bio-16-dUTP (ENZO Diagnostics). Incorporation was approximately 12% as determined by spiking the nick translation mixture with ^{32}P-dATP. Unincorporated Bio-16-dUTP was removed by ethanol precipitation, and the probe was resuspended in 75 µl of hybridization buffer (0.5 M sodium phosphate, pH 6.8, 0.6 M sodium chloride, 5 µM magnesium chloride, 1X Denhardt's). The probe was then boiled and 15 µl were added to the slide. After sealing the hybridization mixture under a glass coverslip, the probe was allowed to hybridize to the chromosomes at 58 °C overnight. Subsequently, the slides were washed with hybridization buffer three times for 20 minutes at 58 °C, and were then incubated with a 1:250 dilution in phosphate buffered saline (PBS) of streptavidin-biotinylated peroxidase (ENZO Diagnostics) for 1 hour at 37 °C. After several PBS washes, a diaminobenzidine (DAB) reaction was performed for 20 minutes at RT in using 0.5 mg/ml DAB in PBS containing hydrogen peroxide (2 µl per ml). Prior to examination in the microscope , the chromosomes were stained for 30 seconds using a 1:20 dilution of Giemsa in 10 mM sodium phosphate, pH 6.8.

Genetic techniques. All deficiency (*Df*) strains used in this work are embryonic lethal. For complementation analysis, males and virgin females of various *Df/Balancer* and single *lethal/Balancer* strains were crossed and the progeny in each phenotypic class were scored. For Southern blot and PCR analyses of deficiency strains, *Df/Balancer* flies were outcrossed to *Canton-S* flies. The *Df/+* F1 males and virgin female siblings were then mated, and eggs were collected on yeasted molasses egg laying plates. Eggs were aged for 48 to 72 hours, during which time any hatched larva were removed. After de-chorionation in 50% bleach, homozygous *Df* embryos were collected by selecting unhatched eggs exhibiting visible signs of development and,

usually, melanization. The *Balancer* chromosome allows for the easy maintenance of recessive lethal mutations as heterozygous stocks (Ashburner, 1989).

Preparation and Southern blot hybridization analysis of *Drosophila* genomic DNA. Genomic DNA was isolated from *Drosophila* embryos or adults as follows: embryos or adults were homogenized in 0.1M EDTA, 1% SDS, 1% DEPC, 0.1 M Tris-Cl, pH9.0 and incubated for 30 minutes at 70 °C. 14 µl 8 M potassium acetate/100 µl homogenate was then added and proteins and debris were precipitated on ice for 30 minutes and removed by centrifugation. DNA was then precipitated by adding 0.5 volumes of isopropanol followed by RT centrifugation. After washing in 70% ethanol and air drying, the DNA pellets were resuspended in 10 mM Tris-Cl, 1mM EDTA, pH 7.4. Following standard protocols, 0.5 - 2.0 µg of genomic DNA was digested with restriction enzymes, electrophoretically separated on 1% SeaKem GTG agarose (FMC Bioproducts), and transferred to membranes. Hybridization to the membranes used the following probes: 1) gel purified dSR-Clp, generated by PCR from pdSR-Cl using the primers sdSR1.1 and asdSR1.1 (Table 1); 2) mixed λe1.1b/λg2.1a probe (see below); and 3) an elongation factor 1 α probe (Walldorf *et al*, 1985) generously provided by C. Wilson and H. Steller. Probes were random primed labeled with ^{32}P-radiolabeled nucleotides, sometimes using the Megaprime labeling kit.

PCR Analysis of dSR-Cl intron/exon structure. Analysis of the intron/exon structure of dSR-Cl was performed using the primer pairs sdSR1.1/asdSR1.1, sdSR1.1/asdSR1.36, sdSR1.5/asdSR1.30, sdSR1.10/asdSR1.25, sdSR1.17/asdSR1.23, sdSR1.25/asdSR1.15, and sdSR1.40/asdSR1.1 in PCR reactions containing 8 pmol of each primer, 1.5 mM magnesium chloride, 1X PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM potassium chloride, 0.001% gelatin), 0.2 mM of each dideoxynucleotide triphosphate (dNTP) - dATP, dCTP, dGTP, dTTP, 0.1 to 0.3 µl Amplitaq DNA polymerase, and either approximately 100 ng of *Canton-S* genomic DNA prepared from adult flies as described above, or less than 10 ng of clone pdSR-Cl (Chapter 3). The reaction parameters were 1 minute each at 94 °C and then 42 °C - 63 °C, depending on the primer pair, followed by 1 or 2 minutes at 72 °C, for 35 or 40 cycles. PCR products were analyzed on a 1% LE agarose gel (FMC Bioproducts) following standard procedures. Primer sequences are listed in Table 1.

Isolation and analysis of genomic clones. Filter lifts from a *Canton-S* genomic library prepared in the lambda dash II vector (generously provided by M. Brodsky and H. Steller) were probed with the random primed ^{32}P-radiolabeled 1101p 5' dSR-Cl probe previously described (Chapter 3). Following standard protocols, plaques corresponding to the 26 positive signals so identified were picked and used to infect *E. coli* strain Q358 (Sambrook *et al*, 1989) for preparation of secondary plates and filters lifts. Upon secondary screening, four (λc4, λd5, λe1, λg2) of the 26 plaques were confirmed positive. Single plaques (denoted λd5.1a, λe1.1b, λg2.1a) were isolated from λd5, λe1 and λg2 by tertiary screening and used to generate frozen stocks and for DNA isolation. Although two secondary λc4 plaques were both confirmed by tertiary screening, it was never possible to grow the tertiary phage generated from

either of these secondaries. Genomic DNA was prepared from isolated lambda clones as follows: 40 ml overnight liquid cultures were started from frozen phage stocks. The next day, bacterial debris was removed by centrifugation and the supernatant was treated with 1200 units of DNase (Boehringer Mannheim) and 4 μg of boiled RNAse (Sigma). The lambda phage were precipitated on ice in 10% PEG/1M sodium chloride and were then resuspended in TM10 buffer (10 mM magnesium sulfate, 50 mM Tris-Cl, pH 7.5). Phage were lysed at 70 °C in the presence of 0.25% SDS, 20 mM EDTA, 0.2 M Tris-Cl, pH 8.5, and the DNA was precipitated on ice for several hours by the addition of potassium acetate to 0.5 M and two volumes of ethanol. After a 70% ethanol wash, DNA was resuspended in 10 mM Tris-Cl, 1mM EDTA, pH 7.4. Restriction mapping of lambda genomic clones followed standard protocols. For Southern analysis, 10 ng - 2 μg of the digested genomic clones were electrophoretically separated on a 1% agarose gel, transferred to filters, and analyzed by hybridization to the following probes: 1) gel purified 244p, generated by PCR amplification from pdSR-CI using T7 and asdSR1.35 (Table 1) primers; 2) the oligonucleotide sdSR1.22, corresponding to dSR-CI sense strand sequence separating the MAM domain and somatomedin B domain coding sequences (Table 1); 3) dSR-CIp; and 4) an oligonucleotide homologous to the T3 promoter sequence present in the lambda dash II vector (Stratagene). Probes were either end-labeled or random primed labeled using ^{32}P-radiolabeled nucleotides, sometimes using the Megaprime labeling kit. Subcloning of genomic clone restriction fragments into the vector pBSKSII+ (Stratagene) was performed following standard molecular biology techniques (Sambrook et al, 1989). DNA was isolated from overnight bacterial cultures using the Qiagen midiprep system, and was sequence by L. Ziaugra of the Whitehead Institute Sequencing Facility.

Isolation of cDNA clones. Colony lifts were made from a 4-8 hour embryo cDNA library (Brown and Kafatos, 1988) generously provided by X. Song and H. Steller. The filters were subjected to hybridization with the ^{32}P-radiolabeled oligonucleotide leas1.45 (Table 1). This probe corresponds to anti-sense strand sequences located in the putative signal sequence coding region of *dSR-CII* (see below) which should be located near the 5' end of the cDNA based on the genomic DNA sequence. 33 hybridizing colonies were detected out of approximately 50,000 colonies screened. Secondary screening of 17 of these positive colonies verified that at least 11 were truly positive. Single colonies corresponding to these clones were grown up for storage. DNA was isolated from the bacterial cultures using the Qiagen midiprep system. A single dSR-CII cDNA clone (clone pII-12) was identified by partial sequencing of eight of these clones. Sequencing was performed by L. Ziaugra of the Whitehead Institute Sequencing Facility.

PCR analysis of *Drosophila* Deficiency and *Canton-S* strains. Single *Canton-S* embryos, or dead homozygous deficiency embryos obtained as described above, were individually placed into 0.3 ml Eppendorf tubes. The embryos were broken open with a sterile pipette tip containing 10 μl of squishing buffer (SB: 10 mM Tris-Cl, pH 8.2, 1 mM EDTA, 25 mM sodium chloride, 200 μg/ml fresh proteinase K) and the SB was then released from the tip. The tubes were placed in a PCR machine at 25 °C, and

were allowed to warm to 37 °C over a 30 minute period. The samples were then heated to 95 °C for 2 minutes, and subsequently were stored at -20 °C until use. In preliminary experiments, it was determined that as little as 1.6 µl of embryonic *Canton-S* DNA so prepared gave strong PCR amplification signals. Two multiplex PCR reactions were performed from each embryo as follows: 1) dSR-CI/STSDm0173/ft PCR - the 60 µl reaction volume contains 1.6 µl of template, 4 pmol primers sdSR1.17, asdSR1.23, sDm0173.15, and asDm0173.25, 8 pmol of primers D1378f and D1378r, 1X PCR buffer, 0.2 mM of each dNTP, 1.23 mM magnesium chloride, and 0.4 µl Amplitaq DNA polymerase (Perkin-Elmer Cetus); 2) dSR-CII/rp49 PCR - the 40 µl reaction volume contains 1.6 µl of template, 4 pmol of primers les1.15 and leas1.25, 16 pmol of primers srp49.2 and asrp49.2, 2.43 mM magnesium chloride, 0.2 mM each dNTP, 1X PCR buffer, and 0.4 µl of Amplitaq DNA polymerase. 40 PCR cycles of 1 minute each at 94 °C, then 58 °C, then 72 °C were performed. Restriction digests of the products were at 37 °C overnight using 1 µl of enzyme added to 9 µl of PCR mixture. PCR and digestion products were analyzed on a 1% LE agarose gel (FMC Bioproducts) following standard procedures. Primer sequences are listed in Table 1.

RNA preparation and Northern blot hybridization analysis. Poly A+ RNA was prepared from strain *Canton-S* at various stages of development. Embryos were dechorionated in 50% bleach and were stored at -80 °C prior to use. Larva, pupa and adults were also stored at -80 °C prior to use. Prior to homogenization, larva, pupa and adults were ground to a fine powder in a mortar and pestle on liquid nitrogen. Embryo's, and the powdered larvae, pupae and adults were homogenized in 8 M urea, 3 M LiCl, 5 mM EDTA, 5 mM dithiothreitol using a Dounce homogenizer. Debris was removed by centrifugation and the supernatant was kept on ice overnight to allow the RNA to precipitate. The RNA was collected by centrifugation, resuspended in TES (10 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.5% SDS) and extracted twice with phenol/chloroform/iso-amyl alcohol (25:24:1). The RNA was then re-precipitated by adding 0.1 volume 3 M sodium acetate, pH 5.3 and 2.5 volumes ethanol and was stored in this state at -20 °C for several days. After centrifugation, the RNA was resuspended in binding buffer (0.4 M sodium chloride, 5 mM EDTA, 10 mM Tris-Cl, pH 7.5) plus 0.5% SDS, and incubated for 1 hour at RT with oligo dT-cellulose. After extensive washing in binding buffer, the poly A+ RNA was eluted with 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, and was precipitated as before. The poly A+ RNA was recovered by centrifugation, was resuspended in deionized water and was stored at -80 °C until use. Following standard procedures, 10 µg of poly A+ RNA was electrophoretically separated on a 1% agarose gel, transferred to membranes, and probed with the following ^{32}P-radiolabeled probes: 1) oligonucleotide leas1.45; 2) gel purified Dm0173p1, generated by PCR amplification *Canton-S* genomic DNA using primers sDm0173.15 and asDm0173.25; and 3) gel purified rp49p (Konugsuwan *et al*, 1985), generated by PCR amplification of *Canton-S* genomic DNA using primers srp49.2 and asrp49.2 (Table 1).

Results

As a first step toward isolating mutants lacking dSR-CI function, the cytological location of the *dSR-CI* gene was determined by *in situ* hybridization of a cDNA derived probe to polytene chromosomes. A single hybridization signal was detected at cytological location 24D4 - 24D8 on the left arm of chromosome 2 (Figure 4-1). This was the only signal detected on multiple chromosomes from two larvae.

A physical map of the 24C - 25A region of 2L is shown in Figure 4-2. The location of the *dSR-CI* gene, as determined by the *in situ* analysis, is indicated by the two-headed arrow. Several deficiency strains have been isolated whose chromosome have breakpoints within this region (Szidonya and Reuter, 1988). The extent of the intact chromosomes in these strains are indicated by the solid lines in Figure 4-2. Note that there is an inherent uncertainty in the assignment of breakpoints, as indicated by the shaded lines. For example, *Df(2L)ed*$^{Sz-1}$ and *Df(2L)M11* appear to have overlapping breakpoints cytologically, but can clearly be separated genetically since the former complements *fat* (*ft*) mutations but not *echinoid* (*ed*) mutations, while the latter complements *ed* but not *ft*. (Szidonya and Reuter, 1988). Several lethal complementation groups have been identified which lie within the 24D4 - 24D8 interval (Szidonya and Reuter, 1988), which indicated by the solid vertical lines in Figure 4-2. These lethal complementation groups are listed above the line of the chromosome, and of these, the *ft* has been cloned (Mahoney *et al*, 1991) and is not *dSR-CI*. In addition, since *l(2)24Db* is a *Minute* mutation, it unlikely to be *dSR-CI* , as these are usually ribosomal proteins (Ashburner, 1989). The previously assigned positions of *ft*, *l(2)24Dc*, *l(2)24De*, and *l(2)24Df* were confirmed by testing the ability of mutants in these genes to complement several of the deficiency strains , which demonstrated that they could complement *Df(2L)ed*$^{Sz-1}$ and *Df(2L)dp*^{h28}, but not *Df(2L)M11* (data not shown). However, *l(2)24Dd* was previously placed incorrectly between the breakpoints of the *Df(2L)ed*$^{Sz-1}$ and *Df(2L)dp*^{h28} chromosomes. In our hands, complementation analysis versus this deficiency gave only two *l(2)24Dd/Df(2L)ed*$^{Sz-1}$ progeny out of 114 scored for one allele, and none out of 84 scored for a second allele. Since one of these alleles also failed to complement *Df(2L)M11* (0/131, the other allele was not tested), *l(2)24Dd* appears to be located in the same cytogenetic interval as *ed*. The ability of *l(2)24Dd* to complement *ed* and *Df(2L)sc*$^{19-1}$ is currently being examined. The assignment of the cytogenetic location of the *l(2)d* and *l(2)g* genes (De Belle *et al*, 1993) has not yet been confirmed. Finally, two genes have been genetically mapped to the 24D region, *angle wing* (*ang*) (Mittler, 1950) and *l(2)G99* (Golubovsky, 1971). The physical locations of these genes have not yet been determined.

Southern analysis of genomic DNA was performed in order to more accurately determine the physical location of the *dSR-CI* gene with respect to the deficiencies illustrated in Figure 4-2. In initial experiments using genomic DNA from *Canton-S* flies, the dSR-CIp probe detected a single EcoRI fragment of approximately 4.8 kbp and two EcoRV fragments of approximately 2.6 kbp and 4.2 kbp (Figure 4-3) consistent with the

presence of an EcoRV site at bp 815 of the dSR-CI cDNA (Chapter 3). Next, genomic DNA isolated from embryos homozygous for several of the region 24D - 25A deficiencies was compared to genomic DNA isolated from their heterozygous *Df/ Balancer* parents by EcoRI and EcoRV digestion and Southern hybridization. As shown in Figure 4-4, hybridization of the dSR-CI probe to an EcoRI fragment of 4.6 kbp was observed in genomic DNA prepared from both *Canton - S* and *Oregon-R* flies in this experiment. This EcoRI fragment is similar in size to that observed in the preliminary experiment shown in Figure 4-3. Interestingly, the *CS* and *OR* genomic DNA did not display the same EcoRV restriction fragment pattern observed previously. While the 2.6 kbp fragment seen in Figure 4-3 was still observed, the 4.2 kbp fragment was now missing. In its place, there was a fragment of approximately 10 kbp length. Thus, there appears to be an EcoRV polymorphism to one side of the *dSR-CI gene.* In order to aid in the discussion below, I will call the two polymorphic variants RFLP-A (2.6 kbp and 4.2 kbp EcoRV fragments) and RFLP-B (2.6 kbp and ~10 kbp fragments). The reason for the discrepancy between the results of these two Southern analyses is unclear, but may be related to the fact that the first experiment (Figure 4-3) used genomic DNA, generously provided by H. Steller, which may have been isolated from a different *CS* line than that used for the second experiment.

Upon analysis of the *Df* strain genomic DNA, it was observed that both the *Df/Balancer* parents and the *Df/Df* embryos from strains *Df(2L)ed*$^{Sz\text{-}1}$ and *Df(2L)dp*^{h2} contained the 4.6 kbp EcoRI fragment and the RFLP-B EcoRV fragments (Figure 4-4). Thus, these two chromosomal deletions do not remove the *dSR-CI* gene from the *Drosophila* genome. Interestingly, the Balancer chromosome appears to be of the genotype RFLP - A, while the chromosome harboring the deletion is of genotype RFLP - B, since both RFLP patterns are observed in the heterozygous parents, but only the RFLP - B pattern is observed in the *Df* homozygotes.

In contrast to the first two *Df* strains, there was no detectable *dSR-CI* signal in genomic DNA obtained from homozygous *Df(2L)M11* embryo's (Figure 4-4). Thus, the *dSR-CI* gene must be contained within the region of the genome defined by the breakpoints of this chromosomal deletion. Genomic DNA isolated from homozygous *Df(2L)ed-dp*^{h1} embryos generated a signal upon hybridization to the dSR-CI probe, although this signal was weaker than that generated by genomic DNA from the parental line and also demonstrated a slight mobility shift (Figure 4-4). This result might indicate that the *dSR-CI* gene is present on the *Df(2L)ed-dp*^{h1} chromosome. However, there are two reasons for believing that the gene is actually deleted from this chromosome. First, it is apparent from the ethidium bromide stained gel used to prepare this Southern blot that genomic DNA from the *Df(2L)ed-dp*^{h1} homozygous embryos is vastly overloaded compared to the genomic DNA from their parents (Figure 4-4 bottom). Thus, the hybridization signal detected may result from a low level contamination of the genomic DNA prepared from the *Df* homozygous embryos with DNA non- *Df* homozygote embryos collected accidentally. Indeed, it is interesting that the parental *Df(2L)ed-dp*^{h1} chromosome appears to be of the RFLP-A genotype, while the weak EcoRV signal detected in embryonic DNA is of the RFLP-B genotype, suggesting that there was in fact a contamination with RFLP-B containing flies.

Second, the EcoRI and EcoRV genomic fragments detected in the homozygous embryonic DNA are mobility shifted to the same degree. This would be unexpected if the *Df(2L)ed-dp*^{h1} was deleting only part of the genomic DNA near the *dSR-CI* gene. Instead, the mobility shift may simply be due to the vast excess of genomic DNA that was run on the agarose gel. Indeed, the genomic fragments detected by hybridization to an elongation factor 1α probe are mobility shifted to the same degree as the fragments recognized by the dSR-CI probe (data not shown).

The results of this Southern analysis are consistent with the localization of the *dSR-CI* gene as determined by *in situ* hybridization. Further, this analysis demonstrated that *l(2)24Dc*, *l(2)24De*, and *l(2)24Df* might correspond to the *dSR-CI* gene since the are known to complement *Df(2L)ed-*^{Sz1} but not *Df(2L)M11* (Szidonya and Reuter, 1988). In addition, these results indicate that *ed* , *l(2)d*, *l(2)g*, and *l(2)24Dd* are not *dSR-CI*, since these genes can complement *Df(2L)M11* , but not *Df(2L)ed-*^{Sz1} (Figures 4-2 and 4-4).

The identification of only a single 4.6 kbp EcoRI fragment containing dSR-CI sequences by Southern analysis suggested that the *dSR-CI* gene was small. PCR amplification of *Canton-S* genomic DNA using primers homologous to the 5' and 3' termini of the dSR-CI cDNA clone yielded a product of approximately 2.3 kbp, when corrected for the faster than expected mobility of the cDNA derived control reaction product (Figure 4-5B, see figure legend for explanation). This result is consistent with the possibility that the *dSR-CI* gene is small, since the cDNA clone is likely represent most, if not all, of the dSR-CI message (the cDNA size is 2.03 kbp and Northern analysis detects a single message of approximately 2.1 kb (Chapter 3)). However, this result does not eliminate the possibility that there is a small exon in the 5' or 3' UTR which is distantly removed from the rest of the *dSR-CI* gene. At least for the region between the two terminal primers I have used, there appear to be only approximately 250 bps of intronic sequence. Additional PCR analysis has identified two introns in the *dSR-CI* gene (Figure 4-5). The first was identified using primers derived from sequences specifying the middle of the first and the middle of the second CCP domains, suggesting that each CCP domain may be the product of a separate exon. The second intron lies between two primers located within the MAM domain coding region. Interestingly, there appears to be no intron separating the sequences coding for the second CCP domain and the MAM domain. It is likely that there is at least one additional intron in *dSR-CI*, since the two introns have been identified are approximately 70 and 100 bps in length respectively.

Prior to mapping the *dSR-CI* gene relative to the 24D - 25A region deletion breakpoints, three genomic clones, λd5.1a, λe1.1b, and λg2.1a, had been isolated from a *CS* library using as a probe the 5' 1101 bps of the dSR-CI cDNA. By digesting these clones with a panel of seven restriction enzymes, it became apparent that there was significant overlap between clones λe1.1b, and λg2.1a (data not shown). These two clones were therefore subjected to further analysis, as described in Experimental Procedures, in order to determine an unambiguous restriction map. Some of the data

used to construct this map is shown in Figure 4-6A, which illustrates the pattern of fragments obtained from several single and double restriction enzyme digests of clone λe1.1b. For example, digestion of this clone with EcoRI generates insert-derived fragments of approximately 0.6, 1.5, 1.7, and 4.6 kbps, and two fragments of approximately 3.9 kbps, as indicated by the increased intensity and width of this band relative to the others. The small 0.6 kbp fragment is barely visible on the ethidium bromide stained gel and did not reproduce well when this figure was generated. ScaI reduces the size of the 4.6 kbp and one of the 3.9 kbp fragments by approximately 1.0 and 0.6 kbps respectively (again, the 0.6 kbp fragment did not reproduce well, but it was obvious on the ethidium bromide stained gel that its intensity was twice that of the band observed in the EcoRI only digest). Some of the restriction digested genomic DNA samples, including those shown in Figure 4-6A, were subjected to Southern hybridization analysis in order to determine which fragments contained sequences homologous to the dSR-CI cDNA. As shown in Figure 4-6B, a probe corresponding to the 5' 244 bps of the dSR-CI cDNA hybridized to the 4.6 kbp EcoRI fragment, and to the 3.6 kbp fragment generated by the EcoRI/ScaI double digest. The same EcoRI, ScaI and EcoRI/ScaI fragments were also identified using an independent dSR-CI probe. The subsequent observation, described above (Figure 4-3 and 4-4), of 4.6 kbp fragments homologous to a dSR-CI cDNA probe in genomic DNA from several different fly strains, described earlier was consistent with the results of the λe1.1b restriction and Southern analyses. Ultimately, a 28 kbp restriction map of the overlapping clones λe1.1b and λg2.1a was constructed by these types of methods (Figure 4-7). Upon completion of the map, it was observed that a PvuI/ScaI fragment of approximately 1.4 kbp (the actual PvuI/ScaI double digest has never been performed) should be homologous to the dSR-CI cDNA. Although this prediction was not tested, it was observed that the dSR-CI cDNA contains each of these sites, separated by a distance of 1.6 kbp, as determined by the cDNA sequence and intron/exon structure analysis presented above. Thus, by this criterion, in combination with the observation of 4.6 kbp EcoRI fragments in both *CS* and λe1.1b genomic DNA which hybridized to dSR-CI probes, the λe1.1b and λg2.1a clones appeared to contain the dSR-CI gene. Furthermore, the PvuI and ScaI sites helped to define the orientation of the gene within the λ clones.

