Human THP-1 Monocyte-Macrophage Membrane Binding Proteins: Distinct Receptor(s) for Triglyceride-Rich Lipoproteins†

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ABSTRACT: An apolipoprotein (apo) E- and lipoprotein lipase-independent, high affinity, saturable and specific binding site and pathway for uptake of certain triglyceride-rich lipoproteins (TGRLP) by human monocyte-macrophages that leads to lipid accumulation and foam cell formation *in vitro* has been reported; two membrane binding activities were identified as receptor candidates with apparent molecular masses of 200 and 235 kDa [Gianturco et al. (1994) *J. Lipid Res. 35*, 1674−1687]. Here we present new evidence that these activities are TGRLP receptors with unique biochemical properties which distinguish them from other lipoprotein receptors. Protease and heparinase susceptibility studies demonstrate that (1) these activities have essential protein, but not heparan sulfate proteoglycan (HSPG) components; (2) the membrane binding proteins (MBPs) are located on the cell surface; (3) HSPGs do not facilitate TGRLP binding to this specific cellular site. Upon reduction, MBP 200 and 235 are both converted into a single, new binding activity of intermediate mobility (MBP 200R); all MBP forms displayed high affinity, saturable TGRLP binding with similar K_ds (1.4−2.2 μg/mL). Notably, MBP 200R retained the combined ligand binding capacity of MBP 200 and 235 prior to reduction, demonstrating that, unlike members of the LDL receptor or the scavenger receptor families, disulfide bonds are not critical for activity. At 65 °C, MBP 235 was converted into MBP 200 without loss of total binding activity, suggesting heat dissociates a small subunit not required for binding from a common large protein subunit that binds TGRLP. Since the MBPs are found on the cell surface, are themselves functionally and structurally related, have distinctly different biochemical properties from members of the LDL receptor and scavenger receptor families, and share all critical characteristics with the cellular binding site, we hypothesize that they represent a new and unique receptor family for apoE- and lipoprotein lipase-independent uptake of TGRLP by human monocyte-macrophages.

The conversion of monocyte-macrophages into lipid-filled "foam cells", characteristic of atherosclerotic lesions, has been a topic of intense research for many years, much of it focusing on mechanisms involved in uptake of modified and oxidized low density lipoproteins (LDL)[1] by the scavenger receptor of differentiated macrophages (Goldstein et al., 1979) and a putative oxidized LDL receptor (reviewed by Steinberg et al., 1989). In addition to these modified LDLs, studies on humans and animals have demonstrated that monocyte-macrophages also take up plasma chylomicrons and very low density lipoproteins (VLDL), triglyceride-rich lipoproteins (TGRLP) respectively derived from the intestine, in response to dietary fat, and from the liver, from endogenous sources (Parker et al., 1970; Hussain et al., 1989a,b). Thus, TGRLP may serve as a nonmodified, native source of lipoprotein lipid for monocyte-macrophage nutrition in the normal state, or in the conversion of monocyte-macrophages into foam cells in pathological states. Indeed, in humans, Parker et al. showed that the monocyte-macrophage-derived foam cells in peripheral tissues of hypertriglyceridemic (HTG), diabetic subjects contained TGRLP core lipids (triglycerides and cholesteryl esters) following chylomicron uptake (Parker et al., 1970). In marmosets and rabbits, Hussain et al. found substantial uptake of chylomicrons *in vivo* by peripheral macrophages, particularly in bone marrow, that was not accelerated by infusion of apolipoprotein (apo) E (Hussian et al., 1989a,b), a somewhat surprising finding since apoE is a necessary ligand for the uptake of large TGRLP by receptors of the LDL receptor gene family. More recently, however, homologous recombinant ("knockout") mice that completely lack apoE were found to accumulate VLDL and remnants in their plasma and develop spontaneous atherosclerosis, which is accelerated by high fat diets (Plump et al., 1992; Zhang et al., 1992). The lesions are characterized by monocyte-macrophage-derived foam cells, as in human lesions, demonstrating unequivocally that apoE is not necessary for the conversion of monocyte-macrophages into foam cells *in vivo* (Nakashima et al., 1994; Reddick et al., 1994). Taken together, these studies *in vivo* suggest the

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[1] Abbreviations: apo, apolipoprotein; TGRLP, triglyceride-rich lipoprotein; HTG-VLDL, hypertriglyceridemic very low density lipoproteins; tryp-VLDL, trypsinized very low density lipoprotein; MBP, membrane binding protein; LDL, low density lipoprotein; PBS, phosphate-buffered saline; HSPG, heparan sulfate proteoglycan; LpL, lipoprotein lipase; LSR, lipolysis-stimulated receptor; LRP, LDL receptor-related protein−α_2-macroglobulin receptor; CR, chylomicron remnant; PMA, phorbol 12-myristate 13-acetate.

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existence of an apoE-independent pathway for the uptake of chylomicrons and VLDL or their remnants by monocyte-macrophages, which could result in foam cell formation.

In vitro evidence for an apoE-independent and lipoprotein lipase (LpL)-independent pathway for chylomicrons and certain VLDL has been reported (Gianturco et al., 1986, 1988, 1994). This route is different from the apoE-mediated receptor binding of TGRLP by the LDL receptor (Gianturco et al., 1982a, 1983; Bradley et al., 1984; Krul et al., 1985; Eisenberg et al., 1988) and other receptors in that gene family (Beisiegel et al., 1989; Kowal et al., 1989; Takahashi et al., 1992) or the apoE- or LpL-facilitated uptake involving heparan sulfate proteoglycans (HSPG) (Rumsey et al., 1992; Williams et al., 1992; Eisenberg et al., 1992; Mulder et al., 1992; Ji et al., 1993). This unique apoE- and LpL-independent pathway and binding site is found in murine macrophages, in human monocyte-macrophages, and in the human monocytic cell line, THP-1, and represents a specific, high affinity, saturable pathway for the uptake and degradation of TGRLP (Gianturco et al., 1988, 1994). Uptake of HTG-VLDL, trypsinized VLDL devoid of apoE (tryp-VLDL), and plasma chylomicrons (S_f >400) via this pathway resulted in rapid (≤4 h), massive lipid accumulation and foam cell morphology of these cells *in vitro* (Gianturco et al., 1982b, 1986, 1988, 1994). Two major TGRLP binding activities with apparent molecular masses of 200 and 235 kDa were identified by ligand blotting analysis in membrane extracts of both normal human blood-borne and THP-1 monocytes and macrophages (Gianturco et al., 1994). Both binding activities demonstrated the lack of sterol regulation, unchanged expression during differentiation, and restricted cellular distribution as did the cellular pathway, characteristics which distinguish this pathway from members of the LDL receptor family. Extensive direct and competitive binding, uptake, degradation, and triglyceride accumulation studies in cells coupled with ligand blotting analyses demonstrated that the cells and the membrane binding activities have identical ligand specificities, further supporting their potential role as monocyte-macrophage receptors for TGRLP. Lipoprotein ligands with high affinity include plasma chylomicrons (S_f >400) from both normal and hyperlipidemic subjects, VLDL S_f 60−400 from many HTG subjects, and a modified TGRLP, trypsinized VLDL (devoid of apoE but retaining most apoB in fragments of <100 kDa). In contrast, VLDL subfractions from subjects with normal plasma triglyceride levels do not bind with high affinity to cells or to the 200 and 235 kDa binding activities in ligand blots of membrane extracts and do not cause rapid, saturable TG accumulation in monocytes or macrophages, as do HTG-VLDL, chylomicrons, or tryp-VLDL (Gianturco et al., 1994). The shared functional and ligand binding characteristics of the cellular pathway and the membrane binding activities strongly support their candidacy as the putative receptors for the apoE-independent uptake of specific TGRLP by monocyte-macrophages (Gianturco et al., 1994).

