Lysine and Arginine Residues in the N-Terminal 18% of Apolipoprotein B Are Critical for Its Binding to Microsomal Triglyceride Transfer Protein†

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ABSTRACT: Apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP) are essential for the efficient assembly and secretion of triglyceride-rich lipoproteins. We have presented evidence for a high-affinity interaction between these proteins [Hussain, M. M., et al. (1997) *Biochemistry 36*, 13060−13067]. In this study, we used chemically modified low-density lipoproteins (LDL) and recombinant human apoB18 to identify amino acid residues in apoB that are critical for its interactions with MTP. Acetoacetylation of 74% of lysine residues and cyclohexanedione modification of 54% of arginine residues completely abolished the interactions between LDL and MTP. Regeneration of lysine and arginine residues by hydroxylamine treatment completely restored the binding of modified LDL to MTP. Carboxyethylation of all the histidine residues decreased, but did not abolish, apoB−MTP interactions. In contrast, glycine methyl ester modifications of aspartic and glutamic acid residues, up to 38−44%, had no effect on LDL−MTP interactions. Furthermore, modification of lysine and arginine, but not the aspartic and glutamic acid, residues in apoB18 also completely abolished its interactions with MTP. These studies indicated that lysine and arginine, but not aspartic and glutamic acid, residues are critical for apoB−MTP interactions, whereas histidine residues are not as critical. Since lysine and arginine residues in apoB are known to interact with the LDL receptors and heparin, we studied the effect of different glycosaminoglycans on apoB−MTP interactions. Glycosaminoglycans had no significant inhibitory effect on apoB−MTP interactions, suggesting that the lysine and arginine residues crucial for apoB−MTP interactions are different from those that interact with the LDL receptor and heparin. The lysine and arginine residues in apoB18 may directly interact with negatively charged residues in the MTP molecule, or they may function to maintain the conformation of the recognition site.

Apolipoprotein B100 (apoB100)[1] is an essential structural protein required for the assembly of triglyceride-rich very low density lipoproteins by the liver. In the plasma, apoB100 interacts with several molecules that play a significant role in the catabolism of apoB-containing lipoproteins. ApoB100 binds to negatively charged sulfate groups of glycosaminoglycans by ionic interactions involving lysine and arginine residues (*1*−*8*). Two high-affinity heparin binding sites corresponding to amino acids 3147−3157 and 3357−3367 have been identified on apoB100 (*1*, *8*, *9*). In addition to these binding sites, delipidated and proteolyzed apoB100 contains several additional heparin binding sites that are dispersed throughout the molecule (*5*, *8*, *9*). Recently, LDL has also been shown to interact with the glycosaminoglycan side chain of decorin, a small proteoglycan, by ionic interactions involving lysine and arginine residues (*4*). In addition to glycosaminoglycans, apoB also interacts with endothelial cell surface-bound lipoprotein lipase (*10*, *11*). In the liver, remnant lipoproteins interact with cell surface proteoglycans and LDL receptors (for review, see ref *12*). Lysine and arginine residues in the apoB100 interact with LDL receptors (*3*, *13*−*16*). This receptor binding site has been localized to the C-terminal half of the molecule and overlaps with two high-affinity heparin binding sites (*3*). Thus, ionic interactions between apoB and LDL receptors can be inhibited by heparin and positively charged proteins (*17*, *18*).

In addition to interacting with molecules that are involved in the catabolism of lipoproteins, apoB has been shown to interact with proteins such as heat shock protein 70, calnexin, and the microsomal triglyceride transfer protein (MTP) that might play a role in the assembly of lipoproteins (*19*−*22*). Using in vitro binding assays, we have demonstrated high-affinity interactions between apoB and MTP (*22*). MTP is a heterodimeric protein consisting of 97 and 55 kDa subunits (*23*). The 55 kDa protein was identified as a ubiquitous ER resident protein, the protein disulfide isomerase (*24*). Several

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[1] Abbreviations: apoB, apolipoprotein B; ELISA, enzyme-linked immunoassays; ER, endoplasmic reticulum; LDL, low-density lipoproteins; MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.05% Tween 20.

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lines of evidence indicate that MTP is essential for the efficient assembly and secretion of triglyceride-rich lipoproteins. Mutations in the 97 kDa subunit result in the near absence of apoB-containing lipoproteins in the plasma of abetalipoproteinemic individuals (*25*−*30*). Co-expression of apoB and the 97 kDa subunit results in the assembly and secretion of apoB-containing lipoproteins in cells that normally do not secrete lipoproteins (*21*, *31*−*34*). Inhibitors of MTP activity decrease the secretion of apoB-containing lipoproteins most likely by increasing cotranslational degradation of nascent apoB (*35*−*38*). How does MTP, a lipid transfer protein, assist in the inhibition of cotranslational degradation and promote the lipidation of apoB? Recently, evidence of a physical association between apoB and MTP has been presented (*20*−*22*). These interactions may impart specificity for the lipidation and assist in the translocation of apoB from the ER membrane. Evidence for chaperone activity comes from co-expression, co-immunoprecipitation, and in vitro binding studies (*20*−*22*, *32*). We have demonstrated that MTP can interact with apoB polypeptides that are not associated with lipids and these interactions decrease with increases in the lipidation of apoB (*22*). Furthermore, it has been proposed that apoB and MTP interact initially by ionic interactions and subsequently by hydrophobic interactions (*22*). In this study, we have identified specific groups in apoB that are critical for its interactions with MTP by using group-specific reagents. Furthermore, the identified amino acid side groups were shown to be different from those involved in the binding of apoB to the LDL receptors and heparin.

