Mapping of Human Apolipoprotein B Antigenic Determinants

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A minimum of 16 epitopes which provide a group of topographical markers to study the conformation of apolipoprotein (apo) B have been mapped in relation to elements of the sequence of apo B-100. Six of these epitopes are identified by monoclonal antibodies (Mabs) directed against low density lipoprotein (LDL) apo B, while at least 10 others react with Mabs obtained by immunization with delipidated and solubilized apo B. Five epitopes which are also expressed on apo B-48 have been assigned to the thrombolytic fragment T4 on the N-terminal side of apo B-100. None of these five epitopes requires the presence of lipids for its expression, suggesting that the conformation of the T4 region of apo B is more dependent on peptide-chain interactions than on peptide-lipid interactions. Four distinct epitopes have been assigned to the median thrombolytic fragment T3 of apo B-100, all of which require the presence of lipids for their expression; those epitopes closer to the C-terminus of T3 require specific interaction with cholesteryl esters. The same lipid dependence also characterizes a cluster of epitopes mapped to the N-terminal region of fragment T2. The epitopes that are close to the T2/T3 cleavage site and depend on the presence of cholesteryl esters for their expression are also those that react with the Mabs that inhibit the binding of LDL to its receptor. Therefore this region, which in addition contains two sequences with structural homology to the apo E receptor binding domain, probably constitutes a physiologically important receptor binding site for apo B. Finally, four other distinct epitopes which do not require the presence of lipids for their expression have been mapped on T2. In conclusion, the present report presents evidence that the immunochemical analogy of apo B-48 and apo B-100 is on the N-terminal half of apo B-100, whereas the apo B receptor binding domain is localized on the C-terminal half of apo B-100 close to the T2/T3 cleavage site. (Arteriosclerosis 7:166-175, March/April 1987)
polyclonal antisera raised against defined synthetic fragments of apo B. Thrombin cleaves LDL apo B into fragments of a similar size to B-74 and B-26, but the primary cleavage site which yields T1 and T2 is located within the B-74 sequence toward the C-terminus. Prolonged exposure to thrombin produces a second cleavage, in which T1 generates two new fragments, T3 and T4. T4 represents the N-terminal fragment of apo B-100 and has a molecular weight comparable to that of B-26 while T3 is the median fragment. The purified apo B fragments generated by the action of thrombin have been used to map more accurately the position of apo B epitopes that are recognized by Mabs against LDL apo B\textsuperscript{14} and lipid-free and solubilized apo B.\textsuperscript{15}

Methods

**Isolation of Lipoproteins**

Sequential ultracentrifugation isolated LDL between the densities of 1.019 and 1.063 g/ml from the plasma of fast-er normalolpemic donors. Phenyimethanesulfonyl fluoride was added to the plasma (1 mM final concentration), and the LDL were dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA and stored at 4°C.

**Monoclonal Antibodies**

The seven hybridoma secreting antibodies used against LDL apo B (anti-apo B\textsubscript{DL}) have been described earlier.\textsuperscript{14} These Mabs have been obtained by immunization and screening with LDL. The antibodies secreted by two clones obtained in the same fusion but not described in the original report are included here, and these Mabs are identified as 4F6 and 6A8. Anti-apo B\textsubscript{DL} 4F6 and 6A8 compete with 

**Synthetic Peptides of Apo B**

Peptides spanning the sequences of interest within the apo B molecule were synthesized by Biosearch, Incorporated (San Rafael, California). The peptides were purified by treating a 50-mg sample with 3 ml of 15 mM dithiothreitol (DTT) in 6 M guanidine hydrochloride, 2.5 mM EDTA (pH 8.5) under nitrogen for 24 hours to reduce the cysteine thiopropyl sepharose column (Pharmacia, Uppsala, Sweden). The samples were recycled for 2 hours and then washed overnight with 0.1 M ammonium acetate, 1 mM EDTA (pH 5.0) (240 ml, 12 ml/h). The columns were then washed with 100 ml of the ammonium acetate buffer plus 0.3 M NaCl and then overnight with ammonium acetate buffer (pH 8.0). The columns were eluted with pH 8.0 buffer that included 20 mM dithiothreitol. The peptide containing fractions were pooled, reduced to about 5 ml by lyophilization, desalted on Sephadex G-10, and then lyophilized.