In this report we directly address the nature of the membrane binding activities, their relationship to each other, and their biochemical properties which distinguish them from members of the LDL receptor and scavenger receptor families. We present evidence that the 200 and 235 kDa binding activities have essential protein but not HSPG components necessary for activity; that both membrane binding proteins (MBPs) are located on the cell surface, a necessary property for interaction with extracellular ligands; and that they share a large common protein component that, unlike previously described lipoprotein receptors, retains full binding activity after exhaustive reduction. These data provide strong biochemical evidence that the related, cell-surface MBPs are apoE-independent TGRLP receptors of human monocyte-macrophages.

MATERIALS AND METHODS

Lipoproteins. HTG-VLDL (S_f 100−400 and 60−100) were isolated from plasma of fasting subjects with types 4 and 5 lipoprotein profiles or in some cases from lipemic plasma obtained from the Red Cross. HTG-VLDL were subfractionated through a discontinuous NaCl gradient from d 1.063 to 1.006 g/mL by a cumulative flotation method (Lindgren et al., 1972) as detailed previously (Gianturco & Bradley, 1986). Protein concentrations of the lipoproteins were obtained by a modified Lowry procedure (Lowry et al., 1951; Helenius & Simons, 1971). Trypsinized VLDL, devoid of immunochemically detectable apoE, was prepared as previously described (Gianturco et al., 1983; Bradley et al., 1984; Gianturco & Bradley, 1986). Functional loss of apoE was demonstrated by lack of binding of tryp-VLDL to partially purified bovine LDL receptors on ligand blot analyses (Brown et al., 1986; Gianturco et al., 1988, 1994). Although tryp-VLDL is devoid of immunochemically detectable apoE, it retains approximately 70% of the original total protein, including most immunochemically detectable apoB, retained as fragments of 100 kDa and less. For binding studies, lipoproteins were iodinated by the iodine monochloride method as described (Bilheimer, 1972) and as used previously (Gianturco et al., 1983; Gianturco & Bradley, 1986). Free iodine was removed by gel filtration followed by extensive dialysis. Samples were filtered (0.45 μm Millex; Millipore Corp.) the day of use; specific activities ranged from ~100 to 250 cpm/ng of protein, as previously described (Gianturco et al., 1988, 1994).

Cells and Cell Culture. THP-1 cells (a human monocytic leukemia cell line) were purchased from the American Type Culture Collection and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin, 100 units/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Cells were maintained in tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air at $<1.0 \times 10^6$ cells/mL. For differentiation, cells (1.5×10^6 cells/well, in 6-well plates) were seeded in complete media; phorbol 12-myristate 13-acetate (PMA) was then added (10^{-7} M final concentration), as described (Gianturco et al., 1994).

Heparinase Treatment of Cells. Cells were seeded in 6-well tissue culture plates (1.5×10^6 cells/well), and phorbol ester (10^{-7} M) was added to induce adherence. After 24 h, cells were washed, preincubated for 2 h at 37 °C in RPMI-1640 with and without heparinase I (Sigma, 5 units/mL), dissolved in sterile saline immediately prior to use, similar to conditions used to demonstrate HSPG-facilitated binding of apoE-enriched lipoproteins to cells (Ji, 1993). The medium was removed, and cells were washed three times with media at 4 °C to remove the heparinase and to cool the cells. Cells were then incubated with RPMI-1640 containing 10 mM Hepes, pH 7.4, and 2 mg of BSA/mL, and indicated amounts of ^{125}I-HTG-VLDL or ^{125}I-tryp-VLDL alone and in the presence of 200 μg/mL unlabeled VLDL for 1.5 h at 4 °C prior to extensive washing with cold buffered saline

containing 2 mg of BSA/mL (Goldstein & Brown, 1974), as previously described (Gianturco et al., 1994). Cells were dissolved in 0.1 N NaOH prior to the measurements of cell-associated radioactivity and cell protein. Dishes with no cells were used to correct for the amount of nonspecific binding to the plastic wells, as described (Gianturco & Bradley, 1986).

Trypsin and Pronase Treatment of Cells. For experiments at 4 °C, THP-1 monocytes (1.5×10^8) were precooled, washed twice with ice-cold PBS in the absence of protease inhibitors, and incubated with 10 mL of buffer A (PBS plus 1 mM EDTA) or buffer A containing either 2 mg/mL trypsin or 2 mg/mL Pronase at 4 °C for 20 min in a rotary shaker. Proteolysis was arrested by dilution with 2 volumes of RPMI containing 2 mg/mL soybean trypsin inhibitor, a protease inhibitor mix [containing 50 units/mL aprotinin, 5 mM benzamidine, 0.1 mM PMSF, 14.5 μM pepstatin, 0.1 mM leupeptin, and 1.9 μM PPACK (520222, Calbiochem, San Diego, CA)], and FBS (10% final concentration). The cells were centrifuged at 1000 rpm for 5 min and washed twice at 4 °C with PBS containing protease inhibitors and then twice with PBS alone. Cells were then resuspended in 6 mL of 20 mM Tris-HCl, pH 8.0 (buffer B), and membrane extracts prepared as described below.

To determine the effects of Pronase pretreatment on the cellular binding of TGRLP, THP-1 cells were seeded and treated with PMA as described for the heparinase pretreatment. Cells were washed once with complete medium and then incubated at 37 °C for 40 min with RPMI-1640 without (control) or with 3 μg of Pronase/mL, as described (Goldstein & Brown, 1974). The cells were washed once with cold RPMI-1640, and one set of control and Pronase-treated cells was then incubated with cold test media containing the indicated concentrations of iodinated tryp-VLDL for 1 h at 4 °C, washed extensively, and processed for determination of the bound lipoprotein as described above. To measure recovery of binding, a second set of control and Pronase-treated cells was further incubated in complete medium for 2−4 h before the binding study or the extraction for ligand blotting analysis was performed. In all protease treatment experiments, cell viability was >95% after incubations with protease, as determined by trypan blue exclusion.

Antibodies. Sheep anti-human apoB antiserum (1001400, Boehringer Mannheim Biochemicals, Indianapolis, IN) was purified by affinity chromatography using an LDL-conjugated Sepharose column, prepared as previously described (Schneider et al., 1982). Immunoaffinity purified rabbit anti-sheep IgG conjugated to alkaline phosphatase was purchased from Jackson Laboratories (West Grove, PA).

Preparation of Membrane and Whole Cell Extracts. THP-1 monocytes (1.5×10^8) were harvested and washed twice with 50 mL of 0.15 M NaCl containing 50 units of aprotinin/mL, 5 mM benzamidine, and 0.1 mM PMSF. For membrane preparations, cells were placed in 6 mL of a hypotonic medium (buffer B) containing the protease inhibitor mix described above for 15 min on ice. The cells were lysed in a Dounce homogenizer with 20 strokes using a B-pestle. The lysate was adjusted to 0.15 M NaCl and 0.1 mM EDTA and centrifuged at 800g for 10 min at 4 °C to pellet nuclei and cell debris. The supernatant was centrifuged at 100000g for 1 h at 4 °C. The resulting membrane pellets were resuspended in buffer C (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1 mM EDTA) containing protease inhibitors, and the proteins were extracted with 40 mM octyl glucoside for 15 min on ice. Insoluble material was sedimented from these detergent extracts at 100000g, and the supernatants were flash frozen in liquid nitrogen after adding glycerol (10%). To prepare detergent extracts from intact cells, the cells were pelleted (1000 rpm, 4 °C, 5 min), resuspended in 2 mL of buffer C containing the protease inhibitor mix, and solubilized with 1% Trion X-114 for 15 min on ice. Aqueous phase extracts were prepared as previously described (Bordier, 1985; Gianturco et al., 1988, 1994) and immediately frozen as described above. Protein content was estimated by the Bradford method using the Bio-Rad Protein Assay reagent (Bradford, 1976).

