Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL

Dominica Calvo,* Diego Gómez-Coronado,† Yajaira Suárez,† Miguel A. Lasunción,† and Miguel A. Vega[1,§]

Hospital de la Princesa,* Madrid; Servicio de Bioquímica-Investigación,† Hospital Ramón y Cajal, Madrid, and Universidad de Alcalá de Henares, Madrid; and Instituto de Parasitología y Biomedicina López-Neyra,§ C.S.I.C., Granada, Spain

Abstract Mouse and hamster SR-BI glycoproteins and their putative human counterpart CLA-I are so far the only scavenger receptors known to bind both native and modified lipoproteins. CD36, a multigland glycoprotein structurally related to SR-BI and CLA-1, has been reported to bind oxidized low density lipoprotein (OxLDL) and acetylated LDL (AcLDL). In this report, we have studied the ability of CD36 to bind native lipoproteins. By transient expression of human CD36 in mammalian and insect cells, we demonstrate that CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, VLDL, and, as previously reported, for OxLDL and AcLDL. The specificity of these interactions is supported by the dose-dependent inhibitory effect of a monoclonal antibody against CD36. Furthermore, at least for HDL, binding to CD36 does not require the presence of apoE. These findings, together with preferential expression of CD36 in tissues performing very active fatty acid metabolism (skeletal muscle, heart, mammary epithelium, and adipose tissue) and its involvement in foam cell formation (macrophages), suggest that binding of lipoproteins to CD36 might contribute to the regulation of lipid metabolism, and to the pathogenesis of atherosclerosis.**—Calvo, D., D. Gómez-Coronado, Y. Suárez, M. A. Lasunción, and M. A. Vega.** Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. *J. Lipid Res.* 1998. **39:** 777–788.

Supplementary key words scavenger receptor • cholesterol • fatty acids • atherosclerosis

Plasma lipoproteins mediate the transport and delivery of lipids, including triglycerides, cholesterol, and certain lipid-soluble vitamins, to body tissues. Chylomicrons and VLDL carry dietary and endogenous lipids, respectively, and mediate the supply of fatty acids to cells through the triglyceride-hydrolytic action of endothelium-anchored lipoprotein lipase (1). This process generates cholesterol-enriched residual lipoproteins (chylomicron remnants, IDL, and LDL) that are removed from the blood by receptors present on liver and peripheral tissues for their subsequent processing, storage, or secretion (1). HDL participates in selective cholesteryl ester delivery to steroidogenic tissues and in reverse cholesterol transport from peripheral tissues to the liver (2).

Alterations of the plasma lipoprotein profile influence the incidence of atherosclerosis. Thus, elevated serum LDL-cholesterol levels are associated with increased risk of atherosclerosis (3), while elevated HDL-cholesterol levels have a protective effect (4). In addition, uptake of structurally modified lipoproteins by macrophages located in the subendothelial space, which leads to the formation of foam cells, is a key event in the initiation and progression of the atherogenic process (5–7).

A variety of cell surface glycoproteins (SR-A, MARCO, CD68, CD36, and SR-BI), collectively designated as scavenger receptors, have been demonstrated to contribute to the uptake of modified lipoproteins (8–12). Scavenger receptors are characterized by their ability to interact with a broad variety of ligands that include modified proteins and lipoproteins, and some polyanionic polysaccharides. In addition, some of them also interact with in vitro oxidized and senescent cells, polyanionic phospholipids, and bacterial components (13).

The scavenger receptor CD36 is a 78 to 88 kDa palmitoylated plasma membrane glycoprotein, possibly anchored to the membrane through two hydrophobic segments located on both N- and C-terminal regions (14, 15), although anchoring through only the C-termi-

Abbreviations: PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OxLDL, oxidized low density lipoprotein; AcLDL, acetylated LDL; DiI, 1,1′-dioctadecyl-3-3-3′-3′-tetramethylindocarbocyanine perchlorate; m.o.i., multiplicity of infection; MIF, median intensity of fluorescence; Lp, lipoprotein.

[1]To whom correspondence should be addressed at: Servicio de Bioquímica-Investigación, Hospital Ramón y Cajal, Crta. Colmenar Viejo Km 9.1, 28034 Madrid, Spain.

nal hydrophobic region has been also suggested (16). It is expressed in monocyte/macrophages, platelets, microvascular endothelial cells, erythroblasts, adipose tissue, and mammary epithelium (14, 17–21). CD36 acts as a receptor for in vitro oxidized LDL (OxLDL) and acetylated LDL (AcLDL) (11, 12), thrombospondin (22, 23), collagens type I (24) and IV (25), fatty acids (20), polyanionic phospholipids (26), apoptotic cells (27, 28), and *Plasmodium falciparum*-infected erythrocytes (14, 17).

CD36 is a member of a novel gene family (29) also constituted by the plasma membrane glycoprotein CLA-1 (30) the putative SR-BI in hamster and mouse (12), and the lysosomal membrane protein LIMPII (31). SR-BI, besides interacting with modified LDL (12), some anionic phospholipids (26), and apoptotic cells (32), also binds native LDL (12) and HDL (33). Through binding HDL, SR-BI participates in selective cellular cholesteryl ester delivery, a role consistent with its restricted expression in steroidogenic tissues and liver in mouse (33). Human CLA-1 interacts with the native lipoproteins, as well as with oxidized and acetylated LDL (34, 35). The function of the third member of the family, LIMPII, is presently unknown.

The contribution of CD36 expressed on monocyte/macrophages in culture to OxLDL uptake and to foam cell formation has been demonstrated (36, 37). Based on the wide lipoprotein specificity found for the CD36-related glycoprotein CLA-1 and SR-BI, in this study we have examined the ability of human CD36 to interact with native lipoproteins. Our results demonstrate that gene transfer of CD36 confers to mammalian and insect cells the ability to bind both native and in vitro modified lipoproteins.

EXPERIMENTAL PROCEDURES

Reagents and cell lines

The cell lines used in this study were: C32 (human melanoma), COS-7 (kidney cells derived from monkey), and Sf9 (ovarian cells derived from the lepidopterm insect *Spodoptera frugiperda*). The fluorescence probe 1,1′-diotadecyl-3-3-3′-3′-tetramethyllindocarbocyanine percholorate (DiI), Bodipy-LDL and Bodipy-AcLDL were obtained from Molecular Probes Inc. (Eugene, OR). The monoclonal antibodies used were FA6-152 (anti-CD36), and TPI/33 (anti-CD69) (38, 39).