An unambiguous restriction map for λd5.1a has not yet been completed, but both the pattern of restriction fragments and Southern analysis indicate that this clone does not appear to overlap with either of the other two clones (data not shown). It has never been possible to obtain PCR amplification products from this clone using several sets of dSR-CI primers. In addition, sequencing of the 1.3 kbp XbaI fragment of this clone which is recognized by an oligonucleotide probe on Southern blots demonstrated no significant homology to *dSR-CI* (data not shown). These results indicate that λd5.1a does not contain dSR-CI sequences, and may have been fortuitously isolated during the initial screen for genomic clones.

After the *dSR-CI* gene was mapped relative to the 24D -25A area chromosomal deletions by Southern hybridization to *Df* strain genomic DNA (Figures 4-2 and 4-4), the λe1.1b and λg2.1a clones were used as probes on the same Southern blots to determine if any of the deletion breakpoints were within 28 kbp genomic interval defined by these clones. Unexpectedly, it was found that every deletion chromosome tested appeared to contain the entire 28 kbp of cloned genomic DNA (data not shown). This result immediately suggested that these genomic clones contained a homolog of the *dSR-CI* gene, and not the gene itself. This hypothesis was strengthened by Southern analysis of restriction enzyme digested *CS* and λe1.1b genomic DNA using a dSR-CI cDNA derived probe. This probe recognized fragments in *CS* genomic DNA that were different from those it recognized in λe1.1b DNA. For example, the cDNA probe recognized two EcoRV bands of 2.8 kbp and approximately 11 kbp in *CS* genomic DNA (Figure 4-8A, left), consistent with previous results. However, this probe recognized only a single EcoRV fragment of greater than 14 kbp in the λe1.1b DNA (Figure 4-8A, right). A similar difference was observed with XmnI digestion: fragments of 1.2 kbp and 1.35 kbp were detected in *CS* DNA, while fragments of 1.6 kbp and 4.9 kbp were detected in λe1.1b DNA. In fact, the only enzyme for which a difference is not detected is EcoRI, in which a single 4.6 kbp fragment is detected in both genomic DNA samples (Figure 4-8A)! This illustrates the importance of using multiple restriction enzymes to analyze genomic DNA: EcoRI was the only enzyme which was used for analysis of *CS* and *Df* genomic DNA which had been mapped in the λ clones.

It is unlikely that genomic polymorphism can account for all of the differences observed between *CS* and λe1.1b genomic DNA since four of five different enzymes tested all show these differences. To verify that these differences were not due to polymorphisms in these restriction site loci, a second blot of the same *CS* DNA was prepared and was hybridized to a mixed λe1.1b/λg2.1a probe. Most of the restriction fragments which gave strong hybridization signals with the cDNA probe did not give strong signals with the mixed λ probe (Figure 4-8B). Thus, the 2.8 kbp and ~11kbp EcoRV fragments, the 1.2kbp and 1.35 kbp XmnI fragments, and the ~12 kbp NcoI fragments detected in *CS* DNA using the cDNA probe are not detected using the mixed λ probe. Instead, the mixed λ probe detected *CS* DNA restriction fragments which were similar in size to restriction fragments that were weakly recognized by the cDNA probe (compare Figure 4-8A left with Figure 4-8B). Interestingly, the mixed λ probe detected only some of the *CS* DNA restriction fragments that were weakly detected by the cDNA probe; for example, two ScaI fragments of around 3.6 kbp and 3.8 kbp are weakly detected by the cDNA probe, but are not detected by the mixed λ probe. This result suggests that there may be additional *dSR-CI* homologs in the *Drosophila* genome. Low stringency Southern analysis of an isogenized *Canton-S* strain should provide a good estimate of the number of homologs.

To ultimately verify that λe1.1b and λg2.1a do in fact contain a homolog of *dSR-CI*, the 1.4 kbp PvuI/ScaI fragment predicted by restriction analysis (Figure 4-6) was subcloned and sequenced. Sequence analysis of this fragment indicated that λe1.1b and λg2.1a do contain a *dSR-CI* homolog. This homolog has tentatively been named *dSR-CII*. The 4.6 kbp EcoRI fragment of λe1.1b was subsequently cloned into pBSIIKS+ and has been partially sequenced (Figure 4-9). Sequence analysis of this clone predicts that, like *dSR-CI*, *dSR-CII* is a small gene which appears to contain three introns ranging in size from 63 to 126 bps. Small introns are quite common in *Drosophila* genes, with 60 to 65 bp introns being the most abundantly represented size class (Mount *et al*, 1992).

Sequencing of a 2066 bp (excluding a short poly A tail) dSR-CII cDNA cloned from a 4 - 8 hr embryonic library (see Experimental Procedures) verified the small size of the *dSR-CII* gene and the presence of all three introns (Figure 4-9). A canonical arthropod transcription start site sequence (Cherbas and Cherbas, 1993) exactly overlaps the 5' end of the cDNA, suggesting that the cDNA represents the entire dSR-CII message. In addition, the 84 bp 5' UTR contains an in frame stop codon 69 bps upstream of the putative initiator methionine codon. There are two polyadenylation signals in the 185 bp 3' UTR, which also contains two ATTTA sequences that may confer instability on this message (Shaw and Kamer, 1986). Comparison of the dSR-CI and dSR-CII cDNA sequences indicates that the two genes are highly homologous, their DNA coding sequences being 67% identical. The locations of the first two dSR-CII introns coincide with the locations of the dSR-CI introns determined by PCR mapping (Figure 4-5). However, the first dSR-CI intron may be significantly smaller (approximately 70 bps) than the corresponding intron in dSR-CII (126 bps). The dSR-CII gene also contains a third intron, which is located between sequences coding for the end of the mucin domain (see below) and the second spacer domain of the dSR-CII protein.

The dSR-CII protein has the same arrangement of domains as the dSR-CI protein (Figure 4-10). The two proteins are quite similar at the amino acid sequence level, having 55% amino acid identity and 70% amino acid similarity overall. A particularly high degree of homology is apparent in the MAM domain (75% identity/ 84% similarity), while the CCP, transmembrane and cytoplasmic domains also share a significant degree of homology. Cleavage of the putative signal sequence of dSR-CII would generate a mature, 581 residue protein of 64.9 kD if unmodified. However, the presence of a serine/threonine rich region indicates that this protein, like dSR-CI (chapter 3), will probably have a greater mass due to significant O-glycosylation. The mature dSR-CII protein will likely have a significantly smaller mass than its homolog, however, since the mucin - like domain of dSR-CII is both shorter and less serine/threonine-rich than the corresponding domain of dSR-CI. The dSR-CII protein may also be N-glycosylated. Three potential N-linked glycosylation sites are conserved with dSR-CI in the MAM domain, and there are four additional sites specific to dSR-CII (Figure 4-10). Both dSR-C proteins have multiple potential serine/threonine phosphorylation sites in their cytoplasmic termini, but again, there

does not appear to be any particular conservation of these sites between the two proteins (Figure 4-10). Finally, it is interesting to note that, whereas an unmodified dSR-CI protein would be essentially neutral (+1 charge overall), an unmodified dSR-CII protein would be slightly acidic (-12 charge overall, calculated pI 6.0).

An additional *dSR-CI* homolog was discovered during a search of the Genbank database. This homolog is represented by the sequence tagged site STSDm0173, which has been generated by the *Drosophila* genome project, and which encodes part of a MAM domain. Comparison of the predicted amino acid sequence encoded by the STSDm0173 sequence with the MAM domains of dSR-CI and dSR-CII (Figure 4-10) shows that the STSDm0173 protein is significantly more divergent than the other two proteins (46% amino acid similarity/54% identity to dSR-CI and 40%/55% to dSR-CII). The MAM domain intron in STSDm0173 is located at the same position as an intron in dSR-CII , but at 60 bps, is shorter than those of dSR-CI (approximately 100 bps) and *dSR-CII* (111 bps). Given the low quality of the STSDm0173 sequence, an accurate determination of the level of conservation awaits the isolation of genomic and cDNA clones containing the complete STSDm0173 sequence. Importantly, there are no stop codons in the 245 bp of known STSDm0173 sequence. Coupled with the observation that an mRNA which hybridizes to the STSDm0173 sequence can be detected by Northern analysis (see below), this suggests that STSDm0173 marks a functional gene. Confirmation of this hypothesis awaits the isolation and sequencing of a corresponding cDNA, and ultimately, the detection of an STSDm0173 protein.

STSDm0173 defines the distal end of a contig assembled from overlapping 75 - 100 kbp P1 genomic clones by the *Drosophila* genome project (Smoller *et al*, 1991) (Figure 4-11). Interestingly, this contig is located at the 24D region of 2L in the vicinity of the *ft* gene. Prior to the identification of the *STSDm0173* gene as a dSR-CI homolog, PCR analysis of several P1 clones from this contig had indicated that the *dSR-CI* gene mapped to the same P1 clones as STSDm0173 (data not shown). Since the primers used for this analysis were homologous to dSR-CII, this assignment was verified using dSR-CI specific primers. At the same time, the *Drosophila* genome project found new STS (STSDm3565) which also mapped to these clones, and which corresponds to the sequence encoding the cytoplasmic domain of dSR-CI! Thus, it is now very clear that the *dSR-CI* and *STSDm0173* genes lie close together in the *Drosophila* genome. The exact arrangement of these two genes relative to each other is unknown, and is currently under investigation.

The colocalization of *dSR-CI* and *STSDm0173* to the same P1 clones indicates that these two genes are located within approximately 80 kbp of each other in the genome. Southern analysis had already indicated that *dSR-CI* was deleted in *Df(2L)ed-dp^{h1}* and *Df(2L)M11* embryos (Figure 4-4). In order to more precisely determine the location of the *dSR-CI* and *STSDm0173* genes relative to the chromosomal deletion breakpoints in the 24D - 25A region, gene-specific primers were used for single embryo multiplex PCR analysis of deficiency homozygotes as described in Experimental Procedures. The use of single embryo PCR has two advantages over Southern analysis for answering this question: 1) since individual

embryos are analyzed, the PCR method is insensitive to small contaminations of homozygous *Df* embryos with their non-homozygous siblings, and 2) it is significantly easier to obtain 10 dead embryos and prepare their DNA for PCR than it is to obtain several hundred dead embryos and prepare their DNA for Southern analysis. The advantage of multiplex PCR is that it allows for the identification of multiple genes from the same embryo using a minimum number of PCR reactions.

Preliminary PCR experiments established working conditions for the multiplex PCR reactions and demonstrated that each specific primer pair amplified only its corresponding gene (data not shown). Upon analyzing three homozygous deficiency embryos from each deficiency strain, it was found that the control rp49 (the gene encoding ribosomal protein 49 - Kongsuwan *et al*, 1985) and dSR-CII primers amplified products of the expected size in every embryo (Figure 4-12A). Thus, as already indicated by the Southern analysis (Figures 4-2 and 4-4), the dSR-CII gene is not deleted in any of the 24D - 25A region chromosomal deletions examined and must be located elsewhere in the genome. The precise location of *dSR-CII* will be determined by *in situ* hybridization to polytene chromosomes. The ability to amplify dSR-CII sequences from these *Df* strain genomic DNAs suggests that cross-hybridization of the dSR-CIp probe to *dSR-CII* sequences may have contributed to the residual hybridization signal detected in the initial Southern analysis of these *Df* strains (Figure 4-4).

The dSR-CI, STSDm0173 and ft primers amplified products in all $Df(2L)ed^{Sz\text{-}1}$, $Df(2L)M\text{-}z^{B}$ and $Df(2L)dp^{h25}$ embryos, in two of the three homozygous $Df(2L)dp^{h28}$ embryos, and in several additional homozygous $Df(2L)dp^{h28}$ embryos (Figure 4-12A and data not shown). Interestingly, weak dSR-CI sized products were also detected in all of the $Df(2L)ed\text{-}dp^{h1}$ and *Df(2L)M11* embryos (Figure 4-12A). The weak amplification of these products, coupled with the fact that it has not been possible to amplify them in subsequent experiments using the same primers and embryos, suggests that they do not correspond to either the *dSR-CI* or the *STSDm0173* gene. Indeed, restriction analysis of these products indicated that they could not be either dSR-CI or dSR-CII because they were insensitive to digestion by either EcoRV (dSR-CI specific) or ClaI (dSR-CII specific) (Figure 4-12A and 4-12B). Furthermore, the dSR-CI sized products could not correspond to STSDm0173 since they were the wrong size. While the origin of these PCR products is not clear, they may represent yet other members of the dSR-C family of proteins, and I am currently attempting to clone them for sequence analysis.

Single embryo PCR has confirmed and extended the conclusions of the Southern analysis regarding the cytological location of *dSR-CI* (Figure 4-4). The demonstration that the MAM domain coding sequences of *dSR-CI* are not deleted in the $Df(2L)dp^{h28}$ and $Df(2L)M\text{-}z^{B}$ chromosomes (Figure 4-12) suggests that it this gene is not *l(2)24Df*. Further, the *STSDm0173* gene is not separated from the *dSR-CI* gene by any deletion breakpoints, thus indicating that most of the mutants which can not correspond to *dSR-CI* also can not correspond to *STSDm0173*. However, since *STSDm0173* is distal to *dSR-CI*, and since the *STSDm0173* primers may be located

in the middle of the gene, it is possible that *ed*, *l(2)24Dd*, *l(2)d* or *l(2)g* may be *STSDm0173* mutants.

In order to begin elucidating the functions of *dSR-CII* and *STSDm0173*, their expression patterns were analyzed by Northern analysis of poly A+ RNA isolated from various stages of *Drosophila* development (Figure 4-13). In contrast to *dSR-CI*, which is expressed throughout development beginning about 4 hours after egg laying (Figures 3-10 and 3-11), *dSR-CII* expression is restricted to 0 - 8 hour embryos. As predicted from the cDNA sequence, the dSR-CII message is approximately 2.0 kb in length, indicating that the dSR-CII cDNA must be very nearly, if not completely, full length. The expression of *STSDm0173* during development differs from both *dSR-CI* and *dSR-CII*. This gene is expressed only during the larval and pupal stages (Figure 4-13). Interestingly, the STSDm0173 message appears to be only approximately 1.25 kb in length, significantly shorter than the messages of the other two *dSR-C* genes.

Discussion

As discussed in Chapter 3, the properties of the *dSR-CI* gene suggest that it might participate in one or more of several different processes during *Drosophila* development and host defense. The isolation of *dSR-CI* mutants would greatly facilitate the identification of the actual *in vivo* functions of this gene. As a first step in this process, the gene was mapped to the region 24D4 - 24D8 on the left arm of chromosome 2. This places *dSR-CI* close to the *ft* gene (Figure 4-1). Using this information, *dSR-CI* was further mapped with respect to several chromosomal deletions. The results of Southern analysis (Figure 4-4), PCR (4-12) and the *Drosophila* genome project are all consistent with the *in situ* localization of the *dSR-CI* gene. These results indicate that *dSR-CI* is located less than 100 kbp to the distal side of the *ft* gene.

In order to facilitate the isolation, analysis and transformation-mediated rescue of *dSR-CI* mutants, it is necessary to have genomic clones containing the dSR-CI gene. Although genomic clones were isolated using the 1101p probe derived from the dSR-CI cDNA, analysis of two of these clones indicated that they actually harbor a close homolog of dSR-CI, which has been named *dSR-CII*. At the same time that *dSR-CII* was identified, database searching revealed that a sequenced tagged site (STSDm0173) generated by the *Drosophila* genome project was moderately homologous to part of the MAM domain coding sequence of *dSR-CI*. While *dSR-CII* does not appear to be located in the near vicinity of *dSR-CI* (i.e. the region from 24A to 25B, based on Southern and PCR analysis), the *STSDM0173* gene is located on the same P1 genomic clones as the *dSR-CI* gene (Figure 4-11), indicating that it is within 100 kbp of *dSR-CI*. Both *dSR-CII* and *STSDm0173* appear to be functional genes, since the dSR-CII cDNA has no stop codons through its entire region of homology with dSR-CI, while STSDm0173 contains no stop codons in the partial open reading frame which has been sequenced. In addition, transcripts corresponding to each of these open reading frames can be detected by Northern analysis (Figure 4-13).

Two additional genomic clones were identified during the initial screen with the 1101p probe. It has never been possible to isolate DNA from one of these clones, so it is not known whether this clone contains the *dSR-CI* gene. The other clone is distinctly different from the *dSR-CII* containing clones and does not appear contain the *dSR-CI* gene by both PCR analysis and sequencing. Since the sequenced portion of this clone was isolated using a *dSR-CI* oligonucleotide probe which may have hybridized to a different region than the 1101p probe initially used to isolate the clone, it remains possible that this clone contains yet another *dSR-CI* homolog.

There are now numerous examples of gene families in *Drosophila*. In some cases, the various members of these families are scattered throughout the genome. Examples include genes encoding secreted proteins (TGF-ß homologs: *decapentaplegic* , *screw*, *60A* - Arora *et al*, 1994; Wharton *et al*, 1991), receptors (type II receptors for TGF-ß: *thick veins*, *saxophone* - Brummel *et al*, 1994), and cytoplasmic proteins (kinesin like proteins: *klp* genes Dalby *et al*, 1993). Often, these genes are

less conserved with each other than they are with corresponding homologues in vertebrates, suggesting that they may have arisen by gene duplication events pre-dating the divergence of the vertebrate and arthropod lineages during evolution.

Structurally related genes have also been found in *Drosophila* which are tightly linked to each other. Examples of such genes include several different transcription factor families (*Antennapedia-complex* and *Bithorax-complex* genes - McGinnis *et al*, 1984; *sloppy-paired 1/slp2* - Grossniklaus *et al*, 1992; *knirps/knirps-related/Embryonic-gonad* - Rothe *et al*, 1989; *invected/engrailed* - Coleman *et al*, 1987; *gooseberry/gooseberry-neuro* - Baumgartner *et al*, 1987; *BarH1/BarH2* Higashijima *et al*, 1992; and *odd-skipped/sister-of-odd-and-bowel/bowel* - Hart *et al*, 1996) and secreted proteins (BMP-1 homologs: *tolloid/tolloid-related-1* - Nguyen *et al*, 1994; the antibacterial peptide *cecropin* genes - Samakovlis *et al*, 1990; *L71* genes - Wright *et al*, 1996). Two members of a gene pair (e.g. *odd/sob* or *kni/knrl*) may be divergently transcribed (Wright *et al*, 1996; Baumgartner *et al*, 1987), convergently transcribed (Coleman *et al*, 1987) or transcribed in the same direction (Grossniklaus *et al*, 1992; Nguyen *et al*, 1994; Hart *et al*, 1996). In the case of the *cecropin* locus, which contains three equally spaced genes separated from one another by two pseudogenes, two of the genes are tandemly transcribed while the third is convergently transcribed relative to the second (Samakovlis *et al*, 1990).

The exact organization of *dSR-CI*, *dSR-CII* and *STSDm0173* within the genome is currently under investigation using *in situ* polytene chromosome hybridization to localize *dSR-CII*, and restriction analysis of P1 clones to determine the distance of separation between and relative orientation of *dSR-CI* and *STSDm0173*. It is possible that these three genes will be organized in a manner similar to the *kni/knrl/Egon* and *odd/sob/bowl* gene families (Hart *et al*, 1996; Rothe *et al*, 1987). Two members of these families are tightly linked to each other (e.g. in the 24A region, < 10 kbp separates *sob* from *odd*), while the third is several lettered divisions away (e.g. *Egon* is in 24C). The *odd/sob/bowl* family is particularly interesting because the two most closely linked genes, *odd* and *sob*, are less related to each other than *sob* is to *bowl*, which is further away in the genome (Hart *et al*, 1996). This is strikingly reminiscent of the sequence relationships between *dSR-CI*, *dSR-CII* and *STSDm0173* (see below).

Tightly linked homologous genes in *Drosophila* often have overlapping temporal and spatial patterns of expression, suggesting that they are regulated by common *cis*-- acting sequences. At one extreme, both members of the *BarH1/BarH2* and *kni/knrl* gene pairs have been found to be expressed identically both temporally and spatially (Rothe *et al*, 1992; Higashijima *et al*, 1992). More often, linked homologous genes have similar, but non-identical, patterns of expression (Samakovlis *et al*, 1990; Nguyen *et al*, 1994; Coleman *et al*, 1987; Grossniklaus et al, 1992; Wright *et al*, 1996; Hart *et al*, 1996; Li and Noll, 1994). This suggests that, in addition to common *cis*-acting sequences, there may be gene-specific *cis*-acting sequences which control the expression of linked homologous genes. It was previously established that *dSR-CI* is expressed during throughout development starting in embryonic stage 10 (Chapter 3). Northern analysis indicates that *dSR-CII*

expression, on the other hand, is restricted to 0 - 8 hour embryos (Figure 4-13). Furthermore, unlike *dSR-CI*, *dSR-CII* is not expressed in the mbn-2 hemocyte cell line (data not shown). Thus, the regulation of *dSR-CII* expression appears to differ significantly from that of *dSR-CI*. The lack of detectable dSR-CII message in females suggests that it is not a maternal gene, and is probably first expressed after cellularization of the embryo occurs around 2.5 hours after egg laying. In contrast to *dSR-CII*, *STSDm0173* expression in larva and early pupa temporally overlaps, but is more restricted than, *dSR-CI* expression. Thus, *STSDm0173* may be regulated in part by the same *cis* -acting elements which control *dSR-CI* expression. It will be interesting to determine the cell and tissue specificity of both *dSR-CII* and *STSDm0173* expression.

The STSDm0173 message is significantly shorter than the dSR-CI and dSR-CII messages (Figure 4-13). This may suggest that the gene represented by STSDm0173 simply encodes a MAM domain containing protein which is not actually a dSR-CI homolog. That MAM domains are found in a variety of otherwise functionally and structurally unrelated proteins demonstrates that they are basically molecular building blocks which can be used in a variety of functionally unrelated proteins. Alternatively, this gene may be a homolog in which one or more of the *dSR-CI* encoded domains are not present. There are precedents for such a relationship between two or more members of a tightly linked gene family in *Drosophila*. For example, one of the *L71* encoded proteins contains a 75 residue C-terminal extension which almost doubles its size relative to the other *L71* encoded proteins (Wright *et al*, 1996). In addition, the *tlr-1* encoded protein contains a large N-terminal extension relative to the *tld* encoded protein (Nguyen *et al*, 1994). An interesting possibility in regard to *STSDm0173* is that it contain sequences homologous to those in *dSR-CI* which encode the CCP, MAM and somatomedin B domains, but lacks the more 3' sequences. Such a gene would generate a 1.2 kb message and would encode a secreted protein. It will be important to determine the actual sequence of the *STSDm0173* gene at the genomic and cDNA levels in order to determine whether it is a true *dSR-CI* homolog.

As has already been mentioned, sequence comparison indicates that *dSR-CI* and *dSR-CII* are closely related genes, while *STSDm0173* is a more distant relative (Figure 4-10). The locations of the first two introns in *dSR-CI* and *dSR-CII* are very similar and may be identical. Consistent with this hypothesis, STSDm0173 contains a putative intron sequence located in precisely the same position as the *dSR-CII* intron (Figure 4-9). PCR analysis indicates that *dSR-CI* must contain at least one additional intron, which may be located in the same region as the third intron of the *dSR-CII* gene.

dSR-CI and dSR-CII are also well conserved at the level of amino acid sequence, with STSDm0173 again being more distantly related. The MAM domains of *dSR-CI* and *dSR-CII* are particularly well conserved, and are more closely related to each other (75% amino acid identity/84% similarity) than to any other known MAM domains (best match: 30% identity/45% similarity to the MAM domain of rat apical endosomal protein (Speelman et al, 1995). The reason for the high level of sequence

conservation between the MAM domains of *dSR-CI* and *dSR-CII* is not known. Perhaps these domains play a similar role in both of these proteins. Although less well conserved than the MAM domains, the CCP domains of dSR-CI and dSR-CII are also more closely related to each other than to other CCP domains (data not shown). In addition, even though it is significantly less well conserved, the MAM domain of STSDm0173 is also more similar to those of dSR-CI and dSR-CII than to any other known MAM domains. The greater homology between *dSR-CI* and *dSR-CII* than between either of these genes and *STSDm0173* suggests that the duplication event which distinguished *STSDm0173* from *dSR-CI/dSR-CII* occurred prior to that which distinguished *dSR-CI* and *dSR-CII* from each other. It is also possible that after a duplication event which generated the *dSR-CI/STSDm0173* gene pair, a second event duplicated both members of this pair and was followed by a translocation event which moved one of the gene pairs to a different region of the genome. Thus, it is possible that there is an *STSDm0173* homolog tightly linked to *dSR-CII* which has not yet been identified. This possibility has not yet been tested.

