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Recombinant Glutathione *S*-Transferase/CD36 Fusion Proteins Define an Oxidized Low Density Lipoprotein-binding Domain*

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CD36 is a multifunctional cell-surface receptor that binds adhesion molecules such as thrombospondin-1 and collagen and modified lipids and/or lipoproteins. It participates in cellular uptake of photoreceptor outer segments and scavenging of apoptotic cells and oxidized low density lipoprotein (Ox-LDL). Recognition and internalization of Ox-LDL by mononuclear phagocytes may play an important role in the development of atherosclerotic lesions. We have utilized a series of recombinant bacterial glutathione *S*-transferase/CD36 fusion proteins that span nearly all of the CD36 molecule to characterize the structural domain on CD36 that recognizes Ox-LDL. We found that the Ox-LDL-binding domain is different from the thrombospondin-1-binding domain located at amino acids 93–120. A fusion protein containing the region extending from amino acids 5 to 143 formed specific, saturable, and reversible complexes with Ox-LDL. As with intact CD36, binding was blocked by excess unlabeled Ox-LDL and antibodies to CD36. The stoichiometry and affinity of the fusion protein for Ox-LDL were similar to those of the intact protein. We also demonstrated that this fusion protein competitively inhibited binding of Ox-LDL to purified platelet CD36 and to CD36 expressed on peripheral blood monocytes and CD36 cDNA-transfected melanoma cells. The use of smaller peptides and fusion proteins including those spanning amino acids 28–93 and 5–93 has further narrowed the binding site to a region from amino acids 28 to 93, although participation of a sequence in the noncontiguous region 120–155 cannot be excluded. This study, for the first time, demonstrates unique regions of the scavenger receptor CD36 that bind the Ox-LDL ligand. Our structural analysis of the receptor provides information as to potential control of the trafficking of modified lipoproteins into the blood vessel wall.

Atherosclerosis is characterized by the formation of intimal plaque with cholesterol deposition, fibrosis, and cellular infiltration in the vessel wall (1). The lesions develop initially with migration of monocyte-derived macrophages, platelets, T-lymphocytes, and lipoproteins (1–4) across the vessel wall. Considerable experimental evidence suggests a model whereby oxidation of low density lipoproteins plays a critical early role in atherosclerosis (4, 5). Oxidized low density lipoprotein (Ox-LDL)[1] is present in human atheroma and can be a proximal source of lipid that accumulates within the cells of the atherosclerotic lesion (6–8). In addition, Ox-LDL may be a pathogenic agonist for vascular cells, including monocytes, platelets, and endothelial cells (9–18), and thus contribute to lesion propagation. LDL particles are presumably subjected to oxidative modification in the vessel wall by reactive oxygen metabolites produced in response to vascular injury in the developing lesion by monocytes, neutrophils, and other cells (19).

Several cellular receptors that bind and internalize modified LDL particles, including Ox-LDL, have been identified and termed "scavenger receptors" (20–24). The molecular mechanisms by which Ox-LDL particles enter macrophages and activate cells, however, remain undefined. CD36, also known as GPIV, has recently been identified as a monocyte/macrophage scavenger receptor with specificity for Ox-LDL (25, 26). Under certain conditions, *e.g.* exposure to monocyte colony-stimulating factor, CD36 may account for as much as 70% of lipid accumulation and foam cell formation (27). CD36 is an 88-kDa transmembrane glycoprotein (28) expressed on platelets (28, 29), monocytes (30, 31), certain microvascular endothelia (32), erythroid precursors (33), specialized epithelia (breast (34) and retina (35)), and adipocytes (36). It functions as a endothelial cell adhesion receptor for *Plasmodium falciparum* malaria-parasitized erythrocytes (37) and sickle cell erythrocytes (38) and also as a cellular receptor for thrombospondin-1 (TSP) (39), and thus participates in platelet-monocyte adhesion (31, 40), platelet-tumor cell adhesion (41), cell-substratum adhesion (42), and angiogenesis (43). Savill and Hogg (44) have shown that macrophage binding and uptake of apoptotic neutrophils are mediated by the specific interaction of TSP on the surface of apoptotic neutrophils with CD36 and $\alpha_v\beta_3$ integrins on the macrophage surface. We have also demonstrated that CD36 is expressed on the surface of retinal pigment epithelium, where it mediates binding and uptake of shed photoreceptor outer segments (ROS) (35). ROS do not express TSP, and anti-TSP antibodies did not block phagocytosis, suggesting that the ligand on the ROS surface for CD36 is not TSP. Rigotti *et al.* (45) have shown that CD36 binds anionic phospholipids. We have shown that phosphatidylserine and phosphatidylinositol liposomes block ROS uptake (46), consistent with the possibility that anionic phospholipids exposed on effete ROS may be the recognition ligand for CD36. These observations and the findings of Abumrad *et al.* (36) related to binding of fatty acids to

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[1] The abbreviations used are: Ox-LDL, oxidized low density lipoprotein; LDL, low density lipoprotein; TSP, thrombospondin-1; ROS, rod outer segment(s); GST, glutathione *S*-transferase.

CD36 indicate that there may be discrete binding sites for acidic phospholipids, fatty acids, and the Ox-LDL moiety on the CD36 molecule.

CD36 belongs to a small gene family that is highly conserved and, in vertebrates, includes at least three members: CD36, LIMPII (a lysosomal membrane protein with >66% sequence homology to CD36) (47), and CLA-1 or SR-B1 (48). Rigotti *et al.* (45) have shown that SR-B1 functions in binding high density lipoproteins, anionic phospholipids, and modified LDL. Since CD36 is a multifunctional protein involved in adhesion, scavenging, and cell signaling, characterizing its structural organization is critical to understanding its complex biological function.

Although the structure of the Ox-LDL-binding domain on CD36 has not been completely characterized, reported data suggest that the domain(s) that bind TSP, *P. falciparum*-parasitized erythrocytes, and Ox-LDL are probably not identical (49). A large group of monoclonal antibodies raised in different laboratories against human CD36 (*e.g.* 8A6, OKM5, FA6-152, L103, 5F1, Smϕ, ESIVC7, and 10/5) have been shown to interact with an immunodominant domain spanning amino acids 155–183 (50, 51, 54) and to block numerous unrelated CD36 functions including TSP binding (52, 53), collagen binding (55), malaria cytoadhesion (56), uptake of apoptotic cells (44) and ROS (35), and Ox-LDL binding (25, 26). Similarly, a monoclonal antibody raised against domain 155–183 expressed on vaccinia virus also blocked multiple functions (50, 58, 59). Whereas these data could be interpreted to indicate that this immunodominant domain functions as both a TSP- and Ox-LDL-binding site, other studies suggest otherwise; TSP, for example, did not compete efficiently with Ox-LDL for binding to CD36 (26). We (53) and others (52) have shown with synthetic or recombinant CD36 peptides that the domain that binds TSP extends from amino acids 93 to 120, with possible participation by a regulatory region from amino acids 139 to 155. The region that interacts with malaria-parasitized erythrocytes has been reported to span amino acids 87–99 (60) or 139–155 (61). These results suggest that monoclonal antibodies may not be adequate tools to dissect structure-function relationships. In this study, we now report the use of a series of GST/CD36 fusion proteins to define the domains of CD36 that specifically bind the Ox-LDL ligand.