Iodination of cells and immunoprecipitation

Cells were surface radioiodinated on ice, lysed, and immunoprecipitated basically as described (40). Briefly, cell lysates were first precleared with protein-A Sepharose (Pharmacia), and with the monoclonal antibody P3X63, followed by incubation with protein-A Sepharose. Human CD36 was immunoprecipitated with 0.2 μg of purified anti-CD36 monoclonal antibody FA6-152 and protein-A Sepharose. Finally, samples were run on 7.5% SDS-PAGE under reducing conditions and analyzed by autoradiography.

DNA constructs

The pBacPAK-CD36 construct was generated by subcloning the EcoRI-XbaI fragment from the human CD36 cDNA, which spans nucleotides 1 to 1725 (14), into the pBacPAK9 vector digested with EcoRI and XbaI. pCEXV-3-CD36 was generated as described (30).

Transfection of COS7 cells

cDNAs of human CD36 and ICAM-3 cloned into the expression plasmids of pCEXV-3 and pCR3, respectively, were transfected into COS-7 cells by the DEAE-dextran procedure as described (30). Transfected cells were used for experiments 48 h after transfection. Expression of CD36 was checked by the CD36-specific antibody FA6-152, which does not react with untransfected or mock-transfected COS7 cells.

Expression of human CD36 and CLA-1 on the surface of insect Sf9 cells

Recombinant baculoviruses containing the cDNA of CD36 (BP-CD36) were produced following the instructions of the BacPAK™ Baculovirus Expression System (Clontech, Palo Alto, CA). Baculoviruses for expression of human CLA-1 were used as a negative control (35). Unless otherwise directed, a multiplicity of infection (m.o.i.) of 10 viruses per cell was used to infect Sf9 cells with viruses BacPAK6 (a baculovirus containing the β-galactosidase cDNA, and used as control), BP-CD69 (a baculovirus containing the cDNA of CD69, kindly provided by Dr. F. Sánchez-Madrid, Hospital de la Princesa), BP-CD36, and BP-CLA-1. Cells were used for experiments 36–48 h after infection.

For immunofluorescence, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, and immunostained by the indicated specific antibody followed by fluorescein-labeled second antibody, essentially as described (30). Expression of CD69 and CD36 on the plasma membrane of infected Sf9 cells was checked by fluorescence flow cytometry analysis using the antibodies TP1/33 and FA6-152, respectively.

Isolation, modification, and labeling of lipoproteins

Native plasma lipoproteins VLDL, LDL, and HDL were isolated by preparative sequential ultracentrifugation of pooled normolipidemic sera from humans after

an overnight fast. Na_2EDTA (0.5 g/l) and Trasylol (300 I.U./ml) were added as preservatives. Purified lipoproteins were extensively dialyzed at 4°C against 0.15 m NaC1, 0.01% (w/v) Na_2EDTA, pH 7.4. AcLDL was prepared from LDL by the addition of acetic anhydride (41). OxLDL was prepared by incubating 3 mg of LDL-protein/ml in 20 μm $CuSO_4$ during 20 h at room temperature. All lipoproteins were filtered through 0.4-μm filters and stored in 0.15 m NaCl, 0.01% Na_2EDTA, pH 7.4, at 4°C. HDL was subfractionated in apoE+ and apoE− fractions by heparin-Sepharose affinity chromatography as described (42).

Labeling of lipoproteins with the fluorescence probe 1,1′-diotadecyl-3-3-3′-3′-tetramethylindocarbocyanine perchlorate (DiI) was carried out according to Via and Smith (43), with some minor modifications. Briefly, lipoproteins were incubated with the DiI probe in lipoprotein-deficient serum for 12 h at 37°C, using the following relative amounts: 300 μg of DiI/3 mg of lipoprotein-lipid/2 ml of lipoprotein-deficient serum. Subsequently, the labeled lipoproteins were re-isolated by ultracentrifugation. All lipoproteins were stored at 4°C in the dark, and used within 2 weeks after their preparation. Unlabeled LDL and DiI-LDL showed no evidences of oxidative modification as assessed by agarose gel electrophoresis (Paragon, Beckman Instruments) and measurement of their lipoperoxide contents by a iodometric method (44, 45). As it was not possible to measure lipid peroxides in DiI-LDL because of the interference of the fluorochrome, sham-labeled LDL was used instead. For sham-labeled LDL, the concentration of lipid peroxides was 27–36 nmol/mg protein, which was within the range reported for fresh isolated, native LDL (44, 46, 47).

Lipoprotein binding assays

Binding and cell association of lipoproteins to cells were carried using lipoproteins labeled with the fluorescence probe DiI, a technique extensively used (33, 48, 49). Cell association of DiI-labeled lipoproteins to COS-7 cells was performed by incubating 5×10^4 cells grown on coverslips with 5 μg/ml of DiI-labeled lipoproteins in PBS containing 1 mm $CaCl_2$, 1 mm $MgCl_2$ for 1 h at 37°C. Binding of DiI-labeled lipoproteins to Sf9 cells was carried out by incubating cells in 100–200 μl of PBS containing 1 mm $CaCl_2$, 1 mm $MgCl_2$ with DiI-lipoproteins at 5 μg/ml for 2 h at 4°C or 1 h at room temperature. After incubation, cells were washed with cold PBS, fixed with 3% formaldehyde in PBS for 10 min at room temperature, and analyzed under fluorescence microscopy or by fluorescence flow cytometry using a FACScan from Becton Dickinson. For flow cytometry analysis, forward-angle light-scatter gates were established to exclude cellular debris and cellular aggregation. At least 5,000 cells were analyzed for each sample. Cell-associated and bound lipoprotein was expressed as median intensity of fluorescence (MIF) assessed using fluorescence windows (channel numbers). Fluorescence emitted by cells alone (autofluorescence) was subtracted. To determine binding dissociation constants (K_d), the cells and the DiI-lipoproteins were incubated for 2 h at 4°C in the presence or absence of a 50-fold excess of the corresponding unlabeled lipoprotein to determine non-specific binding. Experiments were done several times using different lipoproteins isolates. K_d values were obtained by analyzing the data with the InPlot-4 software (GraphPad, San Diego, CA). For inhibition experiments, antibodies or the unlabeled lipoproteins and the labeled-lipoprotein were added simultaneously to the cells.