Ligand Blotting. The ligand blotting assay was performed essentially as described earlier (Gianturco et al., 1988, 1994) with minor modifications. Aliquots of the detergent extracts were electrophoresed on 5% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970) under nonreducing conditions in a Bio-Rad minigel apparatus and electrotransferred to nitrocellulose. For two-dimensional electrophoresis, detergent extracts were electrophoresed in the first dimension on a slab minigel; 1-cm-wide strips containing the MBPs were removed, treated with sample buffer, pH 6.8, with or without 2% 2-mercaptoethanol for 10 min at room temperature, placed lengthwise on a second 5% slab minigel (perpendicular to the direction of electrophoresis in the first dimension and adjacent to a single well on the left for molecular weight markers), electrophoresed, and electrotransferred to nitrocellulose as described above. 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 and incubated with HTG-VLDL or tryp-VLDL S_f 100−400 in ligand buffer or in TEA buffer (0.1 M triethylamine, pH 11.5, 90 mM NaCl, and 2 mM $CaCl_2$) containing 0.5% milk for 3 h. Ligand incubation in the latter buffer reduced background compared to the former buffer used previously (Gianturco et al., 1988, 1994). The blots were washed 3 times for 5 min each with 0.5% milk in ligand buffer and then incubated with affinity purified sheep anti(h)-apoB antibody in 0.5% milk, pH 8, for 3 h. After three 5-min washes, bound antibody was detected by incubation with rabbit anti-sheep IgG conjugated to alkaline phosphatase for 2 h followed by the substrates, BCIP and NBT (Bio-Rad laboratories, CA). All incubations were carried out at room temperature on a rotary shaker. The specificity of ligand binding to the MBPs was confirmed by control incubations in which the primary antibody, the ligand, or both were omitted. Detection and sensitivity of tryp-VLDL and HTG-VLDL were equivalent as determined by densitometric analysis of known concentrations of each lipoprotein applied to nitrocellulose. Ligand blots were scanned on an optical scanner (Hewlett-Packard, Atlanta, GA), and binding activity was quantitated using the Image Quant software (Molecular Dynamics densitometer, Sunnyvale, CA).

RESULTS

Nature of the Membrane Binding Activities. Although receptors in the LDL receptor and the scavenger receptor families are proteins, cellular HSPG also bind TGRLP through interaction with apoE and apoB. Thus, to determine the nature of the ligand binding activities from membrane and whole cell extracts of THP-1 monocytes, extracts were incubated with proteases and heparinase and then analyzed for binding activity by ligand blotting analysis. As shown

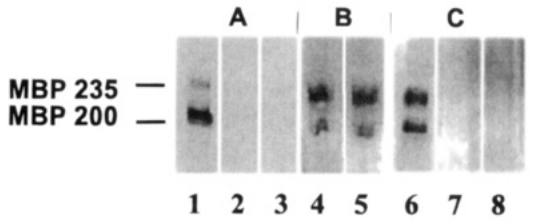

FIGURE 1: Effect of protease and heparinase treatment of THP-1 monocyte detergent extracts and the effects of cell surface proteolysis of intact THP-1 monocytes on MBP 200 and 235 activity. (A) Extracts (both membrane and cell) from THP-1 monocytes were prepared as described in Materials and Methods and incubated with buffer C alone (control) (lane 1) or with buffer C containing 2 μg/mL trypsin (lane 2) or Pronase (lane 3) for 10 min at 4 °C or (B) with 5 units of heparinase I/mL of RPMI-1640 (lane 5) or RPMI-1640 alone (lane 4) for 2 h at 37 °C. Aliquots (~50 μg of protein) were separated on SDS−polyacrylamide gels and transferred to nitrocellulose. Binding activities were determined by ligand blotting using 20 μg/mL HTG-VLDL (S_f 100−400), detected by antibody staining, and quantitated by densitometry of optical scans (see Materials and Methods). The data are representative of three independent protease and two heparinase experiments; no activity was detected after proteolysis, and heparinase had no significant effects on blotting activity of MBP 200 or 235 as determined by scanning densitometry. (C) THP-1 monocytes were washed with ice-cold PBS and incubated with buffer A alone (lane 6) or containing either 2 mg/mL trypsin (lane 7) or 2 mg/mL Pronase (lane 8) for 20 min at 4 °C. After the incubation, the cells (>95% viable) were harvested and washed and extracts prepared as described in Materials and Methods. Ligand blot analysis was performed as described above using HTG-VLDL (20 μg/mL) and antibody detection.

in Figure 1A, both MBP 200 and 235 binding activities were lost when cell extracts were incubated with 2 μg/mL trypsin or Pronase (lanes 2 and 3), when compared to buffer alone (lane 1), for 10 min at 4 °C. In contrast, when extracts were incubated with heparinase (5 units/mL for 2 h at 37 °C), under conditions where heparinase was shown to be active, no loss in binding activity was detected (Figure 1B, lanes 4 and 5). Since protease treatment abolished or greatly reduced all ligand blotting activities in membrane extracts whereas heparinase treatment had no effect on the 200 and 235 kDa binding activities, we designated the binding activities as membrane binding proteins, MBP 200 and MBP 235.

Effects of Cell-Surface Proteolysis on MBP 200 and 235 Activities and Cellular Binding of TGRLP. For receptors to bind extracellular ligands, they must be located, in part, on the cell surface. To demonstrate that the candidate receptors MBP 200 and 235 satisfy this basic criterion, we first carried out cell surface-specific proteolysis of THP-1 monocytes at 4 °C, to preclude internalization of proteases, and under conditions where cells remain intact and viable after treatment. Pretreatment of intact cells with trypsin or Pronase (2 mg/mL) at 4 °C for 20 min abolished the binding activity of both MBP 200 and 235 in membrane extracts (Figure 1C, lanes 7 and 8), compared to an extract from control cells treated with PBS (Figure 1C, lane 6), in two out of three experiments. In the third experiment, Pronase again abolished MBP 200 and MBP 235 activity and trypsin caused approximately an 80% decrease in the ligand binding activity of both MBP 200 and 235. Treatment of THP-1 monocytes with trypsin (0.5 mg/mL) at 37 °C for 5 min, conditions used to demonstrate cell surface localization of the acetyl LDL receptor (Kodama et al., 1988), also resulted in loss of all detectable ligand binding activity of MBP 200 and 235 compared to cells treated with buffer only (not shown).