**Antisera against Synthetic Peptides of Apo B**

Fifty mg of keyhole limpet hemocyanin (KLH) were dissolved in 10% glycerol and 50 mM sodium phosphate (pH 6.0). Five mg of m-maleimidobenzoyl-N-hydroxysuccinimide (MBS) were dissolved in 0.25 ml of dimethyl formamide. The MBS solution was added by drops to the KLH solution and incubated for 30 minutes at room temperature. The MBS-KLH complexes were isolated from the free MBS by gel filtration chromatography on Sephadex G-25 equilibrated with 50 mM sodium phosphate (pH 7.4). About 6.8 mg of MBS-KLH were added to 4 mg of the peptide, and the solution was incubated for 3 hours at 22°C, with occasional mixing. For antibody production, 1 mg of cross-linked peptide was mixed with an equal volume of Freund's complete adjuvant and injected into multiple sites in the rabbit. Freund's incomplete adjuvant was used for boosters. After four injections the antisera titer and specificity were examined by solid phase enzyme linked immunosorption assay\textsuperscript{a} and by Western blot analysis against apo B-100.

**Purification of Thrombolytic Fragments of Apo B**

Thrombin-digested LDL were delipidated by addition of deoxycholate, and the resulting fragments were electrophoresed on SDS-polyacrylamide gradient gels.\textsuperscript{11} The separated fragments were subsequently isolated by electro-elution as previously described.\textsuperscript{11}

**Incorporation of Apo B Fragments Into Lipid Microemulsions**

Microemulsions of dimyristoylphosphatidylcholine and cholesteryl oleate were prepared as described by others\textsuperscript{16} at a molar ratio of 1 and sonicated above the order-disorder transition temperature of the lipids. The microemulsion isolated by sequential centrifugation was incubated with purified apo B fragments which had been made up to 2% sodium dodecyl sulfate (SDS) and heated for 10 minutes at 65°C. The mixture of proteins and lipids at the weight ratio of 0.1 was incubated for 10 minutes at 51°C and for 60 minutes at 37°C, and then dialyzed extensively against PBS.\textsuperscript{17}

**Tryptic Digestion of LDL**

The conditions for digestion of LDL with trypsin have been described.\textsuperscript{15} In this study, digestion was allowed to proceed for either 30 minutes or 24 hours using a protein-to-enzyme ratio of 50.

**Solid Phase Radiolmmunoassay of Apo B and Apo B Fragments**

The radioimmunoassay (RIA) previously described for the anti-apo B\textsubscript{DL} Mab\textsuperscript{14} was used here for the assay of LDL apo B, apo B\textsubscript{DL}, and apo B fragments. The optimal
dilution for each antibody was determined in preliminary experiments. In certain experiments, LDL, apo B_{mol}, or purified apo B thrombolytic fragments dissolved in 5 mM glycine (pH 9.2), were adsorbed to the polystyrene Removawells by overnight incubation with 200 µl of the appropriate antigen solution. The proteolipid recombinants of apo B fragments and microemulsion were assayed in competitive RIA using LDL as the immobilized antigen and as described earlier.17 Recombinants made up with apo HDL and microemulsions were used as negative controls.17

**Polyacrylamide Gel Electrophoresis and Immunoblotting**

Apo B thrombolytic fragments were electrophoresed in 5% polyacrylamide gel using the system of Laemmli.19 Peptides in tryptic digest of LDL were separated in the same SDS system with linear gradient gels of 5% to 15% acrylamide.18 Electrophoretic transfer from SDS-polyacrylamide gels to nitrocellulose paper have been described.2

**Results**

**Reaction with Apo B Fragments Adsorbed on Plastic Wells**

All anti-apo B_{mol} Mabs reacted with the control LDL and solubilized apo B (Table 1), although, as noted earlier, antibody 16 reacted weakly with both preparations. Because the levels of nonspecific binding varied for each antibody, the binding of a given Mab to an apo B fragment was considered significant when the radioactivity bound was at least 50% above that noted with the other fragments. With this restriction, the epitopes for Mabs 6 and 8 were found on fragment T1 and within T1 on the secondary fragment T3; the epitopes for antibodies 7, 15, 20, and 22 were located on fragments T2; and those for antibodies 9, 13, 14, and 17 were on T4.

