Amino terminus of apolipoprotein B suffices to produce recognition of malondialdehyde-modified low density lipoprotein by the scavenger receptor of human monocyte-macrophages

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Abstract Malondialdehyde, a product of lipid peroxidation, produces threshold conversion of low density lipoprotein (LDL) to a form recognized by the type I and type II scavenger receptors of monocyte-macrophages. To investigate whether localized domains of human apoB-100 protein provide recognition determinants, we tested the ability of several different apoB-bearing particles to interact with the scavenger receptor of human monocyte-macrophages. Genetically engineered, carboxyl-terminally truncated apoB proteins assembled into lipoprotein form were labeled by fluorescent dye. Fluorescence microscopy and quantitative fluorescent spectrophotometry showed that purified particles containing as little as 23% of the apoB amino-terminus were internalized by the scavenger receptor after, but not before, malondialdehyde modification. There was no recognition of the particles by the LDL receptor. Similar results were obtained with human plasma LDL homozygous for carboxyl-terminally truncated apoB-45.2. Liposome-incorporated fusion protein containing apoB residues 547–735 displayed specific uptake by the scavenger receptor without modification by malondialdehyde. In contrast, fusion proteins containing apoB residues 3029–3132 or a short amino terminal segment failed to interact. Thus, primary sequence presented by residues 1–1084 sufficed to produce recognition of modified LDL by the scavenger receptor. These receptor-combining domains were sequestered when secreted in lipoprotein form and were expressed upon malondialdehyde modification. When packaged exogenously in liposome form, fusion protein containing apoB residues 547–735, containing approximately 4% of the primary sequence, mediated scavenger receptor-dependent uptake and hydrolysis. Our findings provide an additional function for the amino-terminal region of apoB and demonstrate that primary sequence presented by the first 23% of apoB-100 protein suffices to produce recognition of malondialdehyde-modified LDL by the scavenger receptor of human monocyte-macrophages.—**Kreuzer, J., A. L. White, T. J. Knott, M-L. Jien, M. Mehrabian, J. Scott, S. G. Young, and M. E. Haberland.** Amino terminus of apolipoprotein B suffices to produce recognition of malondialdehyde-modified low density lipoprotein by the scavenger receptor of human monocyte-macrophages. *J. Lipid Res.* 1997. **38:** 324–342.

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Apolipoprotein (apo) B-100, a protein of 4,536 amino acids, is the predominant protein constituent of low density lipoprotein (LDL) and contains the binding domains responsible for cellular uptake mediated by the LDL receptor (1). Modification of LDL protein lysines by selective chemical derivatization or transition metal-induced oxidation converts the lipoprotein to anionic forms recognized by scavenger receptors of macrophages (2). While the uptake of LDL by the LDL re-

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate; PPACK, D-phenylalanine-L-proline-L-arginine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBS/0.1, TBS/0.3, and TBS/0.5, TBS containing 0.1%, 0.3%, and 0.5% Tween-20, respectively; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; flu, fluorescence units.

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ceptor is regulated by intracellular cholesterol levels, the internalization of modified LDL by scavenger receptors is not. Consequently lipoprotein-derived cholesterol accumulates and generates cholesteryl ester-laden macrophages with histological features closely resembling the foam cells of the atherosclerotic lesion. Goldstein and coworkers (3), based upon seminal studies conducted with acetyl-LDL in 1979, hypothesized that conversion of plasma LDL in vivo to forms recognized by macrophage scavenger receptors accounted for foam cell formation in familial hypercholesterolemia. In 1980 Fogelman and coworkers (4) proposed that modification of arterial wall LDL by malondialdehyde and other products of lipid peroxidation accounted for, and was a prerequisite to, accumulation of lipoprotein cholesterol in cells of the atherosclerotic reaction. These early proposals have evolved into the broad concept that lipoprotein alterations by products of lipid peroxidation, generated during oxidative modification of LDL, play both early and sustaining roles in the pathogenesis of atherosclerosis (reviewed in refs. 5, 6).

The cloning of the type I and type II macrophage scavenger receptors (7, 8), differing only in a cysteine-rich extracellular carboxyl-terminus, has provided insight into the predicted structure and function of these cellular surface proteins. The type I and type II macrophage scavenger receptors display broad but circumscribed specificity for anionic molecules, and may not only function in the development of atherosclerosis but also participate in host defense (reviewed in ref. 9) and macrophage adherence (10, 11). In studies with the inhibitor polyinosinic acid, Pearson, Rich, and Krieger (12) have demonstrated that formation of base-quartet-stabilized four-stranded helices accounts for receptor binding, and have provided compelling evidence that discrete, organized patches of anionic clusters of ligands show electrostatic interaction with the receptor collagenous domain.

Despite rapid progress in the structure and function of the type I and type II scavenger receptors, the determinants that trigger scavenger receptor recognition of modified LDL have yet to be fully characterized. This is due, on the one hand, to the broad ligand specificity of the type I and type II scavenger receptors and, on the other hand, to the chemical approaches taken to generate modified LDL. Transition metal-dependent, oxidative modification of LDL has provided considerable insight into the potential role of lipid peroxidation in atherogenesis, and has served as the principal model of arterial-modified LDL (reviewed in ref. 5). Oxidative modification produces covalent derivatization of lysyl residues as well as complex chemical, biochemical, and physical changes in lipoprotein structure. Steinbrecher et al. (13), Zhang, Yang, and Steinbrecher (14) and Alaiz and coworkers (15) have demonstrated that lysine derivatization by autooxidation products of polyunsaturated fatty acids accounts for ligand conversion by oxidative modification. Parthasarathy and coworkers (16) have shown that scavenger receptors bind detergent-solubilized peptide fragments of copper oxidized-LDL. These reports together point to the apoB-100 protein component of oxidatively modified LDL as the carrier of receptor combining determinants. Zhang et al. (14) and Alaiz et al. (15) have proposed that production of anionic array(s) alone, independent of primary sequence, mediate receptor interaction. Interpretation of these investigations has been complicated by evidence that nonidentical but partially interacting sites on the type I and type II scavenger receptors account for binding of oxidatively modified LDL and the classic acetyl-LDL ligand (17). Moreover, several macrophage binding sites distinct from type I and type II scavenger receptors also show high affinity binding of oxidatively modified LDL, and ligand interaction with these additional sites depends upon the degree of LDL lysine derivatization (18). Cloning by expression has identified CD36 (19) and the Fc gamma RII-B2 receptor (20) as functional macrophage receptors for oxidatively modified LDL, and cell biology studies have implicated macrophage sites for oxidatively damaged erythrocytes as receptors for oxidatively modified LDL (21).

Elucidation of determinants governing interaction of oxidatively modified LDL with the type I and type II scavenger receptors thus has presented a number of challenges. We have chosen a discrete product of lipid peroxidation to examine this question. Malondialdehyde, a three carbon product of lipid peroxidation, is generated during transition metal-dependent oxidation of LDL (22). Chemical analyses have shown that malondialdehyde derivatizes the apoB-100 protein of LDL through intramolecular crosslinking of lysyl residues and generates a more negatively charged lipoprotein particle through neutralization of the ε-amino group of lysyl residues (23). Malondialdehyde readily converts LDL in vitro to generate threshold recognition by the scavenger receptors (23, 24). Malondialdehyde acts in vivo to modify apoB-100-containing lipoproteins selectively retained within the atherosclerotic lesion (25, 26). In contrast to oxidatively modified LDL, malondialdehyde-modified LDL competes mole-for-mole with acetyl-LDL (27, 28) for binding to the type I and type II scavenger receptors. The ability of diverse polyanionic compounds to inhibit receptor interaction of malondialdehyde- and acetyl-LDL further indicates that a specific array of negative charges is required for ligand binding (12, 28). Thus, molecular features that typefy interaction of chemically modified, acetyl- and malondi-

aldehyde-LDL with the type I and type II scavenger receptors include abolition of positive charge through lysine derivatization and/or a surface cluster of anionic charge. Whether discrete domains or primary sequence of apoB-100 provide specificity for interaction with the type I and type II scavenger receptors has not been established.

In this report, we provide evidence that the amino terminus of apoB-100 suffices to induce receptor recognition and ligand uptake by the type I and type II scavenger receptors of human monocyte-macrophages.

MATERIALS AND METHODS

Materials

Sodium (^{125}I)iodide (15 mCi/μg) and ECL kits were purchased from Amersham, Arlington Heights, IL; goat anti-human apoE, benzamidine and D-phe-L-pro-L-arg chloromethyl ketone (PPACK) from Calbiochem, La Jolla, CA; sheep anti-human apoB antiserum, secondary species-specific horseradish peroxidase-labeled antibodies, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and aprotinin from Boehringer Mannheim, Indianapolis, IN; and 1,1′ dioctadecyl-3,3,3′,3′ tetramethylindo-carbocyanine perchlorate (DiI) from Molecular Probes, Eugene, OR. The rat liver tumor cell line McArdle-Rh 7777 stably transfected with genetically engineered, carboxyl-terminally truncated human apoB-53 (pB53, (29)) was provided by Drs. Z. Yao and B. J. McCarthy, Gladstone Institute for Cardiovascular Disease, San Francisco, CA. Plasma from a patient homozygous in apoB-45.2 (30) was a gift from Dr. B. Jacotot, Hôpital Henri-Mondor, Creteil, France and Dr. B. Bihain, Universitè de Rennes, Rennes, France. β-galactosidase-apoB fusion proteins (31) purified by immunoaffinity chromatography with immobilized polyclonal antibody to LDL and rabbit antisera to murine apoA-I (32) were provided by Dr. A. J. Lusis, UCLA. Rabbit antiserum to human apoB peptide 890–908 (33) was provided by Dr. T. L. Innerarity, Gladstone Institute of Cardiovascular Disease, San Francisco, CA. All other supplies and reagents were obtained from sources previously reported (23, 34).

