Antipeptide antibodies reveal interrelationships of MBP 200 and MBP 235: unique apoB-specific receptors for triglyceride-rich lipoproteins on human monocyte-macrophages

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Abstract **Two human monocyte-macrophage (HMM) membrane binding proteins, (MBP) 200 and 235, are receptor candidates that bind to the apolipoprotein (apo)B-48 domain in triglyceride-rich lipoproteins for uptake independent of apoE. Microsequence analysis of the purified reduced MBP 200R characterized tryptic peptides of MBP 200R. A synthetic peptide mimicking a unique, unambiguous 10-residue sequence (AEGLMVTGGR) induced antipeptide antibodies that specifically recognized MBP 200, 235 and 200R, in 1- and 2-dimensional analyses, indicating *1*) the ligand binding protein was sequenced and *2*) MBP 200 and 235 yielded MBP 200R upon reduction. These antibodies identified the MBPs in human blood-borne, THP-1, U937 MMs, and endothelial cells (EC) but not in human fibroblasts or Chinese hamster ovary (CHO) cells. Fluorescence activated cell sorting (FACS) analysis located the MBPs on the MM surface as necessary for receptor function. The 10-residue, unambiguous MBP 200-derived sequence is unique, with no matches in extant protein databases. Antipeptide antibodies bind to the MBPs in reticuloendothelial cells that have this receptor activity, but not to proteins in cells that lack this receptor activity. These studies provide the first direct protein sequence and immunochemical data that a new, unique apoB receptor for triglyceride-rich lipoproteins exists in human monocytes, macrophages, and endothelial cells.**—Bradley, W. A., M. L. Brown, M. P. Ramprasad, R. Li, R. Song, and S. H. Gianturco. **Antipeptide antibodies reveal interrelationships of MBP 200 and MBP 235: unique apoB-specific receptors for triglyceride-rich lipoproteins on human monocyte-macrophages.** ***J. Lipid Res.*** **1999.** 40: **744–752.**

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Monocyte-macrophage-derived foam cells are characteristic of atherosclerotic lesions and are present in the peripheral tissues of hypertriglyceridemic (HTG) subjects that have persistent plasma chylomicrons (types 1, 3, and 5) and in subjects with diabetes with grossly elevated triglyceride-rich lipoproteins (TGRLP) (type 5 pattern). In animal studies, apolipoprotein E-deficient mice developed extensive atherosclerotic lesions without, as well as with, dietary insult (1–4), indicating that apoE itself, while a primary mediator of receptor-mediated cellular uptake of TGRLP by members of the LDL receptor gene family, is not necessary for the production of foam cells in vivo and, indeed, may play a protective role when specifically expressed in the arterial intima (5).

In this regard, we have described an apoE-, lipoprotein lipase (LpL)-, and heparan sulfate proteoglycan (HSPG)-independent, high affinity receptor pathway for the receptor-mediated uptake of TGRLP in both murine macrophages (6–8) and human monocyte-macrophages (MM) and THP-1 cells (9) that leads to lipid accumulation and foam cell formation in vitro. Two candidate receptor membrane binding proteins, MBP 200 and 235, were identified in human monocytes and macrophages (8, 9). Studies in THP-1 monocyte-macrophages indicated that *a*) MBP 200 (M_r ~200 kDa) and MBP 235 (M_r ~235 kDa) are located on the cell surface; *b*) MBP 235 contains MBP 200 as the ligand binding subunit along with noncovalently associated smaller subunit(s) of ~35 kDa; and *c*) both MBP ligand binding activities are converted into a single ligand binding species with intermediate mobility, MBP 200R,

Abbreviations: HTG, hypertriglyceridemic; TGRLP, triglyceride-rich lipoproteins; MBP, membrane binding protein; apo, apolipoprotein; Cys, cysteine; VLDL, very low density lipoprotein; LDL, low density lipoprotein; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; LpL, lipoprotein lipase; HSPG, heparan sulfate proteoglycan; HMM, human monocyte-macrophage; EC, endothelial cell; FACS, fluorescence activated cell sorting.

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upon reduction (10). The purification of MBP 200R (11) and the biochemical and ligand binding characteristics which distinguish the monocyte TGRLP/apoB receptors (MBP 200, 235) from other known lipoprotein receptors have been reported (8–12). We hypothesized that these MBPs are related and represent a new and unique receptor family for apoE-, LpL-, and HSPG-independent uptake of TGRLP by human monocyte-macrophages. Moreover, we recently demonstrated that the MBPs bind to a region of apoB-48 (or equivalent domain in apoB-100) making these the only known receptors for apoB-48 (12).

We now present the initial peptide sequence data and immunochemical data that directly support this hypothesis. Microsequence analysis of tryptic peptides of purified MBP 200R indicated that MBP 200R contained a unique unambiguous internal 10-residue sequence that had no matches in protein databases, verifying that MBP 200R is a new, previously undescribed protein. A polyclonal antibody was raised against a 10-residue synthetic peptide, Cys-10-NH_2, whose sequence mimicked that derived from the HPLC-isolated tryptic peptide of MBP 200R. The antipeptide antibody data provide direct support for our conclusions from earlier biochemical studies that the MBPs are structurally and functionally related receptor proteins for the apoB-mediated (12) uptake of hepatic and intestinally derived TGRLP by monocyte-macrophages and endothelial cells. Excessive uptake by this pathway may contribute to foam cell formation and endothelial dysfunction, providing a common mechanistic link between the atherosclerosis and thrombosis risks associated with hypertriglyceridemia.

METHODS

Lipoproteins

HTG-VLDL were isolated from plasma of fasting subjects with types 4 and 5 lipoprotein profiles or from lipemic plasma obtained from the Red Cross. HTG-VLDL were subfractioned by cumulative flotation as previously described (13, 14). Biotinylated VLDL were prepared as described elsewhere (12). Protein concentrations of the lipoproteins were determined by a modified Lowry procedure (15, 16).