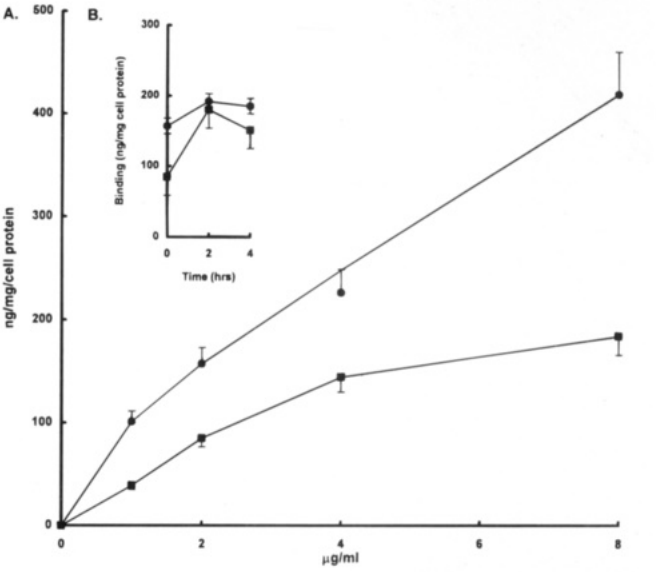

FIGURE 2: Cellular binding of ^{125}I-tryp-VLDL to THP-1 macrophages after pretreatment with Pronase. (A) THP-1 macrophages, 1 day after PMA-induced adherence, were incubated in RPMI-1640 without (closed circles) or with 3 μg of Pronase/mL (closed squares) at 37 °C for 40 min. The cells were then washed and incubated with the indicated levels of ^{125}I-tryp-VLDL for 1 h at 4 °C and processed as described in Materials and Methods. The amount of VLDL bound, in ng/mg of cell protein, is plotted as a function of the amount of ^{125}I-tryp-VLDL to which the cells were exposed. (B) Insert: After incubation with or without Pronase, cells were washed and incubated with complete medium at 37 °C for the indicated times prior to a 4 °C binding study with ^{125}I-tryp-VLDL, 2 μg/mL, as described in (A). Each data point is the average of values from duplicate wells; the range of values is as indicated.

To determine if Pronase treatment also affected the high affinity, apoE-independent binding of tryp-VLDL to cells, THP-1 macrophages were treated with RPMI-1640 alone (control) or with 3 μg of Pronase/mL at 37 °C for 40 min prior to 4 °C binding studies, conditions shown not to inhibit cell growth, but to alter cell surface receptors (Burger, 1970; Goldstein & Brown, 1974). These binding studies were performed in THP-1 macrophages 1 day after adherence to preclude potential ambiguities introduced when either the LDL receptor or LRP is expressed. Previous studies demonstrated that at 1 day after phorbol ester treatment neither the LDL receptor nor the acetyl LDL receptor is expressed (Gianturco et al., 1994; Via et al., 1989). Likewise, LRP expression is minimal before full differentiation (Moestrup et al., 1990). Use of tryp-VLDL devoid of apoE as a ligand precluded potential binding to the VLDL receptor, as well as to the LDL receptor or LRP. As shown in Figure 2, Pronase pretreatment significantly reduced the binding of ^{125}I-tryp-VLDL by 50−65% compared to controls. Recovery of binding of TGRLP to cells (>80%) occurred within 2−4 h after the Pronase-treated cells were washed and further incubated in complete medium at 37 °C prior to assessment of binding at 4 °C (Figure 2, insert). Similar results were obtained in two separate experiments with both tryp-VLDL and HTG-VLDL. Furthermore, ligand blotting analyses demonstrated that THP-1 monocytes treated with 3 μg of Pronase/mL for 40 min at 37 °C also lost approximately 50−60% of their MBP 200 and MBP 235 activities compared to control cells (Figure 3). After the 4 h recovery period in complete media, total MBP activities in control and Pronase-treated cells were equal and somewhat greater than seen immediately after treatment, similar to the recovery seen in

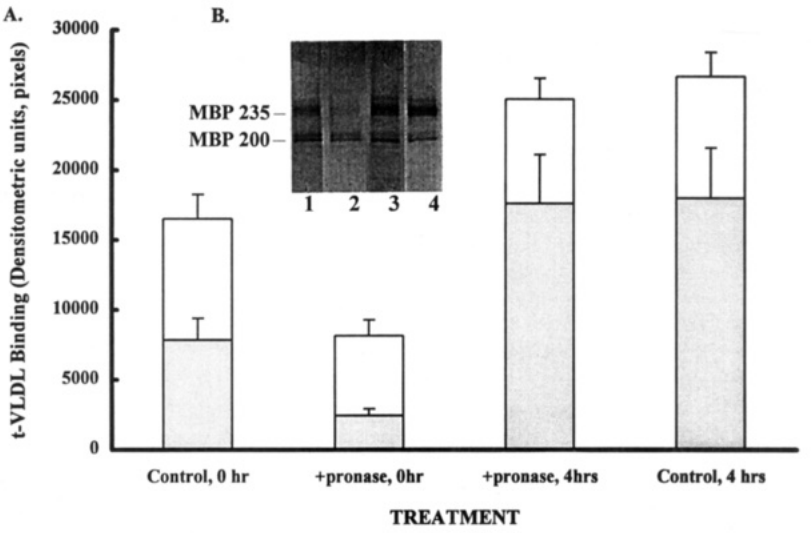

FIGURE 3: Activity of MBP 200 and 235 after pretreatment of THP-1 monocytes with Pronase at 37 °C. (A) Cells treated with Pronase and control cells treated with RPMI alone as described in the legend of Figure 2 were either immediately extracted for ligand blotting analysis (0 h) or washed and further incubated for 4 h at 37 °C in complete media prior to extraction for ligand blotting analysis (4 h) with tryp-VLDL as described in Materials and Methods; densitometry of the ligand binding activities shown in the insert and Image Quant software (Molecular Dynamics), with volume integration, were used to quantify relative binding to MBP 200 and 235. The cells were ≥98% viable after treatment, by trypan blue exclusion. Bars indicate the binding of tryp-VLDL in densitometric units (pixels) to MBP 200 (white bars) and to MBP 235 (shaded bars), and the total binding is represented by the stacked bar for each treatment as indicated. (B) Insert: Ligand blot of representative lanes used in the densitometric analysis: lane 1, control cells; lane 2, cells preincubated with 3 μg/mL Pronase, 40 min at 37 °C; lanes 3 and 4, cells treated with Pronase or control cells were washed, incubated in complete media for 4 h at 37 °C, and extracted for ligand blotting analysis. Analyses were carried out as described in Figure 1. Values are the average of duplicate lanes and are expressed in densitometric units (pixels).

binding of TGRLP to cells (Figure 2, insert). The simultaneous and similar Pronase-induced loss of binding of TGRLP to cells and the loss of MBP 200 and MBP 235 activities in ligand blots coupled with parallel recoveries of cell binding and MBP activities provide strong evidence for the role of these MBPs in the cell surface, apoE-independent binding of TGRLP by monocyte-macrophages.

In all cell surface proteolysis experiments, cell viability was greater than 95%, by trypan blue exclusion, and the cell morphology appeared normal by light microscopy, indicating the treatments did not disrupt the cells, although there was some clumping of monocytes after proteolysis at 4 °C. These experiments indicate that the majority of the detectable ligand binding activity of both MBP 200 and 235 in THP-1 monocytes is sensitive to cell surface-specific proteolysis.