Subjects

Normal subjects at the University of California, Los Angeles were recruited from the staff and student body as donors for blood monocytes and LDL. No one received drugs that might have affected serum cholesterol levels or lipid metabolism. All had normal hematocrits, leukocyte and differential counts, serum cholesterol, and triglycerides. Informed consent was obtained in writing from each subject.

Human monocyte isolation and culture

Five hundred ml of blood was taken after an overnight fast, and the monocytes were separated from 300 ml of venous blood by a modification of the Recalde method (35). The cells were classified and their viability was determined as described. Autologous serum for cell culture was prepared from the remaining 200 ml of blood. Human monocytes were suspended in 30% autologous serum in Iscove's modified Dulbecco's medium supplemented with 2 mM glutamine, 8 μg insulin/ml, and 0.25 μg fungizone/ml (designated medium B). For studies with radioiodinated lipoproteins, samples (0.5 ml) of the cell suspension containing 0.5×10^6 monocytes were transferred to 2.0-cm^2 polystyrene wells. For studies by fluorescence microscopy with DiI-labeled lipoproteins, cells were cultured at a density of 0.28×10^6/cm^2 either on tissue culture inserts (catalogue number 35 3095, Becton-Dickinson, Lincoln Park, NJ) or on 8-well chamber slides (catalogue number 177445, Nunc, Naperville, IL). For other studies by quantitative fluorescence spectrophotometry, samples (5 ml) of the cell suspension containing 5×10^6 monocytes were transferred to 28-cm^2 polystyrene dishes. The cells were maintained 7–10 days at 37°C in a humidified, 5% CO_2 incubator. The medium was aspirated and replaced with fresh medium of the same composition twice weekly. Because of the media changes and the washes, the cells were 99% monocyte-macrophages prior to initiation of each experiment. More than 95% of the cells were viable at the conclusion of experimentation.

Cell culture of continuous cell lines

Permanently transfected McArdle-Rh 7777 rat hepatoma cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Hyclone, Logan, UT), 10% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 (Gibco, Gaithersburg, MD) at 37°C in a humidified, 7% CO_2 incubator (36, 37). G418 was omitted in media for maintenance of the parental cell line (McA-RH 7777, ATCC-CR1.1601).

Isolation of lipoproteins and apoB-containing particles

Human LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated from the serum of individual, fasted human subjects by ultracentrifugation (38), dialyzed against 0.01 M sodium phosphate, pH 7.4, containing 0.15 M sodium chloride and 0.01% EDTA (buffer A) and stored at 5°C. Plasma (5 ml) from a pa-

tient homozygous in apoB-45.2 was incubated with DiI as described by Pitas and coworkers (39), and LDL isolated at d 1.019–1.080 g/ml (30) as described above. The concentration of apoB-45.2 was determined by sandwich radioimmunoassay as described by McCormick and coworkers (40).

Particles containing genetically engineered, carboxyl-terminally truncated human apoB protein, labeled with DiI during cellular culture, were collected from conditioned media. Rat McArdle-Rh 7777 cell lines were grown to 60–70% confluency in 75-cm^2 flasks. The adherent cells were washed three times with phosphate-buffered saline and Dulbelcco's modified Eagle's medium containing 0.01% bovine serum albumin and 3 μM DiI (prepared from a stock solution of 3 mM DiI in ethanol) was added. The conditioned media were removed after an additional 48 h of culture and centrifuged at 1100 *g* for 10 min at 4°C. Protease inhibitor mix, modified after Cardin and coworkers (41), was prepared in phosphate-buffered saline and added to the cell free supernatant to give final concentrations of 2 mM benzamidine, 1 μM PPACK, 0.01% aprotinin, 20 μg/ml lima bean trypsin inhibitor, 20 μg/ml soybean trypsin inhibitor, 0.01% PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 2 mM EDTA.

Aliquots (5 ml) were adjusted with solid potassium bromide to a density of 1.063 g/ml (products of B 36 and pB 53 cell lines) and centrifuged in a 70.1 Ti rotor (Beckman, Fullerton, CA) for 24 h at 40,000 rpm at 4°C to remove rat apoB-100-containing VLDL. The top 2 ml of each tube was removed and the infranatants were adjusted to 1.15 g/ml (product of pB 53 cell line) or 1.21 g/ml (product of B 36 cell line). The product of the B 23 cell line was adjusted to 1.21 g/ml and centrifuged as above. Samples were then collected in equal volumes of supernatant, buffer cushion, and infranatant. The buffer cushions were discarded, and the supernatants and infranatants were dialyzed at 5°C with four changes against 4 liters of buffer A.

β-Galactosidase–apoB fusion proteins were products of apoB genomic DNA cloned into the plasmid expression vector λgt11 containing the inducible β-galactosidase promotor and collected from lysates of isopropyl thiogalactoside-induced lysogens made in *E. coli* Y1089. The clones and their protein products have been previously characterized by Mehrabian and coworkers (31) and nucleotide assignments given by Knott and coworkers (42, 43). The purified fusion proteins were incorporated into stable lipid microemulsions by methods described by Ginsburg and coworkers (44). Microemulsions were prepared by dispersing cholesteryl oleate (NuChek Prep., Inc., Elysian, MN) and dimyristoyl phosphatidylcholine (Sigma, St. Louis, MO) at a 1:1 weight ratio in 0.1 M potassium chloride, 0.01 M Tris-HCl, pH 8.0, and sonicating the mixture for 5 h under a nitrogen atmosphere at a temperature of 52°C. The solution was fractionated by ultracentrifugation and microemulsions were collected between density 1.006 and 1.22 g/ml exactly as described (45). The microemulsions were dialyzed against 0.05 M sodium chloride, 0.05 M sodium carbonate, pH 9 (buffer B) and concentration was determined by colorimetric assay for cholesterol (Boehringer Mannheim) and inorganic phosphate (Sigma). Each fusion protein (0.75–3 mg) was dialyzed against buffer B containing 10 mM deoxycholate and the solution was held in an open-top dialysis bag (Spectrapor 4, Spectrum Medical Industries, Los Angeles, CA). The protein-containing dialysis bag was suspended in buffer B with rapid stirring at a ratio of 1:3,000 (v/v), and the microemulsion was transferred to the fusion protein solution by peristaltic pump at a rate of 0.5 ml/h to give a final ratio of 3:1 (cholesteryl oleate–protein, w/w). Upon completion, the microemulsion-incorporated proteins were dialyzed overnight at 4°C against buffer B to remove residual detergent. Compositional analyses of microemulsion-incorporated, radioiodinated fusion protein were performed after gel chromatography on Sepharose CL-4B (2.5 × 60 cm) in buffer B as previously described (44, 45).

Labeling and chemical modification of lipoproteins

Labeling of LDL with ^{125}I was performed by the method of McFarlane (46) as modified by Bilheimer, Eisenberg, and Levy (47). Radioiodination of apoB fusion proteins with ^{125}I was conducted with Enzymobeads (Bio-Rad, Richmond, CA) as described by Curtiss and Edgington (48). Incorporation of DiI into plasma LDL and HDL was performed in heterologous human lipoprotein-deficient serum, and into homozygote B45.2-LDL was directly performed in autologous plasma, as described by Pitas and coworkers (39). The incorporation of DiI by lipoprotein particles was determined in ethanol by fluorescence after serial dilution over three orders of magnitude, and the concentration was calculated by a standard curve established with stock DiI in ethanol. Measurements were carried out in a SPEX Fluorolog Spectrophotometer (Spex Industries, Inc., Edison, NJ) consisting of a 150 W xenon light source, DM 3000 computer system and 1681 excitation monochrometer. The excitation monochrometer was set at 547 nm and the emission monochrometer at 571 nm. Minimum and maximum fluorescent intensity at 571 nm for each dilution were averaged over 30 sec. The values were expressed as pg DiI per ng protein, and termed specific fluorescence activity.

Derivatization of lipoproteins by 100 mM malondialdehyde was conducted for 3 h at 37°C as previously de-

scribed (23). The malondialdehyde adducts of LDL were quantitated by colorimetric assay at 532 nm with thiobarbituric acid as reported (23).

All labeled and/or modified lipoproteins were immediately dialyzed at 5°C against buffer A. Lipoprotein solutions were sterile filtered (0.2 μm) upon completion of dialysis and stored at 4–5°C. Protein concentrations were determined using the method of Lowry and coworkers (49) or, when material was limited, by quantitative immunoblot for apoB protein. Aliquots were sterile filtered (0.2 μm) just before initiation of the cellular assays.

Cellular assays

Human monocyte-macrophages in culture were washed three times with 1-ml vol of Dulbecco's modified Eagle's medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (medium C). Labeled proteins were added to the cells together with a final concentration of 0.1% bovine serum albumin (Pentex, Miles Laboratory, Kankakee, IL) in Iscove's modified Dulbecco's medium supplemented with 2 mM glutamine (medium D), and the cells were returned to the incubator for the indicated times.

The proteolytic degradation of ^{125}I-labeled protein was measured by assaying the amount of ^{125}I-labeled trichloroacetic acid-soluble (noniodide) material formed by the cells and excreted in the culture medium as described by Goldstein and Brown (50). Corrections were made for the small amounts (<0.01% of total radioactivity added) of ^{125}I-labeled acid-soluble material that was formed in parallel incubations without cells.