EXPERIMENTAL PROCEDURES

Materials—Percoll, Ficoll-Paque, and all other chromatography media were purchased from Amersham Pharmacia Biotech, and Ox-LDL was purchased from PerImmune Inc. (Rockville, MD). LDL was prepared by the method Havel *et al.* as described previously (62, 73). CD36 was prepared from Triton X-114 lysates of outdated human platelets obtained from the New York Blood Center as described previously (53, 55) by sequential chromatography on Q-Sepharose, wheat germ lectin-Sepharose 6MB, and a sizing column of Superose 6. The purified CD36 was stored in phosphate-buffered saline containing 0.05% Nonidet P-40. $Na^{125}I$ was purchased from Amersham Pharmacia Biotech, and 96-well removable strips (Immulon-4 Removawell) were from Dynatech Laboratories Inc. A synthetic peptide spanning CD36 amino acids 77–97 was purchased from Chiron Mimeotopes (Raleigh, NC). Anti-CD36 (8A6) was from Dr. John Barnwell (New York University Medical Center, New York, NY), and FA6 was from the Fifth International Leukocyte Typing Workshop. Rabbit antisera were raised against purified platelet CD36 and were specific as assayed by enzyme-linked immunosorbent assay and Western blotting (53). Radiolabeling of Ox-LDL and fusion proteins was done as described previously (25, 53). Specific activity was determined for each of the labeled reagents prior to each experiment and ranged from 0.02 to 0.05 μCi/μg. Ox-LDL was rhodamine-labeled as described previously (62) using the succinimidyl ester (Molecular Probes, Inc., Eugene, OR). Approximately 8 mol of rhodamine were incorporated per mol of apoB.

Cells and Cell Lines—Fresh human platelets were collected from human blood drawn into citrate anticoagulant. After washing the platelets three times in Tyrode's buffer (10 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, and 5.5 mM glucose, pH 7.4) containing apyrase (5 units/ml), they were resuspended into phosphate-buffered saline containing 0.5% delipidated bovine serum albumin. Some aliquots were activated by addition of 1×10^{-5} M ADP. Bowes melanoma cells stably transfected with human CD36 cDNA (or control plasmid) were prepared and maintained as described previously (41). CD36 expression was confirmed prior to all studies by immunofluorescence flow cytometry. Peripheral blood monocytes were isolated from buffy coats obtained from the American Red Cross (New York, NY) by sequential centrifugation on Ficoll and Percoll gradients (64). Purified monocytes were washed in phosphate-buffered saline and resuspended in RPMI 1640 medium containing gentamicin and supplemented with 5% heat-inactivated human AB serum (Sigma). Monocytes were >98% viable as determined by trypan blue exclusion. More than 90% of the purified cells were monocytes as determined by immunofluorescence flow cytometry using a panel of monoclonal anti-human monocyte antibodies. For some studies, cells were plated on coverslips in 12-well plates and cultured in RPMI 1640 medium and 5% human serum for 4 days to allow differentiation into macrophages.

Preparation of GST/CD36 Fusion Proteins—A series of recombinant GST/CD36 fusion proteins spanning ≈98% of the CD36 sequence were generated in a bacterial expression system (53). These include fusion proteins spanning amino acids 5–143, 67–157, 93–298, 298–439, 118–182, and 93–120. Two additional fusion proteins within region 5–143 were made to further delineate domains within that region that might interact with Ox-LDL. One of these was prepared from region 5–143 fusion protein cDNA by cleaving with restriction enzymes *Sna*BI and *Bam*HI to remove the fragment between amino acids 93 and 143 and then recloning into pGex3T, yielding a fusion protein extending from amino acids 5 to 93. The second construct was made using polymerase chain reaction to generate a fragment spanning amino acids 28–93. An *Eco*RI site was placed at the 5′-end and a *Not*I site at the 3′-end to facilitate cloning into pGex6.1 In addition, a fusion protein from another member of the CD36 gene family, LIMPII, spanning amino acids 75–155 (36,753 Da), was also generated (65). As described previously, all plasmid constructs were mapped, and insertion sites were sequenced to confirm that the fusion protein sequences were correct and in frame. The fusion proteins were prepared according to the method of Frangioni and Neel (66) and purified by affinity chromatography on glutathione-Sepharose beads. They were examined by SDS-polyacrylamide gel electrophoresis, Western blotting, gel filtration chromatography, and enzyme-linked immunosorbent assay to confirm size and to document CD36 immunoreactivity. The molecular masses were within the calculated range with <5% variation from calculated values (53). The fusion proteins did not form dimers or larger multimers, and none of the fusion proteins bound to purified intact CD36 in solid-phase binding assays. The fusion proteins were extensively dialyzed against phosphate-buffered saline to remove any residual detergent present from the preparation procedure. For some experiments, the CD36 peptides were cleaved and eluted from the fusion proteins bound to the agarose beads by treatment with thrombin or factor Xa. The protease was then removed by incubation with benzamidine-Sepharose (53).

Solid-phase Binding Assays—Solid-phase binding assays were used to measure Ox-LDL interactions with CD36 and recombinant GST/CD36 fusion proteins. One of the ligands (*e.g.* CD36 or fusion protein) was immobilized on wells in a detachable 96-microwell plate by overnight incubation at 4 °C. Saturable coating conditions were first determined using radiolabeled proteins. CD36 was thus adsorbed at 4 μg/ml in phosphate-buffered saline, whereas fusion proteins were adsorbed at 10 μg/ml in carbonate buffer (100 mM Na_2CO_3, 1 mM $MgCl_2$, and 0.02% NaN_3, pH 9.8). The amount of protein coated on the wells ranged from 200 to 280 ng. The wells were washed three times with 20 mM Tris and 150 mM NaCl, pH 7.4, containing 0.05% Tween 20 (Tris-buffered saline/Tween) and blocked with Tris-buffered saline/Tween containing 0.5% bovine serum albumin. Radiolabeled Ox-LDL was added in Tris-buffered saline/Tween, and the mixture was incubated for 2 h at 22 °C. The wells were washed thoroughly three to four times with Tris-buffered saline/Tween and dried, and bound radioactivity was measured by γ-counting. Nonspecific binding was determined by carrying out the binding in the presence of excess unlabeled ligand or control GST fusion protein and is indicated in each figure. For competition experiments, the competing proteins (0.1 nM to 0.1 mM) were added along with the labeled Ox-LDL (10 μg/ml) and incubated for 2 h at 4 °C. These data were analyzed using the nonlinear curve fitting program ENZFITTER (72).

Cell Binding and Internalization Assays—Binding of ^{125}I-Ox-LDL to suspensions of peripheral blood monocytes or CD36-transfected Bowes melanoma cells was measured at 4 °C as described previously (26, 41).