The existence of a family of genes related to *dSR-CI* raises the possibility that some of these genes might be functionally redundant. This is a non-trivial issue, especially since *dSR-CI* mutants may have no phenotype if the gene can be functionally replaced by one of its homologs. Functional redundancy of related genes has been observed in yeast (for example, see Kataoka *et al*, 1984) and mammals (for example, see Lohnes *et al*, 1993). Functional redundancy has also been observed in *Drosophila* among some of the linked homologous gene families described earlier. For example, *BarH1* and *BarH2* appear to be completely functionally redundant - only when both genes are deleted from the genome can any phenotypic effect be observed (Higashijima *et al*, 1992). To a lesser degree, *kni* and *knrl* also have redundant functions: during embryonic development, *knrl* can compensate for a loss of anterior *kni* function, but not for a loss of posterior *kni* function, in *kni* mutants. Only when both gene products are absent is anterior head development affected (Gonzalez-Gaitan, 1994). Similarly, *slp1* and *slp2* are almost, but not completely, functionally redundant (Cadigan *et al*, 1994). There may be some degree of functional homology among unlinked homologous genes in *Drosophila* as well. For example, it is possible that the dpp receptor tkv may be functionally redundant with sax in some cell types (Brummel *et al*, 1994).

There are several ways in which the issue of the potential functional redundancy between *dSR-CI*, *dSR-CII* and *STSDm0173* can be addressed. First, having cloned the dSR-CII cDNA, it will now be possible to determine if the dSR-CII protein has scavenger receptor activity in transfected tissue culture cells. This work is currently in progress, and similar studies will be possible once the *STSDm0173* gene is cloned. It has been found, however, that genes with similar activities which can functionally compensate each other under artificial conditions, are not redundant under normal circumstances due to differences in expression or gene structure (Li and Noll, 1994; Rothe *et al*, 1992). Thus, it will also be important to determine the spatial pattern of *dSR-CII* and *STSDm0173* expression in the developing fly. That *dSR-CII* is expressed only in 0 - 8 hour embryos suggests that it may not compensate for a *dSR-*

CI loss except perhaps at this early stage of development. Given the similar temporal pattern of *STSDm0173* and *dSR-CI* expression, determination of both the spatial expression pattern of these genes in late embryos, larva and pupa is of particular importance. Should the STSDm0173 protein turn out to be similar to the dSR-CI protein in structure, function, and expression pattern, it may be necessary to construct chromosomes carrying null mutations of both genes, perhaps by generating small deletions, in order to accurately determine the function of either gene during *Drosophila* development and host defense.

In summary, at least one new gene has been identified which shares significant homology with the entire *dSR-CI* gene, while a second shares significant homology with at least a portion of *dSR-CI*. Are there additional *dSR-CI* homologs in *Drosophila*? There is no *a priori* reason for believing that the answer to this question will be either yes or no, and it will be interesting to analyze *Drosophila* genomic DNA using both PCR and low stringency Southern hybridization analysis in order to answer this question.

Table 4-1. Oligonucleotides used in this study.

Oligonucleotide	Gene	Sequence (5' --> 3')
asdSR1.1	*dSR-CI*	GTTGCATATTTTCTTTTATT
asdSR1.15	*dSR-CI*	CGTTGTTGTTGGCTTCGGCG
asdSR1.23	*dSR-CI*	GCCACTGGGTTCCCTGCTGA
asdSR1.25	*dSR-CI*	CGATAGCAGATGGTAGCTTC
asdSR1.30	*dSR-CI*	GTGGTTGCTCCTTCGACACG
asdSR1.35	*dSR-CI*	TTTCGCCTCGAAGGCGACCA
asdSR1.36	*dSR-CI*	CATCTCGATCGCAAGTTTGC
sdSR1.1	*dSR-CI*	GACCGTATCTATACATTAAG
sdSR1.5	*dSR-CI*	GCGGCTACACTTTGCAGGGA
sdSR1.10	*dSR-CI*	ACACAAGAGATCATTCTTGC
sdSR1.17	*dSR-CI*	CAGATCCCTCACCCTGAAGA
sdSR1.22	*dSR-CI*	GCACCACCACAGAACCAACG
sdSR1.25	*dSR-CI*	TCCCTGCCCCCAACGGTCAC
sdSR1.40	*dSR-CI*	GGCTGTGAGATTCAAGAAGG
leas1.25	*dSR-CII*	GCCATGTGTTCCCTGCGAG
leas1.45	*dSR-CII*	GCGGAATCCAAACTGTAGGC
les1.15	*dSR-CII*	CGAGATCTCTCTGTTTGAATG
asDm0173.25	*STSDm0173*	CTTGATCGCCAGACACGGTG
sDm0173.15	*STSDm0173*	GAACCTCACGGTGGGCCATT
D1378f	*fat*	TTGGACAGGGAGACACAGAAC
D1378r	*fat*	ATAACCATTCAGACCCTCAGG
asrp49.2	*rp49*	GGAGCGACAGCTGCTTGG
srp49.2	*rp49*	CCCAAGATCGTGAAGAAGCG
T3	-	AATTAACCCTCACTAAAGGGA
T7	-	AATACGACTCACTATAGGGC

Figure 4-1. Polytene chromosome *in situ* localization of the *dSR-CI* gene to 24D4 - 24D8.

Polytene chromosomes were prepared and probed with a dSR-CI cDNA derived probe as described in Experimental Procedures. The *dSR-CI* gene maps to the 24D4 - 24D8 region of chromosome 2L.

Figure 4-2 Physical map of the 24D - 25A region of chromosome 2L.

The several known chromosomal deletions, lethal complementation groups, and P element insertions (vertical arrows) are indicated, as are two genes, *angle wing* (*ang*)and *l(2)G99* which have been mapped by recombination to this region of the genome. The location of the *dSR-CI* gene as determined by *in situ* hybridization is indicated by the two-headed arrow, and the locations of the *ft*, *l(2)24Db*, *l(2)24Dc*, and *l(2)24De* genes as determined by complementation analysis, and of the *dSR-CI* and *STSDm0173* genes as determined by PCR analysis of several deficiencies (Figure 4-12) are indicated by the vertical lines. The distal breakpoint of the $Df(2L)ed^{Sz1}$ chromosomal deletion is located in 24A. The proximal breakpoints of the $Df(2L)dp^{h25}$ and $Df(2L)sc^{19-3}$ deletions are located in 25B, and that of $Df(2L)sc^{19-1}$ is in 25C. Except for $Df(2L)\ M\text{-}z^{B}$, the indicated deficiency strains were isolated by Szidonya and Reuter (1988) using either of two chromosomal duplications which cover the four known haplo-insufficient loci located in the 24D - 26 region. One of these duplications, *Dp(2;1)B19*, is indicated by the open horizontal line. This duplication extends to 25F. Two of the haplo-insufficient loci covered by this duplication, *l(2)Db (M(2)LS2)* and *M(2)24F (M(2)z)*, are also indicated. Alleles of most of the indicated lethal complementation groups which are proximal to the distal breakpoint of this duplication were also isolated in the screen of Szidonya and Reuter. Lethal P element insertion loci are indicated by vertical arrows. Those discussed in the text include a - *l(2)k01102*, b - *l(2)k06526*, and c - *l(2)14703*. This figure was compiled from data in Szidonya and Reuter, 1988; De Belle *et al*, 1993; Emmons *et al*, 1995; the Encyclopedia of Drosophila (joint project of the Berkeley Drosophila Genome Project and FlyBase); FlyBase (maintained at Harvard University); and results described herein.

Cytogenetic Map of Region 24C - 25A

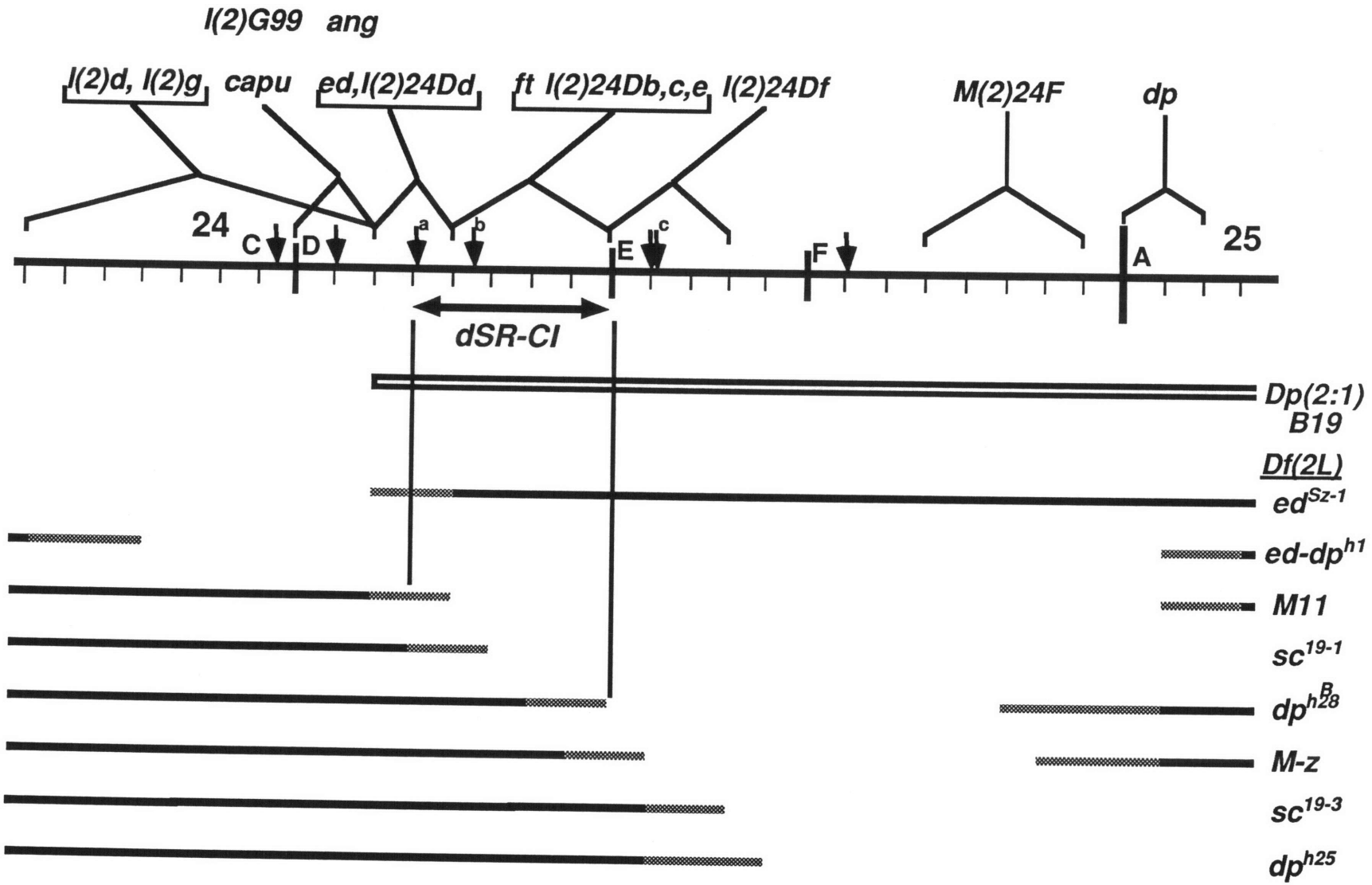


Figure 4-3. Preliminary Southern analysis of *Canton-S* genomic DNA.

Canton-S genomic DNA (0.5 μg) was digested with the indicated restriction enzymes and analyzed by Southern hybridization with the dSR-Clp probe as described in Experimental Procedures. dSR-Clp detects a single BamHI fragment of greater than 14 kbp, a single EcoRI fragment of approximately 4.8 kbp, and two EcoRV fragments of approximately 4.2 kbp and 2.6 kbp. DNA size standards (λBstEII digest, New England Biolabs) are indicated on the side.

Southern Analysis of *Canton-S* Genomic DNA dSR-CI Probe

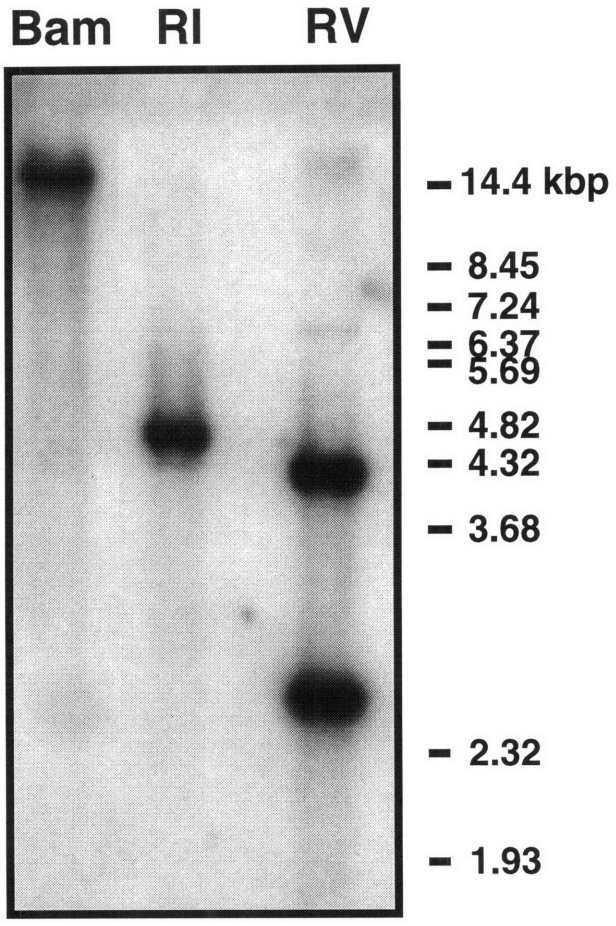


Figure 4-4. Localization of the *dSR-CI* gene by Southern analysis of region 24D - 25A chromosomal deletions.

As described in Experimental Procedures, genomic DNAs were prepared from adult *Canton-S* (*CS*) and *Oregon-R* (*OR*) flies, from *Df/Balancer* heterozygous adults (p), and from unhatched homozygous *Df* embryos (e). Approximately 1.0 μg of each DNA sample (except embryonic DNA from *Df(2L)ed-dp^{h1}*) was digested with EcoRI and EcoRV and then sequentially analyzed by Southern hybridization using a pdSR-CI probe. Strong hybridization to fragments of 4.6 kbp (EcoRI) and 10 kbp, 4.2 kbp and 2.6 kbp (EcoRV) (size markers are λBstEII, only some bands are listed) is detected with the dSR-CIp probe in genomic DNA of from *CS, OR* and all parental *Df* strains, and in the genomic DNA of homozygous *Df* embryos from strains *Df(2L)ed^{Sz-1}*, and *Df(2L)dp^{h25}*. A weak and slightly shifted signal was detected in genomic DNA from homozygous *Df(2L)ed-dp^{h1}* embryos. However, as seen from the ethidium bromide stained gel (bottom) significantly more *Df(2L)ed-dp^{h1}* DNA tested from the homozygous embryos than from their heterozygous parents. Thus, the signal may be due to a small level of contamination of this sample with wild type embryos, rather than to the presence of the *dSR-CI* gene in genomic DNA from this strain. No signal was seen in DNA from homozygous *Df(2L)M11* embryos, which were probed independently of the other *Df* strains.

Southern Analysis of Region 24D - 25A Deficiencies With a dSR-CI Probe

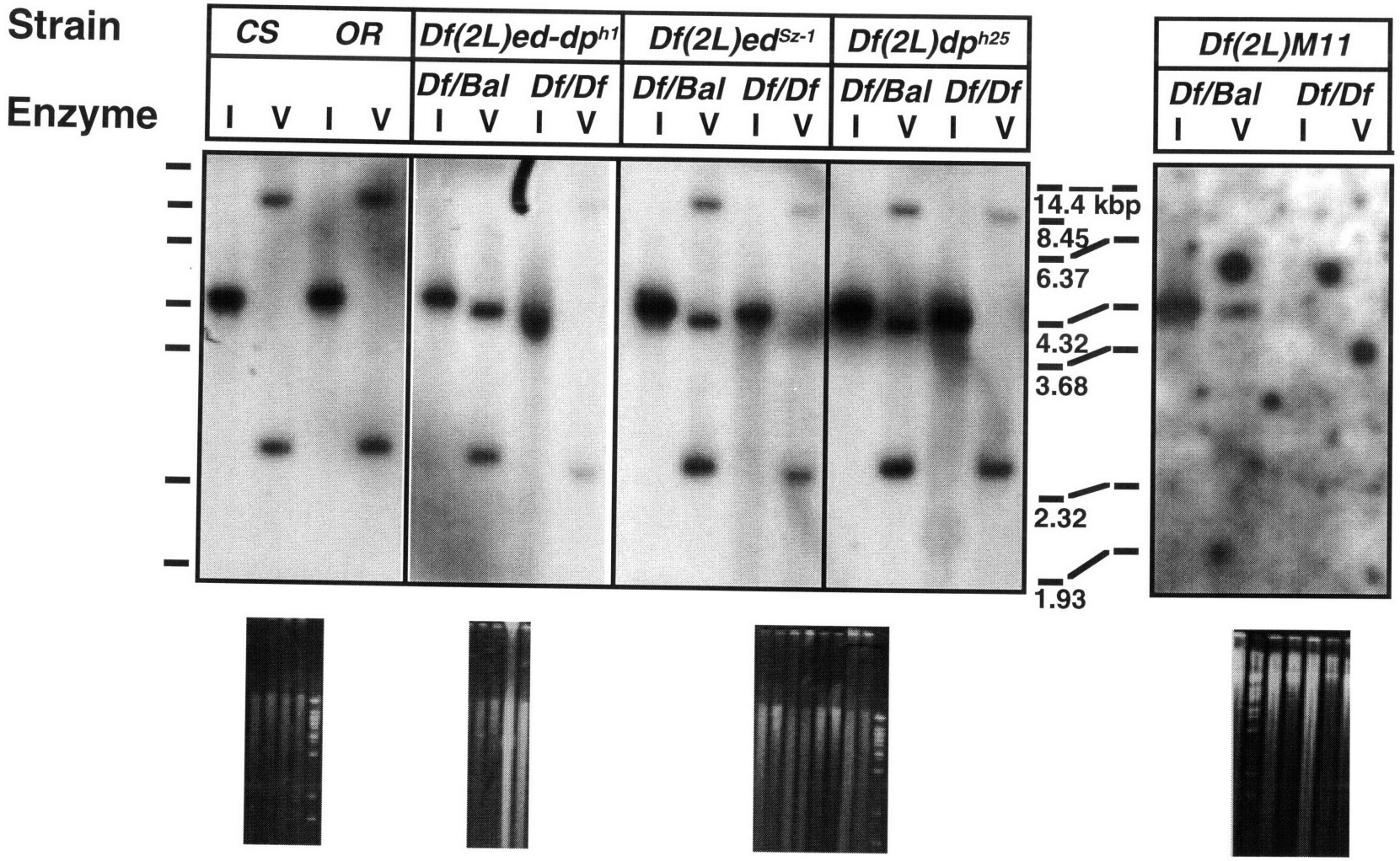


Figure 4-5. PCR analysis of dSR-CI intron/exon structure.

A. A schematic diagram of dSR-CI indicates the positions of the sense (above) and antisense (below) primers relative to the various dSR-CI protein domains encoded by the cDNA.

B. PCR amplification of dSR-CI products from *Canton-S* genomic DNA (*CS*) and the cDNA clone pdSR-CI (c) was performed as described in Experimental Procedures. In most cases, the negative control reactions (no template) are also shown (-). There appeared to be a small contamination in the sdSR1.5/asdSR1.30 PCR reaction, but this did not affect the amplification of an intron containing fragment from *CS* genomic DNA. A negative control reaction was not performed for the sdSR1.10/asdSR1.25 reaction. The sizes, in bps, of the expected amplification products from pdSR-CI are indicated. In each case ϕX174HaeIII markers (New England Biolabs) were used to determine product size, except for the sdSR1.1/asdSR1.1 PCR reaction, which used λBstEII markers. It is evident that larger than predicted amplification products were obtained from the genomic DNA using primers sdSR1.5 and asdSR1.30 (approximately 70 bps larger) and sdSR1.17 and asdSR1.23 (approximately 100 bps larger). These presumably represent introns. The products of the sdSR1.1/asdSR1.1 reaction appear to be running faster than predicted given the size of the cDNA. By correcting for the difference between the expected and observed migration of the cDNA amplified product, it can be estimated that the dSR-CI gene contains approximately 250 bps of DNA not found in the cDNA between these terminal primers.

PCR Analysis of dSR-CI Intron/Exon Structure

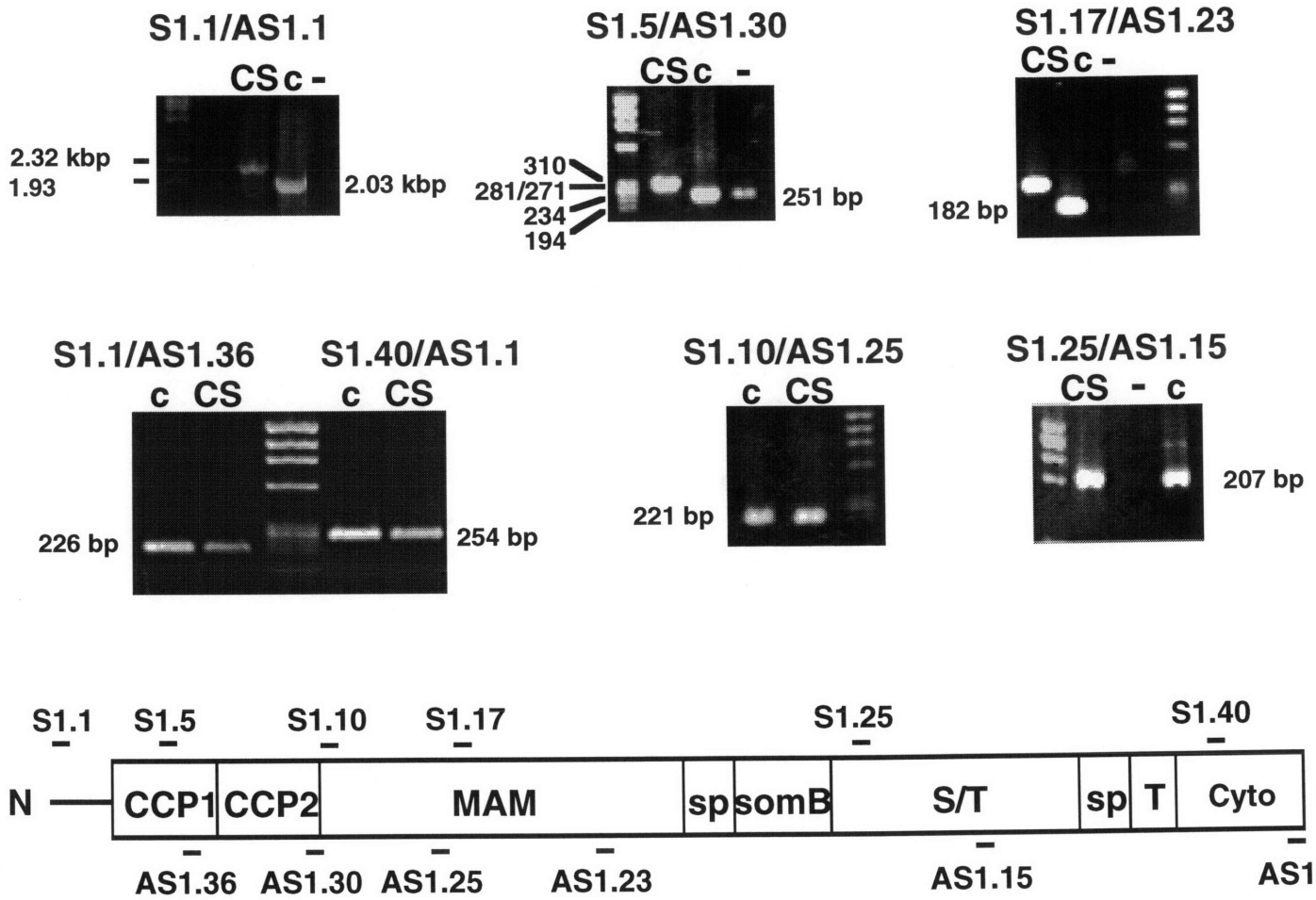
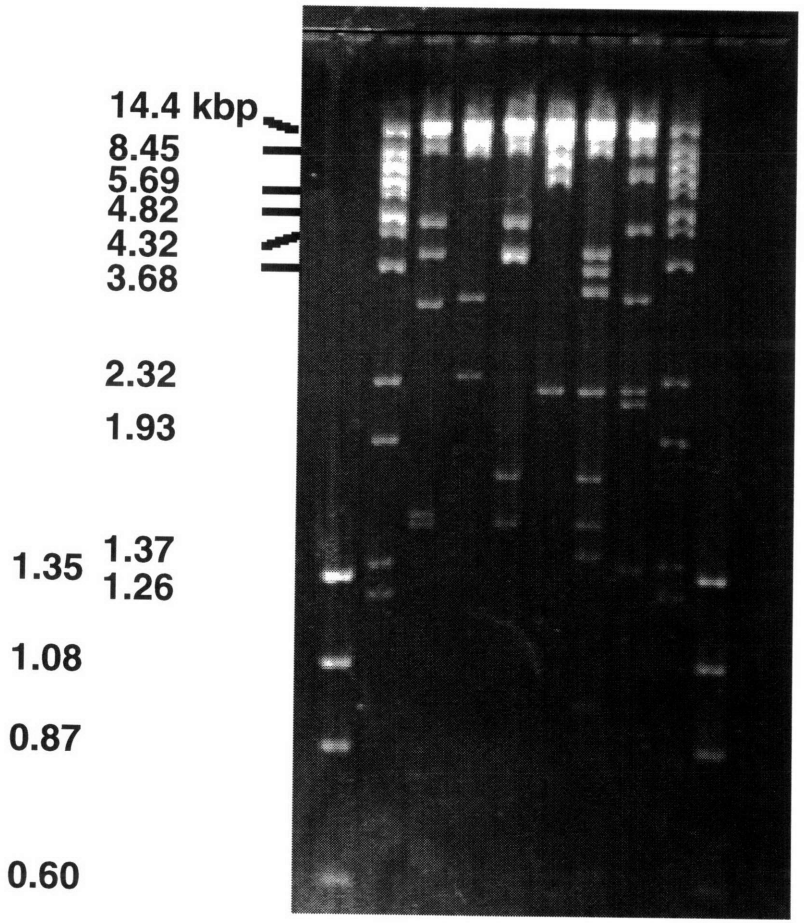


Figure 4-6. Analysis of restriction enzyme digestion products of clone λe1.1b.