Some of the previously described anti-apo B_{DL} Mabs reacted with the thrombin fragments, and the determinant for 1D1 was found on fragment T4, in agreement with its earlier mapping to the B-26 fragment.2 The determinants for 2D8, 3F5, and 4G3 were located on fragment T3, a finding also consistent with their previous assignment to B-74. Antibodies 3A8, 5E11, as well as 4F6 and 6A8, did not react significantly with any of the isolated fragments, a result which may be related to the stringent conformational requirement of the epitopes identified by Mabs such as 3A8 and 5E11.17

**Immunoreactivity of the Apo B-Thrombin Fragments after Incorporation into Microemulsion**

We had previously noted that most of the apo B epitopes recognized by antibodies that could block the binding of LDL to its receptor11 depended on the presence of specific lipids for their expression.18 Assuming that this lipid requirement would be conserved after cleavage of apo B with thrombin, the immunoreactivity of the isolated fragments was studied after equilibration with microemulsions as described earlier.18 The recombinants of fragments T1 and T3 competed with LDL in the immunoblocks with 3F5 and 4G3 (Figure 1), corroborating the presence of the determinants for these Mabs on the fragment T3 that was suggested by the results of the solid phase assay (Table 1). The recombinant-containing fragment T2 competed significantly with LDL in the immunoblocks using 5E11 and 4F6 (Figure 1) or 3A8 and 6A8 (not illustrated). Therefore, the epitopes for 5E11, 4F6, 3A8, and 6A8 can be assigned to fragment T2.

**Immunoblots of Apo B-Thrombin Fragments Separated by SDS-Gel Electrophoresis**

To verify and extend our observations on the reaction of the different Mabs with the thrombin fragments T2 and T4, these thrombin fragments were also separated by SDS-gel electrophoresis and immunoblotted with selected antibodies. Mab 6, which competes with and probably reacts at the same site on apo B as antibodies 4, 5, and 8, reacted very strongly in the immunoblots with fragments T1 and T3.

**Table 1. Reaction of Antibodies against Apo B_{mol} and Apo B_{DL} with Apo B Thrombin Fragments**

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The thrombin fragments, apo B, and LDL were solubilized in 5 mM Tris-glycine, pH 9.2, and were adsorbed onto Removawells at 5, 10, and 30 µg protein/ml, respectively. Each appropriately diluted antibody was incubated overnight, and the bound IgG were measured by addition of labeled anti-mouse IgG (so ±10%).
MAPPING OF APO B EPITOPES

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Figure 1. Competitive radioimmunoassays using the different antibodies between a control LDL immobilized on plastic and various competing antigens. Control LDL (●), microemulsion recombinants containing fragment T1 (△), fragment T2 (○), fragment T3 (●), fragment T4 (□), or apo HDL (○).

Figure 2. Immunoblot of LDL apo B thrombolytic fragments separated by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2. Reaction of Mab 6 with fragments T1 and T3, respectively. Lanes 3 and 4. Reaction of Mabs 2 and 16, respectively, with fragment T2. Lane 5. Reaction of Mab 12 with fragment T4. Protein (15 μg) was applied to each lane.

Position of Epitopes In Relation to the Putative Receptor Binding Site of Apo B-100

Inspection of the amino acid sequence of the C-terminal region of apo B-100 had revealed several potentially important sequences which, by analogy to apo E, could represent the receptor binding domains of the molecule. We have tested the immunoreactivity of the different Mabs with two peptides, residues 39–78 and residues 271–290 based on the sequence of Knott et al. These peptides spanned two of the putative binding regions and are now identified as residues 3120–3156 and 3352–3371, respectively.
Figure 3. SDS-gel electrophoresis of purified apo B-thrombolytic fragments. Lanes 1-5. Coomassie blue stain of apo B-100, fragments T1, T2, T3, and T4, respectively. Lanes A-J. Immunoblots of the purified thrombolytic fragments separated by SDS-gel electrophoresis. Lane A. Reaction of T3 with Mab 8. Lanes B-F. Reaction of T2 with Mabs 5, 15, 20, 22, and 1D1, respectively. Lanes G-J. Reaction of T4 with Mabs 9, 13, 17, and 1D1, respectively. The purified fragments to be transferred were applied across the gel at the concentration of 10 μg protein/cm.