MATERIALS AND METHODS

Materials. All the assays were performed with the purified heterodimeric MTP complex (*22*, *24*, *35*, *39*). Antibodies used for ELISA have been described (*40*, *41*). Human plasma (native) LDL (d = 1.02−1.063 g/L) was prepared by ultracentrifugation and characterized (*41*). Heparin (sodium salt), suramin (sodium salt), chondroitin sulfate, and reagents used for chemical modifications were obtained from Sigma Chemical Co. (St. Louis, MO).

Modification of Acidic Residues in LDL. To modify aspartic and glutamic acid residues (*42*), LDL (6 mg/2 mL) was gently mixed with (modified) or without (control) 200 μL of 10 M glycine methyl ester and adjusted to pH 4.75 with 0.5 or 1 N HCl. Subsequently, 1 mL of 0.4 M carbodiimide dissolved in water was added and the pH adjusted to pH 4.75. The reaction mixture was gently stirred and constantly adjusted to pH 4.75 for 1 h at room temperature. The reaction was stopped by the addition of 6 mL of 5 M acetate buffer (pH 4.75). After 5−10 min, the reaction mixture was dialyzed against 0.001 M HCl for 36 h at 4 °C, followed by extensive dialysis against PBS.

Modification of Histidine Residues in LDL. Histidine modifications were performed using diethyl pyrocarbonate (*43*). LDL (4.5 mg/3 mL) was diluted with 3 mL of 2-(*N*-morpholino)ethanolsulfonic acid (pH 6.0) and incubated with (modified) or without (control) diethyl pyrocarbonate (final concentration, 1 mM) for 30 min at room temperature. The reaction was quenched by the rapid addition of 6 mL of 100 mM histidine in 50 mM Tris-HCl, 100 mM NaCl, and 0.1% poly(ethylene glycol) (pH 7.5). After 15 min, samples were dialyzed extensively against PBS. To regenerate modified histidine residues, ethoxyformylated LDL was incubated overnight with 0.3 M hydroxylamine in 25 mM HEPES buffer (pH 7.5) at 37 °C and dialyzed extensively against PBS before being used.

Modification of Arginine Residues in LDL. For the modification of arginine residues, LDL (5.25 mg/1.5 mL) was incubated with (modified) or without (control) 3 mL of 0.15 M cyclohexanedione in 0.2 M borate buffer (pH 8.1) at 35 °C for 2 h (*14*). The samples were dialyzed extensively and used for amino acid analysis and MTP binding studies. For the regeneration of arginine residues, cyclohexanedione-modified LDL was incubated with equal volumes of 1 M hydroxylamine and 0.3 M mannitol (pH 7.0) for 16 h at 35 °C and dialyzed (*14*).

Modification of Lysine Residues in LDL. For acetoacetylation of lysine residues, LDL (9.33 mg/4 mL) was incubated with (modified) or without (control) diketene [1.15 μmol/(mg of LDL)] in 0.3 M borate buffer (pH 8.1, 2 mL) for 5 min at room temperature and dialyzed extensively (*13*). To regenerate modified lysine residues, acetoacetylated LDL was incubated with 0.5 M hydroxylamine (pH 7.0) for 16 h at 37 °C and dialyzed (*13*). To determine the extent of lysine modifications, modified LDL was reacted with dinitrofluorobenzene (*13*), subjected to an acid hydrolysis, and used for amino acid analysis. For the acetylation of lysine residues, LDL in saturated sodium acetate solution was reacted with acetic anhydride as described by Basu et al. (*44*). LDL was reductively methylated to obtain the maximum modification using formaldehyde and $NaCNBH_3$ as described by Lund-Katz et al. (*45*).

Amino Acid Analysis. Native, control, modified, and regenerated LDL were analyzed in duplicate for amino acid composition by the Wistar Protein Microchemistry Facility (Wistar Institute, Philadelphia, PA). For this purpose, 2 μg of LDL was hydrolyzed with 6 N HCl and 1% phenol for 1 h at 160 °C, followed by manual phenylthiocarbamyl derivatization and high-performance liquid chromatography separation as described by Ebert (*46*).