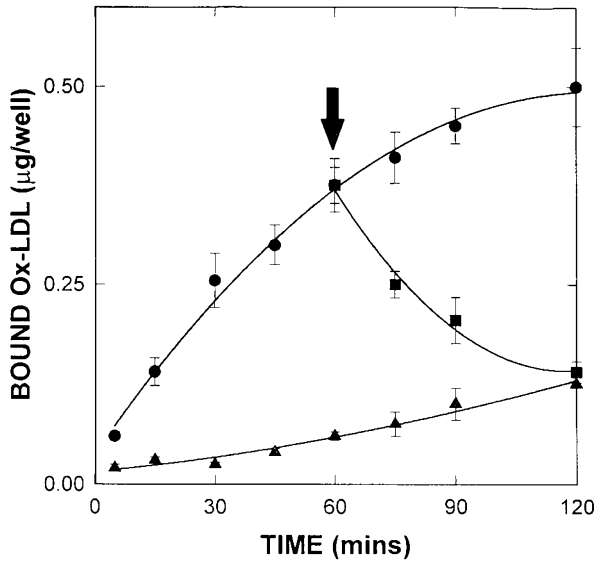

FIG. 1. **Time course of Ox-LDL binding to platelet-derived CD36.** ^{125}I-Ox-LDL (20 μg/ml) was added to immobilized platelet-derived CD36. At timed intervals, wells were washed, and bound radioactivity was measured (●). In some wells (■), bound Ox-LDL was reversed by adding a 10-fold excess of unlabeled Ox-LDL (*arrow*) (n = 4). Binding in the presence of a 50-fold excess of unlabeled Ox-LDL (▲) was measured to determine nonspecific binding. *Error bars* represent means ± S.E.

Inhibition studies were done using 10 μg/ml input concentrations of ^{125}I-Ox-LDL and CD36 or GST/CD36 fusion proteins at concentrations of 5 times the IC_{50} values as calculated from the solid-phase binding assays. For platelet binding studies, freshly washed isolated human platelets (5×10^6 cells) were added to a microcentrifuge tube containing Ox-LDL (20 μg/ml) and, either GST/CD36 fusion proteins at concentrations used for the monocyte assay noted above, or 5-fold excess unlabeled lipoprotein was added and incubated at 4 °C for 1 h. The mixtures were layered onto 50% glycerol and centrifuged for 3 min in a microcentrifuge. After removal of the supernatant, the bottoms of the tubes containing the platelets were clipped and counted for γ-radiation.

Binding and uptake of rhodamine-labeled Ox-LDL to adherent monocyte-derived macrophages were determined by fluorescence microscopy. The cells were preincubated for 15 min with GST/CD36 fusion proteins, and rhodamine-labeled Ox-LDL was subsequently added at a concentration of 10 μg/ml. The cells were placed in a 37 °C incubator for 2 h, washed three times with ice-cold phosphate-buffered saline to remove unbound dye, and fixed with 2% paraformaldehyde for 15 min. The coverslips were mounted, and the cells were examined by fluorescence microscopy. They were photographed using a high speed film (800 ASA) using the same exposure times and F-stops through out the entire experiment for each set.

RESULTS

Ox-LDL Binds to Purified Platelet CD36—At 4 °C, binding of radiolabeled Ox-LDL to immobilized CD36 was specific and time-dependent, reaching equilibrium in ≈1 h (Fig. 1). Binding was complete by 2 h and was completely and rapidly reversed by the addition of a 10-fold excess of unlabeled Ox-LDL. Binding was saturable (Fig. 2*A*) with an apparent affinity of ≈4.1 μg/ml, similar to that previously reported for binding to CD36 expressed on cells (25). Ox-LDL also bound specifically and saturably to GST/CD36 fusion proteins (Fig. 2*A*). One fusion protein (p5–143) bound Ox-LDL with an apparent affinity (4.5 ± 0.6 μg/ml) similar to that of intact CD36, suggesting that the Ox-LDL-binding domain may lie in sequence 5–143. Two other fusion proteins whose sequences lie within region 5–143 (p5–93 and p28–93) bound with only slightly lower affinities (Table I). Fusion protein p67–157 bound Ox-LDL specifically, but with a 3-fold lower apparent affinity (12.2 ± 2.3 μg/ml). The remaining fusion proteins, p118–182, p298–439, and p93–298, including p93–120 (the minimal TSP-binding domain) and peptide 77–97, did not show saturable binding to Ox-LDL. To confirm the specificity of Ox-LDL interaction with immobilized proteins, we demonstrated that binding was blocked by an excess of unlabeled Ox-LDL and by polyclonal anti-CD36 IgG, but not by control nonimmune IgG (Fig. 2*B*).

Inhibition of Ox-LDL·CD36 Complex Formation by Recombinant GST Fusion Proteins—Fluid-phase fusion proteins were used to block binding of Ox-LDL to immobilized platelet CD36 to compare IC_{50} values with apparent affinities (Fig. 3). CD36 itself displaced binding of Ox-LDL to immobilized CD36 with an IC_{50} of 75.3 ± 9.5 nM. Fusion protein p5–143 gave an IC_{50} value of 114.8 ± 12.2 nM, similar to that of intact CD36, whereas the two fusion proteins within this region (p5–93 and p28–93) blocked with a modestly lower IC_{50}. Inhibition by p67–157 and the LIMPII fusion protein L75–155 was not complete at 1 μM, but IC_{50} values calculated by the curve fitting program were 272.9 ± 10.3 and 251.3 ± 11.4 nM, respectively, over the entire range of concentrations used (0.1 nM to 0.1 mM). The slope of the inhibition curve of p28–93 is more representative of a one-site model than the others shown in Fig. 3 for Ox-LDL binding. GST alone or fusion proteins containing amino acids 93–298, 118–182, and 298–439 and the synthetic peptide 77–97 showed no significant inhibition in this assay (Table I).

Inhibition of Ox-LDL Binding to Cells by Recombinant GST/CD36 Fusion Proteins—Inhibition of binding of Ox-LDL to cell-surface CD36 was examined using Bowes melanoma cells stably transfected with the CD36 cDNA, human peripheral blood monocytes, and resting and activated platelets. The fusion proteins with the highest affinities in the solid-phase assays (p5–143, p5–93, and p28–93) inhibited Ox-LDL binding to CD36-transfected cells to the same extent as monoclonal anti-CD36 antibody (Fig. 4). Fusion protein p67–157 and the LIMPII protein L75–155 also blocked binding, whereas fusion proteins p93–298, p118–182, and p298–439, peptide 75–95, TSP, or GST alone showed no significant (10%) inhibition. Similar patterns of inhibition by CD36 peptides were seen using human monocyte-derived macrophages (Fig. 5*A*) and platelets (Fig. 5*B*). Since binding of TSP to platelet CD36 has been shown to be regulated by the activation state of the platelets (49), we also compared binding of Ox-LDL to resting and activated platelets. As reported previously (25), binding of Ox-LDL to resting platelets was saturable with an apparent affinity of 4.1 ± 0.3 μg/ml. Upon platelet activation, we did not observe a change in affinity (apparent affinity was 3.9 ± 0.4 μg/ml) and only a modest 10–15% increase in maximal binding (Fig. 5*B*).