A. 2 μg of DNA from the clone le1.1b was cut with the following enzymes and analyzed on a 1% agarose gel as described in Experimental Procedures: EX - EcoRI/XbaI; X - XbaI; E - EcoRI; S - ScaI; ES - EcoRI/ScaI; SX - ScaI/XbaI; λ - λBstEII markers; ϕ - ϕXHaeIII markers. Unique EcoRI and XbaI sites are located in the polylinkers of λDashII which flank the cloned genomic DNA. The 9.1 kbp and 20 kbp fragments observed upon digestion with these enzymes correspond to the λ arms. ScaI is not located in either polylinker, and generates λ phage DNA fragments of 2.26 kbp and 16.4 kbp, and also fragments containing either 1.3 kbp or 8.8 kbp of λ DNA joined to DNA from the cloned insert. The lengths of the DNA size markers are indicated.

B. The DNA on the agarose gel shown in part A was blotted onto Genescreen and probed with 244p, corresponding to the 5' end of the dSR-CI cDNA. Among the λe1.1b restriction fragments which this probe detects is an EcoRI fragment of approximately 4.6 kbp. The locations of the λBstEII size markers are indicated.

Mapping Data for Clone λe1.1b



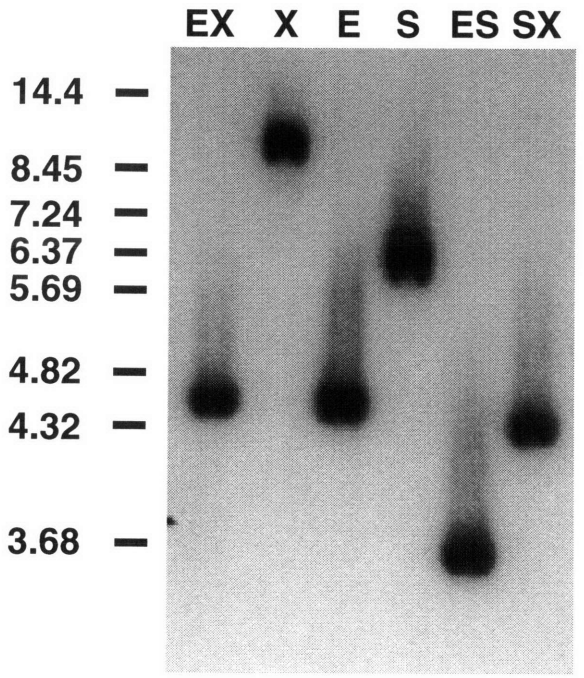
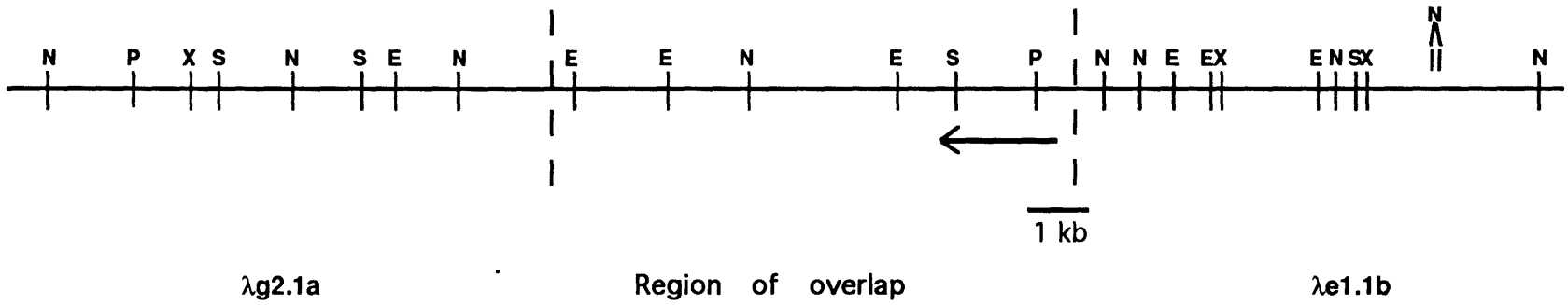


Figure 4-7. Restriction map of the overlapping genomic clones λe1.1b and λg2.1a.

The 28 kbp map was constructed by analysis of the products of restriction enzyme digestion of genomic DNA prepared from clones λe1.1b and λg2.1a as described in Experimental Procedures. Sample data are presented in Figure 4-7. Restriction analysis indicates that sequences homologous to the dSR-CI cDNA are contained within an approximately 4.6 kbp EcoRI fragment of λe1.1b. The orientation of these sequences within the genomic clones was determined by comparing this restriction map to the restriction map of pdSR-CI. Note that the precise location of one NcoI site has not yet been determined.

Restriction Map of Overlapping Genomic Clones λe1.1b and λg2.1a



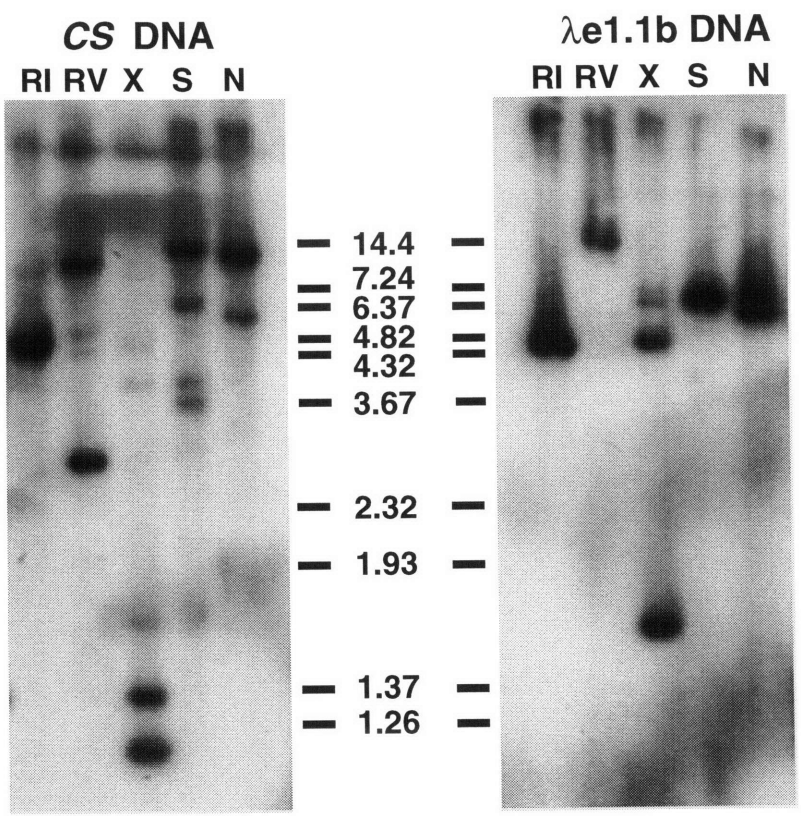
E - EcoRI
N - NcoI
P - PvuI
S - ScaI
X - XbaI

Figure 4-8. Comparative Southern analysis of *Canton-S* and λe1.1b genomic DNA.

Canton-S (2 μg) and λe1.1b (10 ng) genomic DNA were cut with the following enzymes and analyzed by Southern hybridization using either a dSR-Clp probe (A) or a mixed λe1.1b + λg2.1a probe (B): RI - EcoRI; RV - EcoRV; X - XmnI; S - ScaI; N - NcoI. The locations of the λBstEII size markers are indicated.

Comparative Southern Analysis of *Canton-S* and λ Clone DNA Using cDNA and λ Clone Probes

A. cDNA Probe



B. λe1.1b/g2.1a Probe

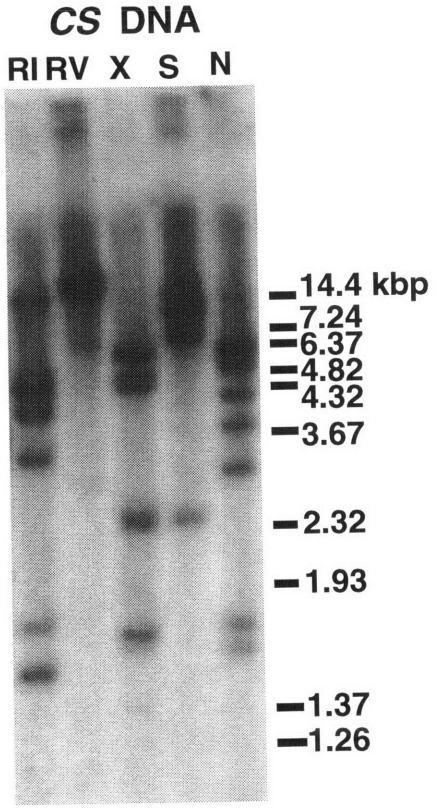


Figure 4-9. Genomic and cDNA sequence of the *dSR-CII* gene.

Genomic clone pDT15-1, containing a 4.6 kbp EcoRI fragment from λe1.1b, was sequenced. Shown are the 2731 bps of contiguous coding strand sequence from this clone which have been verified on both the coding and non-coding strands. The numbering scheme is derived from the complete sequence of the 2066 bp dSR-CII cDNA clone pII-12. The genomic DNA sequences not included in the cDNA (introns, 146 bps of upstream sequence, 263 bps of downstream sequence) are not numbered. The locations of the arthropod transcription initiation consensus sequence (^^^^^), the 5' end of the cDNA (>), the three intron sequences (------), and the two polyadenylation signals (aaaaaa) and two potential mRNA instability signals (####) in the 3' UTR are indicated. Also indicated are the positions of the putative start codon (***), the putative termination codon (TER) and an in frame stop codon in the 5' UTR (STP). Clone pII-12 also contains a 55 bp poly A sequence derived from the dSR-CII poly A tail which is not indicated in this figure.

```
                      GTTGATGTACAAAAACGATTTAATGAACGGTAACATCTTTTT
    TTTGCAGCTGCGAAAAATCAGCTGGTGACGAACCCGCCCTTAAGGACTCGACTTGAAAAT

    >^^^^^          STP
  1 GAGTTGAGCATCAGGTAGCAGTCACTGTCCTTTCGAATCTCCTTAATATACATACAAAAT 60

                               ***
 61 TGCAGAACAAAAAAATTCGGAGCTATGCAATTTTTCTTGGCTCTGACATTGATTTTAGCC 120

121 TACAGTTTGGATTCCGCAAACGGAAGCTGCGAAGGATCGTTAGATTTGGAAAATGGACGA 180

181 TTCTTCACACGATCGAACAATTTAGTGATTTTCCAATGCAATCGCGGATTTGTATTGCAG 240

241 GGAAATTCAATACACACCTGCGATCGGGATGGTCGACTTCGCGAGAAGAAACCATTTTGT 300

        ------------------------------------------------------
301 GCCAGTGAGTGTTGACAGTTTTCGAAAATGTACACCCTTTCAAATCGAACATTTCATATC

    --------------------------------------------------
    ATCATACTAGTTATCAACAAAATTTTGATTTTTTTCATTTTAATTTTAAGGGAAAGGATG 314

315 CGATAAGCCTGAGGATCCCACAAACGGATATGTTTTAGATAATCCTGTAAAGGCGGAAAT 374

375 CGTGTGTTTAGATGGTTTCGTTCTATACGGAAGTCGCACTGCCTTCTGCGATGGAGAAAA 434

435 GTGGAGCACACAGCTGGGAGCTTGCCGGAGAAGCAACCACACGATGGACCACTCCTGCGA 494

495 CTTCGAGAGCGAGGATCAGTGCGGCTGGTCTGCGGAGGAGACCATCTGGTTGCCCTGGAA 554

555 GAGGATCAGTGCGGTGACGGATTTTCACCACCCCAGAACAGGACCACGATACGATCACAC 614

615 CTTTGGAAACGCTTCGGGTGGACACTATATGCGCATGGAGACCCAGATCGAGGCTTATGG 674

675 CAGTTACCACTTCGTATCGCCGGTATATCCGAGATCTCTCTGTTTGAATGCCGCCTGCTG 734

735 CTTTCGGTTTCACTACTTCATGTTCGGAGCTGGAGTGGACAGACTGGTGGTGTCCGTGAA 794

                                                    ------------
795 GCCAGCCTCTCTGCAGATCGATGACATGTGGAACAACTTTAGGTCCAAGTATGTAAACAA

    ------------------------------------------------------------
    CTCGTTTTAGTTTCGTCAAGAAAATGTTATACGAATGAGCTTCTACAACCATATGGTATA

    ---------------------------------------
    CTAGCTGCTTTTATATACTGAAAGTTTATTTTTCTCCAGTTCCAGCAAATTTGAAATAAC 863

864 TGGCTCGCAGGGAACACATTGGCTGGAGCACACAATCACCATCGACAAGATGCATGAGGA 923

924 CTTCCAGGTGGTGTTCACAGCAACGGATGCGAGGTCACAATTCGGGGACATTGCCATCGA 983

984 TGACGTCAAACTGATGACGGGCAGCGAATGTGGAGTGGATGGGTATAGCACCACCACCAC 1043

1044 CACCGAATCAGCATCCTCGGCGATGTCCAGCAGCGAGGAGCCCCTGGTCTTCGACATGAT 1103
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```
1104 GAGCTGTACGTTTCGCTGTGGATCCATTAGTCCGGGCAGTCCACTTTTTTCCGATGAGGG 1163

1164 TATTGTCATGAGCTGCGGCTGTGACGACTCCTGTATTCTAAACAATAATTGTTGTCCCAA 1223

1224 CTATTTCGAAAAGTGTGTGAAGGAGTTGGACATAGGGTCTAATGTTTTTCTAAACTCAGC 1283

1284 GAACACAACAACTACATCAATATCAAGAAATACAACGACGGAAAAAGTATTTAGCATTTC 1343

1344 TTTAGAGGCAAGCAGAAGTTTTACAACACCAAGGACGAGCACAAATTCGACAACATTTCC 1403

1404 AAGAACTCAAATAAGTACTAAAAAGTTACTGAACACCCATGAAACTTCAACAGAAACTTC 1463

1464 AACATTACCAACAAGAAACAAAAAGTTGTCTCAACCTTCAATATTTAGTAGCCAGCACAT 1523

          ----------------------------------------------------

1524 TACAGGTAAGACAAAGAGTTATAATCAATTTTTTTATTACTATATATATATATATATATA

     --------
     TATTCCAGTTACCACTACAACTGATCGTTCGAGAAAGGATTACCACGAGGACCAGGTCAG 1580

1581 TTGGACATGAACACAAATATTCCCCATCCAGCTTATCGTGATAGTCATGTACCTGCTCAT 1640

1641 CGGCGTAATCCTGGTGATAGTTGTGGCCAATCTCAAACAACTGTGTAAAATTCTTACCAA 1700

1701 GAACTCAAGTCGAGATAACGAGAAGGTGGTTAGTTTCAAAAAGGCATTCGAGGGGCTGAG 1760

1761 AAAACCGAGAAGTCCCAAAATTGCAACGGCATGGATCAGCATTTGTGCAGTGCCATTGTT 1820

                                                          ####
1821 CGATGAGGATTTGGATTACTTTGAGGACATGGACATGAACATACGCATGGTGACGGATTT 1880

     #TER           #####
1881 ATGAGGGGTGATTCCATTTACTACCTACAGCCTTACTCAAATTTGAAACCATATCAAATT 1940

                       aaaaaa
1941 AAATGGCATATATTGTGACAATAAATATTTTTAAGAAGTTGATTGTAATCTGTGACCAAA 2000

           aaaaaa
2001 TAGATAATAAATGAGGTACATAAATACATTTAAATGTGTTTTGGGCATTGGTGACCCAAC 2060

2061 TTTGTG                                                      2066


     AAATTCCANATGGGTATTATGTACATTTCAAATTCTCTTTTATTGCCTAAGCTAACCTTT
     TTCAAATGAATGATTTCAAACCCATTTAAGGTGACCCTAATGTAACTCACTTTTAGCATG
     AGGTAACATACAGGTAACTTACTTTTTTCCAAGTTAAAAGAGACTGCGTTTTAAAATACG
     ATTTGTGGCGTTATACTACTTTTACCTATGAGAAAGTATATTAAAGTGTCTTTATCGTTC
     AATTAATTCCTGCTAAGGAATTC
```

Figure 4-10. Comparison of the domain structure and protein sequences of dSR-CI, dSR-CII and STSDm0173.

The complete sequences of each domain of the dSR-CI, and putative dSR-CII proteins, and the partial sequence of the putative STSDm0173 protein MAM domain are aligned. All amino acid identities are boxed. The exact location of the putative signal sequence cleavage sites in dSR-CI and dSR-CII are unclear. Although they have been placed at G20/R21 in dSR-CI and A18/N19 in dSR-CII, there is a second potential cleavage site located at G16/H17 in dSR-CI and G21/S22 in dSR-CII (von Heijne, 1986). The locations of the potential N-linked glycosylation sites in dSR-CI and dSR-CII are underlined. The locations of potential phosphorylation sites in the dSR-CI and dSR-CII cytoplasmic domains are indicated (* protein kinase C, ^ casein kinase 2, # cAMP dependent protein kinase A).

Comparison of dSR-CI, dSR-CII and STSDM0173 Sequences

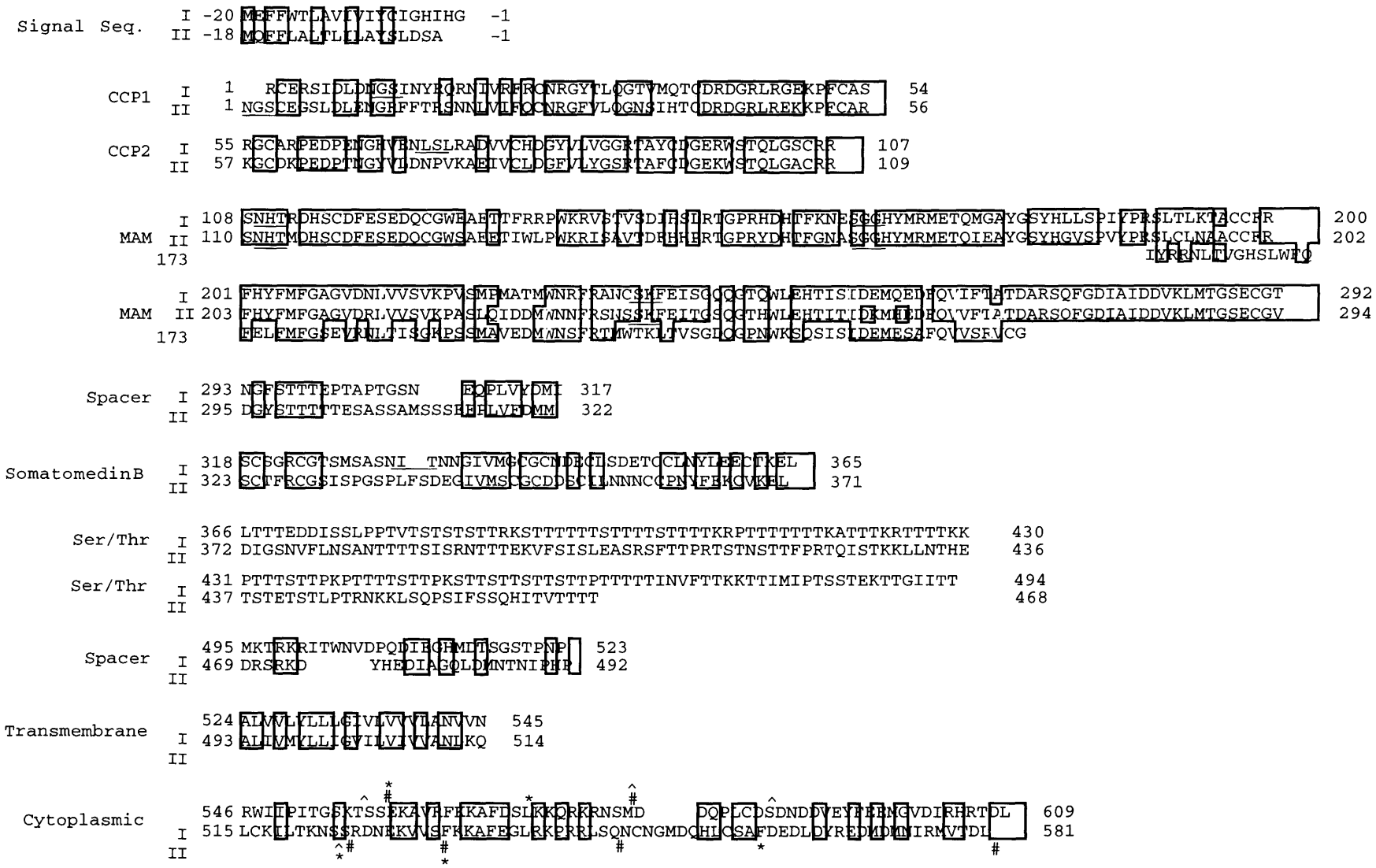


Figure 4-11. Diagram of the fat genomic clone contig of *Drosophila melanogaster.*

This diagram, based on data obtained from the Encyclopedia of *Drosophila*, shows the P1 genomic clones located at the distal end of the fat contig which spans the 24D5 - 25A1 region of chromosome 2L (Hartl et al, 1994; Berkeley Drosophila Genome Project, personal communication). The sequence tagged sites (STS) which have been used to order the P1 clones within the contig are indicated by circles. STSDm0173 defines the distal end of this contig. Other STS sites of note correspond to the *dSR-CI* gene (STSDm3565), the fat gene (STSD1378) and the lethal P element insertion l(2)01085 (STSDm0294, and see Figure 4-2). The physical locations of those P1 clones which have been mapped by *in situ* hybridization to polytene chromosomes are also indicated.