Binding of Native or Modified LDL to the Immobilized MTP. Methods for studying the binding of LDL to immobilized MTP have been described (*22*). It was shown that a method that used native LDL and immobilized MTP gave better binding kinetics compared to other methods that used iodinated LDL (*22*). Since this assay is critically dependent on the recognition of apoB by polyclonal antibodies, we first evaluated the effect of chemical modifications of LDL on the recognition of apoB by polyclonal antibodies. For this purpose, different amounts (0−50 μg) of native and modified LDL were immobilized on microtiter plates. The immobilized LDLs were then exposed to sheep anti-human apoB polyclonal antibodies followed by alkaline phosphatase-labeled rabbit anti-sheep IgGs. The amount of apoB immobilized was quantitated using *p*-nitrophenyl pyrophosphate as the substrate (*22*, *40*, *41*). The optical densities in the wells coated with native, carboxyethylated, cyclohexanedione-modified, acetoacetylated, reductively methylated, and acetylated LDL were not significantly different. Therefore, these chemical modifications affected neither the binding of modified lipoproteins to microtiter wells nor the recognition of modified apoB by polyclonal antibodies. Thus, for the quantitation of these modified lipoproteins, native

Table 1: Quantitation of Amino Acids Modified by Different Chemical Modifications

	amino acid composition (mol %) (% of amino acids modified)					
reagent	Lys	Arg	His	Gly	Ala[a]	n^b
none (control)[c]	9.2 ± 1.2[d]	3.9 ± 0.3	2.8 ± 0.4	5.4 ± 0.3	7.0 ± 0.5	4
hydroxylamine[e]	9.7 ± 1.0	3.8 ± 0.3	2.6 ± 0.1	5.3 ± 0.3	7.0 ± 0.5	4
glycine methyl ester	7.0, 5.4	3.5, 3.2	2.4, 1.2	10.5, 9.8 (44, 38)[f]	6.2, 6.2	2
diethyl pyrocarbonate	7.6	4.2	0.0 (100)	10.0	6.5	1
cyclohexanedione	7.3 ± 1.1	1.8 ± 0.6[g] (54 ± 16)	2.1 ± 0.3	5.3 ± 0.3	6.6 ± 0.2	3
diketene	2.4 ± 0.8[h] (74 ± 9)	4.0 ± 0.5	2.7 ± 0.3	6.5 ± 0.5	8.2 ± 1.0	4

[a] Ala is shown as an example that was not affected by any of the modifications. Ser, Thr, Pro, Tyr, Val, Met, Ile, Leu, and Phe were also not affected. [b] Number of independent experiments performed. In each experiment, amino acid analysis was performed in duplicate and average values were used for statistical analysis. [c] The amino acid compositions of native, control, and modified LDLs were obtained as described in Materials and Methods. The compositions of the native and control LDL were similar (data not shown) to the calculated values obtained from the published nucleotide sequence (accession number X04506) using "peptidesort" analysis of the Genetic Computer Group (Madison, WI) and thus were combined. [d] Mean ± standard deviation. [e] The amino acid compositions of hydroxylamine-treated control and modified LDL were similar. Thus, these values were combined for analysis. [f] The mole percent of acidic residues modified was calculated on the basis of the increase in glycine residues. ApoB contains 11.6 and 5.4 mol % aspartic and glutamic acid and glycine residues, respectively. After the modification, there was an increase in glycine content of 5.1 mol %, which corresponds (5.1/11.6 × 100) to 44% modification of acidic residues. [g] Comparisons were between control and modified amino acids (mol %) using the Student's *t* test ($p = 0.002$). [h] $p < 0.001$.

LDL was used as a standard. In contrast to these modifications, microtiter wells coated with glycine methyl ester-modified LDL developed 60% less color compared to wells with the native and the other modified LDLs, indicating that this modification does affect the recognition of apoB by polyclonal antibodies. Thus, glycine methyl ester-modified LDL was used as a standard to quantitate the binding of these modified lipoproteins to MTP. In control experiments, we found that this modification had no effect on the recognition of apoB by a monoclonal antibody, 1D1 (data not shown).

Human Recombinant ApoB18. McA-RH7777 cells transfected with human recombinant apoB18 cDNA have been described (*40*, *47*, *48*). For experiments, 80% confluent monolayers were incubated with serum-free medium containing 0.2% BSA for 48 h. The conditioned medium was concentrated using Centripreps (Amicon, 30 kDa cutoffs) and was used to determine the amount of apoB18 present and chemical modification of proteins and to study the binding of apoB18 to the immobilized MTP. The apoB polypeptides that interacted with MTP were quantitated by ELISA (*22*, *40*, *41*).

Effect of Glycosaminoglycans and Suramin on ApoB−MTP Interactions. To study the effect of different glycosaminoglycans and suramin, immobilized MTP was incubated with LDL or conditioned medium obtained from McA-RH7777 cells stably transfected with apoB18 in the presence or absence of glycosaminoglycans. After the incubation, wells were washed and the amount of apoB bound was quantified by ELISA.