The cellular association (cell surface bound and internalized) of DiI-labeled lipoproteins was examined 2 h after incubation. For studies by fluorescence microscopy, cells were washed three times with phosphate-buffered saline, fixed with 2% buffered formaldehyde in phosphate-buffered saline, and mounted with glycerol-gelatin mounting medium (Sigma). Cellular fluorescence was immediately visualized using the rhodamine filter set on a Nikon Labphot fluorescence microscope and recorded by color photography. For studies by quantitative fluorescence spectrophotometry, the method of Stephan and Yuracheck (51) was modified as follows. Cell monolayers were washed three times with phosphate-buffered saline and cells were extracted for 1 h with gentle rotation at room temperature with absolute ethanol (2 ml). The extracts were centrifuged at 1400 *g* for 10 min and individually measured by fluorescence spectrophotometry at 571 nm as described above. Corrections were made for cellular autofluorescence (7–20% of total fluorescence) as well as the small amounts (<0.7% of total fluorescence added) of DiI-labeled lipoprotein bound in parallel incubations without cells. All procedures were conducted under reduced light, and samples were foil-wrapped to minimize photobleaching. The amount of cellular-associated DiI-material was calculated on the basis of the specific fluorescence activity of DiI-labeled ligand. To determine the extraction efficiency of DiI-labeled material by ethanol, the cell monolayers were reextracted for 2 h at 37°C with phosphate-buffered saline at pH 7.4 containing 2% sodium dodecyl sulfate, 7 mM dithiothreitol, and 0.33% EDTA (2 ml). Extraction of the cell monolayers with ethanol accounted for >90% of the total DiI-labeled material.

The protein content of cells solubilized by 0.1 M sodium hydroxide was measured by the method of Lowry and coworkers (49) with bovine plasma albumin as a standard. Statistical analyses were conducted by methods previously cited (27).

Electrophoretic and blot analyses

Aliquots of lipoproteins and apoB-containing particles were subjected to electrophoresis under both denaturing and nondenaturing conditions on precast polyacrylamide gels (Novex, Encinitas, CA). Samples for denaturing electrophoresis, containing an average of 1–10 ng of protein, were heated 5 min at 90°C in a solution containing 0.1 M dithiothreitol, 1% sodium dodecyl sulfate, and 0.01 M Tris-HCl, pH 6.8, and electrophoresed on 4–12% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Laemmli (52). Electrophoretic transfer of proteins to Immobilon membrane (Millipore, Bedford, MA) in 25 mM Tris base and 192 mM glycine was conducted with a Bio-Rad Trans-Blot apparatus for 16 h at 50 V and 4°C (53). Blots were soaked in 15% hydrogen peroxide for 15 min, and blocked for 2 h in Tris-buffered saline, pH 7.0 (50 mM Tris-HCl, 150 mM NaCl) (TBS) containing 0.5% Tween 20 and 5% non-fat dry milk. The blots were sequentially incubated with monospecific primary antibody (diluted 1:5,000 in TBS containing 0.1% Tween 20), secondary, species-specific peroxidase-labeled antibody (diluted 1:10,000), and Amersham ECL detection reagents mixed 1:1 as previously described (54). Chemiluminescent products were visualized after 5–10 sec exposure to XAR-5 X-ray film (Eastman Kodak, Rochester, NY) without an intensifying screen and quantitated as necessary by densitometry. Concentrations of apoB were determined by comparison with standard curves of isolated human LDL generated with rabbit primary antiserum to human apoB peptide 890–908 (33) in the above protocol.

Samples for nondenaturing electrophoresis, containing an average of 10–100 ng of protein, were diluted in 0.01 M Tris-HCl, pH 6.8, and electrophoresed on 4–20% gradient polyacrylamide gels essentially as re-

ported (55,56) but without an agarose stacking gel. Electrophoretic transfer of lipoproteins to Immobilon membrane was performed as above. The DiI fluorescence associated with the lipoproteins was visualized using the light source and rhodamine filter device of a Nikon Labphot fluorescence microscope and photographed using a 3-mm thick Schott 06-570 filter. Apoproteins were detected by immunoblot as described above.

TABLE 1. Stable transformants of McArdle Rh 7777 cell lines expressing carboxyl-terminally truncated human apoB assembled in lipoprotein form

Cell Line	Secreted ApoB, % ApoB-100	Predicted ApoB Residues	Calculated Protein Molecular Mass
			kDa
B23[a]	23	1–1084	118
B36[b]	36	1–1720	194
pB53[c]	48; 53	1–2152; 1–2377	240; 265

[a]Originally described in ref. 37.
[b]Originally described in ref. 58.
[c]Originally described in ref. 29; due to mRNA editing activity, secreted particles are heterogeneous for apoB-48 and apoB-53.

RESULTS

Previous investigations (23, 24, 57) have demonstrated that progressive modification of LDL protein by lysine-specific reagents produces a decline in recognition of the lipoprotein by the LDL receptor. This effect has been attributed to abolition of the positively charged ε-amino group of lysyl residues in apoB through derivatization by reagents such as malondialdehyde, acetic anhydride, succinyl anhydride, and diketene. Modification of >15% of the ε-amino groups of lysyl residues in apoB abolishes interaction of the lipoprotein with the LDL receptor and, in the case of malondialdehyde, concomitantly produces threshold recognition by the type I and type II scavenger receptors of human monocyte-macrophages (23, 24). Several strategies were taken to determine whether discrete portions of apoB-100 protein mediated ligand recognition by the type I and type II scavenger receptors of human monocyte-macrophages.

Lipoprotein particles containing genetically engineered, carboxyl-terminally truncated forms of human apoB

The first line of investigation assessed the impact of carboxyl-terminal trunction of apoB protein upon receptor-dependent uptake of lipoprotein particles. ApoB cDNA constructs have been designed to express carboxyl-terminally truncated forms of human apoB (29, 37, 58). The expression in vitro of these constructs assembled together with lipid components in lipoprotein form has been achieved through stable transfection of the rat liver tumor cell line McArdle-Rh 7777. Three stable cell lines secreting lipoprotein particles containing genetically engineered, carboxyl-terminally truncated human apoB proteins were selected for investigation **(Table 1).**

The concentrations of human apoB protein secreted by the McArdle Rh 7777 cell lines were quantitated by immunoblot analyses after density ultracentrifugation and ranged from 150 to 600 ng/ml (data not shown). These levels were insufficient by two orders of magnitude to conduct multiple hydrolytic assays with radioiodinated ligand, and alternative approaches to lipoprotein labeling were considered. The fluorescent dye DiI has previously been successfully used to label human LDL for cell biology studies (e.g., 51, 59). The procedures, however, require exogenous addition of DiI to the lipoprotein in lipoprotein-deficient serum, incubation at 37°C, and subsequent purification of the labeled particles by density ultracentrifugation (39). Given the low concentrations of carboxyl-terminally truncated apoB particles secreted into conditioned media by the rat hepatoma cell lines and need for maximum recovery of material, we considered whether the lipoprotein particles could instead be labeled in situ with the amphiphilic DiI. Each McArdle Rh 7777 cell line tolerated inclusion of the dye in serum-containing medium based upon maintenance of the same rate of cell division as untreated cells over a 2-week period of culture and ability to exclude trypan blue dye.

Conditioned, DiI-supplemented media were collected from the rat hepatoma cell lines expressing human apoB-48/53, apoB-36, and apoB-23 and were subjected to nondenaturing polyacrylamide gel electrophoresis. The incorporation of DiI, visualized at 540 nm, was localized to particles migrating as shown in **Fig. 1A,** and showed distribution not only to lipoproteins but also to albumin and other unidentified proteins present in the conditioned media. Particles containing human apoB protein were isolated by density ultracentrifugation of the individual conditioned media at the following densities: B-48/53 particles, d 1.063–1.15 g/ml; B-36 particles, 1.063–1.21 g/ml; and B-23 particles, d > 1.21 g/ml. As shown in Fig. 1B, isolation yielded fluorescently labeled particles migrating as a single band. Immunoblot analyses confirmed that the migration of apoB protein coincided with the mobility of particles visualized by fluorescence (data not shown).

The apoprotein composition of the isolated lipoprotein particles was analyzed by immunoblot after SDS-PAGE. As shown in **Fig. 2,** the transfected McArdle cell

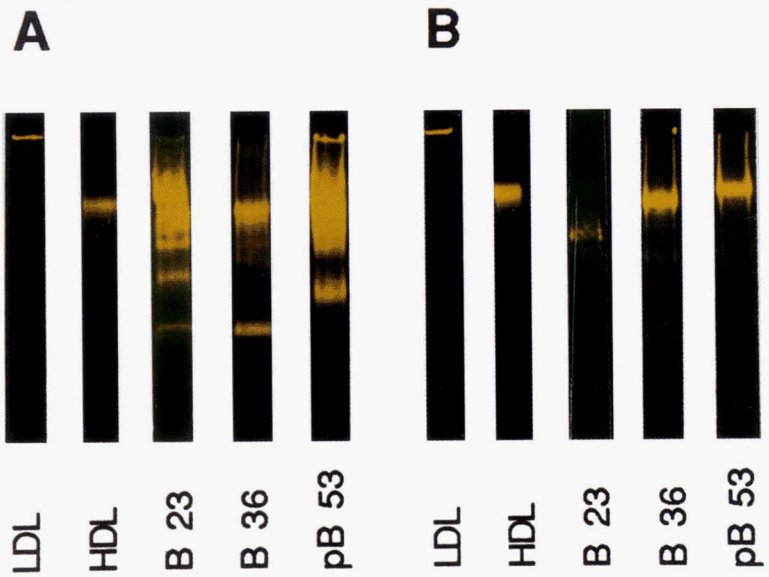

Fig. 1. Visualization of DiI-labeled particles containing human apoB-23, apoB-36, or apoB-48/53 by fluorescence after nondenaturing gel electrophoresis before (A) and after purification (B). Permanantly transfected McArdle Rh 7777 cell lines secreting either human apoB-23 (B 23), apoB-36 (B 36), or apoB-48/53 (pB 53) were grown to 60–70% confluency, washed 3 times with PBS, and incubated for 48 h in serum-free DMEM containing 3 μM DiI and 0.01% BSA. Cellular supernatants were collected as described in Materials and Methods and aliquots were removed for analysis. The remaining supernatants were utilized for isolation of secreted particles containing carboxy-truncated human apoB proteins by density ultracentrifugation as follows: apoB-23, d > 1.21 g/ml; apoB-36, d 1.063–1.21 g/ml; and apoB-48/53; d 1.063–1.15 g/ml. Unfractionated cell supernatants or particles isolated by density ultracentrifugation were concentrated 10-fold by Centricon 30000 filter units (Amicon). Aliquots (25 μl) were subjected to nondenaturing electrophoresis on 4–20% polyacrylamide gradient gels. After electrophoretic transfer onto Immobilon membrane, the blots were visualized by green light at 540 nm. LDL and HDL (1 μg protein) isolated from human plasma and labeled with DiI as described by Pitas and colleagues (39) were included as standards.

lines B23 and B36 secreted carboxyl-truncated apoB protein of the expected molecular weight. In the pB53 transfected cell line, editing of the human apoB messenger RNA (29, 60) led to secretion of particles containing predominantly human apoB-48. The results of Figs. 1 and 2 together confirm previous reports that progressive carboxyl-terminal truncation of apoB protein leads to a progressive shift to smaller, more dense particles and ultimate appearance with water-soluble plasma proteins in the infranatant fraction of d > 1.21 g/ml (29, 37, 58, 61). Immunoblot analyses of the isolated particles showed the absence of apoE protein and, other than a trace amount of apoA-I in particles secreted by the pB53 cell line, the absence of apoA-I protein (data not shown).