GST/CD36 Fusion Proteins Block Uptake of Ox-LDL by Human Macrophages—Human macrophages express Ox-LDL-binding proteins, including scavenger receptor A types I and II, FcγII receptor, CD68 and CD36; in addition, murine macrophages also express SR-B1 and macrosialin, a mouse homolog of CD68 (67). The level of expression of these receptors changes depending on the degree of differentiation of the monocytes in culture and the cytokine milieu. At day 4 in culture, for example, CD36 is highly expressed on monocyte-derived macrophages (27). The cells were seeded sparsely so as to avoid tight clusters of cells, which can nonspecifically trap Ox-LDL among them. Single cells were scored for the presence or absence of surface fluorescence. As intracellular fluorescence was dependent on the development stage from monocyte to macrophage and other scavenger receptors present on the surface, this value was not taken as a criterion for inhibition of binding. These data are represented as a percentage of the control in the figure legend (Fig. 6). As shown in Fig. 6, human monocyte-derived macrophages incubated with rhodamine-labeled Ox-

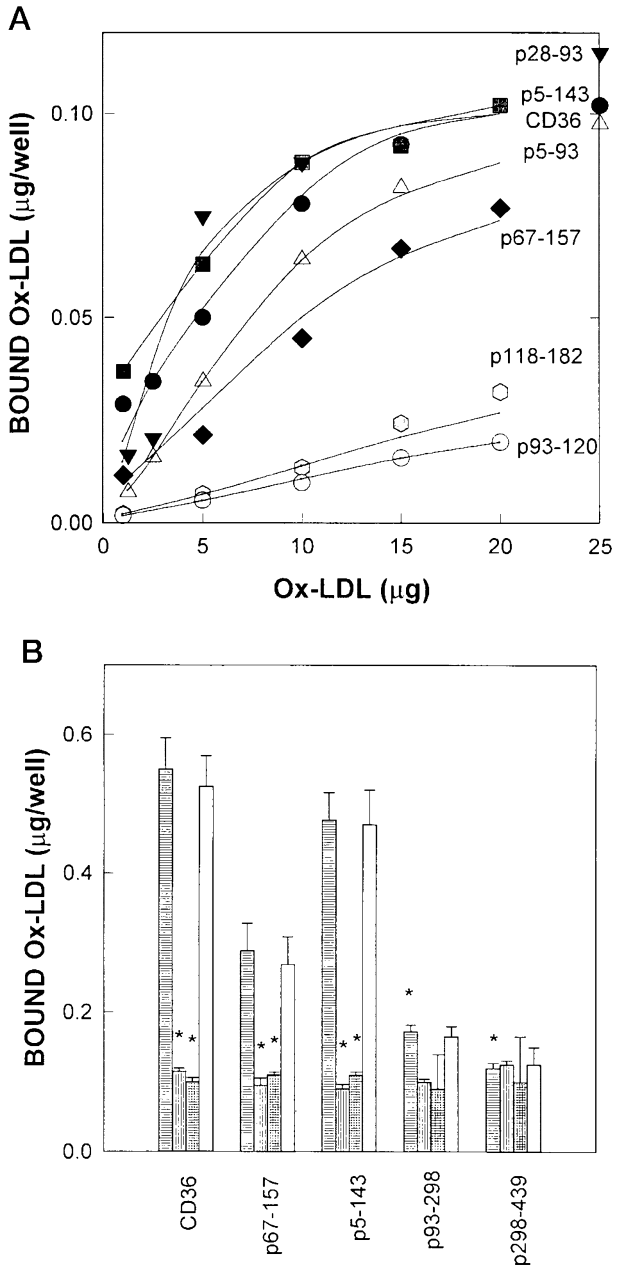

FIG. 2. *A*, binding of Ox-LDL to CD36 and GST/CD36 fusion proteins. ^{125}I-Ox-LDL was added in increasing concentrations (0–50 μg/ml; 0–25 μg/ml shown) and incubated for 2 h at 4 °C with immobilized CD36 (▦) or the GST/CD36 fusion proteins p5–143 (●), p5–93 (▼), p28–93 (△), p67–157 (♦), p93–120 (○), and p118–182 (*shaded hexagon*). Total binding of ^{125}I-Ox-LDL was measured as described for Fig. 1. After subtracting the binding to GST alone (data similar to p93–120), the apparent affinities were calculated. The values for CD36, p5–143, p28–93, and p67–157 were 4.1 ± 0.5, 4.5 ± 0.6, 9.9 ± 2.6, and 12.2 ± 2.3 μg/ml, respectively (n = 10). Fusion proteins p5–93, p93–298, and L75–155 were omitted from the graph for reasons of clarity. *Error bars* were also omitted to avoid crowding; errors calculated as S.D. were no more than 5–10% of the mean. The curves were analyzed using the program ENZFITTER. Molecular masses for p5–143, p5–93, p28–93, p67–157, p93–298, p118–182, p93–120, and p298–439 were 42,426, 37,000, 34,500, 37,598, 50,788, 34,790, 30,683, and 43,605 Da, respectively. *B*, specificity of Ox-LDL binding to fusion proteins. Binding of ^{125}I-Ox-LDL (10 μg/ml) to immobilized fusion proteins was examined in the presence of rabbit polyclonal anti-CD36 antibody (1 μg/ml), a 10-fold excess of unlabeled Ox-LDL, or 1 μg/ml nonimmune rabbit IgG. With CD36, p67–157, and p5–143, the binding was reduced by 75% in the presence of either excess unlabeled Ox-LDL or anti-CD36 antibody, but was unaffected by control IgG (n = 4). Data were analyzed by analysis of variance. *, $p < 0.005$ compared with control binding. Unlabeled Ox-LDL or anti-CD36 IgG did not decrease binding to p93–298 or p298–439 ($p > 0.1$) compared with the control.

TABLE I
Apparent binding affinities

Values of apparent binding affinities were calculated based on a mean from several experiments (n = 6, 10). The binding affinities are reported as μg/ml because the calculation of molarity based on the approximate molecular mass of the Ox-LDL species would only be an approximation. Similarly, the half-maximal constants were calculated as a mean from several experiments. Both these values were fit to the Michaelis-Menten equation with an assumption for a one-site model. Fusion proteins p118–182 and p93–298 gave IC_{50} values >500 nM, whereas p298–439, p93–120, and GST gave values >>1 μM.