RESULTS

Interaction of COS-7 cells expressing human CD36 with native and modified lipoproteins

Cell association of native and modified lipoproteins to COS-7 cells expressing CD36 was examined under fluorescence microscopy after incubating DiI-labeled lipoproteins with COS-7 cells transiently transfected with expression vectors carrying either CD36 (COS-CD36) or ICAM-3 (COS-ICAM-3). Untransfected COS-7 cells (COS) (results not shown), mock (COS-mock) (**Fig. 1A**, **E**, **I**, **M**) and COS-ICAM-3 (results not shown) displayed an internal punctuated staining pattern, possibly reflecting the endosomes and lysosomes containing internalized lipoprotein particles via endogenous lipoprotein receptors. However, when COS-CD36 cells were incubated with either OxLDL (Fig. 1B), HDL (Fig. 1F), LDL (Fig. 1J), or VLDL (Fig. 1N), a subset of cells, whose number was equivalent to the number of cells expressing CD36, as determined by immunostaining with the specific anti-CD36 monoclonal antibody FA6-152 (results not shown), showed an intense plasma membrane staining that overlapped with the staining pattern observed for the COS, COS-mock, and COS-ICAM-3 cells (Fig. 1A, E, I, M). These findings strongly suggest that the expression of CD36 is responsible for the plasma membrane-staining pattern. Specificity of the interactions was proven by the ability of a 50-fold excess of unlabeled lipoproteins to completely inhibit the DiI-lipoprotein-induced cell staining (Fig. 1C, G, K, O). Similar results were obtained when AcLDL was used (results not shown). Moreover, identical staining patterns were observed when using LDL and AcLDL labeled with the structurally unrelated fluorescence

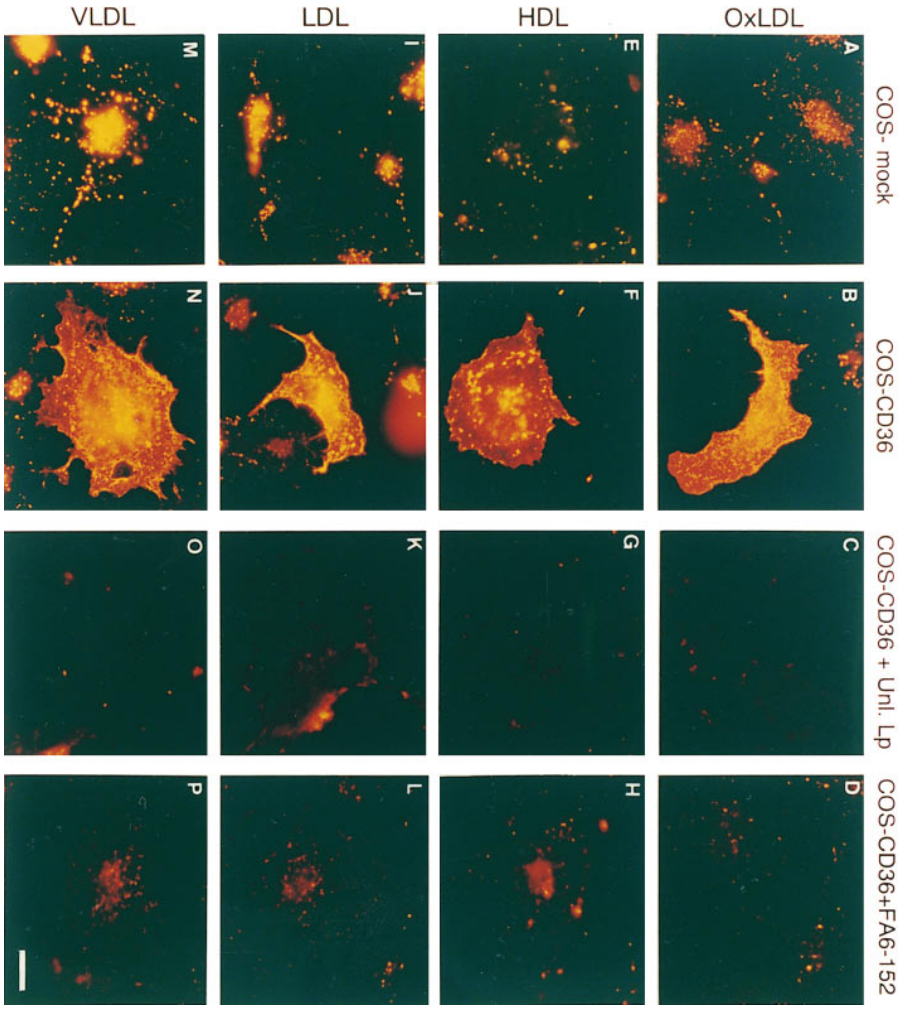

Fig. 1. Binding of DiI-labeled lipoproteins to COS-7 cells expressing CD36. COS-7 cells mock-transfected or transfected with the CD36 cDNA were grown on coverslips and incubated for 1 h at 37°C with 5 μg/ml of the indicated DiI-lipoproteins. For inhibition experiments, a 50-fold excess of the corresponding unlabeled lipoprotein or 5 μg/ml of the CD36-specific antibody FA6-152 was used. Bar, 30 μm.

probe BODIPY instead of DiI (results not shown). Finally, the anti-CD36 specific antibody FA6-152, when added to COS-CD36 cells simultaneously with labeled lipoproteins, eliminated the staining at the plasma membrane and reversed the cell-staining to the pattern displayed by COS, COS-mock, and COS-ICAM-3 cells (Fig. 1D, H, L, P, and results not shown), a finding that provides further solid evidence for the involvement of CD36 in lipoprotein binding. As expected, upon addition of the antibody FA6-152, the punctuated staining pattern displayed by COS, COS-mock, and COS-ICAM-3 cells was not affected (results not shown). Altogether, these results allow us to conclude that CD36 when expressed on COS-7 cells binds native HDL, LDL, and VLDL, besides interacting with the in vitro modified lipoproteins AcLDL and OxLDL.

Expression of CD36 on the plasma membrane of insect Sf9 cells

To reduce the endogenous lipoprotein binding capacity due to the expression of endogenous lipoprotein receptors in most cultured mammalian cell lines and facilitate the performance of quantitative binding experiments, we decided to express CD36 on the plasma membrane of insect Sf9 cells. The structural nature of the lipoproteins recognized by insect cells is different from that present on mammalian cells (50). Moreover, mammalian proteins expressed in this expression system display similar binding and enzymatic activities as those expressed on mammalian expression systems. In fact, and as shown below, Sf9 cells exhibited a negligible human lipoprotein binding activity.