Pilot experiments were performed to validate the specificity of receptor-mediated uptake of DiI-labeled lipoprotein by human monocyte-macrophages (data not shown). Human LDL isolated from the peripheral blood was exogenously labeled with DiI according to the method of Pitas and coworkers (39). Human monocyte-macrophages demonstrated the ability to internalize DiI-labeled LDL and, by comparison with bright field views, lipoprotein uptake over a range of 60 ng–6.25 μg protein/ml was representative of the response of virtually all cells in culture. The role of the LDL receptor in lipoprotein internalization was confirmed by ability of unlabeled native LDL (100 μg/ml) to abolish the fluorescent signal and the failure of polyinosinic acid (30 μg/ml), a competitor of the scavenger receptor, to inhibit uptake. In other experiments, the internalization of DiI-labeled native LDL at 6.25 μg/ml was the same in the absence or presence of reductively methylated LDL added at a 100-fold excess (data not shown). These results demonstrated that DiI remained associated with the original parent molecules rather than redistributing to other lipoprotein particles during the 2-h period of incubation.

The ability of human monocyte-macrophages to internalize DiI-labeled malondialdehyde-LDL was also examined and, by comparison with bright field views, the majority of the cells displayed ability to internalize the labeled, modified lipoprotein over a range of 6 ng–6.25 μg protein/ml (data not shown). The role of the scavenger receptor in uptake was confirmed by the ability of polyinosinic acid to suppress accumulation of the fluorescent lipoprotein and lack of inhibition by unlabeled native LDL.

Other experiments demonstrated that the lower level of sensitivity of detection of lipoprotein internalization in human monocyte-macrophages was approximately 60 ng/ml for DiI-labeled native LDL as a marker of the LDL receptor, and 6 ng/ml for DiI-labeled malondialdehyde-LDL as a marker of the scavenger receptor (data not shown). We attribute the order of magnitude difference in sensitivity for ligand detection to the nearly 10-fold difference in activity of these two receptor pathways in human monocyte-macrophages maintained in culture for 7–9 d (27), an effect made more apparent by the short 2-h period of cell incubation with DiI-labeled lipoprotein.

The ability of the several DiI-labeled particles containing genetically engineered, carboxyl-terminally truncated human apoB protein to interact with human

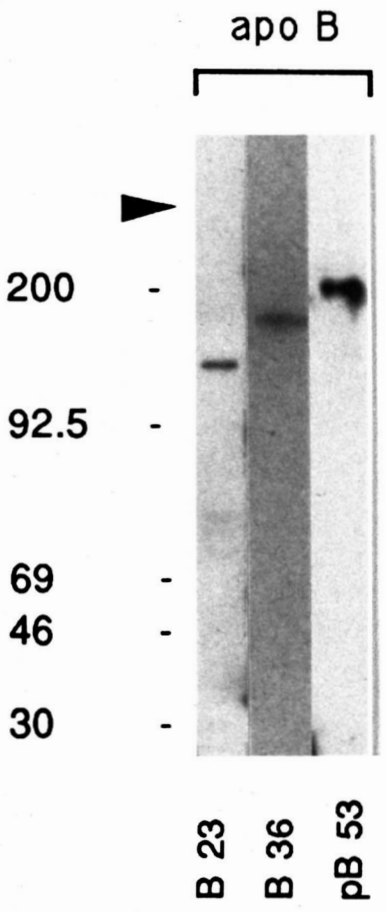

Fig. 2. Immunoblot analyses of particles containing human apoB-23, apoB-36, or apoB-48/53 after fractionation by density ultracentrifugation. The secreted, DiI-labeled lipoprotein particles shown in Fig. 1B were subjected to SDS-PAGE on 4–12% polyacrylamide gels as described in Materials and Methods. After electrophoretic transfer onto Immobilon membrane, individual blots were probed with primary antisera to apoB. The apoproteins were visualized by autoradiography of chemiluminescent product released from peroxidase-tagged immune complex. The large arrowhead indicates the migration of apoB-100 as determined by human LDL.

monocyte-macrophages was then examined. The cells proved uniformly unable to internalize the purified secretory products of the pB53, B36, or B23 cell lines (data not shown). Modification of each preparation by malondialdehyde produced marked fluorescence due to cellular internalization of the in situ DiI-labeled particles **(Fig. 3).** The role of the scavenger receptor in the uptake of malondialdehyde-modified particles containing human apoB-23, B-36, and B-48/53 was confirmed by the specificity of competition by polyinosinic acid (Figs. 3 D, H, L, respectively) and failure of unlabeled native LDL to suppress internalization (Figs. 3 C, G, K, respectively). These findings indicated that recognition sites for the scavenger receptor resided within residues 1–1,184 of apoB sequence common to each of the carboxyl-terminally truncated proteins. Moreover, modification of the lysyl residues by malondialdehyde was necessary to generate expression of the receptor combining sites.

The cellular association of malondialdehyde-modified, DiI-labeled lipoproteins was further assessed by quantitative fluorescent spectrophotometry (51). To provide direct comparison with results obtained by fluorescence microscopy, human monocyte-macrophages were incubated with similar protein concentrations (68 ng protein/ml) of the labeled lipoproteins. As shown in **Table 2,** incubation of the cells with malondialdehyde-modified LDL produced substantial, measurable accumulation of DiI-labeled material. The role of the scavenger receptor in binding and internalization was confirmed by ability of unlabeled, malondialdehyde-modified LDL to suppress >87% of the cellular association of the fluorescent lipoprotein, and lack of inhibition by unlabeled native LDL (data not shown). Incubation of human monocyte-macrophages with DiI-labeled malondialdehyde-modified particles containing human apoB-36 or B-48/53 also produced measurable accumulation of fluorescence (Table 2). Unlabeled, malondialdehyde-modified LDL abolished the cellular association of the modified, carboxyl-terminally truncated apoB lipoproteins. These results gave additional evidence that the amino terminus of apoB protein, after malondialdehyde modification, provided combining sites recognized by the type I and type II scavenger receptors of human monocyte-macrophages.

To compare the levels of cellular association produced by the several malondialdehyde-modified lipoproteins, the data given in Table 2 were also calculated on a molar basis assuming one mol of apoB product per mol particle. Malondialdehyde-modified LDL presented at 0.13 nM produced a 1.3-fold higher level of specific cellular association than either of the modified B-48/53 and B-36 products presented at 0.26 and 0.37 nM, respectively. The higher level may be attributed to higher affinity of binding, owing to optimal expression of the receptor-combining sites in the full length apoB protein and/or to additional receptor binding determinants in the carboxyl terminus of apoB-100 protein (residues 2152–4536). While Zawadzki, Milne, and Marcel (62) have identified an epitope in the carboxyl-terminal 20 amino acids of apoB-100 that, after mild oxidation of LDL by transition metal, shows enhanced immunoreactivity, the relationship of this epitope to expression of domains for the type I and type II scavenger receptors remains unknown. Binding determinations by quantitative fluorescent spectrophotometry to test these possibilities were precluded by the limited numbers of human monocyte-macrophages isolated from individual human subjects.