Fusion proteins	Apparent K_d	IC_{50}
	μg/ml	*nM*
Platelet CD36	4.1 ± 0.5	75.3 ± 9.5
p5–143	4.5 ± 0.6	114.8 ± 12.2
p5–93	9.6 ± 2.6	156.7 ± 11.5
p28–93	9.9 ± 3.5	186.4 ± 12.6
p67–157	12.2 ± 2.3	272.9 ± 10.3
L75–155	13.5 ± 1.3	251.3 ± 11.4

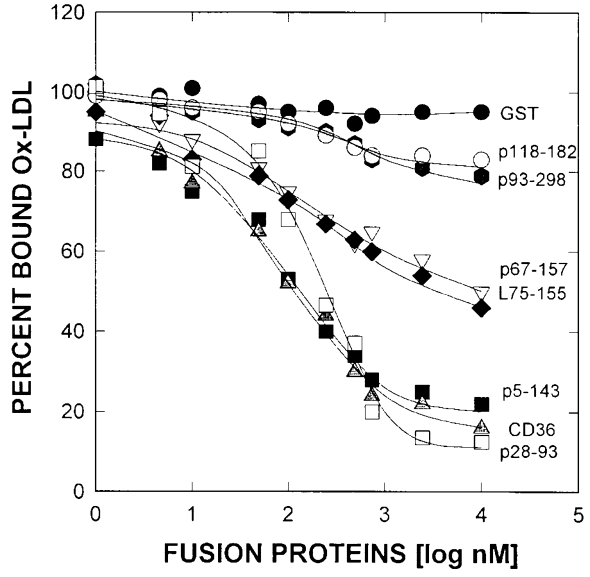

FIG. 3. **Inhibition of Ox-LDL binding to immobilized CD36 by fluid-phase fusion proteins.** Fluid-phase fusion proteins (0.1 nM to 10 μM) were added in increasing concentrations to ^{125}I-Ox-LDL (20 μg/ml) and incubated with immobilized CD36, and bound radioactivity was measured as described for Fig. 1 for CD36 (△), p5–143 (■), p28–93 (□), p67–157 (▽), L75–155 (♦), p118–182 (◍), p93–298 (●), and GST (●) (n = 8). Inhibition is reported as percent binding of Ox-LDL to CD36 in the absence of inhibitor. Similarly, error bars were also omitted to avoid crowding; errors calculated as S.D. were no more than 5–10% of the mean.

LDL showed surface fluorescence as well as intracellular fluorescence indicative of internalized ligand (Fig. 6*A*). Fusion protein p5–143 (Fig. 6*C*) effectively blocked the cell-associated fluorescence, whereas p67–157 (Fig. 6*B*) blocked rhodamine-labeled Ox-LDL binding and uptake (no surface fluorescence) to a lesser extent. Fusion proteins p93–298 (Fig. 6*E*), p118–182 (Fig. 6*D*), and p298–439 (Fig. 6*F*) did not significantly block fluorescence binding and uptake.

DISCUSSION

CD36 is a cell-surface protein that can function as an adhesion molecule with specificity for collagen, TSP, and *P. falciparum*-infected erythrocytes; as a scavenger receptor with specificity for Ox-LDL, high density lipoprotein, apoptotic cells, and effete photoreceptor outer segments (ROS); and as a transport receptor for fatty acids. All of these functions are partially or completely blocked by a wide array of murine monoclonal anti-CD36 antibodies with specificity for a single immunodominant domain between amino acids 155 and 183. Interestingly, a

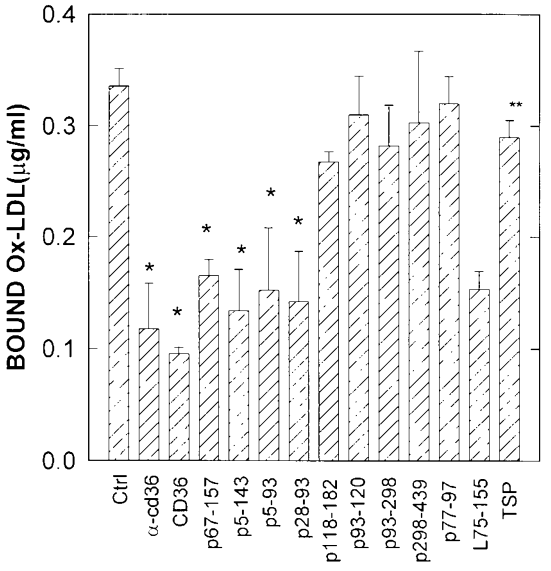

FIG. 4. **Inhibition of Ox-LDL binding to CD36-expressing Bowes melanoma cells by fluid-phase fusion proteins.** ^{125}I-Ox-LDL (10 μg/ml) was added to Bowes melanoma cells for 2 h at 4 °C in the presence of fluid-phase CD36, anti-CD36 antibody, or GST/CD36 fusion proteins at a concentration of five times the IC_{50} value, determined in the solid-phase binding assay. Cell-associated radioactivity was measured, and the amount of bound Ox-LDL was determined. Purified platelet CD36 and monoclonal anti-CD36 antibody (8A6, 2 μg/ml) inhibited 70–72% of the total binding. Fusion proteins p5–143, p5–93, and p28–93 inhibited 87, 88, and 90% of the specific binding, and L75–155 and p67–157 inhibited 74 and 68% of the specific binding, respectively. Fusion proteins p118–182, p298–439, p93–298, and p93–120 and peptide 75–95 (2542 Da) inhibited by <15% and were not statistically significant ($n = 8$). *, $p < 0.05$; **, $p \approx 0.5$, statistically different from the control (*Ctrl*).