Other Analyses. Protein was determined using the Coomassie Plus reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard (*49*). Optical density in ELISA plates was measured using a Dynatech MRX microplate reader (Dynatech Labs, Chantilly, VA). The data were plotted as the mean ± the standard deviation (SD). The molecular masses used for apoB100, MTP, heparin, chondroitin sulfate, and suramin were 512, 146, 16.5, 50, and 1.43 kDa, respectively.

RESULTS

Modification of Aspartic and Glutamic Acid Residues. The carboxylic side chains of aspartic and glutamic acids were modified by glycine methyl esters using water-soluble diimides as catalysts (*42*), the most widely used method for the modification of acidic side chains (*50*). In this method, carboxyl groups are activated by water-soluble diimides and subsequently reacted with nucleophiles, e.g., glycine methyl esters (*42*). The glycine methyl ester modification of aspartic and glutamic acid residues in LDL by 38−44% (Table 1) had no effect on the LDL−MTP interactions (Figure 1).

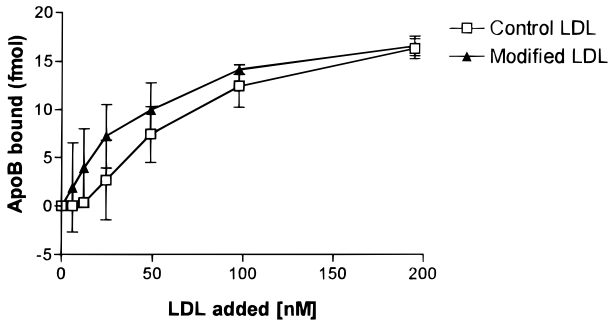

FIGURE 1: Binding of control and aspartic and glutamic acid-modified LDL to immobilized MTP. LDL was treated with (modified) or without (control) glycine methyl esters as described in Materials and Methods. Binding of different amounts of control and modified LDL to the immobilized MTP was performed in triplicate as described in Materials and Methods. The curves represent mean binding of control or modified LDL, and the error bars represent standard deviations. The experiment is representative of two independent experiments depicting the interactions between control and glycine methyl ester-modified LDL and MTP. Control LDL were treated in a manner similar to that of modified LDL, except for the omission of glycine methyl esters.

Modification of Histidine Residues. LDL was modified (Figure 2) using diethyl pyrocarbonate which is a fairly specific and commonly used reagent for the modification of histidine residues in proteins (*43*, *50*−*52*). At slightly acidic pH values, this reagent carboxyethylates one of the imidazole nitrogens (*43*, *50*−*52*). This modification does not usually cause structural changes in modified proteins as determined by fluorescence emission and high-performance exclusion chromatography (*43*, *50*, *52*). Diethyl pyrocarbonate treatment resulted in the modification of all the histidine residues but had no significant effect on other residues in LDL (Table 1). The complete modification of histidine residues in the LDL resulted in decreased binding of carboxyethylated LDL to MTP (Figure 2). At lower concentrations (<25 nM), no significant binding could be detected. However, at higher

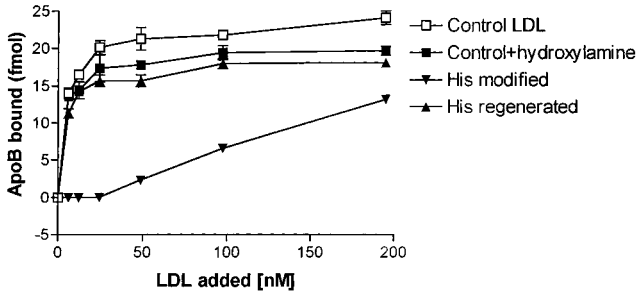

FIGURE 2: Binding of control and diethyl pyrocarbonate-modified LDL to MTP. Histidine residues in the LDL were modified in the presence (modified) and absence (control) of diethyl pyrocarbonate in 1 mM 2-(*N*-morpholino)ethanolsulfonic acid (pH 6.0) as described in Materials and Methods. A portion of control and modified LDL was subjected to hydroxylamine treatment to reverse the modifications. The binding of different LDLs to the immobilized MTP was performed in triplicate. The curves and error bars represent mean and standard deviations of the binding of different LDL at indicated concentrations, respectively. The data are representative of two independent experiments. The amino acid composition was determined in one experiment (Table 1).

