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The Class B Scavenger Receptors SR-BI and CD36 Are Receptors for Anionic Phospholipids*

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The specific recognition of anionic phospholipids in the outer leaflets of cell membranes and lipoproteins by cell surface receptors may play an important role in a variety of physiologic and pathophysiologic processes (*e.g.* recognition of damaged or senescent cells by the reticuloendothelial system or lipoprotein homeostasis). Several investigators have described anionic phospholipid binding to cells, and phosphatidylserine (PS) binding to a partially purified ~95-kDa membrane protein has recently been reported (Sambrano, G. R., and Steinberg, D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1396–1400). Using both direct binding and ligand competition assays in transfected cells, we have found that two class B scavenger receptors, SR-BI and CD36, can tightly bind PS and phosphatidylinositol (PI)-containing liposomes (K_d for PS liposome binding to SR-BI is ~15 μg phospholipid/ml or 0.18 nM (mol PS liposomes/l)), but not phosphatidylcholine, phosphatidylethanolamine, or sphingomyelin liposomes. PS and PI liposomes, but not the others, could effectively compete with PS liposomes and modified or native lipoproteins for binding to these receptors. Phosphatidic acid, another anionic phospholipid, could also compete, but was not as effective as PS or PI. Class B scavenger receptors are the first molecularly well-defined, specific cell surface receptors for anionic phospholipids to be described.

Phospholipids are key structural components of cell membranes and lipoproteins. Based on studies of the red blood cell's and other membranes (reviewed in Schroit and Zwaal, 1991), it is generally assumed that there is an asymmetric distribution of phospholipids in the plasma membranes of eukaryotic cells (Roelofsen and Op den Kamp, 1994). The outer leaflet appears to be composed predominantly of neutral zwitterionic phospholipids, *e.g.* phosphatidylcholine (PC)[1] and sphingomyelin (SM), while the inner leaflet is greatly enriched in negatively charged phospholipids, such as phosphatidylserine (PS) and phosphatidylinositol (PI). Breakdown of this asymmetry, especially the exposure of increased levels of PS on the outer cell surface, occurs in a variety of physiologic and pathologic states. These include platelet activation (Bevers *et al.*, 1983), cell aging (Shukla and Hanahan, 1982), programmed cell death (apoptosis) (Fadok *et al.*, 1992a, 1992b), sickle cell anemia (Kuypers *et al.*, 1994), and *Plasmodium falciparum* infection of erythrocytes (Joshi and Gupta, 1988). Exposure of anionic phospholipids at the external surface of cells can stimulate blood coagulation (Schroit and Zwaal, 1991) and has been proposed to play a critical role in the recognition of damaged or senescent cells by the reticuloendothelial system (Savill *et al.*, 1993).

Several investigators have described specific anionic phospholipid binding to cells, especially to macrophages, using either direct binding or indirect ligand-competition assays (Schroit and Fidler, 1982; Ratner *et al.*, 1986; Allen *et al.*, 1988; Nishikawa *et al.*, 1990; Lee *et al.*, 1992a; Fukasawa *et al.*, 1995; Sambrano and Steinberg, 1995). Several studies have suggested that scavenger receptors which bind modified lipoproteins, such as oxidized low density lipoprotein (OxLDL) or acetylated LDL (AcLDL), may in some cases function as anionic phospholipid receptors (Nishikawa *et al.*, 1990; Fukasawa *et al.*, 1995; for a review of scavenger receptors see Krieger and Herz, 1994). The only receptor for anionic phospholipids to be identified to date is a partially purified ~95-kDa membrane protein from macrophages that was shown to directly bind PS and OxLDL using a ligand blotting assay (de Rijke and van Berkel, 1994; Ottnad *et al.*, 1995; Sambrano and Steinberg, 1995). The primary sequence of this protein has not been reported; thus, it is not known if it is a member of one of the three classes of scavenger receptors, A, B, and C, which have been previously defined based on their distinctive binding activities and primary sequences (Acton *et al.*, 1994; Pearson *et al.*, 1995). Two of these, class A and class C scavenger receptors (SR-A, SR-C), are expressed almost exclusively on mammalian and embryonic *Drosophila melanogaster* macrophages, respectively (Naito *et al.*, 1991; Elomaa *et al.*, 1995; Bell *et al.*, 1994; Pearson *et al.*, 1995). Lee *et al.* (1992b) and Fukasawa *et al.* (1995) have shown that at least two types of class A receptors are unlikely to be involved in the cellular uptake of anionic phospholipid liposomes, and we have recently obtained data suggesting that the *Drosophila* SR-C (Pearson *et al.*, 1995) may not be able to recognize anionic phospholipids.[2]