P1 Genomic Clones at the Distal End of the fat Contig

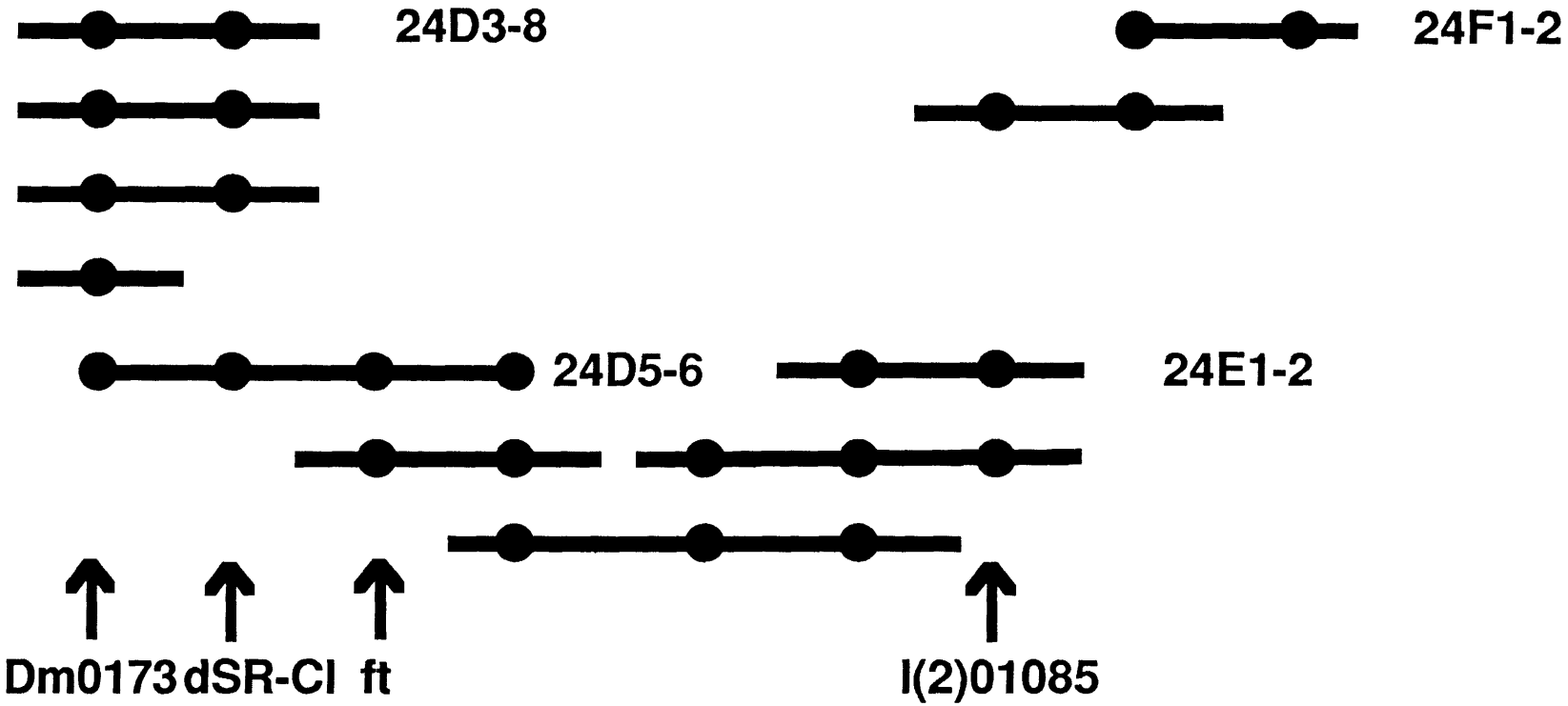
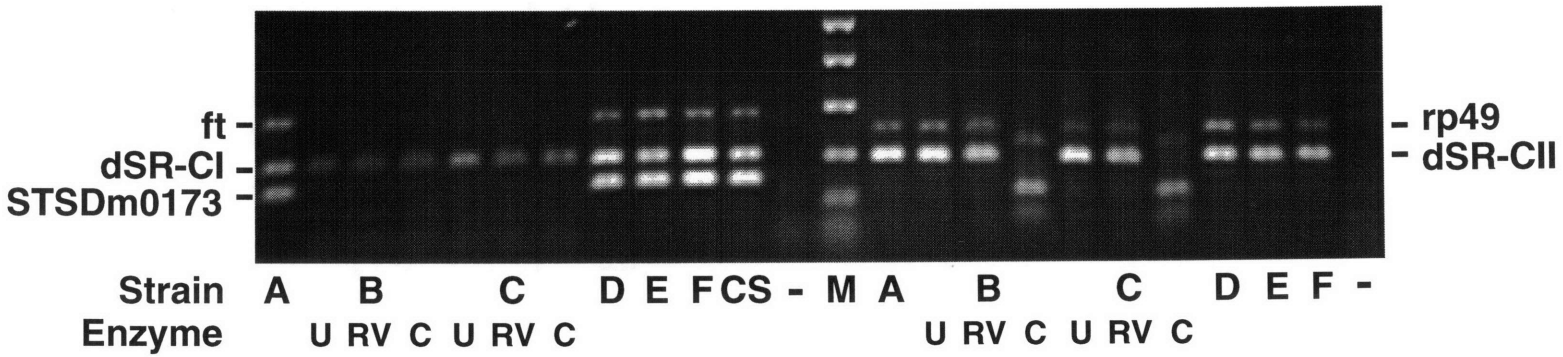


Figure 4-12. Localization of *dSR-CI* and *STSDm0173* by PCR analysis of region 24D - 24E deficiency strain genomic DNA.

A. As described in Experimental Procedures, three individual *Canton-S* (*CS*) embryos and three or more individual embryos homozygous for each of several deficiencies were tested for the presence or absence of sequences corresponding to *dSR-CI*, *STSDm0173*, and *ft* (left), and to *dSR-CII*, and *rp49* (right) by multiplex PCR. The results obtained from a single representative embryo from each strain are shown. These results have been verified in multiple embryos from each strain. The following deficiency strains (see Figure 4-2) were examined: *Df(2L)ed*$^{Sz\text{-}1}$ (A), *Df(2L)ed-dp*^{h1} (B), *Df(2L)M11* (C), *Df(2L)dp*^{h28} (D), *Df(2L)M-z*^{B} (E), and *Df(2L)dp*^{h25} (F). The sizes of the expected PCR amplification products are: dSR-CI - approximately 280 bp, dSR-CII - 293 bp, ft - 474 bp, STSDm0173 - 225 bp, rp49 - 460 bp. The dSR-CI sized PCR products obtained from amplification of the *Df(2L)ed-dp*^{h1} and *Df(2L)M11* strain genomic DNA were subjected to restriction digestion with no added enzyme (un) and with EcoRV (V) or Cla I (I). EcoRV digestion should cut only dSR-CI amplification products, reducing their size by 25 bps, while ClaI digestion should cut only dSR-CII amplification products, yielding two fragments of 119 bp and 174 bp. Lane M is the PCR Marker size ladder (Gibco BRL) containing fragments of size 1.0 kbp, 750 bp, 500 bp, 300 bp, 150 bp, and 50 bp. (-) Lanes are the no template negative control PCR reactions. Note that the two positive control primer pairs did not generate any amplification products in the *CS* control embryo shown here. This outcome was specific to this particular *CS* embryo, since both products were generated in subsequent *CS* control reactions (not shown). Since all of the *Df* embryos tested in this experiment gave PCR products of the expected size using these two primer pairs, the lack of products in this *CS* embryo, which was simply a control for the efficacy of the PCR reaction, does not affect the interpretation of the experimental results.

B. Additional restriction analysis of the multiplex PCR reaction products obtained from *Df(2L)ed*$^{Sz\text{-}1}$, *Df(2L)ed-dp*^{h1} and *Df(2L)M11* embryos using the dSR-CI, STSDm0173 and ft primers. This analysis better illustrates the lack of EcoRV digestion of the dSR-CI sized product obtained by PCR from *Df(2L)M11*, and shows that EcoRV does cut the dSR-CI sized product obtained by PCR from *Df(2L)ed*$^{Sz\text{-}1}$.

PCR Analysis of Region 24D - 25A Deletions



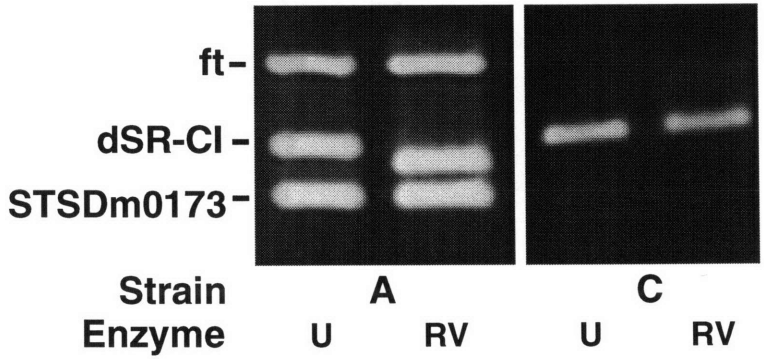
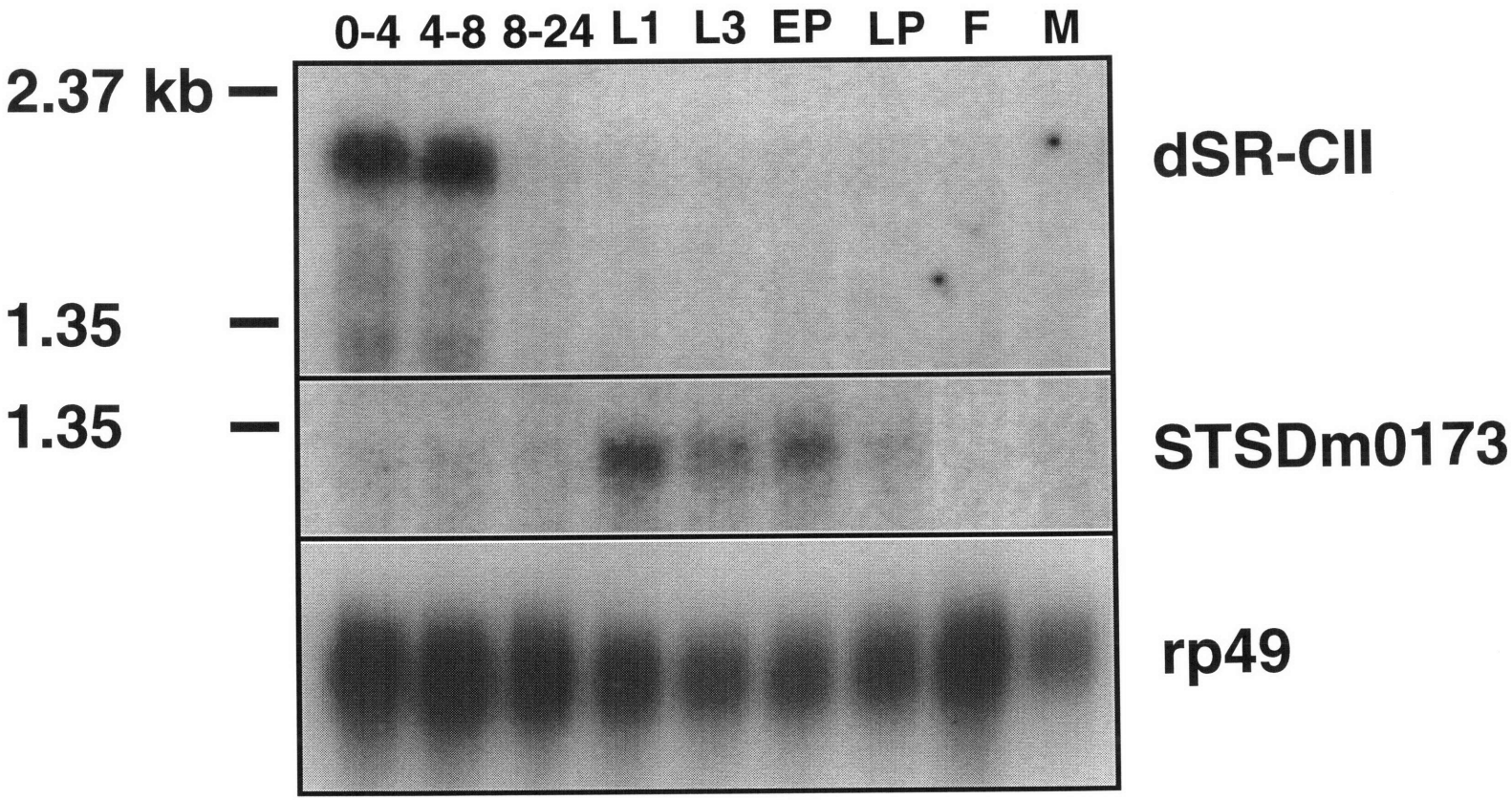


Figure 4-13. Northern hybridization analysis of *dSR-CII* and *STSDm0173* expression during *Drosophila* development.

Poly A+ RNA was obtained from *Canton-S* strain *Drosophila* at various stages of development and analyzed by Northern hybridization using a dSR-CII specific probe, an STSDm0173 specific probe, and an rp49 probe as described in Experimental Procedures. The dSR-CII probe detected a single 2.0 kb message expressed in 0 - 4 and 4 - 8 hour embryos. The STSDm0173 probe detected a single 1.25 kb message expressed during larval and pupal stages. rp49 (0.6 kb) is included to normalize for RNA loading. Abbreviations: L1 - first and second instar larva, L3 - wandering third instar larva, EP - early pupa, LP - late pupa, F - adult female, M - adult male.

Developmental Northern Analysis of dSR-CII and STSDm0173 Expression



Chapter 5

Concluding Remarks

As discussed in Chapter One, the broad ligand binding specificities of the scavenger receptors, combined with their expression patterns, suggest that one or more of these receptors might be pattern recognition receptors active in the innate immune reactions of vertebrates and invertebrates. Many hypotheses have been offered concerning the potential function(s) of these receptors. Unfortunately, there have only been a few studies which have actually addressed whether scavenger receptors play a significant role in any of these processes *in vivo*. Furthermore, interpretation of the results from these studies is complicated by the fact that they have usually involved the use of compounds which can affect the activities of multiple scavenger receptors.

This situation is likely to change in the near future, as mice deficient in the expression one or another of the various scavenger receptors are generated. Indeed, mSR-AI/II deficient mice have already been generated (H Suzuki, Y Kurihara, T Kodama, personal communication). Since homozygous SR-AI/II deficient mice are born and live to adulthood, the SR-AI/II gene does not appear to be essential for normal health and development. However, preliminary data suggest that SR-AI/II participates at some level in apoptotic cell engulfment and liver-mediated LPS clearance from the blood. That it does not play an essential role in either of these processes may indicate that SR-AI/II simply enhances processes mediated by other receptors. It is also possible that SR-AI/II may be part of a system of receptors, all of which provide small but incremental contributions to these phagocytic processes, such that the loss of any one receptor has only mild phenotypic effects. Thus, it will be interesting to determine the effects of double and triple knockout combinations involving SR-AI/II and other genes of interest, such as CD14 and the SR-Bs. In addition, further scrutiny of the SR-AI/II mice may reveal that this receptor is crucial for certain very specific and unanticipated functions.

The powerful genetic tools available in *Drosophila melanogaster* have led me and others at MIT to investigate the possibility that scavenger receptors also exist in this organism. If flies did have scavenger receptors, these genetic tools could then be used to gain a better understanding of scavenger receptor function *in vivo* . There was reason to believe that such receptors might exist, since innate immunity is evolutionarily quite ancient. Indeed, a scavenger receptor activity was identified in *Drosophila* embryos and in the hemocyte/macrophage like S2 cell line. Chapter 3 of this thesis has described the cloning and initial characterization of the *dSR-CI* gene, whose protein product can at least account for the scavenger receptor activity. Although not at all related structurally to the class A or class B scavenger receptors, the dSR-CI protein does exhibit the type of broad ligand binding specificity that is a defining characteristic of pattern recognition receptors. Furthermore, the gene is expressed specifically in embryonic hemocyte/macrophages. These properties, and especially the ability of the dSR-CI protein to bind the immunostimulatory microbial surface constituent laminarin, suggest that dSR-CI might participate in *Drosophila* host defense reactions. In addition, preliminary studies have indicated that the dSR-CI protein might participate in the phagocytic engulfment of apoptotic cells during *Drosophila* development.

These findings have made the *dSR-CI* gene attractive for further study. Having originally looked for scavenger receptor genes in *Drosophila* in order to take advantage of the genetic potential of this organism, the obvious thing to do was to isolate *dSR-CI* mutants lacking functional dSR-CI protein. As a first step, the gene was mapped to the 24D4 - 24D8 region of chromosome 2. For the reasons stated in Chapter Four, genomic clones containing sequences homologous to the dSR-CI cDNA were also isolated. However, these clones actually contained a new gene, *dSR-CII*, homologous to *dSR-CI*. In addition, a third *dSR-CI* homolog was identified as a sequence tagged site generated by the *Drosophila* genome project.

Although it is interesting that there is a family of dSR-C genes, for none of these genes is a function known, and for none of these genes have mutants been identified. Determining the functions of these genes, and in particular, the function of *dSR-CI* , is the goal of ongoing research efforts. For the rest of this Chapter, I would like to discuss some strategies for achieving this goal.

As with *dSR-CI*, determining the cytological location of *dSR-CII* will immediately identify pre-existing point mutations, chromosomal aberrations, and P element insertions which should be useful for isolating *dSR-CII* mutants. At some point, however, a decision has to be made concerning which of the dSR-C genes should take priority in future studies. For this reason, it will be necessary both to obtain the complete sequence of the *STSDm0173* gene and to determine the cell and tissue expression patterns of the dSR-CII and STSDm0173 messages. From the perspective of being interested in pattern recognition receptors of the *Drosophila* innate immune system, it would make sense to prioritize those genes which are expressed in hemocytes and the fat body. In addition, it will be important to determine if the dSR-CII and STSDm0173 proteins have scavenger receptor activity.

By these criteria, *dSR-CI* remains the primary focus for future study. Several different strategies for determining the function of the dSR-CI protein are currently either in progress, or are being planned. First, it is important to know where the dSR-CI message and protein are expressed during later stages of development, and particularly if they are expressed in the larval and adult fat body. In addition, given the increased expression of the dSR-CI message during early metamorphosis, and the preliminary observation that the dSR-CI protein can recognize apoptotic cells, it will be interesting to determine the pattern of dSR-CI expression in early pupa. Since there is some evidence that the croquemort transcript is not expressed in the absence of apoptotic cell death (N Franc, personal communication), it would be interesting to see if croquemort is expressed in deficiency strains deleted for *dSR-CI*, since a lack of croquemort expression in these strains would suggest a role for the dSR-CI protein in regulating *croquemort*. Finally, the very high level of dSR-CI message expression in the adult head remains unexplained and the cell type specificity of this expression should be examined.

Work is currently in progress which is aimed at defining the ligand binding domain of the dSR-CI protein. Under my supervision, G. Johnson, an undergraduate in

the Krieger lab, is constructing a series of truncation mutants which progressively eliminate the extracellular domains of this protein. The first such mutant, in which the N-terminal CCP domain is deleted, has already been constructed and will soon be assayed for receptor activity. Other truncations will be constructed once the binding activity of this first mutant is known. These will include truncations from the C-terminal end of the protein, which will generate secreted proteins that can be studied by *in vitro* binding assays using scavenger receptor ligands coupled to agarose beads (Resnick *et al*, 1993). In addition, if the dSR-CII and STSDm0173 proteins lack scavenger receptor activity, one can imagine that domain swapping experiments might help localize those regions of the dSR-CI protein which are important for ligand binding.

While these studies are interesting in their own right, they may be particularly useful for studying *dSR-CI* gene function in the fly. For instance, by expressing these mutant dSR-CI proteins in wild type flies, it may be possible to generate dominant effects on normal dSR-CI function which will lead to detectable phenotypes such as incomplete development or increased or decreased susceptibility to bacterial infection. The power of this type of approach has already been demonstrated in elegant studies of the *Notch*, *sevenless*, *breathless* and *Toll* genes (Basler *et al* , 1991; , Schneider *et al*, 1991; Rebay *et al*, 1993; Reichman-Fried and Shilo, 1995). By placing mutant dSR-CI constructs under different types of regulatory elements (hsp promotor, UAS elements, or regulatory sequences upstream of the dSR-CI gene), it should be possible to express the mutant proteins both ubiquitously and also fairly specifically in hemocyte/macrophages. It may also be interesting to simply overexpress and/or ectopically express the wild-type dSR-CI protein in flies, as overexpression and ectopic expression of wild type genes has been observed to lead to dominant phenotypes in other systems (Ruohola-Baker *et al*, 1993; Noordermeer *et al*, 1992; Chiba *et al*, 1995).

It still remains necessary to identify mutants lacking normal dSR-CI function. Having localized the dSR-CI gene to a small region of the genome by a combination of *in situ* hybridization and Southern and PCR mapping of genomic DNA obtained from deficiency strains, a limited number of candidate mutations have been identified which might affect the dSR-CI gene (Figure 4-2). In addition to *ang* and *l(2)G99* (Figure 4-2), 18 additional lethal genes have been mapped genetically to within ± 6 map units of the *ed - ft - dp* cluster. By determining the ability of these loci to complement *Df(2L)ed-dp*^{h1}, it has already been possible to eliminate six of these loci from consideration as *dSR-CI* mutants (data not shown). The remaining 12 loci, and also *ang* and *l(2)G99,* remain to be tested in this manner. At the moment, however, there are two loci which are very good candidates for the dSR-CI gene, *l(2)24Dd* and *l(2)24De*. It is also formally possible that *l(2)24Df* corresponds to a regulatory mutation affecting *dSR-CI* expression. In order to determine if either of these loci, or any other loci which don't complement *Df(2L)ed-dp*^{h1}, correspond to the *dSR-CI* gene, western blotting is being performed on protein extracts obtained from every known allele of these loci using an anti-dSR-CI polyclonal antibody (three different antibodies have now been generated which recognize the dSR-CI protein - Pearson, Trigatti, and Krieger, unpublished observations). The basis for believing that this approach might

work is Zucker's recent finding that roughly 75% of all mutations in *ninaA* and at least one additional gene lead to protein production at <20% of wild type levels. It is important to point out however, that a negative Western result would not eliminate the possibility that a candidate locus corresponds to *dSR-CI*.

The other approach for isolating *dSR-CI* mutants, which is currently in the planning stages, is P element mediated mutagenesis (Tower *et al*, 1993). Two lethal P element insertion loci (*l(2)k01102* and *l(2)k06526*) are located near the dSR-CI gene Figure 4-2). In the case of *l(2)k01102*, the lack of complementation with $Df(2L)ed^{Sz-1}$ (data not shown) indicates that it does not affect the *dSR-CI* gene. However, it is quite close to dSR-CI since it also fails to complement *Df(2L)M11*. Unfortunately, *l(2)k06526*, which physically maps to the same chromosome bands as *dSR-CI* (Figure 4-1 and Berkeley Drosophila Genome Project, personal communication) does not appear to contain a functional P element (data not shown). Having identified several P1 genomic clones which contain dSR-CI sequences (Figure 4-11), efforts are now underway to isolate a smaller, more usable, clone which can be easily mapped. Once this is accomplished, it may be possible to determine the location of the l(2)k06526 and l(2)k01102 insertions relative to *dSR-CI*.

Even if the l(2)k06526 insertion disrupts *dSR-CI*, since this P element is non-functional, it will not be possible to rescue *dSR-CI* gene function by precise P element excision. Thus, the primary reason for isolating usable genomic clones containing dSR-CI sequences is that they will enable the isolation of useful P element induced mutations in the *dSR-CI* gene. Basically, by analyzing Southern blots of cloned genomic DNA using PCR generated probes containing the sequences flanking the P element insertion sites, it will be possible to accurately map the location relative to *dSR-CI* of P element insertions generated by local hopping of the *l*(2)k01102 element (and/or other nearby elements) (Dalby et al, 1995). The effects of these P elements on the expression of dSR-CI, and on the phenotype of the mutant line, will then be examined. Given the hypotheses that have been presented for dSR-CI function, it will be important to determine if *dSR-CI* mutants exhibit altered survival rates and changes in antibacterial and antifungal gene expression relative to wild type flies when they are challenged by bacterial and fungal infections. It will also be important to determine if these mutants exhibit changes in hemocyte migration or apoptotic cell engulfment. Since *STSDm0173* is close to *dSR-CI*, the P element mutagenesis approach has the added advantage that it should allow for the isolation of mutations in both *dSR-CI* and *STSDm0173* from the same screen. Finally, it may be possible to use the l(2)k01102 and l(2)k14703 P element insertions to generate overlapping chromosomal deletions that, in trans to each other, specifically delete only the *dSR-CI* gene (Preston et al, 1996). Should the isolation of *dSR-CI* point mutations prove to be particularly difficult (which is possible given the apparent small size of the gene), this approach may prove useful for determining the true function of the dSR-CI protein.