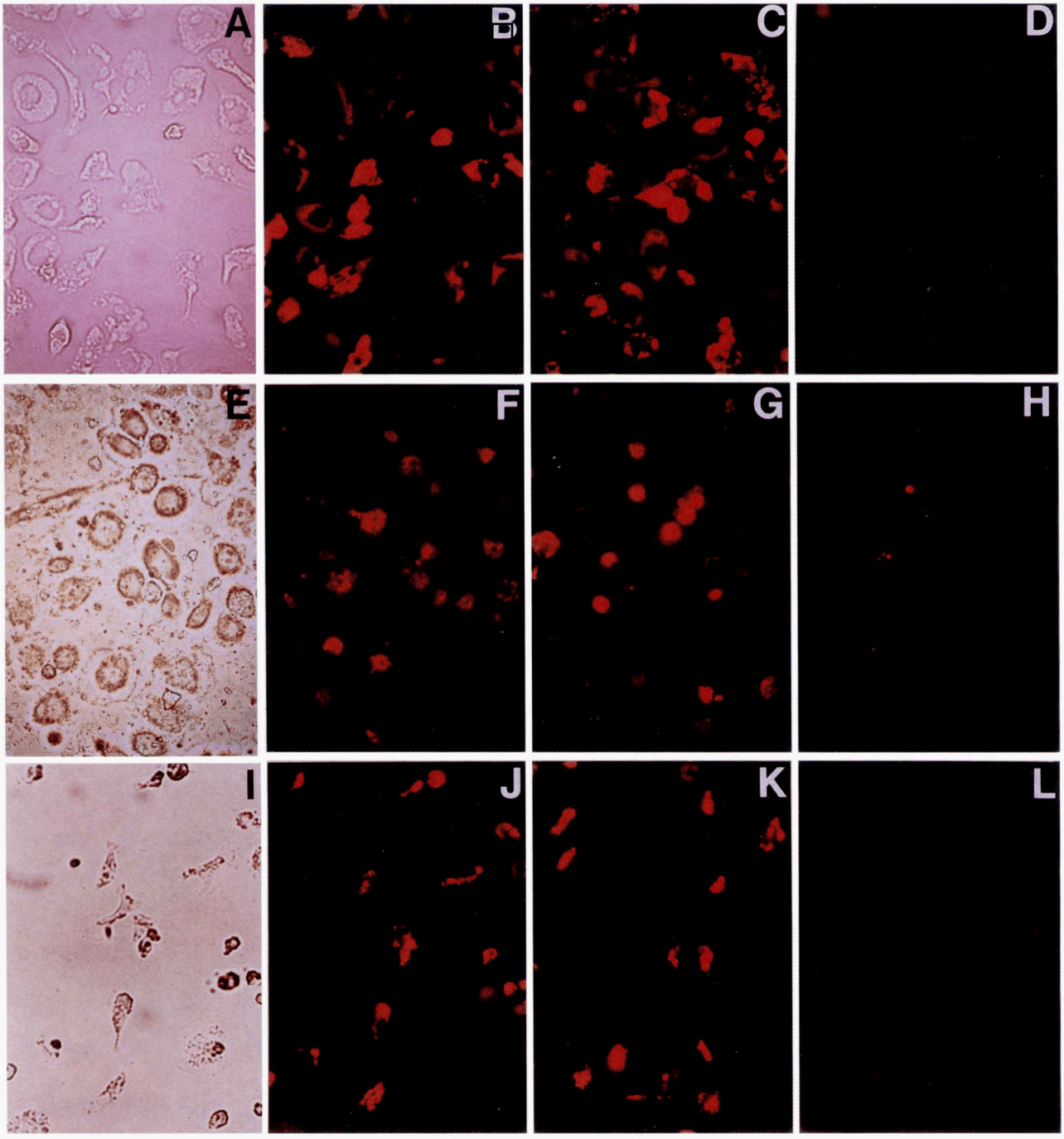

Fig. 3 Ability of human monocyte-macrophages to internalize DiI-labeled, malondialdehyde-modified, carboxyl-terminally truncated particles. The purified particles shown in Fig. 1B were derivatized by malondialdehyde as described in Materials and Methods. Normal human monocytes were cultured on chamber slides (A–D, I–L) or multi-well inserts (E–H) for 7 d in 0.4 ml of medium B containing 30% autologous serum, and were prepared for studies at 37°C as described in Materials and Methods. Each chamber received medium D containing DiI-labeled, malondialdehyde-modified particles containing apoB-23 (A–D), apoB-36 (E–H), or apoB-48/53 (I–L) added in the absence (A, B; E, F; I, J) or presence of 50 μg/ml of unlabeled native LDL (C, G, K) or 30 μg/ml of polyinosinic acid (D, H, L). The final concentrations of the carboxy truncated apoB proteins were estimated by quantitation after immunoblot to range from 30 to 125 ng/ml. After incubation for 2 h at 37°C, the medium was removed and the cells were prepared for microscopic evaluation as described in Materials and Methods. Each field was examined by light as well as fluorescence microscopy and examples of these paired examinations are shown by comparison of A and B, E and F, and I and J. Cellular fluorescence was detected by a Nikon Labphot fluorescent microscope equipped with a rhodamine filter set and recorded on Kodak color daylight film, 1600 ASA. The photographs shown are representative fields selected after a thorough examination of each well and are representative of four (B-36 and B-48/B-53) or two (B-23) experiments conducted with individual particle isolates and with monocyte-macrophages isolated from different human subjects. Magnification, ×90.

TABLE 2. Cellular association of DiI-labeled malondialdehyde-modified plasma LDL and lipoproteins containing carboxyl-terminally truncated apoB

	DiI-MDA-LDL	DiI-MDA-B48/53-LDL	DiI-MDA-B36-LDL
Concentration, DiI-labeled lipoprotein[a]			
ng/ml	68	68	68
pM	132	283	351
Cellular-associated DiI-labeled material in the presence of			
Buffer			
ng lipoprotein associated/mg cellular protein	128	41	33
fmol lipoprotein associated/mg cellular protein	249	171	170
MDA-LDL, 50 μg/ml			
ng lipoprotein associated/mg cellular protein	16	1	1
fmol lipoprotein associated/mg cellular protein	31	4	5

Normal human monocytes were cultured for 8 d in 5 ml of medium B containing 30% autologous serum and were prepared for studies at 37°C as described in Materials and Methods. Each 60-mm dish received 5 ml of medium D containing 68 ng/ml of DiI-labeled, malondialdehyde-modified lipoprotein in the presence of the indicated additions. After incubation for 2 h at 37°C, the medium was removed and the content of DiI-labeled material was determined by fluorescence spectrophotometry as described in Materials and Methods. The values shown are the average of duplicate determinations.

[a]Concentrations of DiI-labeled lipoproteins were determined by protein mass after immunoblot as described in Materials and Methods, and molar concentrations calculated on the basis of molecular weights for apoB products as follows: apoB-100, 515 kDa; apoB-48/53, 240 kDa; and apoB-36, 194 kDa. The incorporation of DiI was determined as described in Materials and Methods to be 52, 23, and 13 pg DiI per ng apoB product after malondialdehyde modification of LDL (DiI-MDA-LDL), and the purified particles secreted by p B53 (DiI-B48/53-LDL) and by B36 (DiI-B36-LDL), respectively.

Plasma LDL homozygous for naturally occurring carboxyl-terminally truncated form of human apoB-45.2

We then turned our attention to localization of scavenger receptor-combining sites in the amino terminus of apoB-100. The studies of particles containing genetically engineered, carboxyl-terminally truncated apoB carried an implicit assumption. This assumption was that lysyl residues contributing to formation of the receptor-combining sites remained accessible to modification by malondialdehyde in the several in vitro lipoprotein-packaged apoB products. Galeano and coworkers (63) have provided convincing evidence that apoB-100 structure and function in plasma LDL are chiefly governed by lipoprotein size, and have proposed that plasticity of protein structure accounts for changes in ability of combining domains to interact with the LDL receptor. The diminished immunoreactivity at an epitope between residues 1031 and 1084 (64) and an epitope near residue 1022 (65) in triglyceride-rich LDL indicates that this plasticity of protein structure extends to the amino terminus. These considerations together raised the issue whether plasma LDL particles, homozygous for carboxyl-terminally truncated apoB and synthesized in vivo, displayed the same ability to interact with the scavenger receptor as particles with genetically engineered, carboxyl-terminally truncated apoB and synthesized in vitro.

Homozygosity for a mutation producing truncated apoB in human subjects has been reported only three times in the world literature (30, 66, 67). Young and coworkers (30) have described the homozygous expression of apoB-45.2 (residues 1–2053) in a human subject with asymptomatic hypolipoproteinemia and have determined that, owing to a nucleotide transversion at apoB cDNA nucleotide 6368, Tyr-2053 is converted into a premature stop codon. Pertinent to the present study, the lipoprotein-associated apoB-45.2 was widely distributed over a density range of d 1.009–1.074 g/ml, and thus was contained in more buoyant, larger particles than either genetically engineered, carboxyl-terminally truncated apoB-48/53 (d 1.063–1.15 g/ml) or apoB 36 (d 1.063–1.21 g/ml).

The second approach to expression of scavenger receptor-combining sites tested whether LDL, homozygous for carboxyl-terminally truncated apoB and synthesized in vivo, displayed the same ability to interact with the scavenger receptor as in vitro particles containing genetically engineered, carboxyl-terminally truncated apoB. As shown in **Fig. 4A,** DiI-labeled LDL homozygous for B-45.2 displayed modest levels of cellular association, and these remained unchanged upon addition of unlabeled native apoB-100-containing LDL. By this criterion, there was virtually no specific, LDL receptor-dependent recognition of LDL homozygous for B-45.2. These results are consistent with localization of the LDL receptor-binding domain to the broad region encompassing residues 3000–4000 (68) as well as identification of two nonlinear domains, residues 3429–3453 and 3507–3523, as key portions of the epitope for

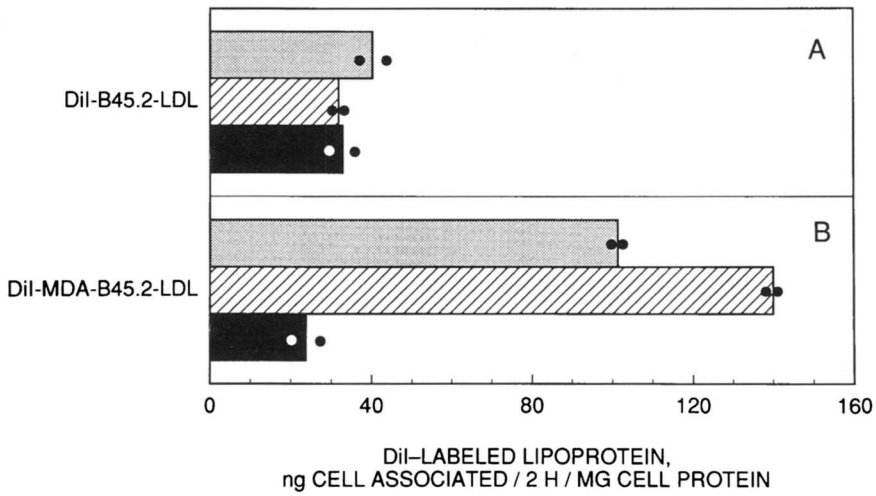

Fig. 4 Cellular association and specificity of receptor-dependent interaction of human LDL homozygous for apoB-45.2 in human monocyte-macrophages. Normal human monocytes were cultured for 8 d in 5 ml of medium B containing 30% autologous serum and were prepared for studies at 37°C as described in Materials and Methods. In A, each 60-mm dish received 5 ml of medium D containing 175 ng/ml of DiI-labeled B-45.2-LDL (50 pg DiI per ng protein) or B, the malondialdehyde-modified lipoprotein in the absence (stippled bars) or presence of native (50 μg/ml, hatched bars) or malondialdehyde-modified, apoB-100-containing LDL (50 μg/ml, solid bars). After incubation for 2 h at 37°C, the medium was removed and the content of DiI-labeled material was determined by fluorescence spectrophotometry as described in Materials and Methods. The values shown are the average of two determinations.

the LDL receptor-blocking antibody MB 47 (69). Our findings, moreover, confirm the previous report by Young and coworkers (70) that Lp-B37, a naturally occurring lipoprotein containing carboxyl-terminally truncated apoB-37, fails to interact with the LDL receptor.