In the current study, we have examined the binding of phospholipids to class B scavenger receptors (SR-B). SR-Bs are members of the CD36 superfamily of proteins, including CD36 itself and SR-BI (Acton *et al.*, 1994). SR-Bs are expressed in a variety of cells and tissues, including macrophages and endothelial cells (Abumrad *et al.*, 1993; and see Greenwalt *et al.*,

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[1] The abbreviations used are: PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SR-A, class A scavenger receptors; SR-B, class B scavenger receptors; SR-C, class C scavenger receptors; haSR-BI, hamster scavenger receptor class B type I; LDL, low density lipoprotein; AcLDL, acetylated LDL; OxLDL, oxidized LDL; M-BSA, maleylated bovine serum albumin; FAF-BSA, fatty acid free bovine serum albumin; DPPC, dipalmitoyl phosphatidylcholine; CHO cells, Chinese hamster ovary cells; cpm, counts/min.

[2] A. Pearson and A. Rigotti, unpublished data.

1992 for review). The highest levels of expression have been reported to be in adipose tissue (Abumrad *et al.*, 1993; Acton *et al.*, 1994). In addition to binding modified LDL (Endemann *et al.*, 1993; Acton *et al.*, 1994; Nicholson *et al.*, 1995), CD36 binds M-BSA (Acton *et al.*, 1994), thrombospondin (Asch *et al.*, 1987), collagen (Tandon *et al.*, 1989), long chain fatty acids (Abumrad *et al.*, 1993; Nicholson *et al.*, 1995), and *P. falciparum*-infected erythrocytes (Oquendo *et al.*, 1989). Although its physiologic functions are unknown, CD36 may serve as an adhesion molecule, a component in fatty acid transport (Abumrad *et al.*, 1993), a signal transduction molecule (Ockenhouse *et al.*, 1989; Huang *et al.*, 1991), and a receptor for senescent neutrophils (Savill *et al.*, 1993). A striking feature of SR-BI, not shared by CD36 or other well-defined scavenger receptors, is its ability to bind native LDL with high affinity (Acton *et al.*, 1994). Thus, SR-BI may play an important role in lipid metabolism. Here, we show that SR-BI and CD-36 specifically bind anionic PS and PI liposomes with high affinity, and are, therefore, the first molecularly well-defined cell surface receptors for anionic phospholipids to be described.

EXPERIMENTAL PROCEDURES

Materials—Reagents (and sources) were: acetic anhydride (Mallinckrodt, Inc., Paris, KY); egg phosphatidylcholine, egg phosphatidic acid, liver phosphatidylinositol, brain phosphatidylserine, egg phosphatidylethanolamine, and brain sphingomyelin (Avanti Polar Lipids, Inc., Alabaster, AL); polycarbonate membrane filters (Poretics Corp., Livermore, CA); sodium [^{125}I]iodide and 1,2-dipalmitoyl-L-3-phosphatidyl[*N*-*methyl*-^{3}H]choline ([^{3}H]DPPC) (Amersham Corp.); DEAE-dextran (Pharmacia Biotech Inc.); Ham's F-12 medium, Dulbecco's modified Eagle's medium, fetal bovine serum, and trypsin/EDTA (JRH Biosciences, Lenexa, KS); and penicillin/streptomycin, glutamine, and G418 sulfate (Life Technologies, Inc.). All other reagents and supplies were purchased from Sigma or were obtained as described previously (Krieger, 1983). Human LDL, AcLDL, ^{125}I-labeled LDL, and ^{125}I-labeled AcLDL (90–300 cpm/ng protein) were prepared essentially as described previously (Goldstein *et al.*, 1983; Krieger, 1983; Acton *et al.*, 1994).