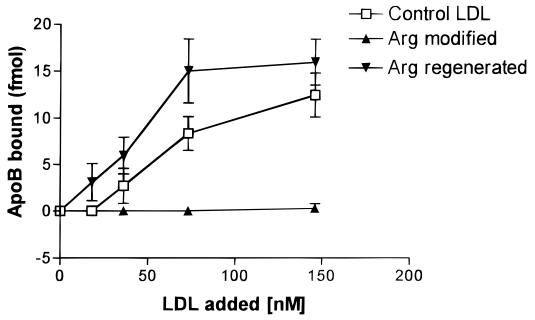

FIGURE 3: Effect of cyclohexanedione modification of arginine residues on the interaction between LDL and MTP. LDL was treated with (modified) or without (control) cyclohexanedione in 0.15 M borate buffer (pH 8.1) as described in Materials and Methods. A portion of the modified LDL was then subjected to hydroxylamine treatment to reverse the modifications (see Materials and Methods). The binding of different concentrations of control, cyclohexanedione-modified, and arginine-regenerated LDL was studied in triplicate. The curves represent the mean binding at indicated concentrations, and the error bars represent standard deviations. The data are representative of four independent experiments. Amino acid analysis was performed on three samples (Table 1).

concentrations (≥50 nM), binding of carboxyethylated LDL to MTP was measurable. At 100 nM, the binding of modified LDL was 70% lower than that of the control LDL (Figure 2). The hydroxylamine treatment resulted in complete regeneration of histidine residues and restored (>80% at 100 nM) the binding of LDL to MTP. The regenerated LDL had a binding similar to that of control LDL treated with hydroxylamine. Hydroxylamine treatment of LDL had no effect on the amino acid composition of LDL (Table 1) but slightly decreased (≈20%) its interactions with MTP. The slightly decreased binding suggests that hydroxylamine does not induce major structural or conformational changes in apoB. Hydroxylamine treatment is known to remove covalently linked fatty acyl groups from apolipoproteins by hydrolyzing ester and thioester bonds (*53*, *54*). Furthermore, it has been used to reverse chemical modifications of lysine, arginine, and histidine residues (*2*, *13*, *14*, *43*, *55*, *56*). Thus, hydroxylamine can be used to reverse various chemical modifications and to determine the importance of different modified side chains in apoB−MTP interactions. These studies indicated that modification of histidine residues decreased, but did not abolish, interactions between apoB and MTP.

Modification of Arginine Residues. 1,2-Cyclohexanedione forms stable adducts with guanido groups of arginine residues that can be reversed by hydroxylamine treatment (*14*, *55*, *56*). The treatment of LDL with 1,2-cyclohexanedione results in selective modification of ≈50% of the arginine residues without affecting the lipid composition, size, or morphology (*14*, *15*). This modification is known to increase the electrophoretic mobility of LDL toward the anode, totally abolish its interactions with the LDL receptors and heparin, and decrease the rate of LDL catabolism (*2*, *14*, *15*). In these studies, cyclohexanedione modified 54% of the total arginine residues (Table 1). Less than 100% modification could be due either to microenvironments around some arginine side chains that lower the pK_a rendering them nonreactive or to the fact that these residues are buried in the molecule and are inaccessible to chemical reagents. Cyclohexanedione modification of arginine residues by 54% completely inhibited interactions between LDL and MTP (Figure 3). Regeneration of arginine residues resulted in complete restoration of the binding of apoB to MTP.

Modification of Lysine Residues. Lysine residues were modified using diketene which specifically acetoacetylates the ϵ-amino groups of lysine residues under certain conditions, and the modified lysine residues can be regenerated by hydroxylamine treatment (*2*, *13*, *50*). Acetoacetylation has no effect on the size, morphological appearance, and chemical composition of LDL but decreases the electrophoretic mobility, inhibits interactions with LDL receptors, and retards the plasma clearance of LDL (*2*, *13*, *16*). Under our experimental conditions, 74% of the lysine residues were modified by acetoacetylation (Table 1). This degree of modification completely abolished the interactions between apoB and MTP (Figure 4A). The loss of interactions could be completely restored by hydroxylamine treatment (Figure 4A).

We then determined whether neutralization of positive charges on the lysine side groups was important for abolishing apoB−MTP interactions by studying the interactions of reductively methylated and acetylated LDL with immobilized MTP. The reductive methylation does not alter the net positive charges, and has no effect on the morphology, size, and electrophoretic mobility of LDL (*13*, *45*). However, it increases the molar ellipticity and decreases the α-helical content of apoB100 in LDL (*45*). This modification has been shown to convert approximately 75% of the ϵ-amino groups into dimethylamino derivatives without reacting with lipids (*45*). In these studies, reductive methylation of lysine residues by 88% did not abolish apoB−MTP interactions (Figure 4B). Thus, modifications of ϵ-amino groups without alterations of the charges do not inhibit apoB−MTP interactions.