Malondialdehyde modification readily produced cellular association of DiI-labeled lipoprotein homozygous for B-45.2 (Fig. 4B). The ability of unlabeled malondialdehyde-LDL, but not native LDL, to suppress cellular association demonstrated that the scavenger receptor pathway accounted for the enhanced accumulation of DiI-labeled material. Thus lysyl residues that contributed to formation of the receptor-combining sites were accessible to modification in the larger, more buoyant LDL homozygous for naturally occurring, carboxyl-terminally truncated apoB-45.2. These findings provided important validation of the studies performed with more dense lipoprotein particles containing genetically engineered, carboxyl-terminally truncated apoB.

Microemulsion-incorporated apoB fusion proteins

We turned to apoB fusion proteins as the final and third line of investigation to localize apoB amino terminal-combining domains for the scavenger receptor. The products of three β-galactosidase-recombinant clones, purified by apoB immunoaffinity chromatography from lysates of induced lysogens made in *E. coli* Y1089 (31), were tested **(Table 3).** Each fusion protein was incorporated into microemulsions of phosphatidylcholine and cholesteryl oleate. This method has previously been used for the reassembly of apoB-100 in stable particles approximately the same size as LDL (44, 45), and appeared uniquely suited to the purposes of this study. Portions of the microemulsion-incorporated fusion proteins were further labeled by radioiodination and subjected to electrophoresis in denaturing SDS-PAGE.

TABLE 3. cDNA clones and their products expressed as β-galactosidase-human apoB fusion proteins

cDNA Clone[a]	ApoB Nucleotides	ApoB Residues	Fusion Protein Molecular Mass
			kDa
A7h	1848–2416[b]	547–735[c]	135
A6c	−114 [5′UT]–3440[b]	1–40[d]	122–124
RP2	9294–9605[b]	3029–3132[c]	127

[a]Originally described in Ref. 31.
[b]Based upon the published nucleic acid sequence (43).
[c]Based upon localization in the published protein sequence (42).
[d]Translation product includes translation of 114 nucleotides at the 5′ end preceding the ATG site as well as in-frame translation of nucleotides in the mature apoB amino terminus (31, 43). Fusion protein from lysates of induced lysogens made in *E. coli* Y1089 displayed a lower molecular mass of 122–124 kDa than the predicted product of 246 kDa generated by the fusion of β-galactosidase (116 kDa) with the protein translation product of apoB nucleotides −114 to 3440 (130 kDa) owing to sensitivity of the product of clone A6c to proteolysis at the carboxyl-terminus (31).

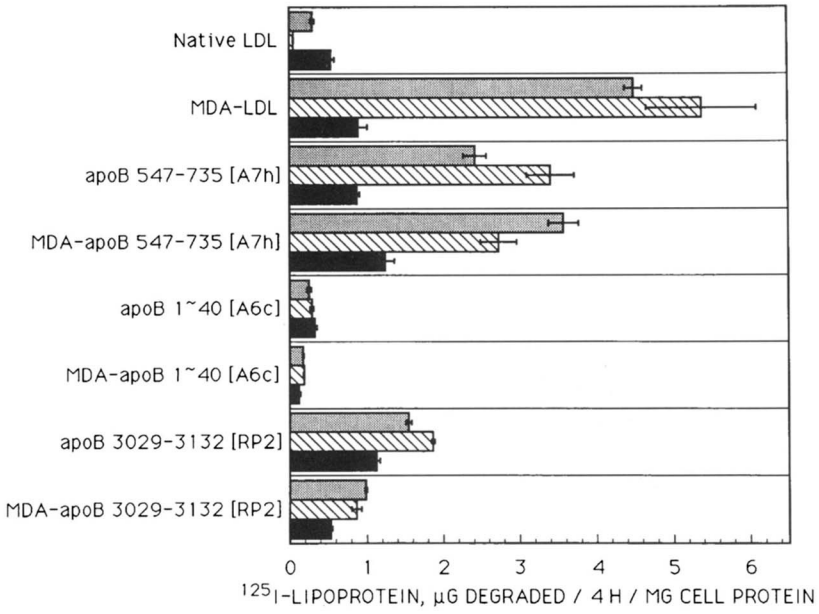

Fig. 5 Comparison of the ability of human monocyte-macrophages to mediate the degradation of microemulsion-incorporated human apoB fusion proteins and apoB-100-containing human LDL. Normal human monocytes were cultured for 9 d in 0.5 ml of medium B containing 30% autologous serum and were prepared for studies at 37°C as described in Materials and Methods. Each 2.0-cm^2 well received 0.5 ml of medium D containing 10 µg/ml of radioiodinated human native LDL (437 cpm/ng) or microemulsion-incorporated fusion proteins containing apoB residues 547–735 (product of clone A7h, 768 cpm/ng) or residues 1–40 (product of clone A6c, 171 cpm/ng) or residues 3029–3129 (product of clone RP2, 40 cpm/ng) or the malondialdehyde-modified derivatives in the absence (stippled bars) or presence of native LDL (750 µg/ml, hatched bars) or polyinosinic acid (100 µg/ml, solid bars). After incubation for 4 h at 37°C, the medium was removed and the content of ^{125}I-labeled acid-soluble material was determined. The values shown are the mean ± 1 SD of quadruplicate determinations.

Each gave a single band after autoradiography and the expected molecular masses of 127 kDa (clone RP2 product, apoB residues 3029–3132), 122–124 kDa (clone A6c product, apoB residues 1–40), and 135 kDa (clone A7h product, apoB residues 547–735) as previously published by Mehrabian and coworkers (31) (data not shown). The microemulsion-incorporated, radioiodinated clone A7h product was also isolated by gel filtration on Sepharose CL-4B as previously described (44, 45). Compositional analyses of the microemulsion-incorporated protein showed an equimolar ratio of cholesteryl oleate:dimyristoyl phosphatidylcholine, and a radioiodinated protein/cholesteryl oleate ratio of 15% (w/w) or approximately 2 mol of clone A7h product per microemulsion particle (data not shown).

The translation product of clone A6c differs in two respects from the other fusion proteins. First, the protein includes the translation of 114 nucleotides at the 5′ end preceding the ATG site as well as in-frame translation of nucleotides in the mature apoB amino terminus (31, 43). Second, the fusion protein from lysates of induced lysogens made in *E. coli* Y1089 displays a lower molecular mass of 122–124 kDa than the predicted product of 246 kDa generated by the fusion of β-galactosidase (116 kDa) with the protein translation product of apoB nucleotides −114 to 3440 (130 kDa). The sensitivity of the product of clone A6c to proteolysis at the carboxyl-terminus accounts for this result (31). We refer to this fusion protein as the truncated product of clone A6c.

The interaction of the radioiodinated, microemulsion-incorporated fusion proteins with human monocyte-macrophages was examined by hydrolytic assay. The specificity of receptor recognition was determined by competition with nonradioactive native LDL as an inhibitor of the LDL receptor, and with polyinosinic acid as an inhibitor of the scavenger receptor. As shown in **Fig. 5,** native human LDL containing full-length apoB-100 displayed modest uptake by the LDL receptor and, after derivatization to generate malondialdehyde-LDL, gave the expected rapid uptake mediated by the scavenger receptor. The product of clone A7h, containing apoB residues 547–735, displayed a rate of hydrolysis considerably greater than that of native LDL that persisted upon addition of a competitor of the LDL receptor. Addition of polyinosinic acid, a competitive inhibitor of the scavenger receptor, readily suppressed degradation of the fusion protein. Derivatization of the radioiodinated, microemulsion-incorporated fusion protein by malondialdehyde further enhanced the rate of hydrolysis. As for the parent fusion protein, polyinosinic acid proved to be an effective competitive inhibitor. These results indicated that the product of clone A7h, containing apoB residues 547–735, mediated recognition by the scavenger receptor pathway without benefit of protein derivatization by malondialdehyde and that modification further enhanced ligand-receptor interaction.