Phospholipid Liposome Preparation—Unilamellar liposomes were made by extrusion through polycarbonate membranes (Szoka *et al.*, 1980). Phospholipid liposomes were prepared containing the indicated phospholipid, phosphatidylcholine, and free cholesterol in a molar ratio of 1:1:1. The lipids were mixed in chloroform and dried by rotary evaporation for 30 min. For preparation of radiolabeled liposomes, 50–75 μCi of [^{3}H]DPPC (62 Ci/mmol) were added to the lipid mixtures before drying. The dried lipids were resuspended in 150 mM NaCl, 0.1 mM EDTA, 10 mM HEPES, pH 7.5 (Buffer A). Once the samples were fully hydrated, they were extruded through 0.1-μm pore size polycarbonate membranes using a mini-extruder device (Avanti Polar Lipids, Inc., Alabaster, AL). After extrusion, liposomes were dialyzed against Buffer A and then stored under nitrogen at 4 °C until use. Liposomes were used within 2 weeks of preparation. The final phospholipid concentration was determined by the method of Bartlett (1959). The average diameters of unlabeled liposomes, which were determined from either two or three independent preparations using light scattering with a Coulter N4 plus light scatterer apparatus (Coulter Electronics Inc., Hialeah, FL), were: PS, 105; PC, 114; PA, 125; PE, 129; PI, 113; and SM, 131 nm. The number of phospholipid molecules/PS liposome was calculated as follows. Cross-sectional areas for cholesterol and phospholipid molecules in hydrated bilayers are assumed to be 0.35 nm^2 and 0.47 nm^2, respectively (Levine and Wilkins, 1971); assuming an homogenous distribution of the components throughout the PS/PC/cholesterol (1:1:1) liposomes, 73% of the surface area ($4\pi r^2 \times 2$ (bilayer) $\times$ 0.73 = 50477 nm^2) was phospholipid, or 107,398 phospholipid molecules/liposome (50477 nm^2/0.47 nm^2). Based on an average phospholipid mass of 785 g/mol, a liposome concentration of 10 μg phospholipid/ml converts to 0.12 nM in liposome particles.

Cell Culture and Transfections—CHO, ldlA (clone 7), ldlA[haSR-BI], and COS M6 cells were grown in culture as described previously (Krieger *et al.*, 1983; Acton *et al.*, 1993, 1994). ldlA (clone 7) is a CHO cell mutant clone whose defective LDL receptor gene results in an essentially LDL receptor-negative phenotype (Kingsley and Krieger, 1984; Sege *et al.*, 1986; Kingsley *et al.*, 1986). CHO and ldlA cells were maintained in monolayer culture with Ham's F-12 medium containing 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (medium A). ldlA[haSR-BI] cells are ldlA cells which express hamster SR-BI (Acton *et al.*, 1994) and were cultured in medium A supplemented with 0.25 mg/ml G418 (medium B). COS M6 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (medium C). All incubations with cells were performed at 37 °C in a humidified 5% CO_2, 95% air incubator unless otherwise noted.

COS cells were transiently transfected with plasmids encoding either haSR-BI (phaSR-BI; Acton *et al.*, 1994) or huCD36 (phuCD36; Oquendo *et al.*, 1989) as described previously (Acton *et al.*, 1994). Cells were plated on day 0, transfected on day 1, and replated on day 2 (1×10^6 cells/well in six-well dishes) in medium C supplemented with 1 mM sodium butyrate. Ligand binding assays were performed on day 3.