Cells and cell culture

THP-1 cells (American Type Culture Collection) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mm glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin and 5×10^{-5} m 2-mercaptoethanol. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air at $<1.0 \times 10^6$ cells/ml (9). Normal human skin fibroblasts were derived from foreskin and grown in DME plus 10% FBS or NuSerum as previously described (17). U937 monocytes were grown in RPMI-1640 containing 10% FBS, 2 mm glutamine, and penicillin and streptomycin as above. Blood-borne monocyte-macrophages were isolated and cultured as previously described (9). The spontaneously transformed human umbilical vein derived ECs (ECVs) were a kind gift from Dr. Suzanne Eskin (Texas Biotechnology, Inc.) and were grown in M199, 10% FBS, 2 mm glutamine, and penicillin and streptomycin as above.

Antibodies

Sheep anti-human apoB antiserum (1001400, Boehringer Mannheim Biochemicals, Indianapolis, IN) was purified by affinity chromatography using an LDL-conjugated Sepharose column. Purified rabbit anti-sheep IgG conjugated to alkaline phosphatase was purchased from Jackson Laboratories (West Grove, PA) or Southern Biotechnology (Birmingham, AL).

Antipeptide antibodies

A 10-residue synthetic peptide was prepared in a UAB peptide core facility and designed from the microsequence data (see below) with an amino-terminal cysteine included and the carboxyterminal amidated to resemble an internal peptide fragment. The synthetic peptide, Cys-10-NH_2, was characterized by its amino acid composition and by mass spectral analysis. Cys-10-NH_2 was linked to keyhole limpet hemocyanin (KLH) by coupling with m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) or by coupling to maleimide-activated KLH according to the manufacture's instructions. The KLH-conjugated Cys-10-NH_2 was injected into three rabbits using a standard protocol for antibody production. The antisera were precipitated with 50% ammonium sulfate, resuspended to approximately half the original volume, and the IgGs were dialyzed extensively against PBS containing 0.1% NaN_3 at 4°C. Purification of the antipeptide antibodies was accomplished by affinity chromatography. Cys-10-NH_2 was coupled to Sulfo-Link (Pierce) Sepharose according to the manufacture's directions and the coupling efficiency was measured by difference in peptide content of equal aliquots before and after coupling. The antipeptide IgGs were equilibrated with the affinity matrix in PBS at 4°C for 30 min to promote binding, then washed with 10 bed volumes of PBS, and finally eluted with 0.1 m citric acid (pH 2.0) and immediately neutralized with 2 m Tris-HCl, pH 7.4 (final). The affinity-purified fractions were pooled and dialyzed exhaustively against PBS. The affinity-purified antipeptide antibodies were aliquoted and stored at 4°C for short-term or frozen at −70°C for long-term storage.

Ligand blotting

Thirty-μl aliquots of the detergent extracts of cells were prepared and electrophoresed on SDS-polyacrylamide (5%) in a Bio-Rad minigel apparatus and electrotransferred to nitrocellulose as previously described (10). After blocking for 1 h with 5% Carnation nonfat dry milk in ligand buffer (50 mm Tris-HCl, pH 8, 90 mm NaCl and 2 mm $CaCl_2$), the blots were rinsed with 0.5% milk in ligand buffer and incubated with 0.5–20 μg/ml HTG-VLDL (S_f 100–400) in ligand buffer containing 0.5% milk for 2 h. The blots were then washed 3 times for 5 minutes each, with 0.5% milk in ligand buffer and then incubated with affinity-purified sheep anti(h) apoB antibody in 0.5% milk for 2 h. After three 5-min washes, bound antibody was detected by incubation of the nitrocellulose strips with rabbit anti-sheep IgG conjugated to alkaline phosphatase for 1 h followed by the substrates, BCIP and NBT (Bio-Rad Laboratories, Richmond, CA). Bound biotinylated VLDL were detected with streptavidin linked to alkaline phosphatase and the preceding substrates. Ligand blots were scanned optically (Hewlett-Packard, Atlanta, GA) and quantitated using the ImageQuant software (Molecular Dynamics densitometer, Sunnyvale, CA) as described (9, 10). The digitized data were used to create the images for Figs. 2–4.

Western (immunochemical) blotting

Proteins were separated as described in the ligand blotting assay. After blocking the nitrocellulose as described above, primary antibodies were diluted into 0.5% milk–ligand buffer and incubated for 4 h at room temperature. Binding of the primary antibody was detected by Goat anti Rb IgG (H + L) conjugated to alkaline phosphatase, using the substrates, BCIP and NBT (Bio-Rad Laboratories) as described (9, 10). The blots were scanned and digitized as above.

Two-dimensional ligand and Western blotting

The 2-dimensional analyses are essentially as detailed elsewhere (10). Briefly, detergent extracts were electrophoresed in the first dimension on a slab minigel apparatus; a 0.6-cm wide region of this gel containing the MBPs was excised, treated with sample buffer at pH 6.8, with or without 2% 2-mercaptoethanol for 10 min at room temperature before it was placed lengthwise on a second 5% slab gel and then run into the second gel. At the end of electrophoresis and electrotransfer onto nitrocellulose, the nitrocellulose was treated as described above and then analyzed for either ligand binding activity with HTG-VLDL S_f 100–400 or biotinylated HTG-VLDL S_f 100–400 or for Western blotting activity with the antipeptide antibodies. The specificity of the ligand and antipeptide antibodies was demonstrated by omitting either the ligand (or primary antibody) for the ligand blot analysis or the primary antibody for the Western blot analysis.

Microsequence analysis

MBP 200R preparations were purified and electrophoresed as previously described (11) and electrotransferred onto either PVDF or onto nitrocellulose in a CAPS-buffer. Coomassie (or Ponceau S)-stained bands on PVDF comigrating with ligand binding activities were excised (0.1 × 1 cm) and submitted for gas-phase Edman degradation directly for N-terminal sequencing. Alternatively, for trypsinolysis, nitrocellulose strips were incubated in 200 μl of 0.25% PVP-360 in 0.1% acetic acid for 20 min. Strips were then washed four times in 200 μl of water and cut into small fragments. Tryptic digestions were performed on the nitrocellulose pieces in a final volume of 50 μl containing 0.1 m NH_4HCO_3, pH 8.0, 10 pmol of trypsin for 24 h at 37°C. Tryptic peptides were isolated by chromatography on a Vydac C-18 column (530 mm ID) using a TFA/acetonitrile ascending gradient from 2% to 70% in 70 min. Peptides were identified by microsequence analysis and mass spectrometry.