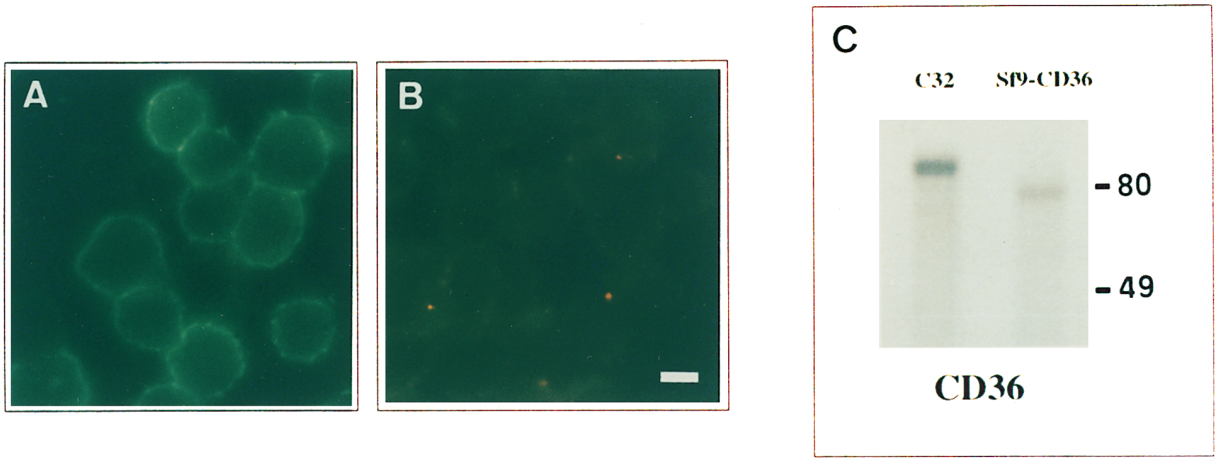

Fig. 2. Expression of CD36 in Sf9 cells. Sf9 cells were infected with the recombinant viruses BP-CD36 (A) or BP-CLA-I (B), and 36–48 h later fixed with paraformaldehyde and immunostained with the anti-CD36 antibody FA6-152. A m.o.i. of 1 was used to avoid infection of all cells in the preparation, and therefore to allow the comparison of the staining between infected versus non-infected cells. When a moi of 10 was used, all cells appeared stained (results not shown). Bar, 10 μm. C) SDS-PAGE analysis of CD36 immunoprecipitated from lysates from cell surface iodinated human melanoma cells C32 and from insect Sf9-CD36 cells. Molecular sizes of some markers are indicated on the right in kDa.

Expression of CD36 on the plasma membrane of Sf9 cells infected by baculoviruses carrying the CD36 cDNA (Sf9-CD36) was explored by cell surface staining using anti-CD36 antibodies (**Fig. 2A**, **B**), and by SDS-PAGE analysis of the CD36 immunoprecipitated from lysates from ^{125}I-surface-labeled Sf9-CD36 cells (Fig. 2C). Importantly, the CD36 expression levels on the plasma membrane that resulted after infection of Sf9 cells, as determined by FACS analysis, were comparable to the levels displayed in some mammalian cell lines (results not shown). The apparent molecular weight size of the CD36 form immunoprecipitated from the infected insect cells was slightly smaller than that observed for the CD36 immunoprecipitated from the human melanoma cell line C32 (Fig. 2C), a feature consistent with the limited processing of core oligosaccharides by Sf9 cells (51). Moreover, variation in the apparent molecular size of CD36 is cell-dependent, a feature that has been attributed to minor changes in its glycosylation degree (19, 52).

Interaction of Sf9 cells expressing CD36 with native and modified lipoproteins

Cell association of OxLDL, HDL, LDL, and VLDL to Sf9-CD36 cells was basically determined as described for the COS-7 cells. Upon incubation with DiI-labeled lipoproteins, Sf9 cells infected with the BacPAK6 baculovirus or with a baculovirus carrying the cDNA of the cell surface molecule CD69 (Sf9-CD69) (used as negative controls) exhibited, under fluorescence microscopy, none or a barely detectable staining that revealed a negligible fluorescence binding activity (**Fig. 3A**, **B**, **D**, **E**, **G**, **H**, **J**, **K**). In contrast, Sf9-CD36 cells displayed an intense fluorescence at the plasma membrane after incubation with the same set of DiI-labeled lipoproteins, consistent with a binding activity (Fig. 3C, F, I, L). For Sf9-CD36 cells, lipoprotein association increased in a linear fashion with the multiplicity of infection, which directly correlated with the cell surface expression levels, and when the temperature was raised from 4°C to 37°C (results not shown). Similar results were obtained when using DiI-labeled AcLDL (results not shown). Therefore, CD36 conferred to Sf9 cells the capability to bind HDL, LDL, VLDL, OxLDL, and AcLDL.

Specific binding of DiI-labeled OxLDL, LDL, HDL, and VLDL to Sf9-CD36 cells increased in a saturable manner (**Fig. 4**). The dissociation constant (K_d) calculated for the binding of OxLDL at 4°C to Sf9-CD36 cells (K_d 1.7 μg/ml) (**Table 1**) agreed with the one reported for the binding of OxLDL to CD36 when expressed on mammalian cells (K_d 1.5 μg/ml) (11), a finding that suggests that CD36 when expressed on insect cells maintains its lipoprotein binding properties unmodified and supports the validity of the binding constants determined using this expression system. The values for the dissociation constants determined from these experiments (Table 1) lie within the range of K_ds of other lipoprotein receptors, and indicate high-affinity interactions.

Specificity of the interactions of CD36 with lipoproteins was further supported by the dose-dependent inhibition of the cell binding of HDL, VLDL, LDL, and AcLDL to Sf9-CD36 cells by the purified CD36-specific antibody FA6-152, but not by a control antibody of the same IgG subclass (**Fig. 5**). Similar results were obtained when OxLDL was used (results not shown).