By contrast, the truncated product of clone A6c, containing apoB residues 1–40, displayed little uptake by human monocyte-macrophages either before or after modification by malondialdehyde (Fig. 5). The failure of the competitive inhibitors to suppress hydrolysis indicated that nonspecific interaction accounted for the

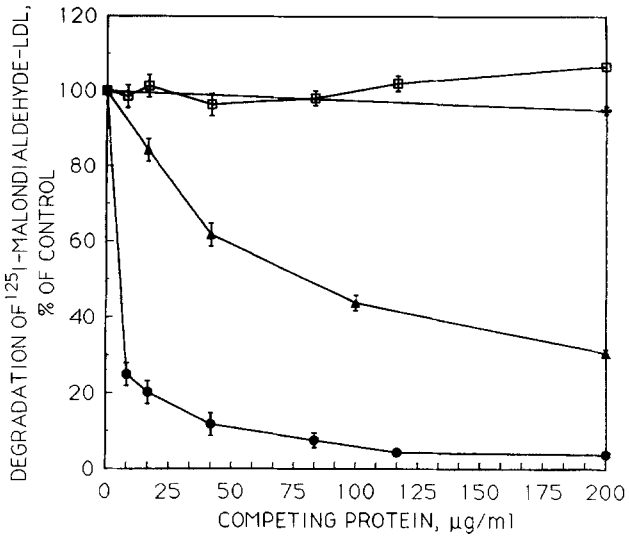

Fig. 6 Ability of microemulsion-incorporated human apoB fusion proteins to compete with the degradation of ^{125}I-labeled-malondialdehyde-LDL in human monocyte-macrophages. Normal human monocytes were cultured for 7 d in 0.5 ml of medium B containing 30% autologous serum and were prepared for studies at 37°C as described in Materials and Methods. Each 2.0-cm^2 well received 0.5 ml of medium D containing 10 µg/ml of radioiodinated human malondialdehyde-LDL (413 cpm/ng) together with the indicated concentrations of nonradioactive malondialdehyde-LDL (●), or fusion proteins containing apoB residues 547–735 (▲) or apoB residues 3029–3132 (□), or native LDL (⊕). After incubation for 4 h at 37°C, the medium was removed and the content of ^{125}I-labeled acid-soluble material was determined. The values shown are mean ± 1 SD of triplicate determinations.

poor rate of degradation. This particular series served, moreover, to validate the overall experimental protocol and demonstrated the absence of potential interactions arising from malondialdehyde modification of β-galactosidase-containing fusion proteins or choice of microemulsions as delivery vehicles for the fusion proteins.

Finally, the product of clone RP2 containing apoB residues 3029–3132, displayed modest uptake that was reduced upon derivatization by malondialdehyde (Fig. 5). Previous investigations have indicated that this portion of primary sequence may play a role in ligand binding by the LDL receptor based on receptor-blocking monoclonal antibody (42). The inability of native LDL to suppress hydrolysis of the fusion protein suggested that either insufficient primary sequence was available for LDL receptor interaction or that the method of presentation was insufficient to allow direct examination of this point. Finally, the addition of malondialdehyde, LDL proved to have some effect upon the hydrolysis of the product of clone RP2 that became more apparent after derivatization of the fusion protein.

Cross-competition analyses were conducted to test the abilities of the clone A7h and clone RP2 products to suppress the interaction of ^{125}I-labeled-malondialdehyde-LDL with the scavenger receptor **(Fig. 6).** The fusion protein containing apoB residues 3029–3132, like native LDL, proved ineffective as a competitor. By contrast, fusion protein containing apoB residues 547–735 inhibited more than 70% of the hydrolysis of ^{125}I-labeled-malondialdehyde-LDL. A greater concentration of the fusion protein, however, was necessary to achieve the same level of suppression produced by competing malondialdehyde-LDL. On a per-particle basis 1 mol of unlabeled malondialdehyde-LDL, but 15 mol of fusion protein, per mol ^{125}I-labeled malondialdehyde-LDL was required to produce 50% suppression of hydrolysis. These data suggested a lower affinity of the fusion protein than malondialdehyde-LDL for the scavenger receptor. This was supported through analyses of the degradation of radioiodinated fusion protein containing apoB residues 547–735 as a function of protein concentration. Whereas the fusion protein showed high-affinity, saturable uptake with half-maximal hydrolysis near 7–10 µg/ml, or approximately 26–37 nM microemulsion-incorporated protein, malondialdehyde-LDL showed half-maximal hydrolysis at 5 µg/ml, or 10 nM (data not shown).

A final cross-competition analysis provided additional evidence that this portion of apoB primary sequence participated in ligand interaction with the scavenger receptor. Unlabeled fusion protein containing apoB residues 547–735 proved able to suppress the hydrolysis of ^{125}-I-labeled malondialdehyde-LDL as readily as the degradation of the radioiodinated fusion protein **(Fig. 7).**

DISCUSSION

The present investigations examined whether discrete portions of the primary sequence of the apoB protien contributed to recognition by the type I and type II scavenger receptors of human monocyte-macrophages. Three lines of evidence were developed with genetically engineered, carboxyl-terminally truncated human apoB protiens assembled into lipoprotiens in vitro, a naturally occuring genetic mutation generating plasma LDL homozygous for carboxyl-terminally truncated human apoB-45.2 in vivo, and microsome-incorporated apoB fusion proteins. These approaches, taken together, localized receptor-combining sites to the amino-terminus of apoB (residues 1–1084).

Pearson and coworkers (12) have provided compelling evidence that spatial arrangement of anionic phosphate groups accounts for binding of polynucleotide quadruplexes to the scavenger receptor, and forms a charged surface complementary to the positively charged surface of the collagenous ligand-binding do-

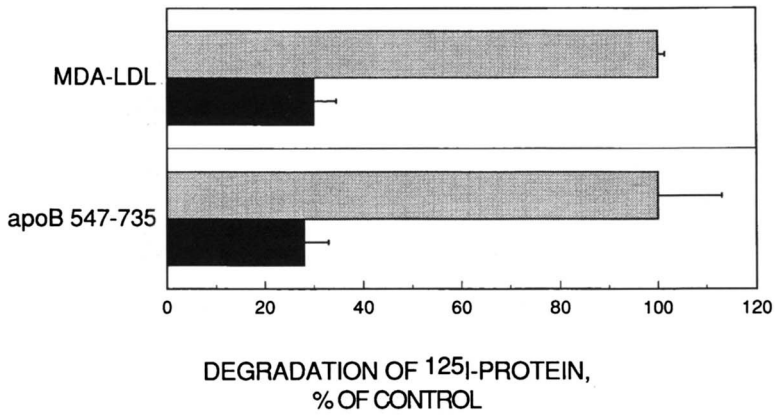

Fig. 7 Ability of microemulsion-incorporated human apoB fusion protein to suppress the degradation of ^{125}I-labeled malondialdehyde-LDL and ^{125}I-labeled microemulsion-incorporated human apoB fusion protein in human monocyte-macrophages. Normal human monocytes were cultured for 7 d in 0.5 ml of medium B containing 30% autologous serum and were prepared for studies at 37°C as described in Materials and Methods. Each 2.0-cm^2 well received 0.5 ml of medium D containing 2 μg/ml of radioiodinated human malondialdehyde-LDL (446 cpm/ng) or microemulsion-incorporated fusion protein containing apoB residues 547–735 (599 cpm/ng) in the absence (stippled bars) or presence of the unlabeled microemulsion-incorporated fusion protein (200 μg/ml, solid bars). After incubation for 4 h at 37°C, the medium was removed and the content of ^{125}I-labeled acid-soluble material was determined. The rates of degradation in the absence of added competitor were 3.3 ± 0.4 and 0.43 ± 0.06 μg/4h per mg cell protein for ^{125}I-labeled malondialdehyde-LDL and ^{125}I-labeled microemulsion-incorporated fusion protein containing apoB residues 547–735, respectively. The values shown are the mean ± 1 SD of triplicate determinations.

main. We propose, by analogy, that apoB protein mediates receptor interaction through favorable, spatial presentation of anionic arrays. The receptor binding ability of liposome-incorporated fusion protein containing apoB residues 547–735 indicates that the tertiary structure of this peptidyl sequence can provide receptor-combining anionic arrays. When this same peptidyl sequence occurs within the larger amino terminus of carboxyl-truncated apoB protein or full-length protein, however, it fails to promote recognition by the scavenger receptor; abolition of the positively charged ε-amino group(s) of lysine may be required to facilitate presentation, and may contribute to formation of the favorable, spatial distribution of anionic arrays.

Segrest and coworkers (71) have recently provided detailed insight into the secondary structure of apoB-100. Computer analyses conducted at high stringency levels predict that, at the amino terminus, residues 58–476 (cluster I) would be comprised predominantly of class G* amphipathic helices and residues 827–1961, a distinct cluster of amphipathic β-strands. The disulfide bond-rich amphipathic helix cluster I is believed to represent one or more folded α-helical globular domains that may be involved in protein–protein interactions, and the distinct cluster of amphipathic β-strands proposed to serve as an irreversible lipid-associating domain. In the present study, microemulsion-incorporated β-galactosidase–apoB fusion protein containing apoB residues 547–735 showed ability to interact with the scavenger receptor prior to derivatization, and displayed enhanced interaction after modification by malondialdehyde. This portion of sequence directly follows the cysteine-rich, disulfide-bonded amphipathic helix cluster I of apoB protein predicted by Segrest and coworkers (71). The segment, flanked by intramolecular disulphide linkages, Cys_{451} to Cys_{486} and Cys_{939} to Cys_{949}, occurs in an area free of potential N-glycosylation sites and heparin binding regions (43, 72–74). The absence of these conformationally constraining features may allow plasticity of protein structure critical to formation of the type I and type II scavenger receptor-combining sites.