FACS, fluorescence activated cell sorting

THP-1 monocytes (2×10^7) cells were washed in sterile media (2 mm EDTA, PBS) at 4°C and resuspended in 1 ml of staining buffer containing 0.1% NaN_3, 0.1% BSA, and PBS, pH 7.4. To 50 μl of the resuspended cells in the staining buffer, 10 μl of human lipoprotein-deficient serum (LPDS) was added and incubated for 30 min at 4°C. Cells were then resuspended in 2 ml of staining buffer, pelleted at 1,000 rpm in a tabletop centrifuge and the step was repeated. The cells were resuspended in 50 μl of staining buffer and 10 μl of the primary antibody (or control antibodies) added, mixed gently, and incubated for 30 min at 4°C. At the end of this incubation, cells were washed twice as described above, resuspended in 50 μl of staining buffer into which a 1:25 dilution of a goat $(Fab')_2$ anti-rabbit IgG (or anti-mouse) labeled with FITC had been diluted. This mixture was incubated for 30 min at 4°C; the cells were washed thoroughly and fixed in 1% formaldehyde. The cells were analyzed in the UAB FACS Core Facility.

RESULTS

Microsequence analysis indicates MBP 200 is a unique protein

MBP 200R was purified from THP-1 monocytes (~70 L of cells) for microsequence analysis as described previously (11). Two independent microsequence analyses of MBP 200R produced no N-terminal amino acids, indicating that the N-terminus of the protein was blocked. As an alternate approach, tryptic peptides were produced and isolated as described in Methods. The longest peptide sequence that was obtained from the gas-phase microsequence analysis was from one 13-residue tryptic fragment whose amino acid sequence was unambiguously identified for residues 4 through 13 (**Fig. 1A**). That the peptide ended with an Arg confirmed its origin as a product of trypsinolysis and indicated it was an internal peptide. The tryptic peptide's major mass fragment, determined by mass spectral analysis of the HPLC fraction (#30), was MH+ = 1333.5. Ongoing cDNA sequence analysis has confirmed this sequence and allowed the positive identification of the first three residues (data not shown) of this tryptic fragment as L, L, D, which, of note, corresponds to the mass of the parent fragment (L) (L) (D) A E G L M V T G G R (Fig. 1; MH+). Two additional tryptic peptides were also identified (fractions 18 and 29), but the lengths of their contiguous sequences were shorter (5 amino acids) and these peptides were not considered for use in antibody production. Back calculations from the yield of tryptic fragments indicated that approximately 50 picomoles of protein had been digested, which corresponded to estimates based upon the Ponceau red staining of the MBP 200R on the nitrocellulose used for the amino acid sequence analysis. The sequence of the peptide itself had no matches in a GenBank search and thus represented a new and unique peptide structure, indicating that the protein from which it was derived was itself new and unique, in keeping with its previously reported unusual structural and functional properties (6–12), particularly that this is the only receptor known to bind specifically to apoB-48 (12).

A. X X X **A E G L M V T G G R**

$MH^+ = 1333.5$

B. HS-C **A E G L M V T G G R**-NH_2

Fig. 1. (A) Amino acid sequence of a tryptic peptide isolated from MBP 200R. First three cycles (designated X-X-X) of the sequencer analysis were ambiguous, however, residues 3–13 were unambiguously assigned as indicated in this figure. From mass spectral analysis this peptide yielded a major mass fragment of 1333.5 which is consistent with a 13-residue tryptic peptide (see text). (B) Amino acid sequence of the synthetic peptide, Cys-10-NH_2, based on the sequence in (A) and designed to resemble internal peptide, used in the production of antipeptide antibodies used in this study.

Strategy for synthetic peptide construction

Because novel protein sequence information was obtained from the microsequence data, we prepared a synthetic peptide to be used in antipeptide antibody production to determine whether the peptide sequenced did indeed arise from trypsinolysis of MBP 200R and not from a potential contaminant. This was critical information to

ensure this sequence information could be used in the design of (degenerate) oligonucleotides for cloning the receptor's cDNA. As indicated above, the 10-residue sequence appeared to be an internal peptide (not N- or C-terminal). Hence, in order to mimic the native state of this peptide, the synthetic peptide was designed with an additional Cys at the amino terminus, which would serve as an attachment site in subsequent reactions of this peptide, and the carboxy-terminus was modified with an amide during peptide synthesis to resemble a peptide bond (Fig. 1B). The peptide, designated Cys-10-NH_2, was purified by HPLC chromatography and characterized by amino acid and mass spectral analysis. The presence of the amino-terminal Cys with a free sulfhydryl group allowed crosslinking of the peptide to the carrier protein, KLH, either by sequential reactions of KLH with m-maleimidobenzoyl-N-hydroxysuccinimide ester and then the peptide or directly with maleimide-activated KLH. The products of both coupling procedures resulted in good antipeptide antibody induction in rabbits.

Antipeptide antibody specificity

Rabbit polyclonal antibodies were generated against the KLH–Cys-10-NH_2 complex and purified as described in Methods. To assess the specificity of the anti-Cys-10-NH_2 antibodies, we passed the total IgG fraction over an immobilized Cys-10-NH_2 affinity matrix and then determined the ability of the IgG fractions that bound, or that failed to bind to the matrix, to detect MBP 200 and 235 by Western blotting. **Figure 2** shows that the antibodies that bound to the immobilized peptide specifically bound to proteins on Western blot analysis (Fig. 2, lanes 2, 3) that had the identical R_fs of MBP 200 and 235 ligand binding activities from detergent extracts of the THP-1 monocyte-macrophages (Fig. 2, lane 1). In contrast, the antibodies that failed to bind to the peptide matrix did not bind to the MBPs (Fig. 2, lanes 4, 5). In this experiment (one representative experiment of five), the unfractionated (starting) ammonium sulfate-precipitated IgGs bound to the MBPs (data not shown). After affinity chromatographic fractionation, however, only those antibodies that specifically bound to the immobilized peptide of the affinity column had MBP reactivity (Fig. 2, lanes 2, 3). That only the antibodies that recognize the peptide domain (AEGLMVTGGR) bind to the MBPs indicates that this sequence is detected within both MBP 200 and 235. Neither the unbound IgG fractions (Fig. 2, lanes 4, 5) nor the washes (not shown) reacted with the MBPs. Additional proteins with this epitope that migrate below MBP 200 in lanes 2 and 3 are probably either MBP precursors or proteolytic degradation products that have lost ligand binding capacity. Here, as previously reported (10), MBP activity is in multiplets which we have shown reflect different oxidation states or disulfide isomers (10). Notably, the multiplets detected by ligand binding activity are exactly replicated by the antipeptide antibodies (Fig. 2, lanes 2, 3). Generally, in all experiments, the antipeptide antibodies reacted better with MBP 200 than with MBP 235, even when the ligand reacted more strongly with MBP 235 than MBP 200. This suggests that the 10-residue peptide sequence is not in the receptor's ligand binding microdomain and/or suggests that the smaller subunit of M_r ~35 kDa that associates with MBP 200 to form MBP 235 (10) partially inhibits antipeptide antibody binding. Thus these experiments indicate that the antipeptide antibodies *1*) specifically recognize the 10-residue peptide and *2*) specifically bind to MBP 200 and 235. These data also indicate that the tryptic peptide was indeed generated from MBP 200R and not from a contaminant; the uniqueness of the peptide's sequence further substantiates the uniqueness of the receptor protein.