Appendix A

Scavenger receptors in innate immunity

(As published in *Current Opinion in Immunology (1996) **8**, 20 - 28.*)

Scavenger receptors in innate immunity

Alan M Pearson

Scavenger receptors are cell-surface proteins expressed by mammalian monocytes and macrophages and by invertebrate hemocytes, among other cell types. They exhibit distinctive ligand-binding properties, recognizing a wide range of ligands that include microbial surface constituents and intact microbes. The ligand-binding properties and expression patterns of these receptors suggest that they may function in one or more host-defense-related processes. Significant advances in scavenger receptor biology have recently been reported, including the identification of several new scavenger receptor genes.

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Abbreviations

AcLDL	acetylated LDL
CCP	complement control protein
EC	endothelial cell
LDL	low-density lipoprotein
LPS	lipopolysaccharides
OxLDL	oxidized LDL
PBM	peripheral blood monocyte
PS	phosphatidylserine
SR	scavenger receptor
SRCR	SR-cysteine-rich
TSP	thrombospondin
VnR	vitronectin receptor

Introduction

The circulating monocytes and tissue macrophages of mammals, and the hemocytes of invertebrates, mediate many of the innate, or non-adaptive, immune responses to microbial infection (see [1–3] as well as other reviews in this issue). In some cases, these evolutionarily ancient phagocytic cells recognize and engulf pathogens that have been opsonized by serum proteins such as complement [1]. Macrophages and hemocytes, however, can also bind pathogens directly, and this direct recognition plays a crucial role in host defense [1,3–5]. Janeway [4,5] has proposed that activation of the innate immune system (and, importantly, of the adaptive immune system also) is initiated when pathogens bind to non-clonally distributed pattern-recognition receptors on immune cells. It was suggested that these receptors exhibit binding specificities for structural patterns typically displayed by the cell-surface molecules of many microorganisms (e.g. lipopolysaccharides [LPS] and glucans), but not normally found on the surfaces of host cells [4,5]. The ability to mediate pathogen binding, should, in principle, enable pattern-recognition receptors to participate both in the activation of innate immune cells and in the effector mechanisms (e.g. phagocytosis) that these cells employ.

Several soluble and membrane-bound mammalian proteins, including the mannose receptor [6], the collectins (Epstein *et al.*, this issue pp 29–35) and the LPS receptor CD14 [7••], exhibit the properties of pattern-recognition molecules. Scavenger receptors (SRs) have also been suggested to be attractive candidates for such non-self recognition receptors [2–4,8,9•,10,11••,12••]. This suggestion is based on several findings. First, SRs exhibit broad ligand-binding specificities (Table 1), a characteristic feature of pattern-recognition receptors. Second, some SRs can bind both microbial surface molecules and intact bacteria. Third, SRs are expressed by macrophages, hemocytes and other immunologically relevant cells. Finally, SR activity is evolutionarily ancient [10]. In addition to their pattern-recognition receptor-like properties, some SRs also exhibit activities typical of cell-adhesion molecules.

Until recently, only two SRs were known, both products of the same gene. Within the past three years, and principally within the past 18 months, several additional SR genes have been reported, helping to define at least three independent SR classes (Fig. 1; see [12••] for a definition of these classes). Scavenger receptors are defined by their ability to bind modified low-density lipoproteins (LDLs), such as oxidized LDL (OxLDL) and acetylated LDL (AcLDL), and they were initially studied primarily for their potential role in atherogenesis [9•]. This review will discuss recent developments in the SR field, in particular as they relate to the potential roles of SRs in host defense.

Scavenger receptor classes

The two class A SRs, SR-AI and SR-AII, were the first macrophage SRs to be identified [13,14] (for a recent review, see [9•]). These receptors comprise six domains, including extracellular α-helical coiled-coil and collagenous regions which oligomerize to form a trimeric glycoprotein (Fig. 1). SR-AI has an additional carboxy-terminal extracellular SR-cysteine-rich (SRCR) domain, which has now been found in thirteen other proteins ([8,15]; D Resnick, personal communication). Nonetheless, SR-AI and SR-AII exhibit nearly identical binding properties, specifically binding a broad array of polyanionic ligands with high affinity. Studies with SR-AI have shown that these ligands include surface constituents of both Gram-positive and Gram-negative bacteria, as well as the intact bacteria themselves (Table 1) [9•,16•,17]. SR-AI and/or SR-AII are expressed primarily by monocytes and by peritoneal and most tissue macrophages. Some dendritic cells, certain specialized endothelial cells, and smooth muscle cells in atherosclerotic lesions also appear to express the protein(s) [18,19•,20–23]. Substantial progress

Table 1

Ligand specificities of the scavenger receptors*

	SR-AI/II	MARCO	SR-BI	CD36	dSR-CI
Lipoproteins/proteins					
AcLDL	+	+	+	±†	+
OxLDL	+	ND	+	+	ND
M-BSA	+	ND	+	+	+
LDL	–	ND	+	–	–
BSA	–	ND	–	–	–
Poly- and oligonucleotides					
Polyl/polyG/oligo dG	+	ND	–	–	+
PolyC/oligo dA	–	ND	–	–	–
Anionic Polysaccharides					
Fucoidan	+	ND	–	–	+
Dextran sulfate	+	ND	–	ND	+
Chondroitan sulfate	–	ND	–	ND	ND
Phospholipids					
Phosphatidylserine	–	ND	+	+	–
Phosphatidylcholine	–	ND	–	–	–
Poly D-glutamic acid	–	ND	ND	ND	+
Lipid IVa/ReLPS	+	ND	ND	–	ND
Other	LTA, *S. aureus*, other Gram-positive bacteria, crocidolite-asbestos	*S. aureus*, *E. coli*	HDL	*P. falciparum*-infected RBCs, collagen, TSP, apoptotic cells, oleic acid	Laminarin (β-glucan)

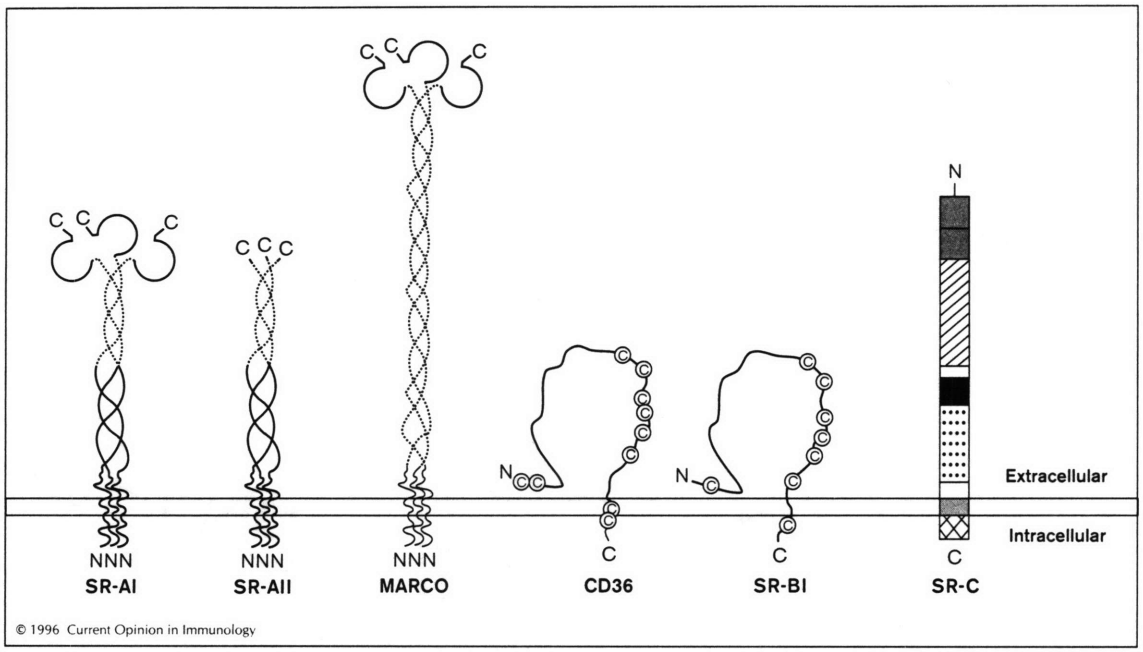
*The ligand specificities reported in the literature have been determined both by directly testing the ability of a given compound to bind to a particular SR, and by testing the ability of a given compound to inhibit the binding of a known ligand to SR-transfected cells. It is important to note that SRs exhibit some unusual binding properties, such as species-specific differences in ligand binding, and non-recipricol cross-competition between two ligands [9•]. Therefore, the inability of compound A to inhibit binding of compound B does not necessarily mean that compound A is not a ligand. Caution must be exercised in interpreting negative data unless direct binding studies have been performed. The data presented are drawn from references presented in the text and from AM Pearson, D Resnick, A Rigotti, M Krieger (unpublished data); [90]. †The binding of AcLDL to CD36 is controversial [12••,27••,36,48•]. As AcLDL is used as an inhibitor to evaluate the contribution of SRs to various macrophage functions, resolution of this issue is important. BSA, bovine serum albumin; HDL, high-density lipoprotein; LTA, lipoteichoic acid; M-BSA, maleylated BSA; ND, not determined; PMN, polymorphonuclear leukocyte (neutrophil); RBC, red blood cell; ReLPS, reduced form of PS.

is being made in defining the regulatory elements that control the macrophage-specific expression of the SR-AI/II gene [24,25].

A third SR-A, termed MARCO, has recently been identified and shown to bind both AcLDL and bacteria [26••]. MARCO expression has been detected only in peritoneal macrophages and in a limited subset of tissue macrophages in the spleen and lymph nodes [26••]. All of these macrophages also express SR-AI/II. The carboxy-terminal portion of MARCO is homologous to the collagenous and SRCR domains of SR-AI, although the MARCO collagenous domain is much longer than that of SR-AI/II. MARCO does not share any other domains with SR-AI/II. In SR-AI/II, and perhaps also in MARCO, the primary ligand-binding region comprises a cluster of basic residues located at the carboxy-terminal end of the collagenous domain [9•,26••].

The search for additional SRs led to the identification of the class B SRs, CD36 [27••] and SR-BI [12••]. These receptors, together with the lysosomal protein Limp II [28], the *Drosophila* emp [29] and croque mort (N Franc, RAB Ezekowitz, personal communication) proteins, and a putative *Caenorhabditis elegans* protein (Genbank accession number 728534), define the CD36 family [30]. The single conserved extracytoplasmic domain shows 25–30% homology between any two family members and comprises an amino-terminal region which contains most of the potential N-glycosylation sites, and a carboxy-terminal region rich in conserved proline, glycine and cysteine residues [30]. Non-conserved putative transmembrane and cytoplasmic domains flank the extracellular domain, but in CD36 only the carboxy-terminal hydrophobic region appears to be embedded in the membrane [31]. Interestingly, croque mort lacks a carboxy-terminal hydrophobic region, suggesting that the CD36 family proteins, including the SR-Bs, may use additional mechanisms to effect association with the cell membrane [28,30]. Initial studies concerning the transcriptional regulation of CD36 gene expression have recently been reported [32,33].

Figure 1



Structures of the class A, class B and class C SRs. The class A SRs, SR-AI and SR-AII, are trimeric glycoproteins generated by alternative splicing of mRNA transcripts from the same gene [9•]. They consist of cytoplasmic (50 amino acids), transmembrane, spacer, α-helical coiled-coil (black) and collagenous (dotted) domains [9•]. The collagenous domain mediates ligand binding [9•]. In addition, SR-AI contains a carboxy-terminal scavenger receptor cysteine rich (SRCR) domain [15]. MARCO is a new SR-A protein comprising amino-terminal cytoplasmic (49 amino acids), transmembrane and spacer domains distinct from those of SR-AI/II. These are followed by a long collagenous domain with carboxy-terminal with homology to that of SR-AI/II, and then a carboxy-terminal SRCR domain [26••]. The model presented for the class B receptors SR-BI [12••] and CD36 [27••] is based on current reports regarding CD36 structure. These experiments indicate that CD36 has a very short carboxy-terminal (<10 residues; 45 residues in SR-BI), a single transmembrane domain, and a single large extracellular domain comprising an amino-terminal region containing most of the N-glycosylation sites and a carboxy-terminal region rich in proline, glycine and cysteine residues (the cysteine residues are indicated). The *Drosophila* class C scavenger receptor, dSR-CI, comprises several domains [11••]. From the amino terminus to the carboxy terminus, these domains are a signal sequence (which is likely cleaved from the mature protein), two complement control protein (ccp) domains (grey), a large MAM domain (stripes), a smaller spacer domain, a somatomedin B domain (black), a serine/threonine rich mucin like domain which may be heavily glycosylated (dotted), another spacer domain, a transmembrane domain, and finally a small cytolplasmic domain (cross-hatched; 64 amino acids). Adapted from figure by M Krieger (unpublished data).

CD36 and SR-BI bind a wide variety of ligands, including *Plasmodium falciparum* parasitized red blood cells (Table 1), and in this way they resemble pattern recognition receptors. At least some of these ligands (for example, phospholipids such as phosphatidylserine [PS] [34••]), must contain negatively charged moieties in order to be recognized by the SR-Bs. Interestingly, the class B receptors (and other CD36 family members?) may primarily be lipid-binding proteins expressed by tissues and cells involved in host defense and/or lipid metabolism (SL Acton *et al.*, unpublished data; see also Note added in proof; [30,35••,36]). These cells include monocytes, macrophages and hemocytes, B lymphocytes, capillary endothelial cells, platelets and adipocytes ([12••,30,33,35••,37,38]; SL Acton *et al.*, unpublished data; see also Note added in proof). The relationship, if any, between host defense and lipid recognition is currently unclear. It has been noted, however, that both mammalian macrophages and insect hemocytes internalize anionic phospholipid vesicles (including PS), and that this may thus be one primitive mechanism by which phagocytes recognize non-self and damaged-self [39]. Perhaps surface expression of anionic phospholipids represents one type of pattern not associated with normal host cells.

After the striking finding that *Drosophila* embryonic hemocytes express SR activity [10], a class C SR gene, *dSR-CI*, was cloned from the hemocyte-like L2 cell line (synonym S2) [11••]. The extracellular portion of the dSR-CI protein contains two amino-terminal complement control protein (CCP) domains. In mammals, CCP domains mediate the binding of complement receptors and complement regulatory proteins to the central component of the complement cascade, C3 [40]. The extracellular region of dSR-CI also contains MAM and somatomedin B domains, and a mucin-like domain which may be

heavily glycosylated. Some of these domains are known to have adhesive properties in mammalian proteins [41–43]. dSR-CI, like SR-AI/II, exhibits high-affinity binding to a broad array of polyanionic ligands (Table 1). Unlike SR-AI/II, however, it also binds uncharged microbial β-glucans, which are potent inducers of the *Drosophila* immune response [3]. These findings suggest that dSR-CI might be able to mediate the direct recognition of microbial pathogens by hemocytes. Indeed, dSR-CI is expressed in the hemocytes of the Drosophila embryo [11••]; whether it is also expressed in larval hemocytes and by the larval fat body, an important tissue for the *Drosophila* immune response [2,3], is not yet known. Because dSR-CI does not appear to account for all of the SR activity found in L2 cells [11••], additional *Drosophila* SRs probably exist; these could potentially include both SR-A and SR-B (e.g. croque mort) homologs.

It is likely that mammals also possess additional, as yet unidentified, SRs. For example, macrosialin/CD68 [44] has recently been shown to be able to bind OxLDL [45]. The roles of this protein as an SR and in host defense are unclear as it is predominantly a lysosomal and endosomal protein [44,45]. Several studies have also suggested the existence in mammals of macrophage and non-macrophage SR activities which may be distinct from those of the SR-As, the SR-Bs and macrosialin/CD68 [46,47,48•,49••,50,51].

Scavenger receptor functions in innate immunity

The potential involvement of SRs in the activation of monocytes, macrophages and hemocytes by microbial products and microbes of various types (Gram-positive bacteria, Gram-negative bacteria, yeast, etc.) [7••,16•,52,53•,54•,55] has not yet been the subject of extensive investigation. Two findings suggest that SR-As are probably not involved in LPS (Gram-negative bacteria)-induced macrophage activation: AcLDL blocks most of the binding of Lipid IVa (an LPS precursor) to murine macrophages, but does not appear to effect Lipid-IVa-induced macrophage activation [17]; and SR-AII-transfected CHO cells do not respond to 100 ng ml^{-1} LPS whereas CD14-transfected CHO cells do [56].

One of the principal functions of SRs may be their participation in the clearance of microbes and highly toxic microbial surface constituents from the body [9•,10,11••,12••, 16•,17,44,57]. The primary reasons for believing this to be the case remain the broad ligand-binding specificities and macrophage/hemocyte expression of the SRs. Several other findings support the involvement of the mammalian SRs in these phagocytic and endocytic processes. First, LPS and other inflammatory stimuli upregulate macrophage SR-AI/II, CD36 and macrosialin/CD68 expression [44,57,58]. Second, peritoneal macrophages from SR-AI/II knock-out mice exhibit a 50% reduction in LPS-binding activity (H Suzuki, Y Kurihara, T Kodama, personal communication). Third, LPS associates with high-density lipoprotein [52,53•], which binds SR-BI (SL Acton *et al.*, unpublished data; see also Note added in proof). Thus SR-BI may facilitate LPS clearance by the liver. Finally, the co-injection of SR ligands (poly I, AcLDL) with lipid IVa significantly reduces hepatic uptake of lipid IVa in mice [17]. SRs may also participate in the clearance by alveolar macrophages of anionic environmental particulates from the lung [59,60].

It has been suggested that some SRs, by participating in microbial clearance processes, may also participate in the activation of the adaptive immune system by mediating antigen uptake and/or processing for presentation to B and T cells [20,44]. In this regard, it has recently been shown that maleylation of proteins, which converts them to SR ligands, enhances their ability to elicit antibody and T-cell responses in the absence of adjuvant *in vivo*, and enhances their presentation to T cells by macrophages *in vitro* [61].

In addition to their potential clearance functions, some SRs may participate in the recruitment of peripheral blood monocytes (PBMs) to infected tissues, a process which requires PBM extravasation through vascular endothelial cells (ECs) (reviewed in [41]). Indeed, it has been suggested that SR-AI/II may participate in this recruitment [19•]. It may be relevant that M-CSF, which induces monocyte to macrophage differentiation and may be released at some infection sites [62], can also upregulate both SR-AI/II expression and SR-AI/II-mediated cell adhesion (see below) [22]. It has also been suggested that macrosialin/CD68, which is upregulated by inflammatory stimuli, may be a ligand for E-selectin [44]. Thus, macrosialin/CD68 might participate in the initial tethering and subsequent adherence of PBM to activated ECs overlying an infected tissue. Interestingly, expression of CD36 on PBM is upregulated upon their E-selectin dependent adherence to activated EC [58]. It has been suggested that this upregulation may be related to the extravasation of PBM through the EC layer and/or to PBM-mediated clearance of apoptotic neutrophils associated with the infection site (see below) [58]. Endothelial cells themselves also transiently upregulate CD36 expression in response to the pro-inflammatory cytokine interferon-γ [38].

Drosophila hemocytes also accumulate at infection sites. In addition to phagocytically clearing pathogens, the hemocytes will often segregate foreign particles (bacteria, parasites) from host tissues by inducing hemolymph clotting and melanization, forming nodules around small pathogens, and encapsulating large invaders within well-organized and multi-layered cellular capsules [2,3,63,64]. These processes, encapsulation in particular, require hemocyte–hemocyte adhesive interactions. dSR-CI has several adhesive domains in its extracellular region, including a MAM domain which has been suggested to regulate homophilic binding interactions in mammalian proteins

[43]. Thus, dSR-CI may be able to participate in hemocyte capsule formation. Actively encapsulating hemocytes also undergo alterations in their surface characteristics [63,64]. Given that insect hemocytes appear to recognize the SR-B ligand PS [39], it would be interesting to know whether one of these alterations involves the cell-surface exposure of PS on activated hemocytes. External exposure of PS has been shown to occur both in activated mammalian platelets during the clotting response [65,66], and in LPS-stimulated monocytes [67].

Scavenger receptor functions in immune-related processes

Establishment of macrophage, lymphocyte and hemocyte populations

In order to effectively participate as sentinel and effector cells in first-line host defense, mammalian monocytes must establish residence throughout the body as permanently differentiated tissue macrophages [1,68]. It has been suggested that SR-AI/II (and MARCO?) might participate in this process as homing and/or retention molecules [19•]. Two recent reports support this hypothesis. First, it has been found that the anti-SR-AI/II monoclonal antibody 2F8 can inhibit cation-independent (non-integrin, non-selectin) adhesion of macrophages to tissue-culture plastic [69] and to frozen splenic, thymic and lymph-node tissue sections [19•]. These adhesion events appear to require, respectively, a serum SR-AI/II ligand [69], and an endogenously expressed tissue ligand and/or homophilic binding interactions between macrophages and SR-AI/II-expressing cells in tissues [19•]. Second, macrophage colony-stimulated factor directs monocyte to tissue macrophage differentiation; it also upregulates both SR-AI/II expression by macrophages and SR-AI/II-mediated macrophage adhesion [22]. Finally, although not part of the innate immune system *per se*, it is worth noting the recent suggestion that SR-AI/II may also participate in the adhesive events that regulate lymphocyte recirculation (see [41]), as it is expressed by lymph node high endothelial venules [20].

It is not known whether any *Drosophila* hemocytes establish permanent residence in specific tissues. They do nonetheless migrate throughout the body of the developing and adult fly, and in some cases they appear to follow specific guidance cues [70•,71•]. dSR-CI is expressed in hemocytes before the onset of migration and contains several domains which participate in the adhesive interactions of other proteins. Thus, dSR-CI might participate in the hemocyte migration process [11••]. Similarly, croque mort might also facilitate hemocyte migration along the extracellular matrix [71•] by recognizing collagen, a mammalian CD36 ligand [30].

Response to wounding

Wounding induces activation of the innate immune system in both mammals and invertebrates [1–3,63,64,72–75] (also reviews in this issue). Monocytes, macrophages and hemocytes are the central mediators of this innate immune response, and play critical roles in guarding against infection, in clearing associated debris, and in wound healing. It has been suggested that wound recognition by these cells may be somewhat analagous to the recognition of infectious microorganisms [2,64,75,76••]; perhaps this is, in part, another manifestation of pattern recognition. Indeed, wounding exposes molecular structures and patterns on damaged cells and tissues, and on recruited platelets, which are not found on normal cells and tissues [64–66].

SRs may participate in wound recognition, both as adhesion molecules and also as pattern-recognition receptors. It has been suggested, for example, that monocyte adhesion to the wound site may be facilitated in part by CD36-mediated binding to thrombospondin (TSP) secreted by activated platelets [77]. Since these platelets also express PS externally [65,66], and as both macrophages and CD36-transfected cells bind PS vesicles [34••,39] (and see below), CD36-mediated recognition of activated platelet PS may also contribute monocyte adhesion. In addition, because both collagen and PS are exposed on wounded tissues and cells themselves [65,66], recognition of these structures by CD36 may contribute to direct binding of monocytes to wound surfaces. Similar mechanisms can be, and in some cases have been ([30], but see [78]), envisioned to contribute to the initial recognition of wounds by both platelets and hemocytes, and to the growth and stabilization of platelet and hemocyte clots. Finally, it has been suggested that SR-AI/II may participate in the clearance of wound-associated debris, as SR-AI/II expression is dramatically upregulated in glial cells and recruited macrophages after optic nerve crush [57]. Other SRs may also be involved in the clearance of damaged cells, since macrophage binding of oxidized red blood cells, which are a model for damaged cells, is completely inhibited by both PS and OxLDL [49••].