Ligand Binding Assays—On day 0, ldlA and ldlA[haSR-BI] cells were plated (2.5×10^5 cells/well in six-well dishes) in medium A or B, respectively, and the assay was performed on day 2. Transfected COS cells were prepared as described above. Binding assays were performed as described previously (Acton *et al.*, 1994), with the following minor modifications. Cells were prechilled on ice for 30 min, incubated with the indicated radiolabeled ligands (^{125}I-LDL, ^{125}I-AcLDL, or ^{3}H-labeled liposomes) in ice-cold medium D (Ham's F-12 containing 0.5% (w/v) fatty acid free bovine serum albumin (FAF-BSA) and 10 mM HEPES, pH 7.4), with or without unlabeled competitors, for 2 h at 4 °C with gentle shaking. Cells were then washed twice with Tris wash buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4) containing 2 mg/ml FAF-BSA, followed by one rapid wash with Tris wash buffer without FAF-BSA. The cells were then solubilized with 0.1 N NaOH, and radioactivity and protein determinations were made as described previously (Acton *et al.*, 1994). The specific, high affinity ligand binding activities shown represent the differences between values obtained in the absence (total binding) and presence (nonspecific binding) of an excess of the indicated unlabeled ligands. Nonspecific binding of [^{3}H]PS liposomes to cells was generally low. For example, for the experiment shown in Fig. 1, nonspecific binding ranged from 10% (at 2.5 μg phospholipid/ml) to 28% (at 100 μg phospholipid/ml) of the total binding. For [^{3}H]PC liposomes, nonspecific binding ranged from 21% (at 2.5 μg phospholipid/ml) to 58% (at 100 μg phospholipid/ml) of the total binding. The binding values are expressed as nanograms of bound ^{125}I-labeled protein or ng of total phospholipids from ^{3}H-labeled liposomes/milligram of cell protein.

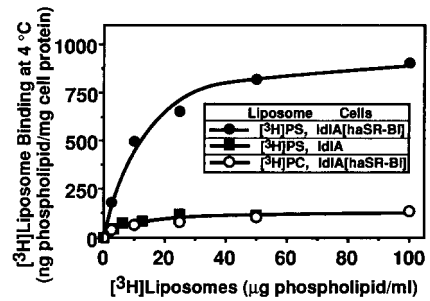

FIG. 1. **Concentration dependence of [^{3}H]PS and [^{3}H]PC liposome binding to transfected ldlA cells expressing haSR-BI (ldlA[haSR-BI]) or to untransfected ldlA control cells.** Transfected ldlA[haSR-BI] cells (*circles*) or untransfected ldlA cells (*squares*) were plated on day 0 and [^{3}H]PS (*filled symbols*) or [^{3}H]PC (*open symbols*) (25–50 cpm/ng total phospholipid) binding at 4 °C was measured on day 2 as described under "Experimental Procedures." The high affinity values shown represent the differences between measurements made with the indicated concentrations of radiolabeled liposomes in the absence (duplicate incubations) and presence (single incubations) of an excess of the corresponding unlabeled liposomes (500 μg of total phospholipid/ml). Data are from two independent experiments in which the data for [^{3}H]PS binding to ldlA[haSR-BI] cells were essentially identical.

RESULTS AND DISCUSSION

To determine if phospholipids could bind to haSR-BI, we prepared 105 nm diameter PS liposomes (PS/ phosphatidylcholine/cholesterol, ratio 1:1:1) radiolabeled with trace amounts of [^{3}H]dipalmitoyl phosphatidylcholine (62 Ci/mmol) and examined their binding at 4 °C to untransfected cells (ldlA) and transfected cells which express haSR-BI (ldlA[haSR-BI]). Fig. 1

TABLE I
Specificity of [^{3}H]PS and ^{125}I-LDL binding to haSR-BI expressed in transfected ldlA cells

ldlA[haSR-BI] cells were plated on day 0. On day 2, the binding of [^{3}H]PS liposomes (10 μg total phospholipid/ml, 28 cpm/ng total phospholipid) or ^{125}I-LDL (5 μg protein/ml, 94 cpm/ng protein) at 4 °C was measured as described under "Experimental Procedures" in the absence ("none") or presence of the indicated phospholipid liposomes (150 μg total phospholipid/ml). The values represent the average of two determinations. The 100% of control values for the binding of [^{3}H]PS liposomes (experiment 1) and ^{125}I-LDL (experiment 2) were 410 and 139 ng/mg cell protein, respectively.