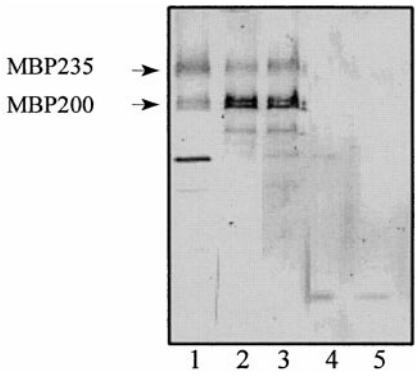

Fig. 2. Specificity of Cys-10-NH_2 antipeptide antibodies. The rabbit polyclonal antisera generated against Cys-10-NH_2 were precipitated with 30–50% $(NH_4)_2SO_4$ and dialyzed extensively against PBS. This fraction was applied to an immobilized Cys-10-NH_2 Sulfolink matrix and allowed to equilibrate for 30 min at 4°C. IgG fractions that did not bind to the immobilized Cys-10-NH_2 did not detect any proteins from a THP-1 detergent extract prepared as described in Methods, lanes 4, 5; after washing, specifically bound antibodies were eluted with citrate buffer, pH 2.0, immediately neutralized, and upon Western analysis (see Methods) detected THP-1 detergent extract proteins (lanes 2, 3; elution fraction 1 and 2, respectively) with the exact mobility of the ligand binding activity, MBP 200 and 235, detected with biotinylated VLDL S_f 100–400 (lane 1). (The sharp lowest band in lane 1 is a nonspecific binding protein sometimes detected when using biotinylated probes.)

All forms of the membrane binding proteins contain the antipeptide epitope

We previously published data that indicated the two major forms of the MBP ligand binding activities convert, upon reduction, into a single binding activity, MBP 200R, with intermediate electrophoretic mobility; MBP 200R possessed the total combined ligand binding activities of the individual MBPs before reduction (10). These findings suggested that MBP 200 and 235 share a large common subunit (MBP 200) that contains the ligand binding domain. Two-dimensional ligand and immunoblotting experiments using the affinity-purified antipeptide antibody corroborate this conclusion. In three separate experiments, the 2-D approach used demonstrates that each MBP, upon reduction, generates equivalent ligand binding species that expresses the peptide epitope (2-dimensional ligand and Western blot analyses with and without reduction in

the second dimension). As detailed in Methods, detergent extracts of THP-1 monocytes were prepared and electrophoresed in the first dimension; 0.6-cm-wide acrylamide strips were then cut from this gel. The acrylamide strips were incubated in buffer in the absence or presence of the reductant, 2-mercaptoethanol (2%), layered perpendicular to the first dimension on top of a second gel and electrophoresed into the second gel. As seen in **Fig. 3A**, when no reductant is added, the ligand binding activities of both MBP 200 and 235 are found on the diagonal with no change in relative mobilities. The identical pattern is obtained after visualization using the antipeptide antibodies (Fig. 3C). Upon reduction, both the ligand binding activities (Fig. 3B) and antipeptide antibody activities (Fig. 3D) have changed mobilities, placing them off the diagonal, demonstrating that both MBPs now have identical mobilities, that of MBP 200R, and identical ligand binding activities and epitope expression. Thus the antipeptide antibodies confirm our previously described model (10) that the reduced form, MBP 200R, is generated from both MBP 200 and MBP 235, and provide new structural evidence that MBP 200 is the major subunit of MBP 235.

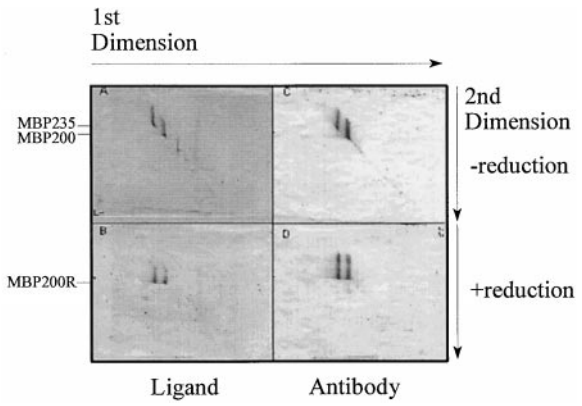

Fig. 3. Two-dimensional ligand blotting and Western analyses of MBP 200 and 235 with and without reduction. THP-1 detergent extracts were prepared as described in Methods. An aliquot using a single notch comb was applied across the top of the first gel, except in the first lane where dye-stained markers were placed to monitor the progress of the electrophoresis. This detergent extract containing the MBPs was electrophoresed under normal conditions. At the completion of the electrophoresis (first dimension), four 0.6-cm strips were sliced the length of the polyacrylamide gel and were either incubated with 2% 2-mercaptoethanol in pH 6.8 sample buffer or in sample buffer alone at room temperature for 5 min. Each of these gel strips was then layered lengthwise on the top of an individual second gel and the electrophoresis was carried out again under the same conditions as in the first and electrotransferred to nitrocellulose for ligand blot and Western analysis. (A) Ligand blot with no reduction prior to the second dimension and no shift in ligand binding activity from the diagonal. (C) Western analysis, with no reduction prior to the 2-dimension, using the Cys-10-NH_2 affinity-purified antipeptide antibodies (diluted 1 to 1000) indicating coincident epitope expression with MBP ligand activity as seen in Fig. 3A. (B) Ligand blot with reduction in the second dimension indicates a shift of both activities off the diagonal with identical R_fs and (D) the corresponding Western blot demonstrating equivalency of ligand binding and epitope expression in the MBP 200R produced from each MBP precursor, MBP 200 and 235.

