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Immunology

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

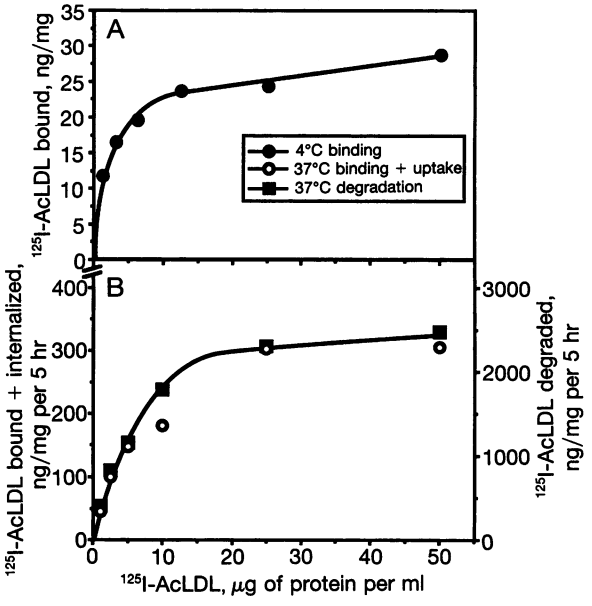

FIG. 1. Concentration dependence of ^{125}I-AcLDL interaction with CHO[dSR-CI] cells at 4°C and 37°C, expressed as ng of cell-associated ^{125}I-AcLDL protein per mg of cell protein. On day 1, CHO[dSR-CI] cells were plated in medium E into either six-well dishes (125,000 cells per well) (*A*) or 24-well 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

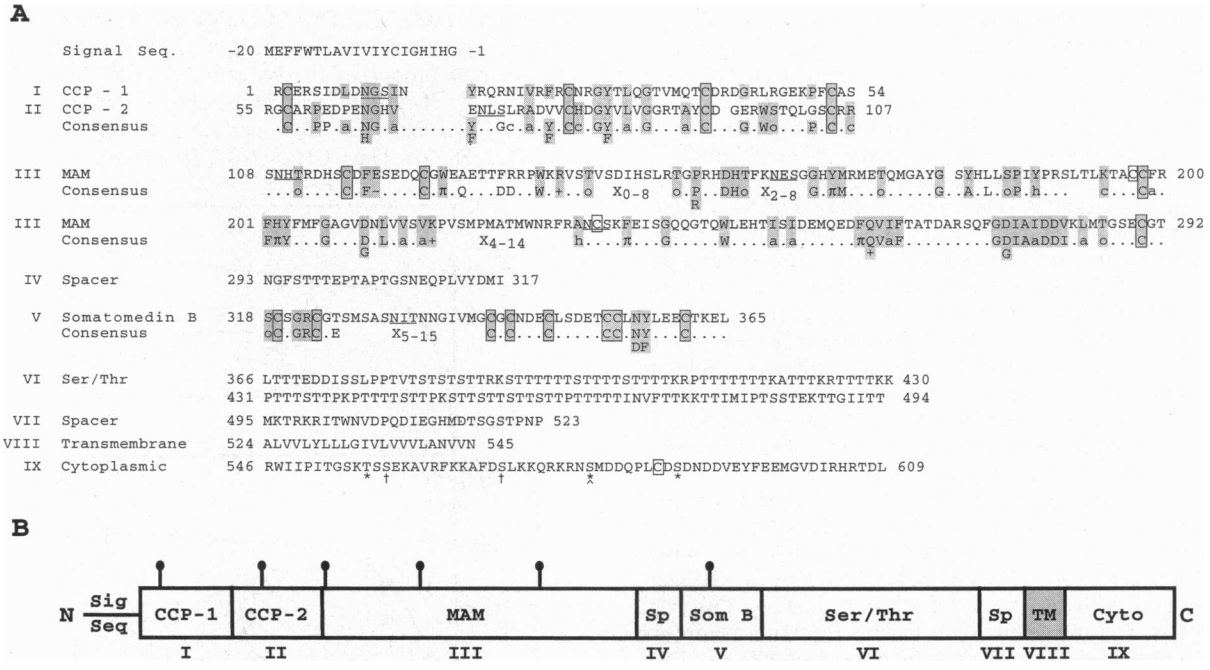

FIG. 2. Predicted protein sequence and domain organization of dSR-CI in single-letter code. (*A*) The cDNA for dSR-CI was cloned and sequenced. The predicted protein sequence is numbered from −20 for the first in-frame methionine in the putative signal sequence; the first residue (arginine) after the 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

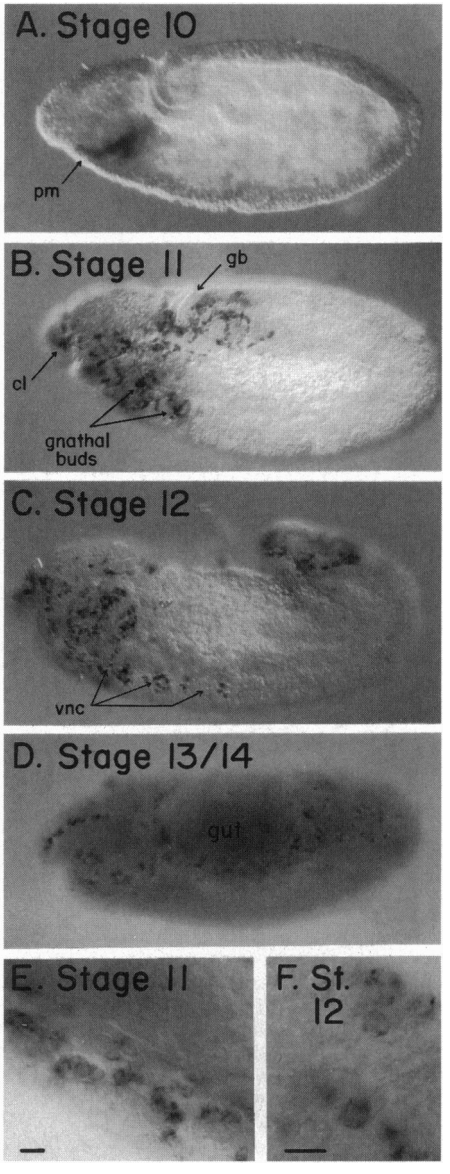

FIG. 3. Expression of dSR-CI mRNA in *Drosophila* embryos. *In situ* hybridization in embryos was performed with a digoxigenin-labeled dSR-CI antisense RNA probe as described in text and was visualized with Nomarski optics. The stage ("St.") of embryonic development is indicated. cl, clypeolabrum; gb, 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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