Inspection of the primary sequence of apoB residues 547–735 reveals two segments within a span of 100 residues that are enriched in acidic amino acids **(Fig. 8A).** The first, comprised of residues 580–596, contains four acidic residues. This segment also contains two pairs of Lys that, if accessible to modification, could form the neutral N, N′-disubstituted 1-amino-3-iminopropene adduct produced by derivatization with the bifunctional malondialdehyde. Thus the anionic nature of this region would be expected to increase upon modification by the lysine-specific reagent. The second segment, comprised of residues 659–675, contains six Glu and one Lys. Abolition of the positively charged ε-amino group of lysine by malondialdehyde derivatization would add to the overall, effective anionic character of this region. As the interaction of fusion protein con-

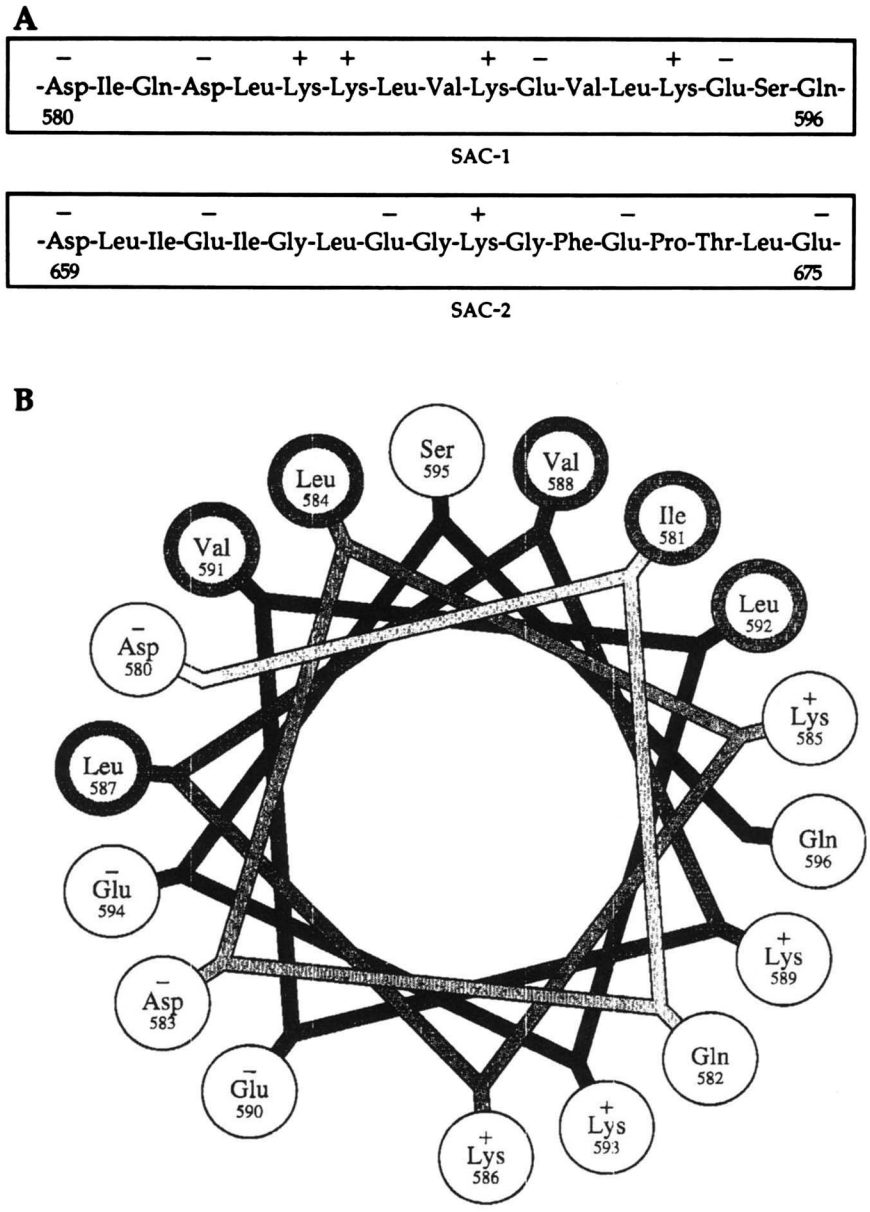

Fig. 8 Proposed binding domains for the scavenger receptor presented by the primary sequence of the amino terminus of mature apoB-100 protein. The putative sites are enriched in acidic amino acids and interspersed with basic lysyl residues which, after neutralization and derivatization by malondialdehyde, would enhance the overall anionic character of peptidyl sequence. In A, primary sequence containing residues 580–596 has been designated SAC-1 (*s*cavenger receptor *a*nionic *c*luster-1) and residues 659–675, SAC-2 (*s*cavenger receptor *a*nionic *c*luster-2). The charge contributions of acidic (−) and basic (+) amino acids, as well as location of amino acid based upon primary sequence of mature apoB-100 protein (42, 71), are indicated. In B, computer analysis of the class B amphipathic helix SAC-1 peptide (residues 580–596) by the program WHEEL (71) which creates a Schiffer-Edmundson helical wheel diagram of a given sequence of amino acids arranged as an ideal α-helix (100* rotation residue) viewed down the long axis. Orientation is by the hydrophobic moment directed toward the top of the page. Hydrophobic moment/residue=2.514625.

taining apoB residues 547–735 with the scavenger receptor was enhanced upon modification by malondialdehyde (Fig. 5), the features of the primary sequence of these regions suggest that one or both segments may participate in formation of the amino terminal-combining site recognized by type I and type II scavenger receptors.

We hypothesize, as first proposed by Goldstein and Brown in 1979 (3), that these anionic arrays function to promote the well-known propensity of the scavenger

receptor to bind anionic molecules. We have designated residues 580–596 as SAC-1 (*s*cavenger receptor *a*nionic *c*luster-1) and residues 659–675 as SAC-2 (*s*cavenger receptor *a*nionic *c*luster-2). Computer-assisted analysis of the secondary structures (75) presented by these clusters, including the preceding and following ten residues, predicts SAC-1 to participate in formation of an α-helical region and SAC-2, in β-sheet formation. SAC-1 has been identified as a fifth member of class B or bipolar amphipathic helical clusters present in full-length apoB-100, and the only member present in the amino terminus (J. Segrest, personal communication). As previously described by Segrest and colleagues (71), the class B motif has the unusual feature of severe asymmetry in distribution of negative and positive amino residue clusters. Computer-assisted analysis shows the marked asymmetrical distribution of polar residues presented by SAC-1 (Fig. 8B). We speculate that replacement of the positively charged ε-amino groups of lysine in SAC-1 by the neutral, hydrophobic N, N′-disubstituted 1-amino-3-iminopropene adduct allows the discrete presentation of spatially oriented, negatively charged side chains. It is noteworthy that fusion protein containing apoB residues 547–735, while containing approximately 4% of the primary sequence of the protein, mediated scavenger receptor-dependent uptake and hydrolysis, and competitively inhibited receptor interaction with malondialdehyde-LDL. The two anionic clusters within residues 580–675 may serve to promote receptor recognition and to orient the ligand during receptor binding. If one or both anionic clusters are critical to ligand recognition by the scavenger receptor, one would expect their expression in various forms of modified LDL including transition metal-oxidized LDL as well as other modified apoB-containing lipoproteins such as β-very low density lipoprotein and lipoprotein[a].

In the intact lipoprotein, both three-carbon malondialdehyde and two-carbon acetic anhydride effectively generate spatially oriented anionic arrays through lysine neutralization. However, charge derivatization of apoB lysine residues per se, as proposed by Zhang et al. (14) and Alaiz et al. (15), apparently does not suffice. For example, the three-carbon acrolein both derivatizes lysine and produces anionic LDL, yet fails to convert the lipoprotein to ligand recognized by the scavenger receptor (76). The introduction of conformational constraint by malondialdehyde derivatization of juxtapositioned lysine residues, and lack of conformational constraint produced by acrolein modification, may account for the difference in ligand conversion between these three-carbon molecules. As another example, lysine derivatization by the four-carbon succinic anhydride generates an anionic adduct whereas lysine modification by the four-carbon crotonaldehyde acts to neutralize the ε-amino group. While both generate more negatively charged LDL, succinyl-LDL is recognized by the macrophage scavenger receptor (24) whereas crotonaldehyde-LDL is not (76). Steric accessibility of the receptor to acidic side chains of the protein may contribute to the difference in lipoprotein conversion. The addition of new anionic adducts by succinylation, and lack of these charged adducts after modification by crotonaldehyde, may account for the formation of receptor-combining determinants.

Several different mechanisms could account for the formation of protein-borne anionic arrays that serve as structural determinants for interaction with the cationic, collagenous domain of the scavenger receptor. *i*) The primary protein sequence (Fig. 8 and ref. 77) may directly provide an appropriate configuration of discrete, negative clusters; *ii*) anionic arrays may be generated through clusters of acidic side chains exposed after abolition of the positively charged ε-amino group of lysine, e.g., derivatization of LDL by malondialdehyde (4) or acetic anhydride (78); and *iii*) negatively charged clusters may be directly formed through replacement of the positively charged ε-amino group of lysine with an anionic adduct (14, 15, 24). Each of these mechanisms, singly or together, appears effective in generating a "topographical consensus" of favorably oriented, discrete patches of negative charge.

Few functions have been assigned to the amino-terminal region of apoB protein. In 1990 Shih and coworkers (79) showed that apoB peptide 1000–1016 accumulates focally at the healing edges of arterial lesions in balloon-catheter deendothelialized rabbit aorta, and have proposed that this arterial wall-binding domain of apoB mediates accumulation of LDL in arterial lesions. In 1994 Sivaram and coworkers (80, 81) provided compelling evidence that the amino-terminal, 116-kDa fragment of apoB binds to lipoprotein lipase and that this interaction facilitates association of the lipolytic enzyme with endothelial cells. In 1995 Bucala and coworkers (82) reported a 67-amino acid domain, residues 1388–1474, that undergoes advanced glycosylation in vitro and in vivo and that, despite its remote location from the LDL receptor binding domain, impairs the binding of LDL modified by advanced glycosylation end products to human fibroblast LDL receptors. Our findings provide yet an additional function for the amino-terminal region of apoB and demonstrate that the primary sequence presented by residues 1–1084, or the first 23% of apoB-100 protein, suffices to produce recognition of malondialdehyde-modified LDL by the scavenger receptor of human monocyte-macrophages. The findings furthermore suggest that modification of lipoproteins such as apoB-48 particles of intestinal origin bearing

carboxyl-truncated apoB protein can contribute to the pathogenesis of atherosclerosis.

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