Reactivity of antipeptide antibodies with extracts of other reticuloendothelial cells but not fibroblasts or CHOs

The antipeptide domain represents a unique sequence, as determined by database searches, but is only a small fraction of the total protein structure of MBP 200. To determine whether this epitope is expressed by other reticuloendothelial cells that express the apoB receptor pathway and not by cells that lack the pathway, a series of ligand and Western blotting studies were carried out comparing THP-1 extracts with membrane extracts from human blood-borne monocyte-macrophages, human U937 monocytic cells, a spontaneously transformed human umbilical vein endothelial cell line (ECV), human skin fibroblasts, Chinese hamster ovary (CHO) cells, and a bovine adrenal cortex membrane DEAE fraction (partially purified LDL R). Results from multiple experiments with and without reduction (**Fig. 4**) demonstrate that all monocytes and the ECVs express the receptor (i.e., have coincident ligand binding and epitope expression) whereas the fibroblasts, CHOs, or adrenal cortex membrane (LDL R) preparation do not. The experiment in Fig. 4, panel A compares non-reduced THP-1 extracts (lanes 1, 4) with the bovine adrenal LDL R preparation (lanes 2, 5), and with fibroblasts (lanes 3, 6) for ligand binding (lanes 1–3) and antipeptide antibody binding (lanes 4–6). The ligand, HTG-VLDL, binds to MBP 200 and 235 in THP-1 extracts (lane 1), to the LDL R in adrenal cortical membrane extracts (lane 2), and to a diffuse, high molecular weight protein in fibroblasts that additional studies identified as the LRP (data not shown). In contrast, the antipeptide antibody binds only to MBP 200 and 235 in THP-1 extracts (Fig. 4A, lane 4) but to nothing in either the adrenal cortex membrane extracts (Fig. 4, lane 5) or the human skin fibroblast extracts (Fig. 4A, lane 6). Panel 4B demonstrates that human U937 monocyte extracts, like THP-1 extracts, express MBP ligand binding activity in non-reduced (Fig. 4B, lane 1) and reduced (not shown) extracts and antipeptide binding activity, i.e., MBP 200R epitope expression, in non-reduced (Fig. 4B, lane 2) and in reduced samples (Fig. 4B, lane 3). Panel 4C shows that the ligand binding activities of THP-1 and HMM extracts (lanes 1, 2) coincide with antipeptide binding (lanes 3, 4) without reduction (Fig. 4C, lanes 1–4). Upon reduction, MBP 200R ligand binding activity is observed in both THP-1 and HMMs (lanes 5, 6) that is coincident with antipeptide antibody binding to HMM (lane 7) and to THP-1 (shown in Fig. 3). In addition, as previously published (10), two lower molecular weight bands are evident in reduced THP-1 extracts that nonspecifically bind any IgG and are therefore detected in ligand blots (Fig. 4C, lane 5, and 4D, lane 1). Finally, Fig. 4, panel D compares ligand binding activity in reduced THP-1 (lane 1) with reduced ECV extracts (lane 2) and antipeptide activity in reduced ECV extracts (lane 3). In contrast to all other cells examined, ECVs express a high molecular weight protein that binds both the ligand (Fig. 4D, lane 2) and the antipeptide antibody (Fig. 4D, lane 3). This protein activity is not affected appreciably by reduction, does not crossreact with anti-LRP (not shown), and may represent either an