Figure 4. Immunoblot of the 30-minute tryptic digest of LDL apo B separated by SDS-gel electrophoresis. The peptides were applied across the gel at the concentration of 70 μg protein/cm. Lane 1. 125I-labelled molecular weight standards (67, 43, 30, and 20.1 kDa). Lanes 2-9. Immunoreaction with Mabs 2, 2D8, 3F5, and 4G3, antiserum to peptide 3120-3156, and Mabs 3A10 and 5E11. The numbers on the right indicate the calculated Mr in kDa of the major immunoreactive bands.

tively, based on the complete apo B sequence.12 None of the antibodies reacted with the peptides in the direct solid-phase binding assay (not illustrated). Because all apo B epitopes recognized by antibodies that interfere with the binding of LDL to the receptor have been found to be conformation-dependent and affected by the presence of specific lipids, the two peptides of interest were also incorporated into proteolipid recombinants using the microemulsions, and these were tested in competitive radioimmunoassays as described above. Neither of these peptides that were preincubated with the microemulsions could react with any of the tested Mabs (not illustrated). However, it was not verified in these experiments that these short peptides did, in fact, bind to the microemulsion.

Finally, LDL was exposed to limited tryptic digestion. The resulting fragments were separated by SDS-gel electrophoresis and were submitted to immunoblotting with the rabbit antisera against each of the above peptides, (antisera 4158 and 4161 against residues 3120-3156 and 3352-3371, respectively) as well as with selected Mabs.

The immunoreactivity pattern of the 30-minute tryptic digestion of LDL apo B was nearly identical for antibodies 3A10, 5E11, and antiserum 4161 directed against peptide 3352-3371 (Figure 4, lanes 7 to 9) and for antibodies 4F6 and 6A8 (not illustrated). These antibodies reacted with a fragment at 101 kDa, with a doublet at 90 kDa and 86 kDa and with a second doublet at 66 kDa and 61 kDa. It is noteworthy that antiserum 4158 directed against the peptide 3120-3156 reacted with the same fragments, with the exception of the two smaller ones at 66 kDa and 61 kDa. However, this antiserum reacts with a distinctive broad band at 62 to 67 kDa, a protein band which is also recognized by antibodies 3F5 and 4G3 (Figure 4, lanes 4 to 6). The immunoreactivity pattern of the 24-hour tryptic digest of LDL apo B confirmed the above results and indicated that antibodies 3A10, 5E11, 4F6, and 6A8 (the reaction of the latter two Mabs is not illustrated) as well as antiserum 4161 reacted with a double protein band at 66 kDa and 61 kDa while antibodies 3F5 and 4G3 and antiserum 4158 to peptide 3120-3156 reacted with a broad band centered at about 65 kDa (Figure 5).

From these results we can conclude that the largest tryptic fragment of apo B that contained the sequences of peptides 3120-3156 and 3352-3371 as well as the epitopes for 3A10, 5E11, 4F6, and 6A8 (but not those for 3F5
Mapping of Determinants Located toward the N-Terminal Thrombin Fragment of Apo B

The synthesis of peptides reproducing selected partial sequences of apo B on the T4 fragments has allowed the production of specific antisera to these peptides; these antisera have been used in the further mapping of the epitopes present on T4. Monoclonal antibodies 9, 13, and 17 have been shown to compete with one another and with 1D1 in competitive binding experiments. In addition, these Mabs react with the same pattern of tryptic peptides of apo B (Figure 6, lanes 3–5). It is therefore probable that they react at the same or at close site(s) on apo B; in the subsequent experiments, Mab 17 was chosen as representative of this group of antibodies. Similarly Mabs 1, 3, 10, and 14 have been shown to compete with one another, and they react with the same tryptic fragment of apo B as Mab 11. Thus, Mabs 3 and 14 were chosen as representative of this group of Mabs in the following experiments. The limited tryptic digest of LDL apo B at 30 minutes generated a number of fragments immunoreactive with the different antibodies (Figure 7). The largest immunoreactive band at 68 kDa contained the epitopes for antibodies 3, 12, 14, and 17 as well as the sequences for peptides 17–33, 158–186, 259–280, 399–415 (Figure 7, lanes 1 to 4 and 6 to 9, respectively). Since the molecular weight of the span between peptide 399–415 and the N-terminus of apo B is 44,610, the fragment of 68 kDa mentioned above most likely covers a 68-kDa span of apo B protein which starts at or close to the N-terminus. The second largest immunoreactive fragment has a molecular weight of about 62,000 and contains the sequences for peptides 158–186, 259–280, and 399–415 but not that of peptide 17–33. The 62-kDa fragment contains also the epitopes for Mabs 3, 14, and 17, but not that for Mab 12. In addition, we have noted that Mab 12 and antiserum to peptide 17–33 have similar patterns of immunoreactivity with the smaller fragments of the 30-minute tryptic digest of LDL apo B when the autoradiograph of the immunoblots are overexposed (not illustrated). Finally, it is significant that the 24-hour tryptic digest of LDL apo B does not react with either Mab 12 or the antiserum to peptide 17–33 (Figure 8, lanes 4 and 6). Based on this evidence, we conclude that the epitope for Mab 12 is close to sequence 17–33 and to the N-terminus of apo B. It is possible that a cleavage of 6 kDa on the N-terminal side of the 68 kDa fragment has generated the secondary 62 kDa fragment and caused the deletion of the signals for both Mab 12 and the 17–33 sequence.