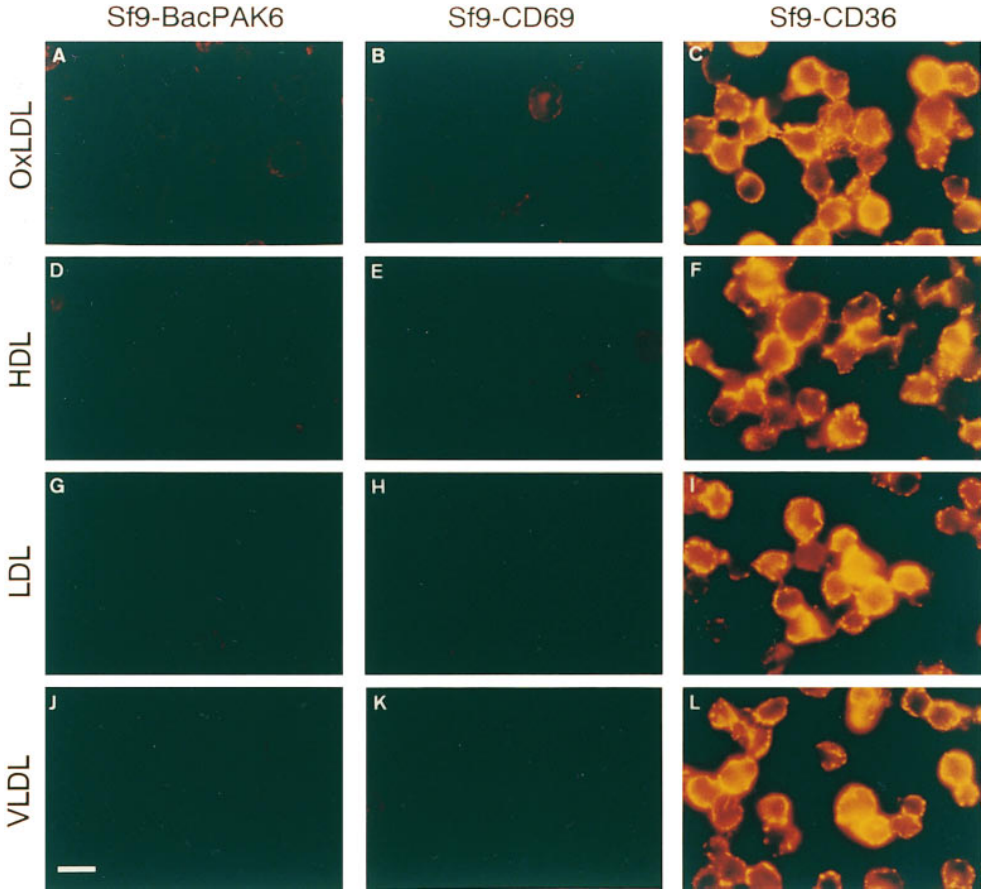

Fig. 3. Binding of DiI-labeled lipoproteins to Sf9 cells expressing CD36. Sf9 cells infected with the baculoviruses containing the cDNAs indicated were grown on coverslips and incubated for 1 h at room temperature with 5 μg/ml of the indicated DiI-lipoproteins. After washing, cell association of DiI-labeled lipoproteins was observed under fluorescence microscopy. Bar, 30 μm.

Cross competition experiments using the distinct LDL forms revealed that while a 40-fold excess of unlabeled LDL, AcLDL, and OxLDL significantly blocked the binding of DiI-LDL to Sf9-CD36 cells, only unlabeled AcLDL and OxLDL, but not LDL, displaced the binding of DiI-AcLDL (**Fig. 6**). A reasonable explanation for this observation concerns the higher binding affinity for CD36 of AcLDL and OxLDL than native LDL. Another explanation would be the existence of nonreciprocal cross competition, a phenomenon previously reported to occur for the scavenger receptor SR-AI regarding its ligands AcLDL and OxLDL (8), and for allosteric enzymes. Differences in ligand binding to different receptor conformations and/or binding sites have been proposed as molecular explanations for nonreciprocal cross competition (8, 53).

The binding specificity of CD36 to HDL was additionally explored by competition experiments with unlabeled HDL, LDL, and OxLDL (**Fig. 7**). As expected, in all cases competition was observed, being the inhibitory capacity of each lipoprotein consistent with its respective K_d value.

Binding of HDL to CD36 is apo E-independent

To examine the requirement of apoE in the binding of HDL to CD36, we tested the ability of unlabeled HDL–apoE+ and HDL–apoE− to compete for the binding of total DiI-HDL. As shown in **Fig. 8**, both HDL–apoE+ and HDL–apoE−, like total HDL, were effective competitors. These results indicate that binding of HDL to CD36 does not require the presence of apoE in the lipoprotein particle. Whether this conclusion applies to other lipoprotein types needs to be investigated.

DISCUSSION

Cell-association and binding studies allowed us to demonstrate that human CD36 is a high affinity recep-