Next, LDL was subjected to acetylation. This modification does not affect the size and configuration of the particle but increases the net negative charges due to neutralization of lysine ϵ-amino groups compared to native LDL (*44*). Acetylation is known to abolish the LDL receptor binding capacity and enhance its interactions with scavenger receptors (*13*, *44*). Acetylation of LDL abolished interactions between

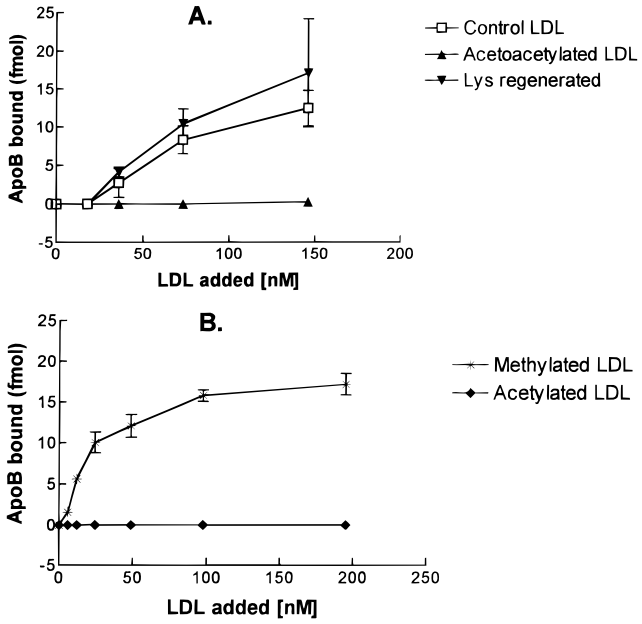

FIGURE 4: Effect of different chemical modifications of lysine residues on apoB−MTP interactions. (A) Acetoacetylation. LDL was reacted with (acetoacetylated) or without (control) diketene in 0.1 M borate buffer (pH 8.1) as described in Materials and Methods. A portion of acetoacetylated LDL was then subjected to hydroxylamine treatment to regenerate lysine residues. Binding of control, acetoacetylated, and lysine-regenerated LDL to the immobilized MTP was performed in triplicate as described in Materials and Methods. The curves represent mean binding at given concentrations, and error bars represent standard deviations. The data are representative of four independent experiments. (B) Reductive methylation and acetylation of LDL. LDL was subjected to reductive methylation and acetylation as described in Materials and Methods. The binding of reductively methylated and acetylated LDL was performed in triplicate. Acetylated LDL was not subjected to hydroxylamine treatment. The curves and error bars represent the mean and standard variations of the binding of different lipoproteins to immobilized MTP, respectively. The data are representative of three independent experiments.

apoB and MTP (Figure 4B). The inhibition was similar to that observed for the acetoacetylation (Figure 4A) of lysine residues, a modification that also alters the charges. Thus, these studies indicate that the loss of positive charges on ϵ-amino groups of lysine residues is needed to abolish the binding of apoB to MTP.

Effect of Chemical Modifications on the Interactions between ApoB18 and MTP. The data from experiments described above demonstrated that lysine and arginine residues in LDL are critical for its interaction with MTP. In a previous study, we had demonstrated that the C-terminally truncated apoB18 interacts the best with MTP (*22*). To further evaluate whether lysine and arginine residues in the N-terminal 18% of apoB are important in these interactions, we subjected the conditioned medium from stably transfected McA-RH7777 cells that express human recombinant apoB18 to different chemical reactions that modify histidine, lysine, or arginine residues (Table 2). We did not determine the extent of amino acid modifications because such an estimation in the conditioned medium would not provide specific information concerning the extent of amino acids modified in apoB18. Similar to those of LDL, modifications of lysine and arginine residues in the conditioned medium obtained from cells expressing human recombinant apoB18 completely abolished interactions between apoB18 and MTP. Again,

Table 2: Effect of Chemical Modification of ApoB18 on Its Interaction with MTP[a]

modifications	apoB18 bound (fmol)	% of control
histidine modifications		
control	35.54 ± 1.73[b]	100
modified[c]	9.48 ± 2.09	72
regenerated	39.97 ± 2.83	112
arginine modifications		
control	22.53 ± 1.17	100
modified	0	0
regenerated	23.66 ± 1.63	105
lysine modifications		
control	22.53 ± 1.17	100
modified	0	0
regenerated	20.45 ± 1.85	91

[a] Serum-free conditioned medium (see Materials and Methods) from McA-RH7777 cells stably transfected with human recombinant apoB18 cDNA was concentrated and treated with various reagents. The immobilized MTP (1 μg/well) was then incubated in triplicate with control and modified conditioned media containing 25 nM apoB18. Furthermore, modified medium was treated with 0.3 M hydroxylamine to regenerate the original residues as described in Materials and Methods and used for MTP binding studies. The amount of bound apoB18 was determined by ELISA as described before (*22*, *40*). [b] Mean ± standard deviation ($n = 3$). [c] Concentrated conditioned medium (1.5 mL) was reacted with diketene (2 μmol), 0.1 M cyclohexanedione, or 0.6 M diethyl pyrocarbonate to modify lysine, arginine, or histidine residues, respectively, as described for LDL in Materials and Methods.

modification of histidine residues decreased interactions between apoB18 and MTP but did not abolish them. The binding activities of the control and modified conditioned media were restored to various extents (91−112%) of the control after hydroxylamine treatment (Table 2). These data were interpreted to suggest that the lysine and arginine residues present in the N-terminal 18% of apoB play an important role in the binding of apoB to MTP.