Heparinase Effects on Binding of TGRLP by THP-1 Monocytes. Cellular HSPGs mediate binding of certain lipoproteins to cells via apoE, apoB, or LpL, and bridge binding to lipoprotein receptors (Rumsey et al., 1992; Eisenberg et al., 1992; Mulder et al., 1992; Ji et al., 1993). To determine if HSPG were involved directly in the cellular binding of TGRLP, THP-1 macrophages (1 day after adherence to minimize expression of the LDL receptor, LRP, LpL, and apoE) were preincubated with or without 5 units/mL heparinase for 2 h at 37 °C prior to a 4 °C binding study with iodinated HTG-VLDL, alone or with excess unlabeled VLDL to measure total and nonspecific binding and to calculate specific binding. As shown in Table 1, there was no statistically significant ($p < 0.05$) change in total, nonspecific, or specific binding of iodinated HTG-VLDL to THP-1 macrophages after heparinase treatment. Similar results were obtained in a separate experiment with tryp-VLDL devoid of apoE. Control experiments were performed to demonstrate active heparinase under these incubation conditions [release of ^{35}S-glycosoaminoglycans from cells prelabeled with inorganic $^{35}SO_4^{2-}$ (Woods et al., 1993; Ji et al., 1993)]. Thus, experiments with heparinase indicate that HSPGs are not required nor do HSPGs facilitate the apoE-independent pathway and binding of TGRLP to THP-1 macrophages. Moreover, these experiments further indicate that cell-derived LpL or apoE does not mediate binding of VLDL to cells under these conditions, since heparinase treatment also removes surface-bound LpL and apoE (Lilly-Stauderman et al., 1993).

Table 1: Effects of Heparinase Pretreatment of THP-1 Macrophages on the Subsequent Binding of ^{125}I-HTG-VLDL

^{125}I-HTG-VLDL (μg/mL)	heparinase	^{125}I-HTG-VLDL bound (ng/mg of cell protein) total	nonspecific	specific
2	−	60.7 ± 4.8[a,b]	33.1 ± 2.2	27.6
	+	72.0 ± 2.5	42.4 ± 3.9	29.6
4	−	116 ± 5.0	50.3 ± 2.0	65.8
	+	120 ± 6.6	53.4 ± 5.7	66.1

[a] THP-1 macrophages, 1 day after treatment with phorbol ester to induce adherence in 6-well plates, were washed and incubated with RPMI-1640 alone or with heparinase I, 5 units/mL for 2 h at 37 °C, washed 3 times with ice-cold RPMI-1640 containing 10 mM Hepes, pH 7.4, and 2 mg BSA/mL, and then incubated for 1 h at 4 °C in the same media containing the indicated concentration of ^{125}I-HTG-VLDL alone (for total binding) or with a 10-fold excess of unlabeled HTG-VLDL for nonspecific binding. Specific binding was calculated as the difference between the mean total and mean nonspecifically bound VLDL. Values are the mean (±SE) of triplicate dishes of cells, expressed as ng of ^{125}I-labeled HTG-VLDL protein/mg of cell protein. [b] There were no statistically significant ($p < 0.05$) differences between control and heparinase treatments (unpaired, two-sided t tests).

Effects of Reduction on MBP 200 and MBP 235. Since both MBP 200 and 235 are functionally related, we carried out experiments to determine if their physicochemical characteristics are similar to each other but different from the other lipoprotein receptors. Previously reported lipoprotein receptors are characterized by loss of ligand binding activity upon exhaustive reduction, as measured by ligand

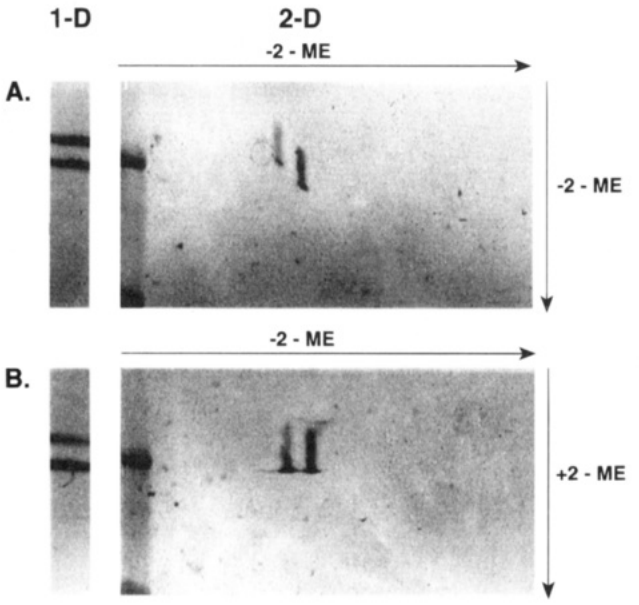

FIGURE 4: Two-dimensional SDS−PAGE of MBP 200 and MBP 235 with and without reduction. Detergent extracts were electrophoresed in the first dimension on a slab minigel; four 1-cm strips containing the MBPs were removed, and two strips treated with Tris-HCl buffer, pH 6.8, without (panel A) and two strips treated with (panel B) 2% 2-mercaptoethanol for 10 min at room temperature. One set of strips was directly transferred for ligand blotting (single lanes on left, 1-D). The other strips were placed lengthwise on a second 5% slab minigel (perpendicular to the direction of electrophoresis in the first dimension), electrophoresed, and electrotransferred to nitrocellulose, and ligand binding was detected as described in Materials and Methods. The 2-D panels demonstrate that, without reduction (panel A) prior to the second electrophoresis, the activities remain on the diagonal with no change in apparent molecular weight, whereas with reduction (panel B) both the activities are found off the diagonal, each migrating with a mobility intermediate to their initial mobilities and identical to each other. The single lane on the far left of panels A and B, 2-D, shows internal prestained molecular mass markers: ~200 and 97 kDa, respectively.

blotting analyses. Therefore, we first tested the ligand binding activities of MBP 200 and MBP 235 after reduction with 2-mercaptoethanol. Initial experiments demonstrated that upon reduction both MBP 200 and MBP 235 binding activities were completely lost; however, there appeared to be a conversion of one or both of their activities into a new, single binding activity designated MBP 200R (described below). Since the new binding activity was intermediate in mobility between MBP 200 and MBP 235 on SDS−PAGE, we questioned if MBP 200R arose from either MBP 200 or MBP 235, or if both activities were converted into the new species. Two-dimensional (2-D) SDS−PAGE was used to separate the MBPs on the basis of size, both before and after reduction with 2-mercaptoethanol. In a representative experiment shown in Figure 4, THP-1 monocyte membrane extracts were electrophoresed over the entire width of the gel in the first dimension without prior reduction. One-centimeter-wide strips containing the MBPs were cut from the gel and incubated in sample buffer (pH 6.8) without (Figure 4, panel A) or with (Figure 4, panel B) 2% 2-mercaptoethanol for 10 min at room temperature. The gel strips were placed on top of a second 5% slab minigel for electrophoresis in the second dimension and then electrotransferred to nitrocellulose for standard ligand binding analyses. Equivalent gel strips from the first dimensional separations served as controls, shown at the left of each panel (Figure 4, 1-D), and were transferred and blotted after treatment with sample buffer plus (Figure 4B) and minus (Figure 4A) reductant, demonstrating that this step did not alter MBP binding activities, either due to reduction itself or due to activity loss during the 10-min incubation.

In three independent experiments, both MBP 200 and MBP 235, separated in both the first and the second dimension without reduction, retained their original, distinct mobilities and activities (seen on the diagonal) (Figure 4, panel A, 2-D). In contrast, when the second dimension was run after reduction, both MBP 200 and MBP 235 mobilities were altered relative to the ~200 kDa standard and migrated with identical mobilities (see off the diagonal), intermediate between MBP 200 and 235 (Figure 4, panel B, 2-D). This demonstrates unequivocally that MBP 200R arises both from MBP 200 and from MBP 235. These changes in electrophoretic mobility reflect reduction of intramolecular disulfide bonds and, in the case of MBP 235, where its electrophoretic mobility increased upon reduction, the possible loss of a small subunit not involved in ligand binding, further addressed below.