Recognition and engulfment of apoptotic cells

The induction of apoptosis in various cells during the immune response is an important effector mechanism for host defense in higher eukaryotes from plants to animals [79–82]. In animals, apoptosis also plays an important role in the normal development and turnover of organs, tissues and cells, including those of the immune system [79,83,84]. The phagocytic clearance of apoptotic cells, and of damaged and senescent cells, is a primary function of monocytes, macrophages and hemocytes [1,71•,79,83]. These phagocytes may engulf effete cells in part using pattern-recognition mechanisms similar to those used for the recognition and engulfment of infectious microorganisms [1,75,76••]. Indeed, apoptotic cells, like damaged cells and tissues, express surface characteristics not usually found on normal self cells [49••,79,85].

Multiple mechanisms are associated with apoptotic cell engulfment [49••,79], a finding which is not surprising, given

the considerable heterogeneity displayed by macrophages of different origins [1,68], and the importance of clearing apoptotic cells from the body [79]. It has repeatedly been suggested that SRs may participate in some of these mechanisms [10,11••,19•,34••,49••]. Evidence to support these suggestions is now accumulating, although the precise roles, and sometimes the actual identities, of the SRs involved have not yet been fully established.

Strong support for the direct involvement of SR-AI/II in apoptotic cell engulfment has been provided by two recent findings. First, the anti-SR-AI/II monoclonal antibody 2F8 inhibits 50% of apoptotic thymocyte uptake by thymic macrophages and elicited peritoneal macrophages (N Platt, S Gordon, *J Cell Biochem Suppl* 1995, 19B:300). Second, thymic macrophages from SR-AI/II knockout mice (H Suzuki, Y Kurihara, T Kodama, personal communication) exhibit a 50% reduction in apoptotic cell uptake (N Platt, H Suzuki, Y Kurihara, T Kodoma, S Gordon, personal communication). Other receptors, perhaps including other SRs, are also involved in apoptotic cell engulfment by these macrophages (N Platt, S Gordon, *J Cell Biochem Suppl* 1995, 19B:300).

Some SR-Bs also appear to be involved in the clearance of apoptotic cells. It has been demonstrated that both PBM-derived macrophages and bone-marrow derived macrophages use a combination of CD36, the vitronectin receptor (VnR, $a_v\beta_3$ integrin), and TSP to engulf apoptotic leukocytes [86,87]. A direct role for CD36 in VnR/TSP dependent apoptotic cell uptake was recently demonstrated by transfecting a CD36 cDNA into Bowes melanoma and COS cells, both of which constitutively express the VnR. The transfected cells, but not the untransfected cells, were able to phagocytose apoptotic cells in a VnR/TSP/CD36 dependent manner [88••].

Resident and elicited peritoneal macrophages recognize and engulf apoptotic cells and damaged red blood cells by VnR-independent mechanisms. At least some of these mechanisms clearly require the involvement of SRs, as OxLDL can partially or completely inhibit the binding and engulfment of these cells, depending on the nature of the target [49••,89•]. The identity of the relevant SRs is currently unknown, but they could include SR-AI/II (N Platt, S Gordon, *J Cell Biochem Suppl* 1995, 19B:300), CD36 [27••], SR-BI [12••], and macrosialin/CD68 [49••] (however, see [45]), all of which bind OxLDL.

At least some of the VnR-independent mechanisms involve the recognition of PS exposed on the surface of the target cells. Both putative PS receptors and SRs appear to participate in this PS dependent process [49••,85,87,89•]. Several findings suggest the intriguing possibility that these SRs may include the SR-Bs, and perhaps other CD36 family members. First, CD36 is expressed by both elicited and resident peritoneal macrophages [27••,30,89•]. Second, PS vesicles are SR-B ligands and can inhibit the binding of native and modified LDLs to the SR-Bs [34••]. Indeed, CD36 and SR-BI are the only PS receptors that have been molecularly well defined. Third, the same CD36-transfected Bowes cells that engulf apoptotic cells in a VnR/TSP-dependent manner (see above) can also bind and engulf shed photoreceptor rod outer segments, which externally express modified phospholipids, via a VnR/TSP-independent mechanism (SW Ryeom, JR Sparrow, RL Silverstein, *Clin Res* 1995, 42:113A). It will be interesting to determine whether SR-Bs do indeed participate in the PS-dependent engulfment of apoptotic, damaged and senescent cells.

Conclusions

A common characteristic of infectious microorganisms, damaged cells and tissues, and apoptotic and senescent cells is the cell-surface expression of molecular structures and patterns not found on normal host tissues and cells. That the mechanisms used by vertebrate and invertebrate immune cells for the recognition of and response to these diverse targets may be related is an old concept [1,2,57,75,76••]. These mechanisms may include the recognition of target cells mediated via pattern-recognition receptors, as first proposed by Janeway for the recognition of microbial pathogens [4,5].

It has often been suggested that SRs are just such pattern-recognition receptors. This review has discussed the rationale behind these suggestions and has reviewed some of what is currently known about the involvement of the SRs in host-defense-related processes. Among the various SR functions that have been proposed, the strongest evidence currently supports a role for the participation of SRs in LPS clearance and in apoptotic cell engulfment. Many potential functions have been proposed for the SR-Bs, both here and elsewhere. Interestingly, some individuals appear to lack all platelet, monocyte and macrophage CD36 expression, and yet are perfectly healthy [48•]. Given the redundancy built into biological systems, this finding does not rule out a role for CD36, or any other CD36 family members, in any of the processes discussed in this review.

Targeted gene disruption of SR genes should greatly contribute to the elucidation of the roles that SRs play in host defense. SR-AI/II knock-out mice have already been generated (H Suzuki, Y Kurihara, T Kodama, personal communication), and we can look forward to other SR knock-outs in the future. Given the similarities between the innate immune systems of mammals and insects, it is likely that genetic studies in *Drosophila* will also contribute to our understanding of SR functions. Thus, we can hope that, in the coming years, new studies of SR function will provide clear insights into the roles that these receptors play in innate immunity and host defense.

Note added in proof

The study referred to in the text as (SL Acton *et al.*, unpublished data) has now been published [91•]. Fukasawa *et al.* [92] have reported that CHO cells transfected with SR-IB can recognize, and perhaps engulf, apoptotic cells.

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Appendix B

Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*

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Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*

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ABSTRACT **Mammalian class A macrophage-specific scavenger receptors (SR-A) exhibit unusually broad binding specificity for a wide variety of polyanionic ligands. The properties of these receptors suggest that they may be involved in atherosclerosis and host defense. We have previously observed a similar receptor activity in *Drosophila melanogaster* embryonic macrophages and in the *Drosophila* macrophage-like Schneider L2 cell line. Expression cloning was used to isolate from L2 cells a cDNA that encodes a third class (class C) of scavenger receptor, *Drosophila* SR-CI (dSR-CI). dSR-CI expression was restricted to macrophages/hemocytes during embryonic development. When expressed in mammalian cells, dSR-CI exhibited high affinity and saturable binding of ^{125}I-labeled acetylated low density lipoprotein and mediated its chloroquine-dependent, presumably lysosomal, degradation. Although the broad polyanionic ligand-binding specificity of dSR-CI was similar to that of SR-A, their predicted protein sequences are not similar. dSR-CI is a 609-residue type I integral membrane protein containing several well-known sequence motifs, including two complement control protein (CCP) domains and somatomedin B, MAM, and mucin-like domains. Macrophage scavenger receptors apparently mediate important, well-conserved functions and may be pattern-recognition receptors that arose early in the evolution of host-defense mechanisms. Genetic and physiologic analysis of dSR-CI function in *Drosophila* should provide further insights into the roles played by scavenger receptors in host defense and development.**

It has been more than 100 years since Metchnikoff's observations of phagocytosis in invertebrates led him to propose the cellular theory of immunity (1). Since that time, studies in vertebrates have shown that phagocytosis plays a key role in both adaptive immunity, which involves clonally selected antibody and cellular responses, and nonadaptive, or innate, immunity (2). Interestingly, invertebrate innate immunity, which comprises both humoral and cellular components (3–7), is strikingly similar to that in vertebrates (3, 6). For example, complement-like serine protease cascade reactions in invertebrates are activated in response to infection (8, 9). Some of the molecules used in these cascades, such as *Limulus* coagulation factor C, are structurally homologous to mammalian complement proteins. Factor C contains complement control protein (CCP) domains (8), which are also found in a large number of mammalian complement and complement regulatory proteins, clotting proteins, and leukocyte cell adhesion proteins (10). Invertebrates also use a variety of macrophage-associated processes to respond to microbial infection (3–6, 9), including phagocytosis of both opsonized and unopsonized pathogens (3–5, 9, 11).

The immune responses of invertebrate macrophages and other hemocytes are induced by intact microorganisms and by exposure to isolated microbial surface constituents, such as lipopolysaccharide (LPS) and laminarin (3, 8, 9, 12). It has been proposed that direct recognition of these inducers by both vertebrate (13–15) and invertebrate macrophages (4, 5) is mediated by pattern-recognition receptors (2, 3, 7). Such receptors are predicted to exhibit broad ligand-binding specificity for molecular structures common among microbial pathogens. Thus, they may mediate the self/nonself discrimination required to initiate and regulate innate host-defense responses (2). Macrophage-specific class A scavenger receptors (SR-A) are characterized by broad polyanionic ligand-binding specificity (16, 17) and, thus, may serve as pattern-recognition receptors for innate host defense (17, 18). Indeed, LPS and lipoteichoic acid, which are toxic shock-inducing surface constituents of Gram-negative and Gram-positive bacteria, bind with high affinity to the collagenous ligand-binding domains of SR-A (14, 15, 18, 19). Scavenger receptors may also be involved in the recognition of atherogenic lipoproteins (16, 20); the phagocytic clearance of damaged, senescent, or apoptotic host cells (18, 21, 22); and in cell–cell or cell–matrix adhesive interactions (23).

We have recently demonstrated that *Drosophila* embryonic macrophages and the macrophage-like *Drosophila* Schneider L2 cell line exhibit a scavenger-receptor activity resembling that of the mammalian macrophage-specific SR-A (21). We have now used an expression cloning method to isolate a cDNA‡ from L2 cells that encodes a previously unidentified protein, dSR-CI, which defines a third class of scavenger receptor. This *Drosophila* class C scavenger receptor is distinct from both class A and the recently identified class B (SR-B, ref. 24) mammalian scavenger receptors. dSR-CI is expressed virtually exclusively in macrophages/hemocytes during embryonic development, and its broad polyanionic ligand-binding properties are almost identical to those of mammalian SR-A. Nevertheless, there is no significant sequence homology between SR-A and SR-C. SR-C is a multidomain protein containing several sequence motifs, including the CCP domain, found in numerous mammalian host-defense proteins.

MATERIALS AND METHODS

cDNA Library Construction and Expression Cloning of dSR-CI. Poly(A)$^+$ RNA (30 μg) was prepared from *Drosophila* Schneider L2 cells as described (25), except that the cells were homogenized and the DNA was sheared with a Brinkmann Polytron disrupter (PT10S probe; three times for 5 s at setting 4). cDNA was synthesized by using an oligo(dT) primer ligated

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Abbreviations: dSR-CI, *Drosophila* macrophage scavenger receptor class C type I; LDL, low density lipoprotein; AcLDL, acetylated LDL; CCP, complement control protein; DiI-AcLDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled AcLDL; SR-A, macrophage scavenger receptor class A.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U17693).

to phosphorylated *Bst*XI linkers [pCTTTAGAGCACA and pCTCTAAAG (Research Genetics, Huntsville, AL)], size-selected into either >1.5-kbp or >2.0-kbp fractions, and ligated into the *Bst*XI site of the expression plasmid pcDNAI (Invitrogen) (26). DNA pools (1600–4000 clones per pool) from transformed MC1061/p3 cells (Invitrogen) were transfected into COS-M6 cells, the transfected cells were visually screened for uptake of fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated low density lipoprotein (DiI-AcLDL), and a single clone (S0o24.11c) was isolated from one positive pool, all as described (24, 27). This clone, designated pdSR-CI, was sequenced on both strands by using the Sequenase 2.0 (United States Biochemical) and the GIBCO/BRL PCR-based sequencing kits. Sequence comparisons and data-base surveys were performed with the Genetics Computer Group sequence analysis software package (versions through 7.3) (28), and BLAST from the National Center for Biotechnology Information (29). RNA blot analysis of L2 and Kc poly(A)$^+$ RNA (30) was performed with a full-length PCR-amplified dSR-CI cDNA.

***In Situ* Hybridization to *Drosophila* Embryos.** Clone p6-5, which contains the 5′-terminal 815 bp of the dSR-CI cDNA, was constructed as follows. pdSR-CI was double-digested with *Eco*RV and *Xba* I and blunted with the Klenow fragment; the large vector-containing fragment was purified and recircularized. Two digoxigenin-labeled RNA probes were generated from *Apa* I (sense strand)- and *Spe* I (antisense strand)-digested p6-5 DNA by *in vitro* transcription from the phage T7 (sense) and phage SP6 (antisense) promoters by using digoxigenin-dUTP (Genius 2.0 DIG RNA labeling kit; Boehringer Mannheim). Canton S strain embryos (0–16 hr) were collected and processed, *in situ* hybridizations were performed, and the embryos were staged, all as described (31, 32).

Cell Culture. Cells were maintained at 37°C in a humidified 5% CO_2/95% air incubator (mammalian cells) or at 25°C in tightly capped flaskettes (*Drosophila* cells). Wild-type CHO cells were grown in medium A (Ham's F-12 supplemented with PSG (100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine) and 5% (vol/vol) fetal bovine serum), COS-M6 cells in medium B (Dulbecco's modified Eagle's medium supplemented with PSG and 10% fetal bovine serum), *Drosophila* Schneider L2 cells in medium C (Schneider's medium supplemented with PSG and 10% heat-inactivated fetal calf serum), and *Drosophila* Kc cells in medium D (D22 medium supplemented with PSG without glutamine). CHO[mSR-AII] cells, which express murine SR-A type II (33), were grown in medium E [medium A containing 0.5 mg of geneticin (G418; GIBCO) per ml]. A stable transfectant (clone 2.6a) expressing dSR-CI, designated CHO[dSR-CI], was isolated by transfecting 1×10^6 CHO cells with 0.5 μg of pSV2neo and 9.5 μg of pdSR-CI by the Polybrene method (30, 34), selecting transfectants in medium E, and cloning a receptor-positive (uptake of 1 μg of protein per ml of DiI-AcLDL) colony by dilution plating (34).

Assays and Reagents. Scavenger receptor activities in mammalian cells at 4°C (measured in six-well dishes) and 37°C (24-well dishes) and in *Drosophila* L2 cells at 25°C were determined as described (21, 33, 35). Cell surface binding of ^{125}I-labeled acetylated low density lipoprotein (^{125}I-AcLDL) (2 hr, 4°C) and its binding plus internalization (5 hr, 37°C) are expressed as ng of cell-associated ^{125}I-AcLDL protein per mg of cell protein. Degradation activity is expressed as ng of ^{125}I-AcLDL protein degraded in 5 hr per mg of cell protein. Protein determination was by the method of Lowry *et al.* (36). For competition experiments, cells were incubated with ^{125}I-AcLDL for 5 hr in the absence (triplicate incubations) or presence (duplicates) of competitor during the assays. Stock solutions of competitors [e.g., laminarin (Sigma)] were prepared in Dulbecco's complete phosphate-buffered saline (4–10 mg/ml). These and other reagents (e.g., sodium butyrate from Pfaltz & Bauer) were obtained and/or prepared as indicated or as described (21, 24, 35).

RESULTS AND DISCUSSION

To identify the gene responsible for the macrophage scavenger receptor-like activity in *Drosophila* Schneider L2 cells, we prepared a cDNA expression library from L2 cell poly(A)$^+$ mRNA, divided the library into small pools, transfected the pools into COS-M6 cells, and visually screened the transfected cells for endocytosis of fluorescent DiI-AcLDL. A single receptor-positive pool was identified (≈350,000 clones screened) and was subdivided repeatedly to obtain a single functional plasmid (designated pdSR-CI for plasmid encoding *Drosophila* scavenger receptor class C, type I) [for nomenclature, see Acton *et al.* (24)]. Northern blot analysis of poly(A)$^+$ RNA showed that the dSR-CI message (≈2.1 kb) was expressed in receptor-positive L2 cells at a level >50-fold higher than that in scavenger receptor-negative *Drosophila* Kc cells (not shown).

A CHO cell line stably transfected with pdSR-CI, CHO-[dSR-CI], was generated. These cells exhibited at 4°C (Fig. 1*A*) high-affinity, saturable ^{125}I-AcLDL binding ($K_d \approx 2$ μg of protein per ml) and at 37°C (Fig. 1*B*) high-affinity, saturable binding plus internalization and degradation ($K_d \approx 5.5$ μg of protein per ml). The degradation of bound and internalized ^{125}I-AcLDL by CHO-[dSR-CI] cells, which was chloroquine-sensitive (not shown) and thus presumably lysosomal, was more efficient than that in Schneider L2 cells (21). This difference may be due to differences in the assay conditions (e.g., temperature, medium) or in the fundamental properties of the cultured cells. The affinity of dSR-CI for ^{125}I-AcLDL was comparable to the affinities of the receptors in *Drosophila* Schneider L2 cells and of mammalian SR-A (21, 38, 39). Thus, CHO[dSR-CI] cells express a scavenger

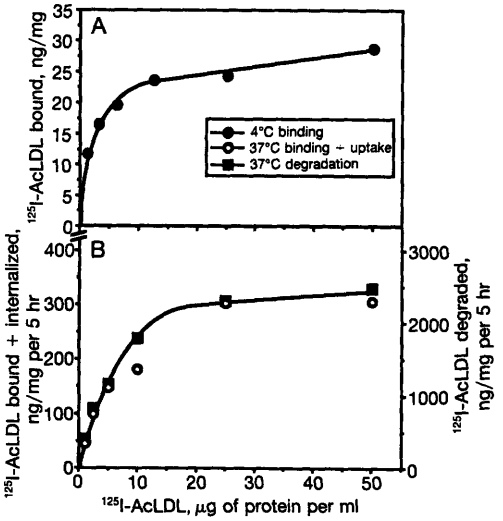

FIG. 1. Concentration dependence of ^{125}I-AcLDL interaction with CHO[dSR-CI] cells at 4°C and 37°C, expressed as ng of cell-associated ^{125}I-AcLDL protein per mg of cell protein. On day 1, CHO[dSR-CI] cells were plated in medium E into either six-well dishes (125,000 cells per well) (*A*) or 24-well dishes (60,000 cells per well) (*B*). On day 3, the indicated amounts of ^{125}I-AcLDL in medium A were added and binding for 2 hr at 4°C (*A*) or binding plus internalization and degradation for 5 hr at 37°C (*B*) were determined. The high-affinity values shown represent the differences between measurements made in the absence (duplicate incubations) and presence (single incubations) of excess unlabeled AcLDL (400 μg protein per ml). Untransfected CHO cells exhibit virtually no scavenger receptor activity (37).

receptor-mediated endocytic pathway that is similar to those of L2 cells and mammalian macrophages (16, 17, 21, 38, 39).

A hallmark of SR-A and L2 cell scavenger receptors is their broad polyanion binding specificity, usually assessed by measuring inhibition of ^{125}I-AcLDL binding and subsequent uptake and degradation (16, 18). Using such an assay, we found that numerous SR-A polyanionic ligands, in addition to AcLDL itself, were effective inhibitors/competitors of ^{125}I-AcLDL degradation by both CHO[dSR-CI] and L2 cells. These included the modified protein M-BSA (maleylated bovine serum albumin), the four-stranded polynucleotides poly(I) and d(A_5G_{37}), and the polysaccharide dextran sulfate. At concentrations of 400 μg/ml [100 μg/ml for d(A_5G_{37})], they all reproducibly inhibited scavenger receptor activity by >85% in both cell types. Furthermore, all were high-affinity competitors for both cell types: their concentrations that gave half-maximal inhibition ranged from ≈0.1 to 5 μg/ml (data not shown). As with SR-A, single-stranded dA_{37} (100 μg/ml) and unmodified LDL and BSA (400 μg/ml) did not compete (<15% inhibition). Surprisingly, poly(D-glutamic acid) inhibited dSR-CI (see below), although it is not an SR-A inhibitor (16, 17). These results suggest that expression of dSR-CI could account for L2 cell-scavenger receptor activity.

While there were many similarities in the scavenger receptor activities of CHO[dSR-CI] and L2 cells, two notable differences were observed. First, the apparent K_i values for poly(I) and d(A_5G_{37}) were lower by a factor of ≈10 for CHO[dSR-CI] than for L2 cells. Second, poly(D-glutamic acid) inhibited both cell types with relatively high affinities (apparent K_i values ≤ 25 μg/ml) but with substantially different maximal levels of inhibition (measured with 400 μg/ml). While poly(D-glutamic acid) inhibited most of the ^{125}I-AcLDL degradation by CHO-[dSR-CI] cells (75%), it inhibited only about 20% of the activity in L2 cells. These disparities between the CHO[dSR-CI] and L2 cells may be due to differences in the assay conditions or in the properties of the receptors expressed in dissimilar cells from different species. Alternatively, they raise the possibility that L2 cells may be like mammalian macrophages (17) and express multiple types of scavenger receptors, some of whose specificities may differ from that of dSR-CI [e.g., insensitive to poly(D-glutamic acid)].

Because of the broad polyanion specificity of CHO[dSR-CI] and L2 cell scavenger receptor-mediated ^{125}I-AcLDL degradation, it was surprising to find that laminarin, an uncharged (β1–3)-linked D-glucose polymer, was also a highly effective inhibitor (apparent K_i values ≈ 2 and 6 μg/ml, respectively; >85% inhibition at 400 μg/ml). In contrast, dextran (400 μg/ml), another uncharged glucose polymer, did not inhibit ^{125}I-AcLDL degradation (not shown). The mechanism of laminarin inhibition of dSR-CI activity and its relationship to laminarin-induced immune responses in cultured *Drosophila* cells (12) have not yet been established.