Lysine and Arginine Residues in ApoB That Interact with MTP Are Different from Those That Interact with the LDL Receptor or Heparin. Lysine and arginine residues in LDL have been shown to be important for the interactions of apoB100 with the LDL receptor or heparin (*2*−*4*, *6*−*9*, *13*−*15*). Heparin competes with the LDL receptor for interactions with apoB100, indicating that the heparin and receptor binding sites are the same or juxtaposed (*17*). To test whether the same site also interacts with MTP, we studied the inhibition of LDL−MTP interactions by heparin and chondroitin sulfate. Heparin and chondroitin sulfate had no significant inhibitory effect on the interactions between LDL and MTP (Figure 5A). In addition, we studied the effect of suramin, a highly charged polysulfated polycyclic hydrocarbon, that inhibits the binding of LDL to LDL receptors (*57*). In this study, suramin inhibited LDL−MTP interactions by 20−40% (Figure 5A), indicating that interactions between LDL and MTP are ionic. Differences between glycosaminoglycans and suramin may be due to differences in the amount of sulfated groups and the density of negatively charged groups in these molecules. We had demonstrated that taurocholate treatment of LDL results in its enhanced interaction with immobilized MTP (*22*). Consideration was given to the possibility that taurocholate treatment may expose lysine and arginine residues that may then interact with heparin and MTP. Heparin and chondroitin sulfate did not inhibit the interactions between taurocholate-treated LDL and MTP (data not shown). These data indicate that the

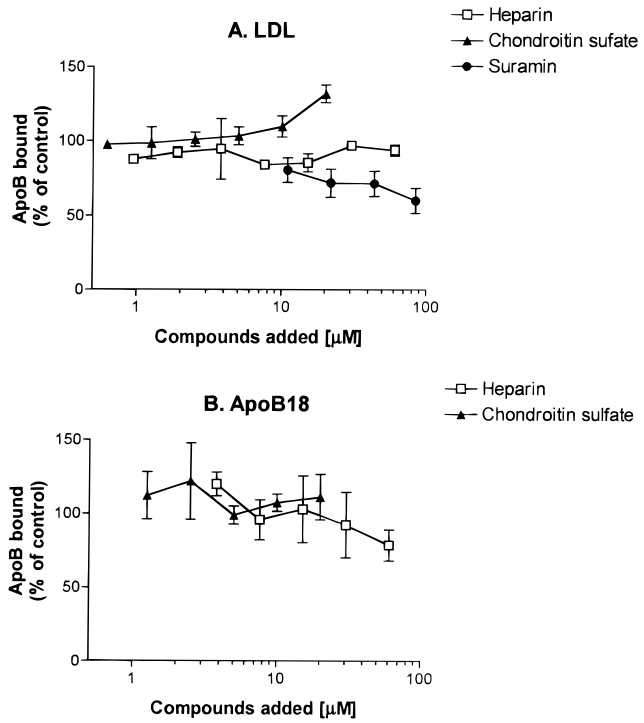

FIGURE 5: Effect of glycosaminoglycans and suramin on apoB−MTP interactions. (A) Effect on LDL−MTP interactions. Immobilized MTP (1 μg/well) was incubated in triplicate with LDL (50 nM) in the presence and absence of different indicated concentrations of different glycosaminoglycans and suramin for 2 h at 37 °C. The amount of apoB bound was quantified as described in Materials and Methods. The amount of apoB bound (100% control) in the absence of any competitor was 8.3 ± 0.6 fmol. (B) Effect on apoB18−MTP interactions. Concentrated conditioned medium (100 μL) from McA-RH7777 cells expressing human recombinant apoB18 was incubated in triplicate with immobilized MTP, and the amount of apoB18 bound was quantitated as described in Materials and Methods. The 100% control corresponded to 43.8 ± 4.7 fmol of apoB18 bound. The curves represent mean binding of apoB in the presence and absence of different indicated concentrations of glycosaminoglycans, and error bars represent standard deviations. Some of the error bars are not visible because standard deviations are smaller than the size of the symbols. The data are representative of three independent experiments.

MTP interacting site is different from the LDL receptor−heparin binding site. This is in agreement with our previous studies indicating that the N-terminal 18% of apoB100, a region that lacks the LDL receptor binding site, interacts the best with MTP (*22*). Furthermore, consideration was given to the possibility that heparin binding sites present in the N-terminal portion of apoB that may be masked in the LDL particle due to lipidation may interact with MTP. To address this issue, we studied the ability of glycosaminoglycans to compete for the binding of apoB18 to MTP. Again, heparin and chondroitin sulfate had no significant inhibitory effect on apoB18−MTP interactions (Figure 5B). These studies indicate that lysine and arginine residues in apoB which interact with MTP may be different from those that interact with heparin and LDL receptors.