Ligand Binding Affinities of MBP 200, MBP 235, and MBP 200R. Since the 2-D experiments proved that MBP 200R activity can arise from both MBPs, we then asked if this conversion occurred with or without a loss in the binding activity of the precursors, MBP 200 and 235 (Figure 5). Native HTG-VLDL and tryp-VLDL were used as ligands, and specific binding to the MBPs before and after reduction was detected with anti-apoB antibodies (Figure 5A) and quantified by scanning densitometry (Figure 5B). In the representative experiment shown in Figure 5, MBP 200 and MBP 235 exhibited similar, high affinity, saturable binding, with saturation occurring between 5 and 10 μg of apoVLDL/mL (Figure 5A,B). Scatchard analyses of the data indicate MBP 200 and MBP 235 have similar K_ds of 1.6 and 2.2 μg of apoVLDL/mL and B_{max}s of 96 and 66 ng of apoVLDL/mg of cell protein, respectively (Figure 5C). The binding of HTG-VLDL to MBP 200R exhibits similar, high affinity, saturable binding with a K_d of 1.4 μg/mL and a B_{max} of 160, approximately equal to the sum of the maximal binding activities of MBP 200 and MBP 235 (Figure 5C). Indeed, the theoretical (calculated) curve (dashed line), obtained by adding the amount of VLDL bound to MBP 200 and 235 at each level of lipoprotein, is nearly superimposable on the measured binding curve for MBP 200R (Figure 5B). Similar results were obtained with either HTG-VLDL S_f 60−100 or S_f 100−400 and with the corresponding tryp-VLDL. Notably, the binding affinities of HTG-VLDL and tryp-VLDL for the MBPs in this study are similar to their binding affinities for intact human blood-borne monocyte-macrophages and THP-1 monocytes and macrophages, where K_ds of 2−4 μg of apoVLDL/mL were determined, as previously reported (Gianturco et al., 1994). This striking similarity in ligand affinities provides additional supporting evidence that these MBPs are responsible for the apoE-independent, high affinity, and saturable binding of TGRLP to monocytes and macrophages.

Thermal Conversion of MBP 235 into MBP 200 Binding Activity. The reduction data indicated that MBP 200 and 235 share a common protein component of approximately 200 kDa which retains all the ligand binding activity. Since the mobility of MBP 235 actually increased upon reduction, whereas MBP 200 mobility decreased, this suggested that a small subunit may be present in MBP 235 that is lost upon reduction, either due to reduction of an intermolecular

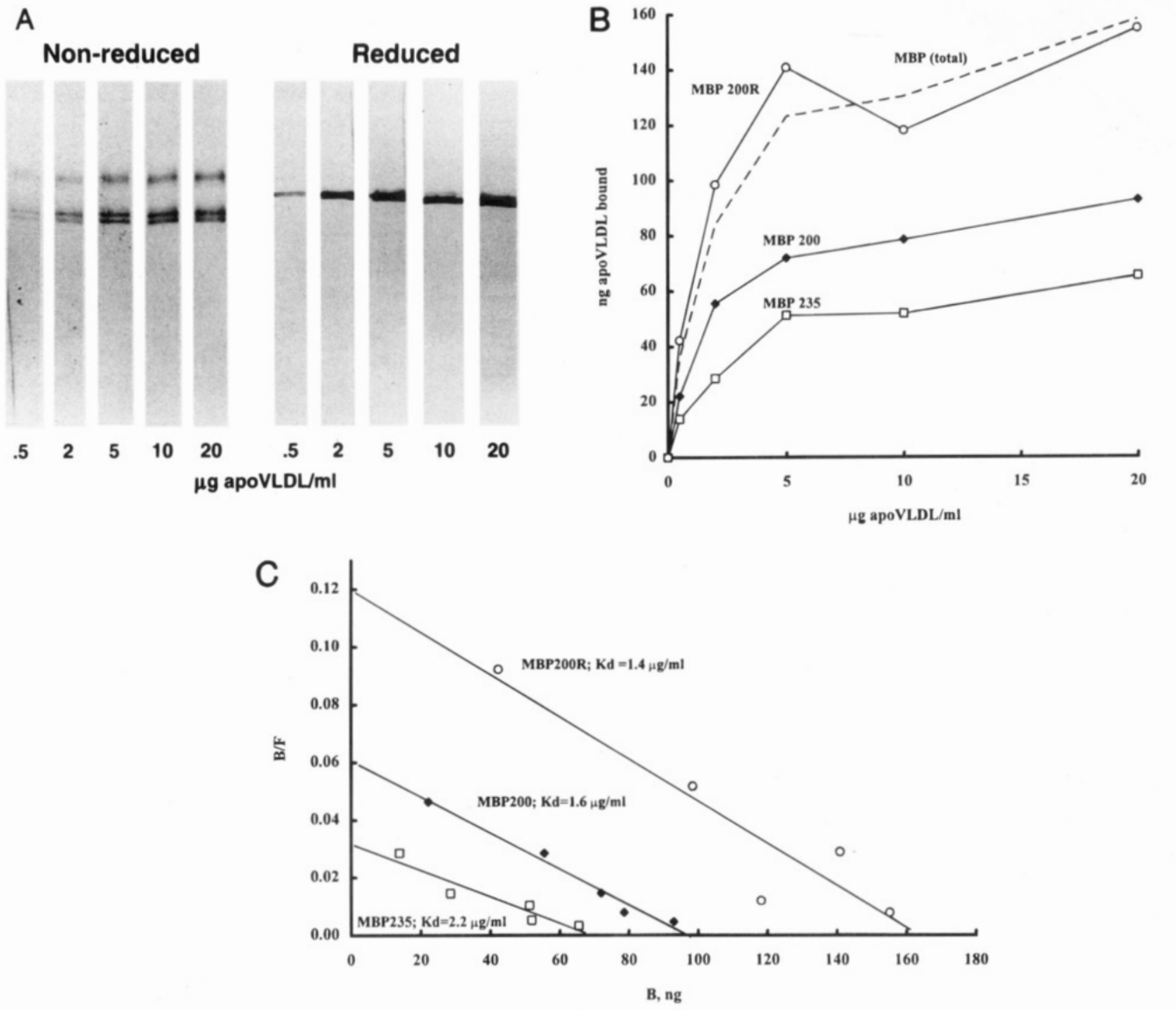

FIGURE 5: Saturation binding of HTG-VLDL to MBP 235, MBP 200, and MBP 200R. (A) Triton X-114 aqueous phase extracts of THP-1 monocytes were prepared as described in Materials and Methods. Equal aliquots of detergent extracts (25 μL; 12.5 μg of protein), before (first five lanes on left; nonreduced) and after reduction with 2-mercaptoethanol (last five lanes; reduced), were electrophoresed and transferred to nitrocellulose. Individual nitrocellulose strips were incubated with the indicated concentrations of HTG-VLDL (0.5, 2, 5, 10, and 20 μg of apoVLDL/mL) and detected with apoB antibodies as described in Materials and Methods. (B) The amount of VLDL bound to MBP 200, 235, and 200R activities in panel A was determined by densitometric scanning of each MBP region in the ligand blots using a calibration curve generated from known amounts of the same VLDL applied to nitrocellulose in an immunochemical dot blot assay quantified with a purified goat anti-human apoB antibody. The amount of ligand bound to each MBP region, expressed as ng of apoHTG-VLDL S_f 60−100 bound, is plotted as a function of the amount of HTG-VLDL to which the nitrocellulose was exposed: MBP 200 (♦), MBP 235 (□), MBP 200R (○). The broken line represents the calculated sum of binding of HTG-VLDL to MBP 200 plus its binding to MBP 235 at each level of VLDL. (C) The data from panel B were plotted after transformation by the method of Scatchard to determine K_ds of the MBPs, expressed as μg/mL.