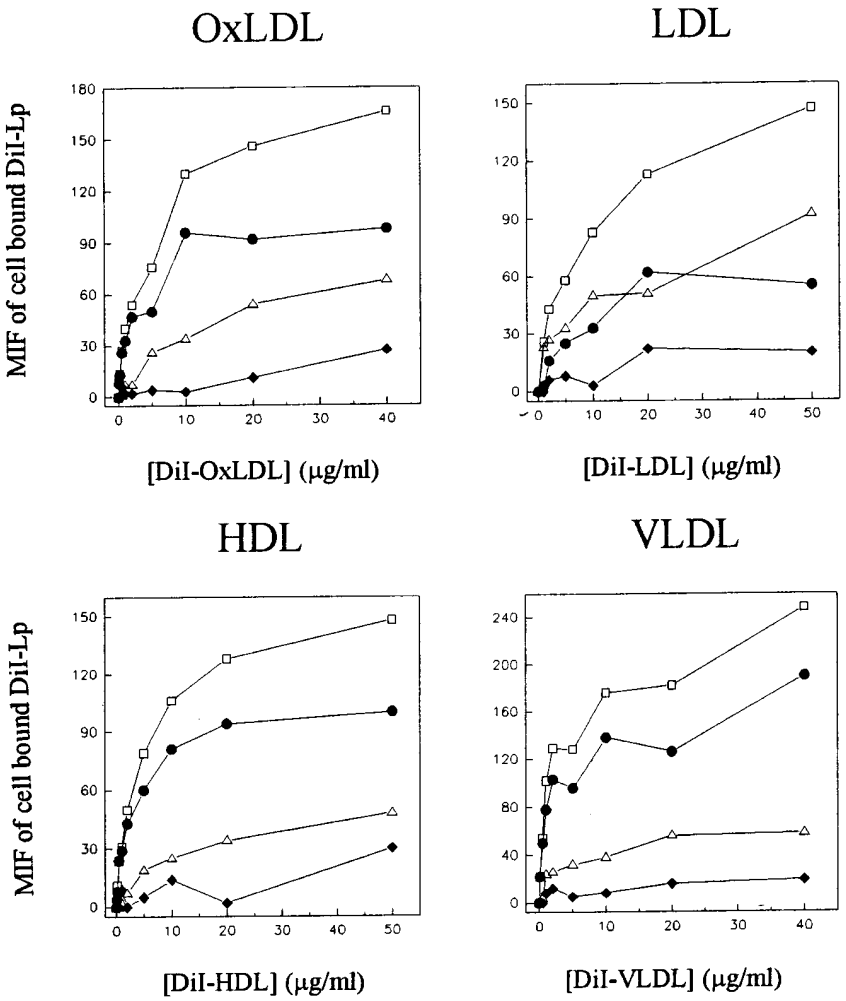

Fig. 4. Saturation curves of OxLDL, LDL, HDL, and VLDL binding to Sf9-CD36 cells. BacPAK6, CD69, and CD36 infected Sf9 cells (infected with a m.o.i. of 10) were incubated for 2 h at 4°C with increasing amounts of DiI-labeled OxLDL, LDL, HDL, and VLDL. After washing, cell-associated fluorescence was measured and expressed as MIF values of lipoprotein (Lp) bound. The specific binding values (filled circles) of each lipoprotein to Sf9-CD36 cells represent the differences in the median fluorescence values between the total binding (open squares) and the nonspecific binding (open triangles) obtained in the presence of a 50-fold excess of the corresponding unlabeled lipoprotein. For Sf9-BacPAK6 cells, only the specific binding, calculated as indicated above, is shown (filled diamonds). Sf9-CD69 cells exhibited binding profiles similar to those obtained for the Sf9-BacPAK6 cells. Duplicate determinations were done for each experiment. Data shown correspond to a representative experiment of two (for OxLDL) and three (for LDL, HDL, and VLDL) independent experiments using different lipoprotein preparations.

tor for the plasma lipoproteins HDL, LDL, and VLDL, and for the in vitro LDL modified forms OxLDL and AcLDL. Although the ability of CD36 to bind modified lipoproteins had been already documented (11, 12), so far no description of the capacity of CD36 to interact with native HDL, LDL, and VLDL has been reported.

There is some controversy concerning the LDL forms that bind to CD36. While Endemann et al. (11) showed that neither AcLDL nor LDL competed for the binding of OxLDL to CD36 expressed on the human

TABLE 1. Constant dissociation (K_d) values for the binding of OxLDL, LDL, HDL, and VLDL to CD36

	OxLDL	LDL	HDL	VLDL
K_d (μg of lipoprotein / ml)	1.7 ± 0.6	6.3 ± 0.2	2.9 ± 1.6	1.2 ± 0.4

Values used to obtain the K_ds for the binding of each lipoprotein to CD36 were determined by subtracting the values of the specific binding obtained with Sf9-BacPAK6 cells from the values of the specific binding obtained with Sf9-CD36 cells (Fig. 4). To determine K_ds, the data were analyzed by the InPlot-4 software package.

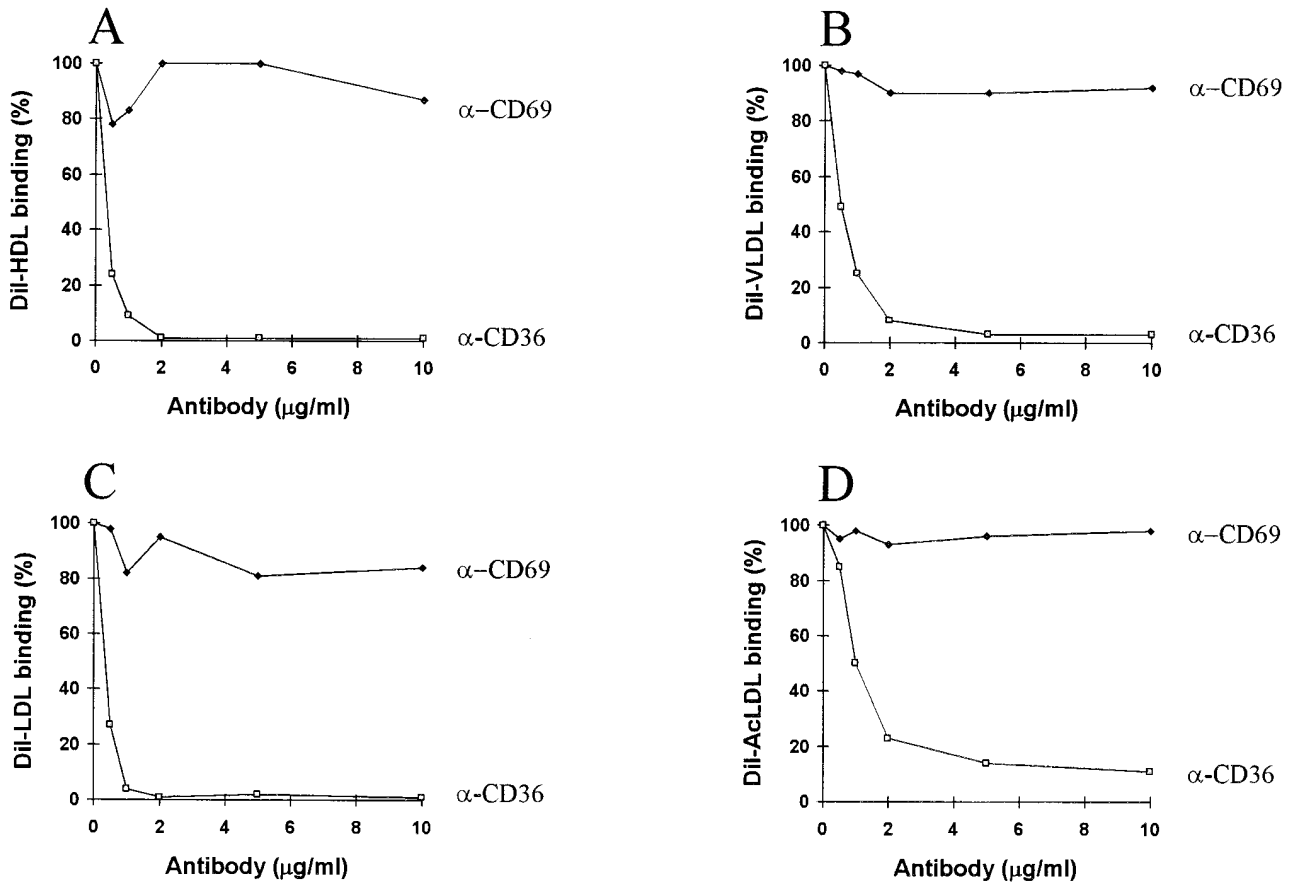

Fig. 5. Effect of specific antibodies against CD36 on the binding of HDL, VLDL, LDL, and AcLDL to Sf9-CD36 cells. Sf9-CD36 cells were incubated for 1 h at 4°C with 5 μg/ml of the indicated DiI-labeled lipoproteins in the presence of the indicated amounts of purified antibodies reacting with either CD69 (TP1/33, IgG1 subclass) or CD36 (FA6-152, IgG1 subclass). After incubation, lipoprotein binding to cells was determined by fluorescence flow cytometry. The results of the experiments are represented as percentages of binding with respect to the experiment performed in the absence of any competitor. Data shown correspond to a representative experiment of two independent experiments using different lipoprotein preparations.