DISCUSSION

We have recently presented evidence that the initial protein−protein interactions between apoB and MTP are ionic (*22*). In this study, we have identified the charged residues in apoB molecules that are critical for these interactions by selective chemical modification of different residues in the apoB100 and human recombinant apoB18. No attempts were made to modify different residues in MTP. Although chemical modifications are not absolutely specific for individual residues, reagents have been developed that predominantly modify certain amino acid side groups. For example, acetoacetylation, cyclohexanedione modifications, and diethyl pyrocarbonate treatments have been shown to be fairly specific for lysine, arginine, and histidine residues, respectively (*13*, *14*, *42*, *50*, *52*). Furthermore, the chemical modification approach has been used to identify amino acid residues in apoB that are involved in interactions with LDL receptors and glycosaminoglycans (*4*, *5*, *13*, *14*).

In this study, each modified LDL was compared with its own control rather than native LDL because the maximum binding of control LDL (Figures 1−4) and apoB18 (Table 2) to MTP was variable. For example, the binding of control LDL and apoB18 in histidine modification experiments (Figure 2 and Table 2) was higher than the controls in arginine and lysine modification experiments (Figures 3 and 4 and Table 2). The differences may represent subtle structural changes in apoB due to different experimental conditions (ions, salt concentration, pH, etc.) for various modifications.

In this study, the neutralization of negatively charged side groups of aspartic and glutamic acid residues in LDL by glycine methyl ester modification had no effect on apoB−MTP interactions (Figure 1). It is highly unlikely that the methyl ester groups would function in a manner similar to that of the carboxyl groups of aspartic and glutamic acids. Thus, the lack of an effect on apoB−MTP interaction by this modification most likely indicates that acidic residues in apoB do not play a critical role in these interactions.

Modification of all the histidine residues in LDL did not completely abolish apoB−MTP interactions. Below 25 nM, modification of histidine residues resulted in undetectable binding of apoB to MTP. At higher concentrations, however, the binding between modified LDL and MTP was detectable, indicating that the modification of histidine residues did not abolish MTP binding but reduced the affinity of the interactions. Thus, histidine residues may be involved but are not critical for apoB−MTP interactions.

On the other hand, the loss of apoB interaction with MTP was closely related to the loss of positive charges on the arginine and lysine residues (Figures 3 and 4). The loss of apoB binding was not due to denaturation of LDL as demonstrated by the complete reversal of inhibition of apoB−MTP interactions after hydroxylamine treatment of modified LDL. Thus, positively charged ϵ-amino and guanido groups in apoB appear to be critical for its interactions with MTP. The chemical modifications of lysine and arginine residues may prevent the binding of apoB to MTP by several mechanisms. (1) Modifications may directly abolish the positive charges required for the proposed ionic interactions (*22*) between these proteins. (2) The conformation of the recognition site may be disrupted by altering the spatial distribution of the positive charges or other yet unidentified critical residues in these interactions. (3) The modifications may directly interfere with chemical reactivity, other than charge. (4) The bulky groups introduced during modification may prevent interactions between proteins.

Similar explanations have been put forth to explain the loss of activity of other proteins after chemical modifications in several systems (*13*, *14*). In the case of apoB−MTP interactions, we favor the first possibility because initial apoB−MTP interactions are inhibited by high salt concentrations (*22*) and suramin (Figure 5) and because the apoB binds to LDL receptors and heparin via ionic interactions.

There are some similarities between the interactions of apoB with the LDL receptor, heparin, and MTP. All these interactions are completely abolished after the modification of lysine and arginine residues by acetoacetylation and cyclohexanedione modification, respectively. Also, apoB interactions with MTP and heparin are not affected by reductive methylation. However, there are some important differences. Reductive methylation of lysine residues, which does not alter the positive charges, abolishes the binding of apoB to LDL receptors (*13*) but not to MTP (Figure 4). The addition of methyl groups by reductive methylation may induce conformational changes that do not affect apoB−MTP interactions but inhibit apoB−LDL receptor interactions. The LDL receptor interacting site on apoB100 has been localized to the C-terminal 30% of the molecule (*3*), whereas the MTP interacting site appears to reside in the N-terminal 18% of the molecule (*22*). In contrast, heparin binding sites are dispersed all over the molecule (*1*−*8*). The interaction between the LDL receptor and LDL are inhibited by heparin (*17*), but those between apoB and MTP are not, indicating that heparin and LDL receptor binding sites may not overlap with the MTP binding site. Thus, it appears that lysine and arginine residues, different from those involved in LDL receptor and heparin interactions, are crucial for the interaction of apoB with MTP.

In summary, we have demonstrated that ϵ-amino groups of lysine and guanido groups of arginine residues in the N-terminal 18% of apoB100 are critical for its interaction with MTP. These residues may be involved in ionic interactions with the negatively charged acidic amino acids of MTP. Further characterization of individual residues that interact with MTP can be achieved with site-directed mutagenesis of lysine and arginine residues in the N terminus of apoB.

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