disulfide bridge(s) or by an allosteric process caused by the disruption of the intramolecular disulfides during reduction of the large 200 kDa protein. To distinguish between these possibilities, we used heat to disrupt potential noncovalent interactions in MBP 235, since the MBPs were stable in the denaturing detergent SDS as well as in the nondenaturing detergents Triton X-114, Triton X-100, and octyl glucoside (Gianturco et al., 1994). Monocyte extracts were heated at several temperatures for up to 10 min prior to quantitative ligand blot analysis. There were no significant changes at 37 or 45 °C, but, as shown in Figure 6, at 55 and at 65 °C there was a progressive loss of MBP 235 activities (lower, shaded bars) and a compensatory increase in MBP 200 activities (upper white bars) with time so that the total combined activities remained approximately constant and not significantly different from control. Thus, temperature studies indicate that MBP 235 can be converted into MBP 200 with full retention of ligand binding activity. Furthermore, these data suggest that MBP 235 has a noncovalent subunit of approximately 35 kDa that is lost upon heating or disruption of the intramolecular cystines of the parent backbone shared by MBP 235 and MBP 200 and that is not involved directly in ligand binding. Additional temperature dissociation data indicated that when heated to 90 °C, MBP 235 binding activity was not detectable after 1 min and there was a decrease in the ligand binding activity of MBP 200 with time, due most likely to its denaturation (data not shown). Interestingly, in the presence of 2% SDS, the ligand binding activities of MBP 200 and MBP 235 were stable for at least 20 min at room temperature and showed slower

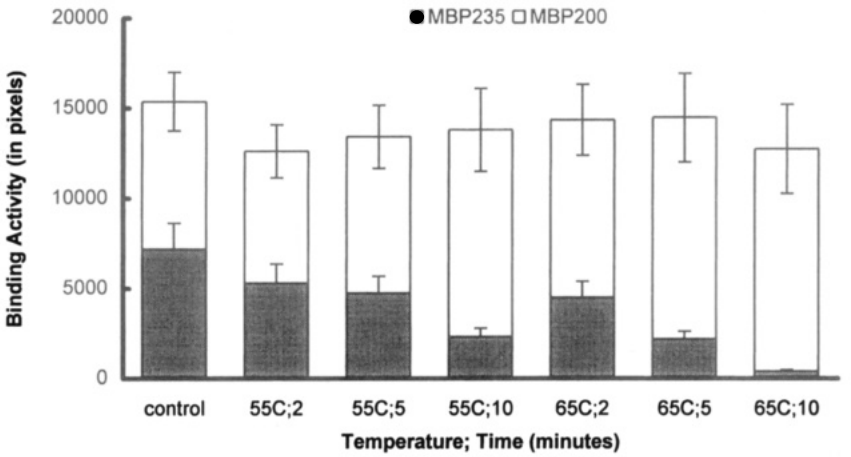

FIGURE 6: Temperature effects on MBP 200 and MBP 235. Detergent extracts of THP-1 monocytes were prepared as described in Materials and Methods. Aliquots were placed in preheated baths at 55 and 65 °C and removed at the indicated times, quenched in SDS sample buffer (2% final concentration) without reductant, and immediately electrophoresed for ligand blotting analysis. Control incubations at room temperature and up to 45 °C indicated that the binding activities were unchanged over this time period. The ligand blots were performed as described in Materials and Methods and quantitated as described in Figure 5. Ligand binding activities are expressed in pixels, quantitated by volume integration using Image Quant software; the upper, white bars represent the activity of MBP 200, and the solid bars represent the activity of MBP 235 at the indicated times and temperatures. The combined activities are represented by the sums of the individual bars.

inactivation at 90 °C than in the absence of 2% SDS. This suggested that SDS stabilized the binding activities and the association of the putative small subunit with the common MBP 200.

DISCUSSION

The current study was undertaken to define the molecular nature and biochemical characteristics of the membrane binding activities of human monocytes and macrophages that previous studies indicated are likely receptor candidates for a unique pathway for the apoE- and lipoprotein lipase-independent binding, uptake, and degradation of TGRLP by both blood-borne and THP-1 monocytes and macrophages (Gianturco et al., 1994). Protease susceptibility studies demonstrate that both MBP 200 and MBP 235 have protein components essential for ligand binding activity. Surface-specific proteolysis of intact cells, under conditions where viability was maintained at >95%, led to a significant, reproducible loss of both MBP activities (Figures 1C and 3) as well as a similar loss of the high affinity binding of TGRLPs to cells (Figure 2). Both TGRLP cellular binding and MBP 200 and 235 activities increased in parallel when cells were allowed to recover in complete medium after protease treatment. These experiments established the cell surface location of both MBPs. The parallel loss and recovery of MBP binding activity and cellular binding of TGRLP (Figures 2 and 3) strongly support their role as TGRLP receptors.

Protease susceptibility, however, did not rule out the possibility that the activities were proteoglycans. Moreover, proteoglycans, specifically HSPG, augmented and/or facilitated lipoprotein binding to the LDL receptor (Rumsey et al., 1992; Mulder et al., 1992) and to LRP (Nykjaer et al., 1993). Heparinase treatment of THP-1 extracts had no effect on either of the MBP activities, as measured by ligand blotting methods (Figure 1B), indicating these MBPs are not themselves HSPGs. Likewise, there was no decrease in the total, nonspecific, or specific binding of HTG-VLDL or tryp-VLDL to THP-1 macrophages pretreated with active heparinase prior to 4 °C binding studies (Table 1), indicating cellular HSPGs are not necessary for binding to this receptor. Moreover, since heparinase treatment would remove any HSPG-bound LpL or apoE, these studies indicate that under these conditions cell-derived LpL or apoE is not involved in the cellular binding of TGRLP.

The LDL receptor, LRP, and the VLDL receptor have multiple cysteine-rich repeats in their ligand binding domains. Upon reduction, the LDL receptor loses its ability to bind lipoprotein ligands (Daniel et al., 1983). The recently cloned VLDL receptor has homologous ligand binding domains (Takahashi et al., 1992) and would be expected to lose binding activity upon reduction, although this has not yet been reported. Although from a different gene family, the scavenger receptor was also susceptible to inactivation by exhaustive reduction. Its trimeric structure (MW ~ 260 kDa) could be selectively reduced with thiols so that the monomeric form (MW ~ 78 kDa) retained full ligand binding activity, although upon exhaustive reduction (5% 2-mercaptoethanol or 100 mM DTT), all ligand binding activity was lost (Via et al., 1992). In contrast, reduction of MBP 200 and MBP 235 generated a new active species, MBP 200R, which remained active even under exhaustive reduction conditions (boiling in 100 mM DTT or 5% 2-mercaptoethanol). Thus, although disulfides were present in both MBPs (both mobilities changed upon reduction), these disulfides were not essential for ligand binding activities. Interestingly, several permissible oxidation states and/or disulfide isomers of the MBPs were detected in some blots; e.g., in Figure 5A, doublets of activities for both MBP 200 and MBP 235 were detected that, upon reduction, were converted into the single protein binding activity, MBP 200R. It is not known whether the different forms observed in some but not all ligand blots of unreduced extracts are handling artifacts or reflect an as yet unidentified functional role, such as in regulation or transport.