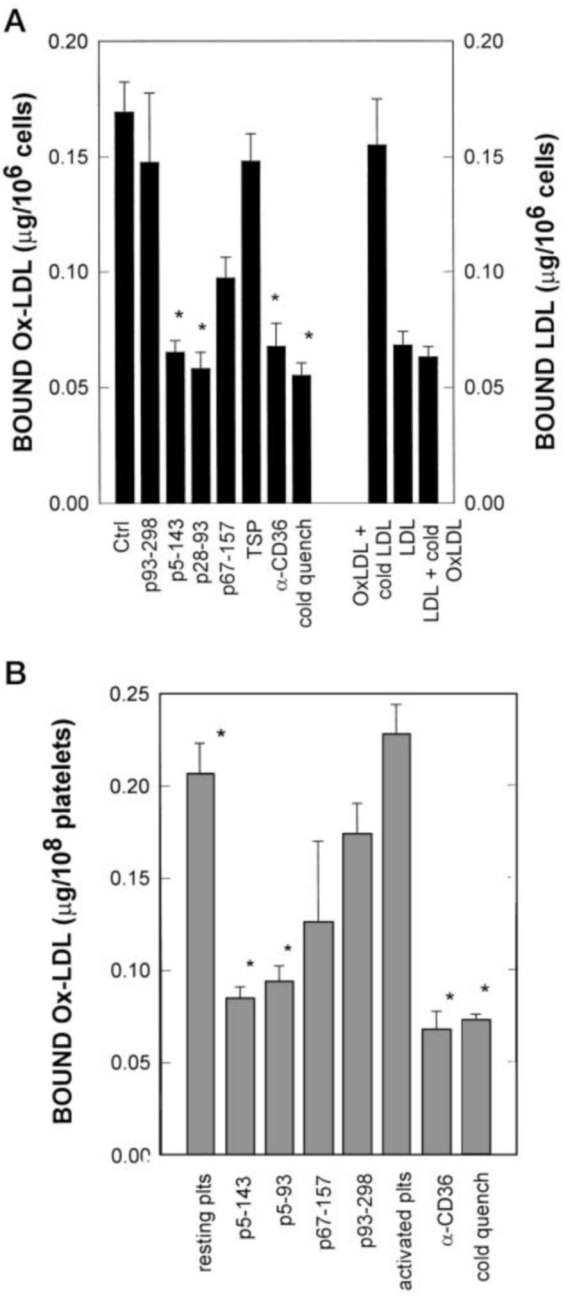

FIG. 5. **Inhibition of Ox-LDL binding to surface CD36 on peripheral blood monocytes and platelets by fluid-phase fusion proteins.** *A*, peripheral blood monocytes maintained in culture for 4 days were treated with ^{125}I-Ox-LDL (10 μg/ml) and anti-CD36 antibody (1 μg/ml), GST/CD36 fusion proteins at concentrations of five times the IC_{50} value, or a 10-fold excess of unlabeled Ox-LDL for 2 h at 4 °C. After washing, cell-associated radioactivity was measured, and the amount of bound Ox-LDL was determined ($n = 4$). *, $p < 0.05$ compared with the control (*Ctrl*). LDL binding (20 μg/ml) was also measured in the presence and absence of unlabeled Ox-LDL (100 μg/ml). LDL binding does not affect Ox-LDL binding under these conditions. *B*, resting or activated (treated with 10^{-5} M ADP) platelets (*plts*) were incubated in Broakman's buffer with 1% bovine serum albumin containing ^{125}I-Ox-LDL (10 μg/ml) and anti-CD36 antibody (1 μg/ml), GST/CD36 fusion proteins at concentrations of five times the IC_{50} value, or a 10-fold excess of unlabeled Ox-LDL as described for *A* ($n = 4$).

different region, spanning amino acids 93–120, has been shown by us (53) and others (52) to fulfill all of the requirements of a TSP-binding domain, suggesting that for CD36, the antibody experiments probably do not identify specific functional domains. Perhaps engagement of this immunodominant region by antibodies disrupts the tertiary structure of CD36 so that all functions are lost. In this study, we have used recombinant CD36 fusion proteins to characterize the Ox-LDL-binding domain. We show that the Ox-LDL-binding domain on CD36 is different from the TSP-binding domain. A CD36 fusion protein spanning amino acids 93–120 (the TSP-binding domain) did not bind Ox-LDL or block Ox-LDL binding to CD36 or to cells expressing CD36. These findings are consistent with our earlier observations that TSP does not significantly block Ox-LDL binding to CD36 or CD36-expressing cells (26). Our results also suggest that the Ox-LDL-binding domain is different from the immunodominant domain at amino acids 155–183, as two fusion proteins containing this domain (p118–182 and p93–298) did not significantly bind or inhibit Ox-LDL binding. These results are in contrast to the conclusions of Puente Navazo *et al.,* who showed that expression of a chimeric CD36 construct substituting the murine sequence 155–183 for the human sequence in transfected cells led to decreased Ox-LDL binding. Their data are, however, difficult to interpret since murine CD36 bound Ox-LDL at least as well as human CD36 and since the apparent affinity of Ox-LDL for the murine-human chimera was similar to that for the wild-type receptors (1 μg/ml *versus* 0.5 and 0.7 μg/ml) (57).

Ox-LDL bound to an immobilized fusion protein containing the amino-terminal third of CD36 (p5–143 and p28–93) with approximately similar apparent affinity as intact CD36. Ox-LDL is a complex molecule made from oxidizing LDL, which is composed of apoB protein, fatty acids, triglycerides, cholesterol, and cholesteryl esters. It is possible that any or all of the lipid components of Ox-LDL may interact with CD36. It is therefore not difficult to perceive that the Ox-LDL moiety may bind to separate domains on CD36, and these domains may be discrete noncontiguous regions that may be spatially proximal. A sequence comparison among CD36 proteins from different spe-

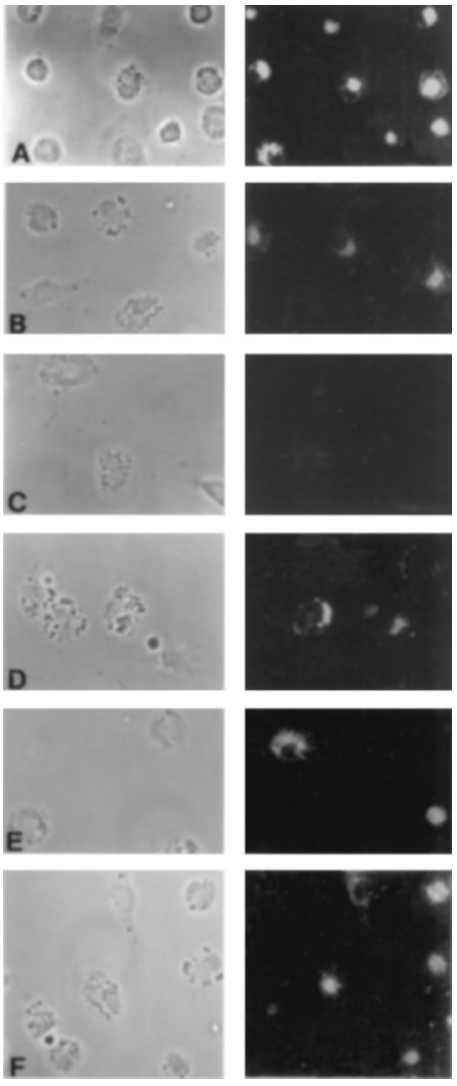

FIG. 6. **Binding of rhodamine-labeled Ox-LDL to 4-day culture-derived macrophages in the presence of fluid-phase fusion proteins.** Rhodamine-labeled Ox-LDL (10 μg/ml) was incubated for 2 h at 37 °C with 4-day culture-derived macrophages in the presence of fluid-phase GST/CD36 fusion proteins. Surface fluorescence represents only CD36-related binding. Internalized ligand may be present due to non-CD36-related uptake and the level of maturation of the macrophage. Cells were scored in terms of surface fluorescence with respect to the control. *A* shows a phase fluorescence pair in the absence of fusion proteins (100% of the cells were fluorescent); *B* shows cells with p67–157 (52 ± 4.9% positive); *C* with p5–143 (19.5 ± 5.3% positive); *D* with p118–182 (81.7 ± 7.5% positive); and *E* and *F* with p93–298 (83 ± 6.2% positive) and p298–439 (96 ± 4% positive), respectively (n = 4).

cies and with other members of the CD36 gene family, including SR-B1 and LIMPII, shows significant homologies in the N-terminal region, especially from amino acids 31 to 190. This is consistent with our experimental data showing that the LIMPII peptide L75–155 also bound Ox-LDL. Interestingly, using the pattern-induced multisequence alignment program PIMA (68, 69), amino acid sequences of other fatty acid- and/or Ox-LDL-binding proteins, such as human fatty acid-binding protein and mouse macrosialin (70, 71), could be lined up with the CD36 family members, *i.e.* human CD36 and LIMPII overlapping residues 45–140 and then again residues 151–299 (sequential branching clustering).