Competing liposomes	[^{3}H]PS binding	^{125}I-LDL binding
150 μg/ml	*% of control*	
None	100	100
Phosphatidylserine	14	20
Phosphatidylinositol	17	19
Phosphatidic acid	44	56
Phosphatidylcholine	82	88
Phosphatidylethanolamine	115	86
Sphingomyelin	109	98

shows that there was substantial, high affinity (K_d ~15 μg phospholipid/ml) and saturable binding to the transfected cells (*filled circles*), but relatively little binding to the untransfected cells (*filled squares*). Assuming that the phospholipid and cholesterol were uniformly distributed in homogenous liposomes containing approximately 107,400 molecules of phospholipid/liposome (see "Experimental Procedures"), we estimate the K_d (mol of PS liposomes/liter) to be ~0.18 nM. PS binding was apparently independent of divalent cations because it was not inhibited by EDTA (1–10 mM, data not shown). Binding depended on the phospholipid composition of the liposomes. In contrast to that of [^{3}H]PS liposomes, the binding of radiolabeled PC liposomes (PC/cholesterol, 2:1, Fig. 1, *open circles*) was very low and similar to [^{3}H]PS binding to untransfected ldlA cells.

These results indicated that phospholipids can bind to haSR-BI and that this binding might depend on the charge of the phospholipid head group. The specificity of the binding was further assessed by determining the competition for [^{3}H]PS binding by unlabeled liposomes of various compositions (indicated phospholipid/PC/cholesterol, ratio 1:1:1). Table I shows that the anionic phospholipids PS and PI were effective inhibitors while the zwitterionic PC and PE as well as SM were not. PA, another anionic phospholipid, was able to compete, but not as effectively as PS and PI.

We previously established that haSR-BI can bind both native LDL and AcLDL with high affinity (Acton *et al.*, 1994). Fig. 2*A* shows that PS and PI liposomes inhibited virtually all of the binding of ^{125}I-AcLDL to haSR-BI in transiently transfected COS cells (>50% inhibition at concentrations >10 μg phospholipid/ml), while PC had virtually no effect at concentrations as high as 250 μg phospholipid/ml. We also observed that PS, but not PC, liposomes inhibited most of the binding of native ^{125}I-LDL to haSR-BI in ldlA[haSR-BI] cells. Indeed, the specificity of liposome inhibition of ^{125}I-LDL binding was almost identical to that for the inhibition of [^{3}H]PS binding (Table I). The extent of PS inhibition of ^{125}I-LDL binding depended on the relative PS content of the liposomes. Fig. 3 shows that inhibition by 500 μg phospholipid/ml increased substantially as the amount of PS in PS/PC mixed liposomes increased from 0 to 50 mol % of total phospholipid, with greater than 50% inhibition occurring when the PS mol % was >10. These competition experiments suggest that anionic phospholipids bound to haSR-BI at a site close to or identical with the site(s) of native and modified LDL binding and that interaction of the liposomes with haSR-BI may involve polyvalent binding via multiple anionic phospholipid molecules.