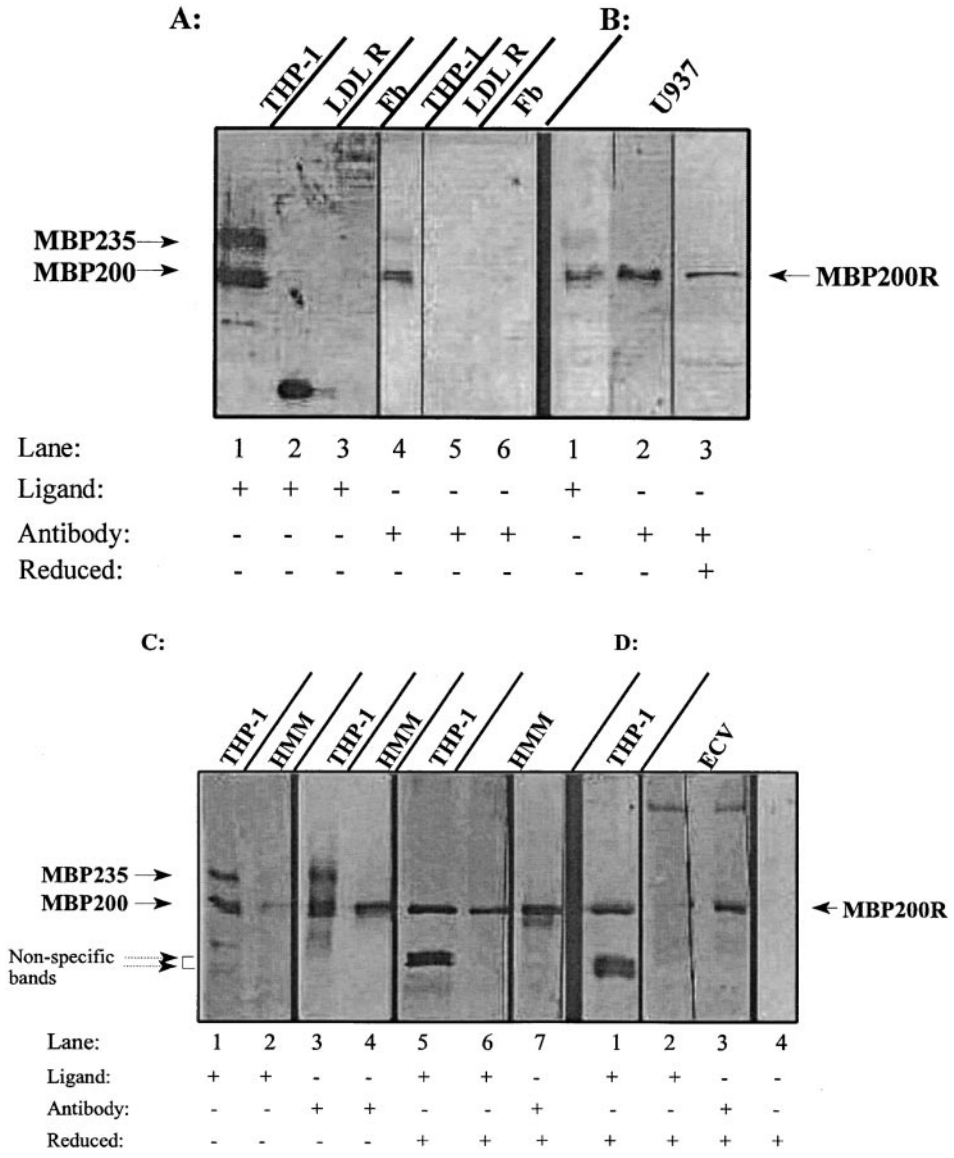

Fig. 4. Antipeptide antibodies recognize MBPs in human blood-borne monocyte-macrophages (HMM), human U937 monocytes, and in a human endothelial cell line, ECV, but not in human fibroblasts or partially purified bovine LDL receptor preparations. Detergent cell extracts were prepared as described in Methods. Panel A: Lanes 1, 2, and 3 represent the ligand binding activities of THP-1 extracts, partially purified bovine LDL receptor preparations, and human fibroblast extracts, respectively, using HTG-VLDL S_f 100–400 (5.0 μg/ml) as a ligand and visualized as described in Methods. Lanes 4, 5, and 6 are the corresponding immunoblots with antipeptide antibody detection. Panel B: Lanes 1 and 2 are U937 extracts without reduction and lane 3 is with reduction as outlined in Methods. Lane 1 demonstrates the ligand binding activity of the U937 detergent extracts, whereas lanes 2 and 3 illustrate the antipeptide antibody detection in the absence (lane 2) and presence (lane 3) of reductant. Panel C: Lanes 1, 3, and 5 are THP-1 detergent extracts; lanes 2, 4, 6, and 7 are detergent (octylglucoside) extracts of HMMs. Ligand binding activities are shown in lanes 1 and 2 for unreduced and in lanes 5 and 6 for reduced detergent extracts of THP-1 and HMMs, respectively. Antipeptide antibody detection is seen in lane 3 (THP-1, unreduced) and lanes 4 (HMMs, unreduced) and 7 (HMMs, reduced). Panel D: All lanes are reduced detergent extracts from THP-1s and an endothelial cell line, ECV. Lane 1 (THP-1) and Lane 2 (ECV) demonstrate the ligand binding activities. Lane 3 (ECV) is detection by the antipeptide antibody. Lane 4 is a control where the primary antipeptide antibody is not included, which indicates the proteins detected by the antipeptide antibody in lane 2 are specifically recognized by this antibody.

aggregate of MBP 200 or may be another related TGRLP binding protein that also contains the same peptide sequence. MBP 200R ligand binding activity is barely evident in the reduced ECV extract (lane 2) but MBP 200R is readily detected with the antipeptide antibodies in reduced ECV extracts (lane 3). Lane 4, Fig. 4D is an ECV reduced control lane which was incubated with neither the ligand nor the antipeptide antibody, only with the final AP linked goat anti-rabbit IgG. This demonstrates that the ECVs, unlike THP-1 reduced extracts, contain no non-

specific IgG-binding proteins and that the MBP 200R and the high molecular weight, EC-specific ligand and antipeptide binding protein is specifically detected by ligand (lane 2) or antipeptide antibody (lane 3). Thus multiple experiments with each cell type represented in the collage of Fig. 4 demonstrate that the three types of human monocytes and the ECVs but not the LDL receptor preparations, the fibroblasts, or CHOs (not shown) express MBP 200, 235 and/or MBP 200R consistent with previous cell binding and ligand blotting studies which showed the latter two cell types to be receptor negative. Reduction increases the sensitivity of detection by both ligand and antibody (Fig. 4C, lanes 5–7). One possible reason is that the multiple forms of the receptor activity/protein collapse into a single species (see Fig. 3B and D) with additive ligand binding activities as previously documented (10) and with additive antipeptide antibody binding. Second, the antipeptide antibody may also have increased avidity for the reduced form.

FACS analysis using antipeptide antibodies demonstrates the cell surface location of MBPs in THP-1 monocyte-macrophages

For a protein to function as a receptor for an extracellular ligand, it must be located on the cell surface. We previously published indirect evidence for the cell surface location of the MBPs in THP-1 monocyte-macrophages using proteolysis of intact cells, which demonstrated a concomitant loss of high affinity, specific binding of ligand both to cells and to MBPs by parallel cell studies and ligand blot analyses; the specific binding both to cells and to MBPs recovered in parallel with time (10). To provide direct evidence for the cell surface location of the MBPs, we used the antipeptide antibodies for FACS analysis of THP-1 monocytes (**Fig. 5**) and HMMs (not shown). CDw49d (VLA-4, α-chain) was included as a known surface antigen for a positive control. As seen in Fig. 5, the THP-1 monocyte population was shifted after THP-1s were preincubated with antipeptide antibodies relative to controls (omission of the primary antibody or replacement with non-immune rabbit IgG). This directly documents the surface location of the antipeptide epitope. Thus FACS analysis confirms the cell surface location of the apoB receptor on monocytes.

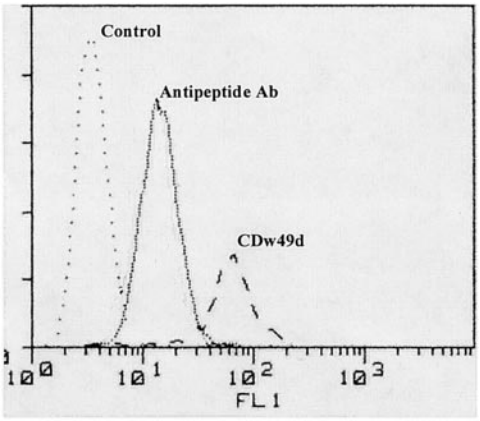

Fig. 5. Fluorescence-activated cell sorting analysis demonstrates that the antipeptide antibody detects the presence of TGRLP/apoB R (MBPs) on the surface of THP-1 monocytes. Anti-CDw49d was used as a positive control and no primary antibody served as the negative control. Incubations are described in Methods.