An important finding in the current study is that the MBPs possess the biochemical characteristic of high affinity binding for TGRLP, a necessary property for a receptor. The MBPs have K_ds between 1.4 and 2.2 μg of apoVLDL/mL (Figure 5), similar to the binding of lipoproteins to the LDL receptor, and ~10-fold higher affinity than ligands for the scavenger receptor. The MBPs' K_ds are strikingly similar to those

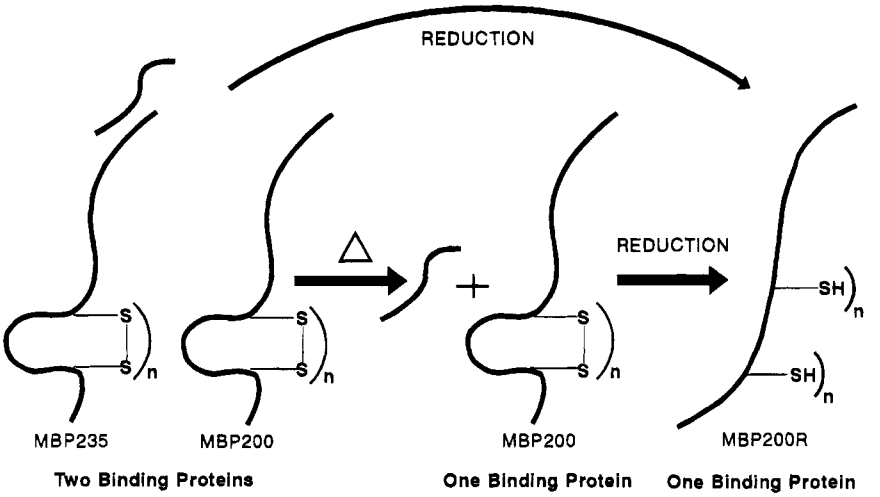

FIGURE 7: Model of the interrelationship between MBP 200 and MBP 235. A schematic model illustrating the potential structural relationships of the MBPs from monocytes and macrophages and changes that occur during reduction or heating.

measured for specific, apoE-independent binding of TGRLP to human blood-borne and THP-1 monocytes and macrophages previously reported (Gianturco et al., 1994), again reinforcing their suitability as candidate TGRLP receptors and illustrating another difference from the low affinity, high capacity HSPG binding and slow uptake of lipoproteins by the HSPG-assisted cellular pathway (Rumsey et al., 1992; Williams et al., 1992; Mulder et al., 1993).

Thermal dissociation studies indicated that MBP 235 could be converted into MBP 200, suggesting that a small subunit of approximately 35 kDa is associated with the 200 kDa subunit to form MBP 235. The loss of this subunit, either through heat dissociation or through reduction, does not change the binding activity of the 200 kDa protein, indicating the smaller subunit has no direct role in ligand binding, unlike the 39 kDa receptor-associated protein which inhibits ligand binding to LRP (Herz et al., 1991). At this point, it is difficult to determine what role, if any, such a subunit might have, but several possibilities, including regulatory and membrane association functions, are considered for future study.

Our current working model (Figure 7) illustrates schematically the changes in apparent molecular masses of MBP 200 and 235 reflected by changes in mobility seen on SDS–PAGE when membrane extracts are treated with reducing agents or heat. In this model, MBP 235 consists of two subunits of 200 and 35 kDa that can dissociate, upon heating, into an active 200 kDa subunit and an inactive smaller subunit(s). Upon reduction, the MBP 235 complex also dissociates, and its 200 kDa active core protein is extended by the reduction of intramolecular cystines, yielding an active binding protein (MBP 200R) with a slightly slower migration, intermediate between MBP 235 and MBP 200 mobilities (Figures 4 and 5A). MBP 200 does not have a subunit associated, has saturation binding characteristics like those of MBP 235, and upon reduction is extended due to loss of its intramolecular disulfide bonds, yielding a product with identical electrophoretic mobility as that produced by reduction of the MBP 235 complex. This model emphasizes the major finding that a large and similar, if not identical, protein backbone is common to both MBP 200 and 235 and contains the ligand binding domain(s). Thus, the ligand binding domain(s) are not located either in domain(s) involved in the structural changes that occur upon reduction or in domains that bind to the putative 35 kDa subunit. More definitive information on the TGRLP binding domain(s) will be available when the ongoing cloning of this receptor is completed.

As discussed above, MBP 200 and 235 differ from the LDL receptor and its family (LRP and the VLDL receptor) and from the scavenger receptor in a number of respects, including apparent molecular mass, resistance to inactivation by reductants, and ligand specificity. Although the VLDL receptor protein has not yet been described, the homology of its cDNA with the LDL receptor predicts both a similar molecular mass (~130 kDa on SDS–PAGE) and susceptibility to inactivation by reduction; in addition, studies in transfected cells suggest its ligand is apoE (Takahashi et al., 1992). Thus MBP 200 and 235 do not resemble the VLDL receptor in these respects. MBP 200 and MBP 235 and this receptor pathway are also unlike the recently reported lipolysis-stimulated receptor (LSR) pathway in several critical respects (Bihain & Yen, 1992; Yen et al., 1994). The LSR pathway requires free fatty acid stimulation for expression and is present on a wide variety of cell types; the putative candidate receptor proteins for the LSR pathway are 115 and 85 kDa and are only active in the presence of free fatty acids, quite unlike the properties of MBP 200 and MBP 235 reported here and of the pathway reported previously (Gianturco et al., 1994). Neither the interrelationship between the putative LSR proteins nor their stability in the presence of reductants or heat has been reported. However, since the LSR and this TGRLP receptor pathway differ in the other crucial aspects just discussed, they do not appear to be related.

Both LRP and the LSR have been proposed as the chylomicron remnant (CR) receptor of hepatocytes (Brown et al., 1991; Herz, 1993; Yen et al., 1994). Although the TGRLP receptor pathway is not regulated by sterol and even though human postprandial plasma chylomicrons (S_f >400) bind with high affinity to MBP 200 and 235 (Gianturco et al., 1994) and are rapidly internalized by THP-1 monocytes and macrophages, we do not believe that the TGRLP receptor, reported here, is the elusive CR receptor, for two reasons. First, similar MBPs were not detected by ligand blotting analyses of hepatoma cell lines (HepG2 and HepG3B) under conditions where they were readily detected in monocyte-macrophages (Gianturco et al., 1994). Second, we initially detected this pathway and identified the MBPs using TGRLP in which the apoE was intentionally and totally

removed by proteolysis to establish that the uptake by this pathway was apoE-independent. In contrast, numerous studies indicate apoE is the ligand for the CR receptor(s) (for a review see Mahley & Hussain, 1991).

In summary, the new data presented here strongly support the conclusion that MBP 200 and MBP 235 are functionally and structurally related candidate receptor proteins for binding and uptake of TGRLP in human monocyte-macrophages. Since the MBPs share all critical characteristics with the cellular binding site, are found on the cell surface, and have distinctly different biochemical properties from members of the LDL receptor and scavenger receptor families and other lipoprotein uptake mechanisms, we hypothesize that they represent a new and unique receptor family for apoE- and lipoprotein lipase-independent uptake of TGRLP by human monocyte-macrophages.

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