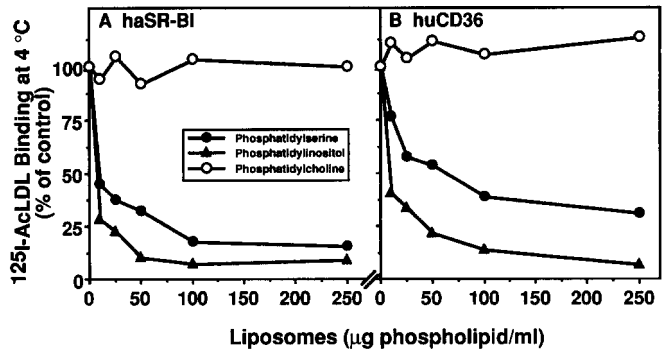

FIG. 2. **Inhibition by PS, PI, and PC liposomes of ^{125}I-AcLDL binding to either haSR-BI (*A*) or huCD36 (*B*) expressed by transfected COS cells.** On day 1, COS cells were transfected with expression vectors for haSR-BI (*panel A*) or huCD36 (*panel B*) as described under "Experimental Procedures." On day 2, transfected cells were plated in six-well dishes in medium C plus 1 mM sodium butyrate. On day 3, medium D containing ^{125}I-AcLDL (5 μg protein/ml, 299 cpm/ng protein) was added, and 4 °C binding was measured in the presence of the indicated concentrations of phosphatidylserine (*filled circles*), phosphatidylinositol (*filled triangles*), or phosphatidylcholine (*open circles*) liposomes. The values represent the means of duplicate determinations. The 100% control values were 119 (*panel A*) or 86 (*panel B*) ng ^{125}I-AcLDL protein/mg cell protein.

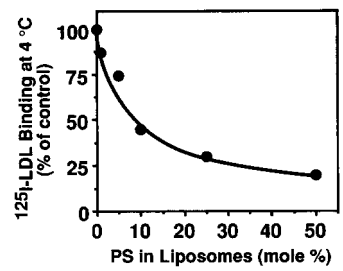

FIG. 3. **Effect of the phosphatidylserine/phosphatidylcholine molar ratio on liposome inhibition of ^{125}I-LDL binding to haSR-BI at 4 °C.** On day 2 of cell growth, the binding of ^{125}I-LDL (5 μg protein/ml, 278 cpm/ng protein) to ldlA[haSR-BI] cells was measured as described under "Experimental Procedures" in the presence of PS/PC/cholesterol liposomes (500 μg phospholipid/ml, total phospholipid/cholesterol ratio of 2:1) with the indicated composition of phosphatidylserine (expressed as mole percentage of total phospholipid). The binding values represent the means of duplicate determinations. The 100% control value was 560 ng ^{125}I-LDL protein/mg cell protein.

haSR-BI is a member of the CD36 superfamily of proteins (Acton *et al.*, 1994). Both haSR-BI and human CD36 (huCD36, 32% amino acid sequence identity to haSR-BI) are class B scavenger receptors which can bind a variety of modified proteins (AcLDL, OxLDL, maleylated BSA); however, they cannot bind to many of the other polyanions which are ligands of the class A and C scavenger receptors (Acton *et al.*, 1994). Fig. 2*B* shows that, as was the case for haSR-BI, the binding of ^{125}I-AcLDL to huCD36 expressed in COS cells was inhibited by PI and PS, but not by PC. PI was a significantly better inhibitor than PS. Thus, CD36, as well as haSR-BI, apparently can serve as a receptor for anionic phospholipids. Except for the apparent differences in the ability to recognize PE, the receptor activity reported by Fukasawa *et al.* (1995) is remarkably similar to that of CD36.

The phospholipid and modified lipoprotein binding specificities of the class B scavenger receptors (Endemann *et al.*, 1993; Acton *et al.*, 1994; Nicholson *et al.*, 1995), the ~95 kDa OxLDL receptor (Sambrano and Steinberg, 1995; de Rijke and van Berkel, 1994), and the AcLDL receptor activity reported by Nishikawa *et al.* (1990) are not identical. Therefore, in addition to SR-BI and CD36 there may be other receptors for phospholipids. Establishing the structural and functional relationships

of class B receptors to previously described anionic phospholipid receptor activities and determining the physiologic significance of anionic phospholipid binding by class B scavenger receptors, particularly with regard to the recognition of lipoproteins and damaged or senescent cells, will require additional studies.

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