DISCUSSION

We have shown previously by cell and biochemical studies that unique membrane binding proteins, MBP 200 and 235, exist for both hepatic and intestinally derived TGRLP in human monocyte-macrophages (8–12). These MBPs comprise an apoE-, lipoprotein lipase-, and heparan sulfate proteoglycan-independent receptor pathway for the binding and uptake of these apoB-containing lipoproteins specifically by reticuloendothelial cells. Uptake of TGRLP by this pathway causes both triglyceride and cholesterol accumulation in THP-1 and blood-borne monocyte-macrophages in a rapid (≤4 h), saturable fashion (9); the lipid that accumulates reflects the composition of the TGRLP particle (6). We also demonstrated recently that the binding of TGRLP to these MBPs is mediated by apoB, more specifically by a region within the N-terminal, apoB-48 domain (12). Moreover, apoB-48 alone is sufficient to mediate binding of TGRLP to MBP 200 and 235; apoB-100-containing TGRLP may bind through an analogous domain in apoB-100 (12). This mode of uptake by monocyte-macrophages is distinct from that for the uptake of these TGRLP particles by members of the LDL R family. The latter is mediated by apoE and is essential for their normal disposal by liver pathways involving the LDLR and LRP (see review, 18). Furthermore, the binding of TGRLP to the apoB/TGRLP receptor is inhibited by LpL (12), in contrast to TGRLPs' enhanced binding to LDLR or LRP via LpL. This inhibition is consistent with the reported binding of LpL to the N-terminal region of apoB-100 (19). Other characteristics that distinguish the ligand binding properties of the apoB/TGRLP receptor from the apoE-dependent lipoprotein receptors are that TGRLP binding is not inhibited by lactoferrin, heparin, or antibodies to apoE (12).

In the current study we report for the first time that a purified, reduced ligand binding form of THP-1 MBPs, MBP 200R, has been partially characterized by gas-phase microsequence analysis and found to contain unique internal peptide sequences. A synthetic peptide was prepared using this initial sequence data. Antibodies developed against the synthetic peptide bind to all active (ligand blotting) forms of the receptors with the identical mobilities in two-dimensional as well as normal SDS-PAGE after electrotransfer to nitrocellulose membranes. Although the antipeptide antibodies bind to all active forms of the MBPs, they do not inhibit binding of TGRLP to the MBPs (data not shown). This is not surprising, however, considering that this epitope represents a small microdomain of MBP 200. This microdomain therefore does not appear to be directly involved in ligand binding although the 2-D studies demonstrate that it is within the ligand binding

subunit MBP 200 and the FACS analyses indicate it is expressed on the surface of MMs, as necessary for a cell surface receptor.

These studies provide the first direct protein data on the unique structure of the apoB/TGRLP receptor of human monocyte-macrophages and the binding characteristics of the first anti-receptor antibodies produced. The peptide sequence is not found in any other known proteins, including lipoprotein receptors of the LDL R family or the scavenger receptor family. These results support the conclusion that these monocyte-macrophage MBPs represent a new family of lipoprotein receptors that bind apoB (12). In addition, the antipeptide antibody data confirmed that the correct protein was isolated from the THP-1 human monocytes and sequenced, as the antipeptide antibody binding corresponded identically to the ligand binding activities both in 1- and 2-D Western blots. The amino acid sequence data served not only to design a peptide for antibody production but also as the basis for the design of degenerate oligonucleotides used to produce a PCR fragment from a THP-1 monocyte-macrophage library which has been used as a probe for the identification of clones from a λgt 10 THP-1 monocyte cDNA library (Clontech, Palo Alto, CA). Completion of the apoB/TGRLP receptor cDNA sequence is in progress. To date the cDNA sequence data confirms the unique structure of the apoB/TGRLP receptor.

The antipeptide antibodies demonstrate the presence of the apoB/TGRLP receptor in the reticuloendothelial cells, i.e., HMMs, THP-1 and U937 monocyte-macrophages and in a human umbilical vein endothelial cell line, ECV, but not in human fibroblasts or CHO cells as had been previously determined indirectly by cell binding, uptake, and lipid accumulation studies. In addition, the partially purified bovine LDL receptor detected by ligand blotting (Fig. 4A, lane 2) was not detected by the antipeptide antibody (Fig. 4A, lane 5). This further demonstrates specificity of the antipeptide antibody and confirms previous cell binding and ligand blotting experiments that indicated that the MBPs are not related to the LDL receptor family (8–12). The diffuse, high molecular weight HTG-VLDL binding activity seen in fibroblast extracts (Fig. 4A, lane 3) likewise does not bind the antipeptide antibodies and appears to be the LRP, as determined by using anti-LRP, kindly provided by Dr. D. Strickland (data not shown). Finally, using the human endothelial cell line, ECV, we demonstrate the presence of MBP 200R and a higher MW species coincident with ligand binding activity (Fig. 4D, lane 2) and antipeptide antibody activity (Fig. 4D, lane 3) in ECV detergent extracts. The higher MW species may be an oligomeric form of the receptor or, more likely because of its discreetness and its reproducibility in ECVs and its absence in MMs and other cells, another closely related, EC-specific apoB receptor, suggesting that an apoB receptor family exists in reticuloendothelial cells. The ability of the antipeptide antibodies to bind to THP-1 monocytes (Fig. 5) and HMMs (not shown), as demonstrated by FACS analysis, indicates the surface location of the apoB/TGRLP receptor and the potential usefulness of these highly specific antibodies in elucidating the cell biology of this macrophage lipoprotein receptor.

As previously suggested (9–12), we hypothesize that this monocyte-macrophage apoB/TGRLP receptor normally serves a nutritional role by delivering essential lipids and lipid-soluble vitamins to circulating monocytes and accessible macrophages, as in the bone marrow and spleen. In pathological states with persistent chylomicrons, this pathway could lead to monocyte-macrophage-derived, lipid-engorged foam cells as seen in vivo (20, 21). Its presence in human endothelial cells may explain, at least in part, the deleterious effects of HTG-VLDL on EC-mediated fibrinolysis that we have observed in vitro (22, 23). If similar phenomena occur in vivo, this reticuloendothelial apoB receptor may be, in part, responsible for the increased risk for cardiovascular disease in hypertriglyceridemia or prolonged postprandial lipemia due to apoB R-mediated foam cell formation and endothelial dysfunction.