Assignment of the precise location of the Ox-LDL-binding domain within this N-terminal region was not as simple as that of the TSP-binding domain. Ox-LDL bound with only slightly lower affinity to fusion proteins containing amino acids 5–93 and 28–93, suggesting that the region downstream of the hydrophobic signal peptide and proximal to the TSP-binding domain beginning at amino acid 93 may contain the core binding site for Ox-LDL. Studies showing that an antibody reactive with amino acids 30–70 did not block Ox-LDL binding and that p67–157 also bound Ox-LDL and blocked Ox-LDL/CD36 interaction suggest that the domain might be present in the sequence between amino acids 67 and 93 (57). However, a synthetic peptide spanning amino acids 77–97 did not bind Ox-LDL or block Ox-LDL binding to CD36, suggesting either that sequences upstream of amino acid 77 are required for Ox-LDL binding or that the shorter synthetic peptide does not adequately fold to present a optimal binding domain. In addition, although CD36 is an integral membrane protein, we have found that the soluble fusion proteins were effective competitors of Ox-LDL binding to cellular CD36 in three different cell types *i.e.* monocytes, platelets, and the Bowes transfected cells, and that the IC_{50} values were similar to the apparent affinity of Ox-LDL binding to CD36 expressed on the cell surface. Thus, the fusion proteins should have a secondary structure similar to that of the native protein.

We observed that Ox-LDL binding was sensitive to pH and ionic strength, indicating that charge/charge interactions may be criteria for binding (data not shown). Maximal binding was seen for CD36 between pH 6.5 and 8 and for p5–143 between pH 6 and 8. Optimal salt concentrations for both CD36 and p28–93 were between 125 and 250 mM NaCl, postulating a reaction between the negatively charged lipid on the Ox-LDL molecule and the positively charged amino acids on CD36. The shorter peptides may therefore not be able to form stable secondary structures with the appropriate charge distribution. Our data do not rule out a role for sequences downstream of amino acid 120 in regulating Ox-LDL binding. Since some reports indicate that CD36 may form dimers in the cell membrane (63), it is possible that amino acid sequences involved in intermolecular as well as intramolecular interactions may also contribute to Ox-LDL binding affinity.

Fusion protein p5–143 contains only one sulfhydryl group, whereas p67–157 contains none, suggesting that disulfide bond formation is not essential for Ox-LDL binding. Acetylation of sulfhydryl side chains with *N*-ethylmaleimide, however, increased the steady-state binding by a small percentage (data not shown), indicating that the longer, more hydrophobic structure may stabilize the binding interaction.

The binding affinity of CD36 for at least one ligand, TSP, has been shown to be regulated by post-translational modifications. Asch *et al.* (49) have shown that extracellular dephosphorylation of a Thr residue at position 92 of CD36 during platelet activation regulates TSP binding, *i.e.* dephosphorylation led to "activation" of CD36 as a functional TSP receptor. Unlike the situation with TSP, however, we did not observe any difference in Ox-LDL binding to resting platelets (with phosphorylated CD36) and activated platelets (with dephosphorylated CD36).

In summary, our studies show that the Ox-LDL-binding domain on CD36 is different from and independent of the TSP-binding domain and is located in the amino-terminal third of the molecule. The critical component of the domain probably lies between amino acids 28 and 93, albeit other possibly noncontiguous regulatory sequences (139–155) may be involved. Inhibition studies show that reagents based on this structure are effective at blocking macrophage binding and uptake of Ox-LDL, suggesting a potential therapeutic approach in limiting the development of lipid-laden cells in the blood vessel wall during atherosclerosis.