embryonic kidney cells 293, Acton et al. (12) reported that COS cells expressing CD36 acquired the ability to bind both AcLDL and OxLDL, and Nicholson et al. (37) did not observe LDL or AcLDL binding activity on mouse fibroblast NIH-3T3 cells expressing CD36 versus the non-CD36 expressing cells. Our data, however, indicate that CD36 expressed on either COS-7 or Sf9 cells is able to mediate the binding of OxLDL, AcLDL (also reported by Acton et al. (12) and suggested by Nozaki et al. (36) and native LDL (Fig. 1, 3, 4, 5). We have also found that AcLDL inhibits LDL binding to CD36-expressing cells but not vice versa (Fig. 6). A similar non-reciprocal cross competition phenomenon has been described for the ligands AcLDL and OxLDL of the SR-AI receptor (8). Moreover, we also observed that LDL was a poor competitor and OxLDL competed partially for HDL binding to Sf9-CD36 cells (Fig. 7). Although the inhibitory capacity of each lipoprotein significantly correlated with its respective K_d value, additional factors may contribute to explain the above observations, such as: *1*) the existence of distinct lipoprotein binding sites on CD36, and/or of additional cell binding sites besides those present on CD36 (for example, endogenous LDL receptors present on COS-7 cells partially mask the binding activity of LDL to CD36); *2*) steric hindrance effects due to the larger size of LDL and OxLDL with respect to that of HDL (through this effect, LDL or OxLDL particles could preferentially impede the binding of larger particles to nearby unoccupied receptors, but not the binding of smaller particles like HDL); and *3*) a cell-type dependent activity of the CD36-lipoprotein binding properties, as it has been suggested (36, 37, 54). In this regard, expression of CD36 on HeLa and CHO-KI cells does not result in a significant increase in the binding of OxLDL or AcLDL as compared to the mock or un-

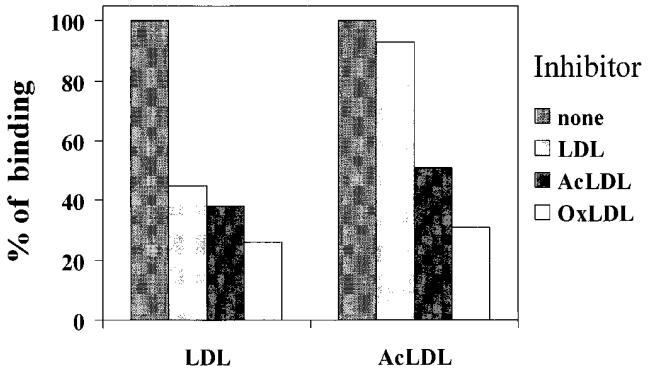

Fig. 6. Lipoprotein displacement of LDL and AcLDL binding to Sf9-CD36 cells. Sf9-CD36 cells were incubated in duplicate with 5 μg/ml of Bodipy-LDL or Bodipy-AcLDL for 1 h at room temperature in the presence of 200 μg/ml of the unlabeled inhibitors LDL, AcLDL and OxLDL. After washing, binding of Bodipy-LDL and Bodipy-AcLDL was determined by fluorescence flow cytometry. Depicted values represent the percentages of binding with respect to the experiment carried out in the absence of any competitor. Data shown were representative of three independent experiments.

transfected cells (results not shown (37, 54)). Finally, some of the differences observed among laboratories concerning CD36-lipoprotein binding activity might be due to differences in the procedures used for lipoprotein labeling. An exhaustive and careful examination of the lipoprotein binding activities in different cell types expressing CD36 and in diverse experimental conditions may help to solve this issue.

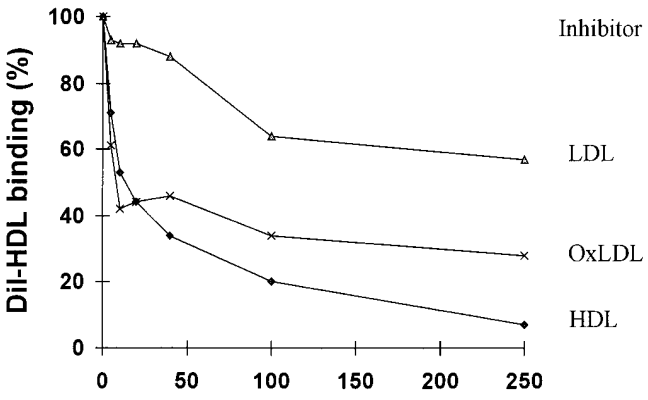

Fig. 7. Lipoprotein displacement of HDL binding to Sf9-CD36 cells were incubated in duplicate with 5 μg/ml of DiI-HDL for 2 h at 4°C in the presence of increasing concentrations of the following unlabeled inhibitors: HDL, LDL, and OxLDL. After washing, binding of DiI-HDL was determined by fluorescence flow cytometry. Depicted values represent the percentages of binding with respect to the experiment carried out in the absence of any competitor. Data shown were representative of two independent experiments.

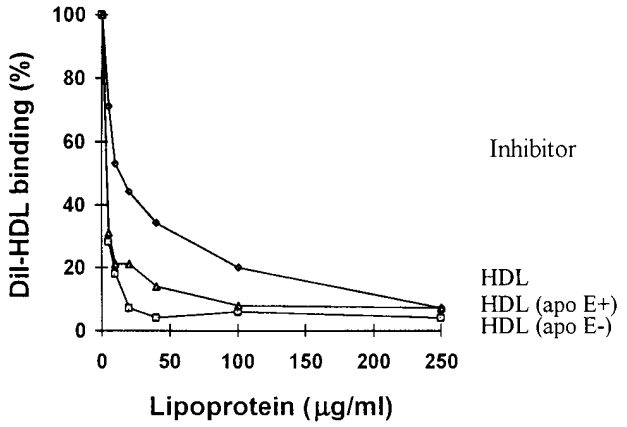

Fig. 8. Effect of HDL, HDL-apoE+ and HDL-apoE− on the association of DiI-HDL to Sf9-CD36 cells. Sf9-CD36 cells were incubated in duplicate with 5 μg/ml of DiI-HDL for 2 h at 4°C in the presence of increasing concentrations (0, 5, 10, 20, 40, 100, and 250 μg/ml) of the following inhibitors: total HDL (diamonds), HDL-apoE− (squares), and HDL-apoE+ (triangles). After washing, binding of DiI-HDL to cells was determined by fluorescence flow cytometry. Depicted values represent the percentages of binding with respect to the experiment carried out in the absence of any competitor. Data shown correspond to a representative experiment of two independent experiments using different lipoprotein preparations.