The 2032-bp dSR-CI cDNA encodes a 629-residue polypeptide (Fig. 2*A*). It has a 39-bp 5′ untranslated region with an

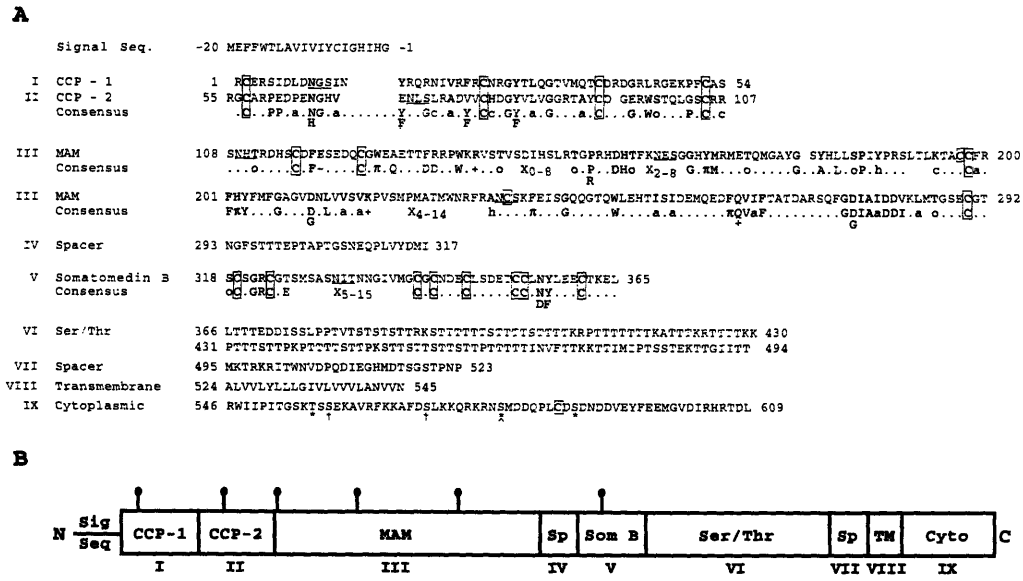

FIG. 2. Predicted protein sequence and domain organization of dSR-CI in single-letter code. (*A*) The cDNA for dSR-CI was cloned and sequenced. The predicted protein sequence is numbered from −20 for the first in-frame methionine in the putative signal sequence; the first residue (arginine) after the predicted cleavage site (40) is designated +1. Cysteines are boxed, and potential N-linked glycosylation sites are underlined. Potential phosphorylation sites in the cytoplasmic domain (IX) are indicated [*, casein kinase II (41); †, protein kinase C (42); ∧, cAMP/cGMP-dependent protein kinase (43, 44)]. The protein is divided into nine domains, some of which belong to previously described motif families (see text). Consensus sequences for those motifs are indicated below the corresponding sequences in dSR-CI. The CCP consensus sequence is that of Perkins *et al.* (45). We generated the MAM and somatomedin B consensus sequences from 13 MAM sequences (7 independent sequences and 6 sequences of homologs from different species), and 15 somatomedin B sequences (8 independent sequences from 6 proteins, and 7 sequences of homologues). MAM consensus criteria were as follows: single amino acids or combinations with aromatic (π = F, W, Y), hydroxyl (o = S, T), or positive or negative (+ = H, K, R; − = D, E) side chains must be present in ≥5 independent sequences; aliphatic (a = A, V, L, I) or charged (c = +, −) residues, in ≥6; and hydrophobic residues (h = a, π, M), in all 7. At positions in which only two amino acids occur in at least 6 of the sequences, both are shown. Somatomedin B consensus residues occur in ≥5.67 of the 8 independent sequences. For these calculations, residues were assigned an appropriate fractional occupancy weight when they occurred in a sequence represented by several species homologues. The MAM consensus sequence differs somewhat from that assembled by Beckmann and Bork (46) when fewer cloned sequences were available. (*B*) Schematic diagram of the domain structure of dSR-CI. The signal sequence (Sig Seq), CCP, MAM, somatomedin B (Som B), spacer (Sp), Ser/Thr-rich putative O-glycosylated (Ser/Thr), transmembrane (TM), and cytoplasmic (Cyto) domains and the potential N-linked glycosylation sites (ball and stick symbols) are indicated. The domains are numbered as in *A*.

in-frame stop codon 15 bp upstream of the putative initiator methionine and a 106-bp 3′ untranslated region containing a poly(A) signal 84 bp downstream of the termination codon. The predicted dSR-CI protein is a multidomain type I transmembrane protein (Fig. 2*B*) that has no significant homology to the mammalian SR-A or SR-B molecules (24, 33). Its N-terminal 20 residues (Fig. 2*A*) represent a putative signal sequence, which is followed by a 609-amino acid (67.6 kDa) mosaic protein comprising nine domains with six potential N-linked glycosylation sites (underlined in Fig. 2*A*). Domains I (54 residues) and II (53 residues) (Fig. 2*A*) are members of the CCP family of domains (10). Their sequences conform to the overall CCP consensus sequence (69% and 65% identities, respectively) about as well as other randomly selected CCP sequences (not shown). Over 160 CCP domains have been found in more than 30 proteins, including many complement proteins (e.g., C1r, C2, DAF, CR1), and other proteins in vertebrates [e.g., clotting factors, selections (47), proteoglycans (48)] and invertebrates [the *Drosophila hikaru genki* gene product (49) and *Limulus* coagulation factor C (8)]. In many cases, these ≈60-residue domains participate directly in binding interactions with other proteins. It is possible that the CCP domains in dSR-CI, which apparently are projected significantly out into the extracellular space (Fig. 2*B* and see below), may play a role in ligand binding.

Domain III (185 residues) is the first known invertebrate member of the MAM family of extracellular domains (initially named for Meprin, A5 antigen and receptor protein tyrosine phosphatase Mu) (46), for which no functions have yet been assigned. A 25-residue spacer (domain IV) separates the MAM domain from domain V, a 48-amino acid somatomedin B-like domain (50). This motif was first described as a fragment of the extracellular matrix molecule vitronectin.

Domain VI is a 129-residue serine/threonine-rich domain, which, by analogy with other cell surface proteins, such as the LDL receptor and the mucins, is presumably heavily O-glycosylated and highly extended (51). Threonine and serine comprise 55% and 12%, respectively, of all amino acids in this domain, and within an 87-residue subregion (positions 381–467), they account for 79% of all residues, while lysine, arginine, and proline compose all but one of the other 18 residues. Thus, this domain is reminiscent of both vertebrate and *Drosophila* mucins (52, 53). Unlike these mucins, domain VI contains no identifiable internal repeat units at the DNA or protein levels. Based on typical mucin lengths of 2.5 Å per residue (51), domain VI might extend >320 Å from the cell surface. This would significantly project the N-terminal domains (I–V) out into the extracellular space, potentially facilitating their interactions with ligands.

The remainder of the protein is composed of a 29-residue spacer segment (domain VII), a 22-residue putative transmembrane domain (domain VIII), and a 64-residue cytoplasmic domain (IX), none of which show significant sequence similarity to other proteins. The cytoplasmic domain contains several potential sites for phosphorylation by various kinases (Fig. 2*A*) (41–44).

To begin to explore the physiological functions of dSR-CI, we used *in situ* hybridization to examine its expression during *Drosophila* embryonic development (Fig. 3). Throughout the stages of development examined, the expression pattern of dSR-CI was essentially identical to the distribution of macrophage/hemocytes (54, 55). For example, dSR-CI expression was seen during developmental stage 10 [4–5 hr after egg laying (ael)] in the procephalic mesoderm (Fig. 3*A*), which gives rise to all of the embryonic macrophages/hemocytes (55). During stage 11 (5–7 hours ael; Fig. 3*B*), the stained cells appeared to migrate posteriorly into the gnathal buds and into the tail end of the germ band (which is adjacent to the head due to germ-band elongation) and anteriorly into the head and clypeolabrum. During stage 12 (7–9 hr ael), germ-band retraction carried the tail region cells to the posterior end of the embryo (Fig. 3*C*). In addition, dSR-CI-expressing cells appeared to migrate both posteriorly and anteriorly along the ventral and dorsal surfaces of the ventral nerve cord (not shown), so that by late stage 12 (Fig. 3*C*), punctate single-cell staining could be seen in the grooves of the ventral nerve cord. By stages 13/14 (10–11 hr ael, Fig. 3*D*), stained cells appeared to have migrated not only throughout the head and tail regions of the embryo but also around the gut. By stage 11, some dSR-CI-positive cells were found in cavities (Fig. 3*E*), where macrophages/hemocytes accumulate (55, 56). In later stages, stained cells, rather than being integrated into defined tissues, were found scattered throughout the hemocoel. Here, macrophages/hemocytes both deposit extracellular matrix (54) and phagocytose apoptotic cells (32, 55, 56). Expression of dSR-CI precedes the onset of apoptosis in stage 11 (ref. 56). Finally, we observed that dSR-CI was expressed in multivesicular macrophage-like cells, which presumably contained apoptotic corpses (Fig. 3*F* and refs. 55 and 56). Therefore, we

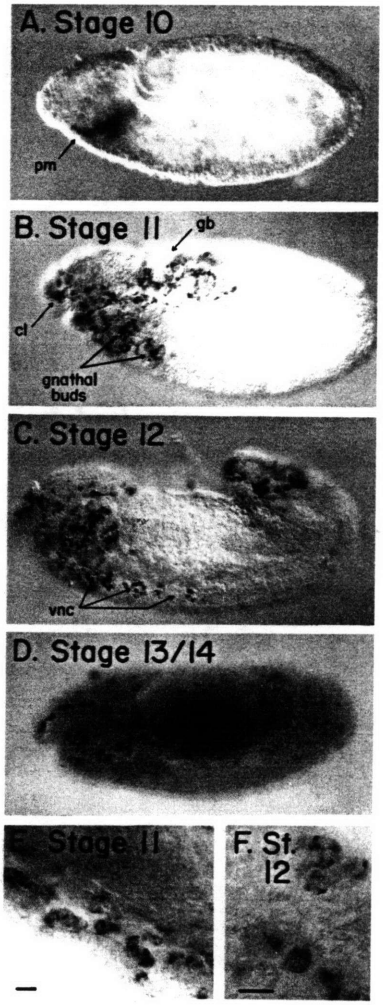

FIG. 3. Expression of dSR-CI mRNA in *Drosophila* embryos. *In situ* hybridization in embryos was performed with a digoxigenin-labeled dSR-CI antisense RNA probe as described in text and was visualized with Nomarski optics. The stage ("St.") of embryonic development is indicated. cl, clypeolabrum; gb, germ band; pm, procephalic mesoderm; vnc, ventral nerve cord. (Bars in *E* and *F* = 10 μm.)

conclude that dSR-CI expression in embryos is primarily, if not exclusively, restricted to macrophages/hemocytes. Occasionally we observed unstained cells with a macrophage-like morphology. It is not clear whether this was due to low sensitivity of the staining assay or to the presence of a distinct population of dSR-CI-negative macrophages.

The broad polyanionic binding specificity, mosaic structure, and macrophage/hemocyte-specific expression of dSR-CI suggest that this receptor may participate in a variety of macrophage/hemocyte functions. These include host defense (e.g., pathogen recognition and phagocytosis) (3, 7, 18, 21), cell–cell or cell–matrix adhesion (18, 54), wound healing (4, 5), and possibly recognition and clearance of apoptotic and senescent cells (18, 21, 22, 56). Macrophage scavenger receptors, such as dSR-CI, are attractive candidates for the pattern-recognition receptors that help confer the polyspecificity and self/nonself discrimination required for innate immunity in both vertebrates and invertebrates (2, 3, 7, 18, 21). Furthermore, the presence of CCP domains in dSR-CI places this receptor in a superfamily of proteins, many of which are involved in vertebrate and invertebrate host defense. It should be useful to determine if there are vertebrate homologues of dSR-CI and if there are additional classes of scavenger receptors in invertebrates (e.g., homologues of mammalian SR-A and SR-B). The application of genetic techniques available in *Drosophila*, along with additional molecular and physiologic studies, should provide a powerful approach for the investigation of scavenger receptor structure and function.

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Appendix C

The SRCR superfamily: a family reminiscent of the Ig superfamily

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The paper presented in this appendix, of which I am the second author, describes the Scavenger Receptor Cysteine Rich (SRCR) domain superfamily of proteins. The first known SRCR domain was defined by the C-terminal sequence of the class A, type I mammalian macrophage scavenger receptors. The domain has since been found in more than one dozen other proteins, many of which are expressed by cells of the immune system (see also Resnick, 1996). The first known function for an SRCR domain has recently been described (Bowen et al, 1996) - mediating the binding of the SRCR domain-containing protein CD6 to its Ig domain containing ligand, the activated leukocyte cell adhesion molecule (ALCAM). The primary author of this paper was David Resnick. My contribution to this work was limited to assisting with the amino acid sequence alignment of the SRCR domains and recognizing the higher-order repeat structure of SRCR domains in the WC1 and M130 proteins, as described herein.

The SRCR superfamily: a family reminiscent of the Ig superfamily

Many proteins are molecular mosaics composed of a wide variety of conserved sequence motifs which comprise structurally distinct domains[1]. In many cases, these motifs are characterized by short, disulfide-stabilized domains present in the extracellular portions of membrane proteins and in secreted proteins[1,2]. Examples include the immunoglobulin repeat[3,4], kringle domains, epidermal growth factor (EGF)-like repeats, complement C9/LDL-receptor domains, Ly-6 repeats[5,6] and the P-domain[7]. These 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. Proteins containing as few as one and as many as 36 copies of any one single type of domain have been reported[8,9].

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[10,11]. This class of domain, designated the SRCR (scavenger receptor cysteine-rich) domain, was initially defined by the presence of one to four copies per polypeptide chain of an approximately 101-residue motif in the type I scavenger receptor, the speract receptor, CD5/Ly1 and complement factor I (CFI) (a total of 13 sequences, including nine independent sequences and four sequences of homologs from different species). The recent cloning of genes encoding 24 additional, independent SRCR domains, and ten 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[12], are those of CD6 (Ref. 13), the cyclophilin-C-binding protein[14] and its homolog the MAC2-binding protein[15] (CyCAP/MAC2-bp), the WC1 antigen[16], M130 (Ref. 17), a new CFI homolog (EMBL accession No. S15468), and two new homologs each of CD5/Ly1 (Refs 18, 19) and the scavenger receptor[20,21]. Thus, the superfamily of SRCR-domain-containing proteins includes eight different members derived from five mammalian (human, bovine, murine, rat and rabbit), one amphibian (*Xenopus laevis*) and one invertebrate (sea urchin) species (Table I). The sequences of the SRCR domains are shown in Fig. 1.

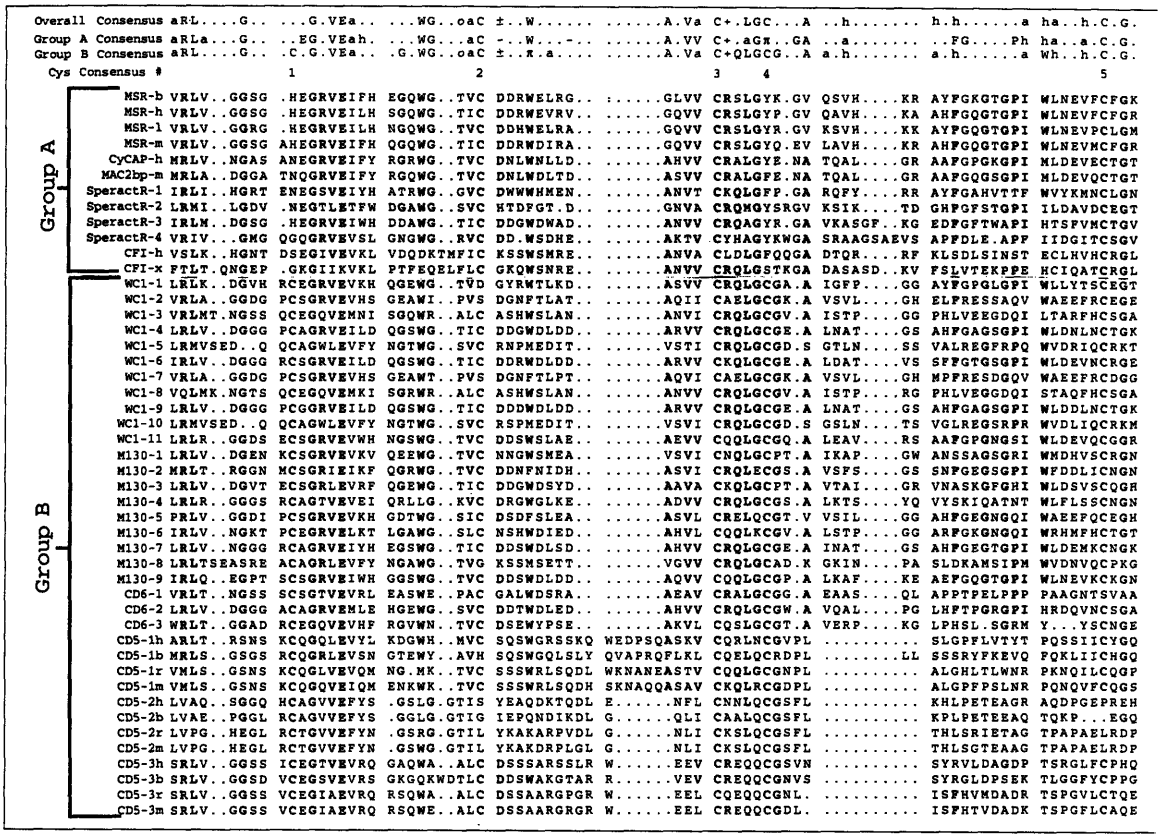
An alignment of all 33 independent SRCR domains (total of 47 sequences) is shown in Fig. 1. Positions at which there is greater than 50% sequence identity are shaded. To simplify the sequence comparisons, we have divided the SRCR superfamily into two groups, A and B, based primarily on the differences in the spacing pattern between their cysteine residues (Fig. 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 Fig. 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.

Table I. Proteins containing SRCR domains[a]

Protein (abbreviation)	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 plasma
WC1	11	Mammalian $CD4^-$, $CD8^-$, gd 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 Ref. 26.



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 (Fig. 1). In the case of CD5-2, although the amino-terminal half of this SRCR domain clearly conforms to the consensus sequence, most of the residues in the carboxy-terminal half of the domain differ substantially from the consensus (Fig. 1). Because of this lack of sequence homology, we did not attempt to align two of the three cysteines in the carboxy-terminal half of CD5-2 with the consensus cysteine positions; any particular alignment would be somewhat arbitrary. Because the CFI and CD5 sequences played important roles in the initial analysis of the SRCR domain[10], the alignment of sequences and the definition of the SRCR consensus shown in Fig. 1 differ 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[22] 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[14,15]. Based on the known structures of other cysteine-containing domains (such as 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 (Fig. 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 and C^6–C^8, C^3–C^6 and C^5–C^8, or C^3–C^8 and 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 Fig. 3. All of the members of this group are cell-surface or secreted polypeptides containing between one and 11 SRCR domains. Other than the scavenger receptor trimer[22], the oligomeric states of these proteins are unknown, although MAC2-bp forms a large multimer[15]. Proteins containing multiple SRCR domains in a single polypeptide chain have short interdomain spacers whose median length is six residues. Our dendrigraphic[23] and visual analysis of the sequences of two such proteins, WC1 and M130, revealed an interesting higher-order repeat,

```
E...a..C.. .......... ....C..... ..A.VhC.
E...L.-C.h .......... ....C..... .DAGV.C.
E...aW.C.. .......... ....C..... ..A.VhCS
       6                    7          8

ESS.IEECRI RQWGVRA... ....CSHD.. EDAGVTCT
ESS.IEECKI RQWGTRA... ....CSHS.. EDAGVTCT
ESS.IEECKI RQWGVRV... ....CSHG.. EDAGVTCT
ESS.IENCKI NQWGVLS... ....CSHS.. EDAGVTCT
ESS.LASCRS LGWMVSR... ....CGHE.. KDAGVVCS
EAS.LADCKS LGWLKSN... ....CRHE.. RDAGVVCT
ETR.LEDCYH RPYGRPWL.. ....CNAQ.. WAAGVECL
EAH.ITECNM PVTPYQHA.. ....CPYTHN WDVGVVCK
EDR.LIDCIL RDGWTHS... ....CYHV.. EDASVVCA
ENETLSQCQM KVSADMT... ....CAT... GDVGVVCE
ETS.LAECTF TKRRT..... ....MGYQ.. DFADVVCY
ENS.LAECAL RKLP ..... ....MQDN.. QVAKVTCY
EST.VSDCEH SNIADYRNDG ....YNHG.. RDAGVVCS
EPE.LWVCPR VPCPGGT... ....CHHS.. GSAQVVCS
ESF.LWSCPV TALGGPD... ....CSHG.. NTASVICS
ESH.VWRCPS RGWGQHN... ....CRHK.. QDAGVICS
DTS.LWQCPS DPWNYNS... ....CSPK.. EEAYIWCA
ESQ.VWRCPS WGWRQHN... ....CNHQ.. EDAGVICS
EPE.LWSCPR VPCPGGT... ....CLHS.. GAAQVVCS
ESF.LWSCPV TALGGPD... ....CSHG.. NTASVICS
ESH.VWRCPS RGWGRHD... ....CRHK.. EDAGVICS
DTS.LWQCPS GPWKYSS... ....CSPK.. EEAYISCE
ESS.LWDCVA EPWGQSD... ....CKHE.. EDAGVRCS
ESA.LWDCKH DGWGKHSN.. ....CTHQ.. QDAGVTCS
ESA.LWNCKH QGWGKHN... ....CDHA.. EDAGVICS
EPA.VWQCKH HEWGKHY... ....CNHN.. EDAGVTCS
ETS.LWDCKN WQWGGLT... ....CDHY.. EEAKITCS
ESH.LSLCPV APRPEGT... ....CSHS.. RDVGVVCS
EQH.MGDCPV TALGASL... ....CPSE.. QVASVICS
ESR.IWQCHS HGWGQQN... ....CRHK.. EDAGVICS
PDT.LWQCPS SPWEKRL... ....ASPS.. EETWITCD
ESS.LWDCPA RRWGHSE... ....CGHK.. EDAAVNCT
NAT.LAGAPA LLCSGAEWRL ....C..... EVVEHACR
EAY.LWDCPG LP.GQHY... ....CGHK.. EDAGVVCS
ELT.LSNCSW RFNNSNL... ....CSQS.. LAARVLCS
LGS.FSNCSH SRNDM..... ....C..... HSLGLTCL
LGS.FSNCSL NRGRQV.... .......... DSLALICL
PWS.FSNCST SSLGQ..... ....C..... LPLSLVCL
PWS.ISNCNN TSSQDQ.... ....C..... LPLSLICL
QPLPIQWKIQ NSSCTSLEHC FRKIKPQKSG RVLALLCS
RPLPIRWEIQ NPKCTSLEQC FRKVQPWVGG QALGLICS
RPLPIRWEAQ NGSCTSLQQC FQKTTVQEGS QALAVVCS
RPLPIRWEAP NGSCVSLQQC FQKTTAQEGG QALTVICS
K...LSQCHE LWERNSY... ....C..... KKVFVTCQ
I...LSRCHK LEEKKSH... ....C..... KRVFVTCQ
K...LSQCYQ L.QKKTH... ....C..... KRVFITCK
K...LSQCYH L.QKKKH... ....CN.... KRVFVTCQ
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Figure 1

Comparison of the sequences of 47 SRCR domains from eight 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 0.25 to the total count; for two homologs, each sequence contributed 0.5 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. A similar, independently derived, consensus sequence has already appeared[17]. 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 type I; 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 are used: b, bovine; h, human; m, murine; r, rat; l, rabbit (lapin); and x, *Xenopus laevis*.

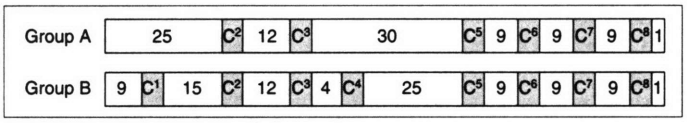

Figure 2

Consensus spacing of cysteines in group A and group B SRCR domains, based on the alignment in Fig. 1; the average number of residues between the cysteines is indicated.

which had previously been identified as an internal repeat in WC1 at the DNA sequence level[16] and was independently identified in a somewhat different form by Law and colleagues[17]. This repeat is defined by a cassette of five SRCR domains, designated [b–c–d–e–d]. The amino-to-carboxy-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–d]; and M130 (domains 1–9), h–i–j–k–[b–c–d–e–d]. The [b–c–d–e–d] 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 might 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

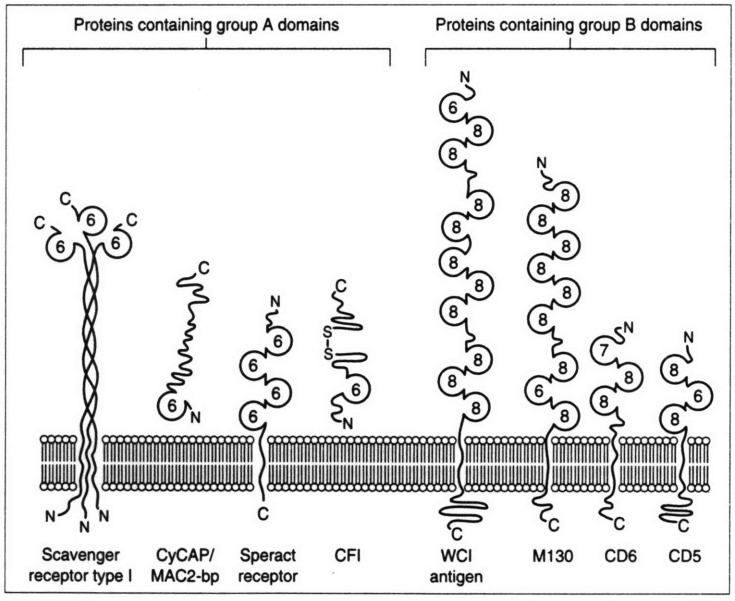

Figure 3

Models of proteins in the SRCR domain superfamily. Models illustrating the number and relative positions of SRCR domains (open circles) in eight proteins are shown. Also shown are the numbers of cysteines in each domain.

other cell-surface or extracellular molecules. In the case of the speract receptor, the SRCR domains form 91% of the extracellular portion of the molecule, and thus probably mediate the binding of its ligand, the sperm-activating peptide speract[24]. Similarly, the SRCR domains form most of the extracellular sequence of CD5, and probably define the binding site for CD72, the only known ligand for CD5 (Ref. 27). The carboxy-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 carboxy-terminal SRCR domain[11]. 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 and macrophages) or are secreted and known or suspected of being involved with host defense (CFI and 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 that play critical roles in host defense[3,4]. Additional studies will be required to determine the detailed molecular structures of SRCR domains and their functions.

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