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REFERENCES

1. Ross, R. (1993) *Nature* **362,** 801–809
2. Katsuda, S., Coltrera, M. D., Ross, R., and Gown, A. M. (1993) *Am. J. Pathol.* **142,** 1787–1793
3. Fagiotto, A., Ross, R., and Harker, L. (1984) *Arteriosclerosis* **4,** 323–340
4. Navab, M., Berliner, J. A., Watson, A. D., Hama, S. Y., Territo, M. C., Lusis, A. J., Shih, D. M., Van Lenten, J., Frank, J. S., Demer, L. L., Edwards, P. A., and Fogelman, A. M. (1996) *Arteriosclerosis* **16,** 831–842
5. Parthasarathy, S., Steinberg, D., and Witzum, M. D. (1992) *Annu. Rev. Med.* **43,** 219–225
6. Witztum, J. L., and Steinberg, D. (1991) *J. Clin. Invest.* **88,** 1785–1792
7. Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) *J. Clin. Invest.* **84,** 1086–1095
8. Quinn, M. T., Parthasarathy, S., Fong, L. G., and Steinberg, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2995–2998
9. McMurray, H. F., Parthasarathy, S., and Steinberg, D. (1993) *J. Clin. Invest.* **92,** 1004–1008
10. Khan, B., Parthasarathy, S., Alexander, R., and Medford, R. (1995) *J. Clin. Invest.* **95,** 1262–1270
11. Kume, N., Cybulsky, M., and Gimbrone, M. (1992) *J. Clin. Invest.* **90,** 1138–1144
12. Rajavashisth, T., Andalibi, A., Territo, M., Berliner, J., Navab, M., Fogelman, A., and Lusis, A. (1990) *Nature* **345,** 254–257
13. Fei, H. H., Berliner, J. A., Parhami, F., and Drake, T. A. (1993) *Arterioscler. Thromb.* **13,** 1711–1717
14. Yla-Herttuala, S., Lipton, B. A., Rosenfeld, M. E., Sarkioja, T., Yoshimura, T., Leonard, E. J., Witztum, J. L., and Steinberg, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5252–5256
15. Galis, Z. S., Sukhova, G. K., Kranzhofer, R., Clark, S., and Libby, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 402–406
16. Clinton, S. K., Underwood, R., Hayes, L., Sherman, M. L., Kufe, D. W., and Libby, P. (1992) *Am. J. Pathol.* **140,** 301–316
17. Deigner, H. P., and Claus, R. (1996) *FEBS Lett.* **385,** 149–153
18. Cushing, S., Berliner, J., Valente, A., Territo, M., Navab, M., Parhami, F., Gerrity, R., Schwartz, C., and Fogelman, A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5134–5138
19. Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., and Steinberg, D. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3883–3887
20. Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., and Krieger, M. (1990) *Nature* **343,** 531–535
21. Parthasarathy, S., Fong, L., Otero, D., and Steinberg, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 537–540
22. Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori, H., Aburatani, H., Takaku, F., Suzuki, H., Kobari, Y., Miyai, T., Takahashi, K., Cohen, E. H., Wydro, R., Housman, D. E., and Kodama, T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9133–9137
23. Ottnad, E., Parthasarathy, S., Sambrano, G. R., Ramprasad, M. P., Quehenberger, O., Kondratenko, N., Green, S., and Steinberg, D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1391–1395
24. Goldstein, J. L., Ho, Y. K., Basu, S. K., and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 333–337
25. Endemann, G., Stanton, L. W., Madden, K. S., Bryant, C. M., White, R. T., and Protter, A. A. (1993) *J. Biol. Chem.* **268,** 11811–11816
26. Nicholson, A. C., Pearce, S. F. A., and Silverstein, R. L. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 1–7
27. Huh, H.-Y., Pearce, S. F. A., Yesner, L. M., Schindler, J. M., and Silverstein, R. L. (1996) *Blood* **87,** 2020–2028
28. Oquendo, P., Hundt, E., Lawler, J., and Seed, B. (1989) *Cell* **58,** 95–101
29. Clemetson, K. J. (1985) in *Platelet Membrane Glycoproteins* (George, J. N., Nurden, A. T., and Phillips, D. R., eds) pp. 51–80, Plenum Press, New York
30. Knowles, D. M., Tolidijian, B., Marboe, C., Agati, V. D., Grimes, M., and Chass, L. (1984) *J. Immunol.* **132,** 2170–2173
31. Silverstein, R. L., Asch, A. S., and Nachman, R. L. (1989) *J. Clin. Invest.* **84,** 546–552
32. Greenwalt, D. E., Watt, K. W. K., So, O. Y., and Jiwani, N. (1990) *Biochemistry* **29,** 7054–7059
33. Edelman, P., Vinci, G., Villeval, J. L., Vainchender, W., Henri, A., Miglierina, R., Rouger, P., Reviron, J., Breton-Groius, J., Sureau, C., and Edelman, L. (1986) *Blood* **67,** 56–63
34. Clezardin, P., Frappart, L., Clerget, M., Pechoux, C., and Delmas, P. D. (1993) *Cancer Res.* **53,** 1421–1430
35. Ryeom, S. W., Sparrow, J. R., and Silverstein, R. L. (1996) *J. Cell Sci.* **108,** 387–395
36. Abumrad, N. A., El-Maghrabi, R., Ez-Zoubir, A., Lopez, E., and Grimaldi, P. A. (1993) *J. Biol. Chem.* **268**, 17665–17668
37. Handunnetti, S. M., Van Schravendijik, M. R., Hasler, T., Barnwell, J. W., Greenwalt, D. E., and Howard, R. J. (1992) *Blood* **80,** 2097–2104
38. Brittain, H. A., Eckman, J. R., Swerlick, R. A., Howard, R. J., and Wick, T. M. (1993) *Blood* **81,** 2137–2143
39. Asch, A. S., Barnwell, J., Silverstein, R. L., and Nachman, R. L. (1987) *J. Clin. Invest.* **79,** 1054–1061
40. Silverstein, R. L., and Nachman, R. L. (1986) *J. Clin. Invest.* **79,** 867–874
41. Silverstein, R. L., Baird, M., Lo, S. K., and Yesner, L. M. (1992) *J. Biol. Chem.* **267,** 16607–16612
42. Asch, A. S., Tepler, J., Silbiger, S., and Nachman, R. L. (1991) *J. Biol. Chem.* **266,** 1740–1745
43. Tolsma, S. S., Volpert, O. V., Good, D. J., Frazier, W. A., Polverini, P. J., and Bouck, N. (1993) *J. Cell Biol.* **122,** 497–511
44. Savill, J., and Hogg, N. (1992) *J. Clin. Invest.* **90,** 1513–1522
45. Rigotti, A., Anton, S. L., and Krieger, M. (1995) *J. Biol. Chem.* **270,** 16221–16224
46. Ryeom, S. W., Silverstein, R. L., Scotto, A., and Sparrow, J. R. (1996) *J. Biol. Chem.* **271,** 20536–20539
47. Vega, M. A., Segui-Real, B., Garcia, J. A., Cales, C., Rodriguez, F., Vanderkerckhove, J., and Sandoval, I. V. (1991) *J. Biol. Chem.* **266,** 16818–16824
48. Acton, S., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) *J. Biol. Chem.* **269,** 21003–21009
49. Asch, A. S., Liu, I., Barnwell, J., Kwakye-Berko, F., Dokun, F., and Goldberger, J. (1993) *Science* **262,** 1436–1440
50. Daviet, L., and McGregor, J. L. (1996) *Platelets* **7,** 117–124
51. Daviet, L., Buckland, R., Puente Navazo, M. D., and McGregor, J. L. (1995) *Biochem. J.* **305,** 221–224
52. Leung, L. L. K., Li, W.-X, McGregor, J. L., Albrecht, G., and Howard, R. J. (1992) *J. Biol. Chem.* **267,** 18244–18250
53. Pearce, S. F. A., Wu, J., and Silverstein, R. L. (1995) *J. Biol. Chem.* **270,** 2981–2986
54. Alessio, M., Greco, N. J., Primo, L., Ghigo, D., Bosia, A., Tandon, N. N., Ockenhouse, C. F., Jamieson, G. A., and Malavasi, F. (1993) *Blood* **82,** 3637–3647
55. Tandon, N. N., Kralisz, U., and Jamieson, G. A. (1989) *J. Biol. Chem.* **264,** 7576–7583
56. Barnwell, J., Ockenhouse, C., and Knowles, D. (1985) *J. Immunol*. **135,** 3494–3497
57. Daviet, L., Craig, A. G., McGregor, L., Pinches, R., Wild, T. F., Berendt, A. R., Newbold, C. I., and McGregor, J. L. (1997) *Eur. J. Biochem.* **243,** 344–349
58. Puente Navazo, M. D., Daviet, L., Ninio, E., and McGregor, J. L. (1996) *Arterioscler. Thromb. Vasc. Biol*. **16,** 1033–1039
59. Puente Navazo, M. D., Daviet, L., Savill, J., Ren, Y., Leung, L. K., and McGregor, J. L. (1996) *J. Biol. Chem.* **271,** 15381–15385
60. Asch, A. S., Silbiger, S., Heimer, E., and Nachman, R. L. (1992) *Biochem. Biophys. Res. Commun.* **182,** 1208–1217
61. Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F., and Howard, R. J. (1995) *Cell* **82,** 77–87
62. Stanton, L. W., White, R. T., Bryant, C. M., Protter, A. A., and Endermann, G. (1992) *J. Biol. Chem.* **267,** 22446–22451
63. Rhinehart-Jones, T., and Greenwalt, D. E. (1996) *Arch. Biochem. Biophys.* **326,** 115–118
64. Wright, S. D., and Silverstein, S. C. (1982) *J. Exp. Med.* **156,** 1149–1164
65. Crombie, R., and Silverstein, R. (1998) *J. Biol. Chem.* **273,** 4855–4863
66. Frangioni, J. V., and Neel, B. G. (1993) *Anal. Biochem.* **210,** 179–187
67. Yoshida, H., Quehenberger, O., Kondratenko, N., Green, S., and Steinberg, D. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 794–802
68. Smith R. F., and Smith T. F. (1992) *Protein Eng.* **5,** 35–41
69. Argos, P. (1994) *Curr. Opin. Biotechnol.* **5,** 361–371
70. Baillie, A. G. S., Coburn, C. T., and Abumrad, N. A. (1996) *J. Membr. Biol.* **153,** 75–81
71. Ramprasad, M. P., Terpstra, V., Kondratenko, N., Quehenberger, O., and Steinberg, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14833–14838
72. Leatherbarrow, R. (1989) ENZFITTER, Elsevier Biosoft, Cambridge, UK
73. 73. Havel, R. J., Felts, J. M., and van Duyne, C. M. (1962) *J. Lipid Res.* **3,** 297