One of the major points of the present study is the binding of native LDL to CD36. Lipoproteins were isolated and labeled under conditions to prevent peroxidation. A certain modification during the process cannot be completely excluded; however, in our hands the level of lipoperoxides in sham-labeled LDL was of 27–36 nmol/mg protein, which is in the range reported by others for fresh isolated, native LDL (44, 46, 47), and about ten times lower than that observed in LDL stored for 2 months at 4°C in the dark and supplemented with α-tocopherol. Furthermore, lipoperoxide concentrations in LDL that were isolated and sham-labeled in the presence or absence of 20 μm BHT were equivalent (results not shown). DiI-labeled LDL showed a K_d value of 1.5 μg/ml for the binding to the LDL receptor in isolated human peripheral blood lymphocytes (results not shown), which is similar to the values reported in the literature (K_d = 2 μg/ml) for ^{125}I-labeled LDL (55). In addition, in experiments using Bodipy-LDL and Bodipy-AcLDL (obtained from Molecular Probes) we obtained results similar to those obtained using DiI-LDL and DiI-AcLDL. Therefore, all these results support that the DiI-LDL and the unlabeled LDL used in the present study displayed the properties of native LDL.

CD36 is structurally related to CLA-1, SR-BI, and LIMPII. Interestingly, SR-BI (the putative hamster homologue of human CLA-1) has been shown to interact with both modified LDL and native LDL and HDL (12,

33). CLA-1 binds the same lipoprotein set that SR-BI does (34, 35), and like human CD36, also interacts with VLDL (35). Therefore, the CD36 and CLA-1 (SR-BI) receptors are unique with respect to their ability to interact with both native and oxidized lipoproteins. In addition to sharing similar lipoprotein binding specificities, they both participate in the recognition of apoptotic cells (28, 32) and act as receptors for the anionic phospholipids phosphatidylserine and phosphatidylinositol (26). Altogether, these data indicate that the ligands for CD36 and CLA-1 largely overlap. The region of CD36 comprising amino acids 155–183 has been shown to contribute to the binding of OxLDL (56), and apoptotic cells (57). Whether this multifunctional domain also contributes to the interaction with native lipoproteins needs to be investigated.

The fact that CD36 interacts with the major lipoprotein classes, which differ in their apolipoprotein constituents, raises the question of the structural nature of the ligand recognized on each lipoprotein type. Several experimental observations shed light on this question: *1*) the characterization of rat CD36 as a long-chain fatty acid binding/transport protein (20); *2*) the partial inhibitory effect of oleic acid on the binding of OxLDL to CD36 (37); and *3*) the ability of phosphatidylserine and phosphatidylinositol to interact with CD36 and to compete for the AcLDL binding to CD36 (26). These features strongly suggest that fatty acids present in lipoproteins may contribute to the structural moieties that are recognized by the CD36 receptor. Our data showing the apoE-independent recognition of HDL by CD36 are compatible with the above suggestion.

Lipid loading of macrophages as a consequence of the binding of modified lipoproteins to CD36 has been addressed (36, 37). Interestingly, CD36, like lipoprotein lipase (58), is mainly present in tissues performing active fatty acid metabolism, such as skeletal muscle, heart, adipose tissue, and mammary epithelia (20, 21, 35, 54). In these tissues, triglyceride-rich lipoproteins are hydrolyzed by endothelium-anchored lipoprotein lipase, and the released fatty acids are subsequently taken up by the underlying cells, where they are oxidized for energy production (muscle tissue and heart), reesterified for storage (adipose tissue), or secreted (mammary epithelia). The ability of CD36 to bind/transport fatty acids (20), the coexpression and physical association of CD36 with the cytoplasmic protein FABP (fatty-acid binding protein) in rat heart and skeletal muscles (59) and in bovine mammary gland (60), and the correlation between heart muscle capillary endothelial cell CD36 expression and blood triglyceride levels (54) support the participation of CD36 in fatty acid metabolism, and even the existence of a functional interplay between lipoprotein lipase and CD36. Thus, in analogy to SR-BI, which upon binding lipoproteins participates in the selective delivery of cholesteryl esters to steroidogenic tissues (33), CD36 could mediate delivery of fatty acids to cells active in fatty acid metabolism. Moreover, this latter observation is at a physiological level compatible with the ability of CD36 to mediate the binding of VLDL. In this regard, it should be noted that the in vivo relevance of the VLDL receptor (61) in lipoprotein metabolism has recently been questioned because of the absence of alterations in the lipoprotein profiles in mouse deficient in this receptor (62).

The elucidation of the events that take place once a given lipoprotein binds to CD36 (internalization of the lipoprotein, selective delivery of some of its components, lipid uptake, lipoprotein recruitment that may indeed facilitate the subsequent action of other lipoproteins receptors and/or enzymes, triggering of cell signalling, etc.) and the study of lipoprotein metabolism in CD36 transgenic and deficient animals will facilitate the determination of the in vivo contributions to lipid metabolism and atherosclerosis of the interactions between CD36 and lipoproteins.

This work was supported by grants from the Ministerio de Educación y Ciencia of Spain PB93/1020 to M. A. V., F.I.S. 94/0540 to D. G. C., and SAF96/0011 to M. A. L. D. C. is recipient of a predoctoral fellowship from the Comunidad Autónoma de Madrid. We thank Drs. F. Sánchez-Madrid and José L. Alonso for their generous gifts of reagents (ICAM-3 expression vector, the CD69 baculovirus, and the monoclonal antibody TP1/33), and Dr. Brian Seed for kindly providing the CD36 cDNA. We also thank Dr. Angel Corbí for critical reading of the manuscript.

Manuscript received 11 September 1997 and in revised form 18 December 1997.

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