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REFERENCES

1. Plump, A. S., J. D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J. G. Verstuyft, E. M. Rubin, and J. L. Breslow. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71:** 343–353.
2. Zhang, S. H., R. L. Reddick, J. A. Piedrahita, and N. Maeda. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* **258:** 468–471.
3. Nakashima, Y., A. S. Plump, E. W. Raines, J. L. Breslow, and R. Ross. 1994. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* **14:** 133–140.
4. Reddick, R. L., S. H. Zhang, and N. Maeda. 1994. Atherosclerosis in mice lacking apoE evaluation of lesional development and progression. *Arterioscler. Thromb.* **14:** 141–147.
5. Bellosta, S., R. W. Mahley, D. A. Sanan, J. Murata, D. L. Newland, J. M. Taylor, and R. E. Pitas. 1995. Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. *J. Clin. Invest.* **96:** 2170–2179.

6. Gianturco, S. H., W. A. Bradley, A. M. Gotto, Jr., J. D. Morrisett, and D. L. Peavy. 1982. Hypertriglyceridemic very low density lipoproteins induce triglyceride synthesis and accumulation in mouse peritoneal macrophages. *J. Clin. Invest.* **70:** 168–178.
7. Gianturco, S. H., S. A. Brown, D. P. Via, and W. A. Bradley. 1986. The β-VLDL receptor pathway of murine $P388D_1$ macrophages. *J. Lipid Res.* **27:** 412–420.
8. Gianturco, S. H., A. H-Y. Lin, S. C. Hwang, J. Young, S. A. Brown, D. P. Via, and W. A. Bradley. 1988. Distinct murine macrophage receptor pathway for human triglyceride-rich lipoproteins. *J. Clin. Invest.* **82:** 1633–1643.
9. Gianturco, S. H., M. P. Ramprasad, A. Lin, R. Song, and W. A. Bradley. 1994. Cellular binding site and membrane binding proteins for triglyceride-rich lipoproteins in human monocyte-macrophages and THP-1 monocytic cells. *J. Lipid Res.* **35:** 1674–1687.
10. Ramprasad, M. P., R. Li, W. A. Bradley, and S. H. Gianturco. 1995. Human THP-1 monocyte-macrophage membrane binding proteins: distinct receptor(s) for triglyceride-rich lipoproteins. *Biochemistry.* **34:** 9126–9135.
11. Ramprasad, M. P., R. Li, S. H. Gianturco, and W. A. Bradley. 1995. Purification of the human THP-1 monocyte-macrophage triglyceride-rich lipoprotein receptor. *Biochem. Biophys. Res. Commun.* **210:** 491–497.
12. Gianturco, S. H., M. P. Ramprasad, R. Song, R. Li, M. L. Brown, and W. A. Bradley. 1998. Apolipoprotein B-48 or its apolipoprotein B-100 equivalent mediates the binding of triglyceride-rich lipoproteins to their unique human monocyte-macrophage receptor. *Arterioscler. Thromb. Vasc. Biol.* **18:** 968–976.
13. Gianturco, S. H., and W. A. Bradley. 1986. The role of apolipoprotein processing in receptor recognition. *Methods Enzymol.* **129:** 319–344.
14. Lindgren, F. T., L. C. Jensen, and F. T. Hatch. 1972. The isolation and quantitative analysis of serum lipoproteins in blood lipids and lipoproteins. *In* Blood Lipids and Lipoproteins. G. J. Nelson, editor. Wiley Interscience, New York. 181–274.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
16. Helenius, A., and K. Simons. 1971. Removal of lipids from human plasma low density lipoprotein by detergents. *Biochemistry.* **10:** 2542–2547.
17. Gianturco, S. H., A. M. Gotto, Jr., R. L. Jackson, J. R. Patsch, H. D. Sybers, O. D. Taunton, D. L. Yeshurun, and L. C. Smith. 1978. Control of 3-hydroxy-3-methylglutaryl-CoA reductase activity in cultured human fibroblasts by very low density lipoproteins of subjects with hypertriglyceridemia. *J. Clin. Invest.* **61:** 320–328.
18. Cooper, A. D. 1997. Hepatic uptake of chylomicron remnants. *J. Lipid Res.* **38:** 2173–2192.
19. Choi, S. Y., P. Sivaram, D. E. Walker, L. K. Curtiss, D. G. Gretch, S. L. Sturley, A. D. Attie, R. J. Deckelbaum, and I. J. Goldberg. 1995. Lipoprotein lipase association with lipoproteins involves protein-protein interaction with apolipoprotein B. *J. Biol. Chem.* **270:** 8081–8086.
20. Fredrickson, D. S., J. L. Goldstein, and M. S. Brown. 1978. The familial hyperlipoproteinemias. *In* The Metabolic Basis of Inherited Diseases. J. G. Stanbury, M. F. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, New York. 604–655.
21. Parker, F., J. D. Bagdade, G. F. Odland, and E. L. Bierman. 1970. Evidence for the chylomicron origin of lipids accumulating in diabetic eruptive xanthomas: a correlative lipid biochemical, histochemical, and electron microscopic study. *J. Clin. Invest.* **49:** 2172–2187.
22. Li, X., J. C. Koons, R. L. Benza, M. Parks, V. K. Varma, W. A. Bradley, S. H. Gianturco, K. B. Taylor, J. R. Grammar, E. M. Tabengwa, and F. M. Booyse. 1996. Hypertriglyceridemic VLDL decreases plasminogen binding to endothelial cells and surface localized fibrinolysis. *Biochemistry.* **35:** 6080–6088.
23. Li, X., H. E. Grenett, R. L. Benza, S. Demissie, S. L. Brown, E. M. Tabengwa, S. H. Gianturco, W. A. Bradley, G. M. Fless, F. M. Booyse. 1997. Genotype-specific transcriptional regulation of PAI-1 expression by hypertriglyceridemic VLDL and Lp(a) in cultured human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3215–3223.