On the 30-minute tryptic digest of LDL apo B, a 48 kDa band appeared which reacted slightly with Mabs 3, 14, and 17 and with the antiserum to peptide 399–415 (Figure 7, lanes 1, 3, 4, and 8). On the 24-hour digest, the same band of 48 kDa was predominant and reacted strongly with Mabs 3, 14, and 17 and with the antiserum to peptide 399–415 and gave a slight reaction with the antiserum to peptide 259–280 (Figure 8, lanes 1–3, 8, and 7, respectively). We would therefore conclude from these results that the epitopes for Mabs 3, 14, and 17 are located on a 48-kDa segment that includes and extends downstream from the peptide 259–280. Finally, the appearance on the 24-hour tryptic digest of two bands at 18 and 22 kDa that contain the sequences of peptides 158–186 and 259–280 but do not react with Mabs 3, 14, and 17 (Figure 6) sug-
Figure 7. Immunoblots of the 30-minute tryptic digest of LDL apo B separated by SDS-gel electrophoresis. The peptides were applied across the gel at the concentration of 70 μg protein/cm. Lane 5, 125 I-labeled molecular weight standards (67, 43, 30, and 20.1 kDa). Lanes 1–4. Immunoreaction with Mabs 3, 12, 14, and 17, respectively. Lanes 6–9. Immunoreactions with the antisera against synthetic peptides 17–33, 259–280, 399–415, and 158–186, respectively. The numbers on the right indicate the calculated Mr in kDa of the major immunoreactive bands.

Figure 8. Immunoblots of the 24-hour tryptic digest of LDL apo B separated by SDS-gel electrophoresis. Experimental conditions and identification of Lanes 5–9 are as described for Figure 7. Lanes 1–4. Immunoreactions with Mabs 17, 3, 14, and 12, respectively.

gests that the epitopes for these Mabs are more likely to be located downstream of the peptide 399–415 than between peptides 259–280 and 399–415.

Discussion

Our initial map resulted from the cotitration of antibodies and had defined the relative position of antigenic determinants on LDL apo B and specifically in the region of the LDL receptor binding site.14 Our second map identified the position of epitopes on the delipidated and denatured apo B species and fragments.5 This approach allowed us to discover that apo B-100 and apo B-48 were antigenically related, whereas the fragments B-74 and B-26 were not.2 These observations could then be resolved into an assignment of the epitopes that fitted logically with the original characterization by Malloy, Kane, and colleagues3,5,6 of the hepatic and intestinal apo B species. This second map introduced the concept of the two distinct regions that exist on apo B-100: one common to apo B-100 and apo B-48 which also includes the fragment B-26, the other exclusive to apo B-100 which contains the putative LDL receptor binding site.2 The present report extends and corroborates these initial assignments of epitopes; they are now localized in relation to elements of the apo B sequence.

The series of Mabs against apo B used in this study have identified 16 or more different epitopes. These series included: the seven Mabs directed against LDL apo B which identify six distinct epitopes that have been extensively characterized;5,14,17,18 two other previously unreported Mabs that originate from the same fusion and block LDL receptor binding; and finally, 19 Mabs directed against delipidated and solubilized apo B, whose specificity has been recently described15 and which react with at least 10 different epitopes. These 16 epitopes should provide a group of topographical markers to study the conformation of apo B as it appears on LDL and other apo B-containing lipoproteins.

Five epitopes have been assigned to the fragment T4 on the amino-terminal side of apo B, and each of these epitopes is expressed on apo B-48.15 Monoclonal antibody 12 L is found close to the N-terminus, probably within 6 kDa, and three other groups of Mabs react with epitopes that appear located within 48 kDa downstream of residue 259 (Figure 9). Anti-apo B_{DL}, 1D1 and anti-apo B_{apo} 13 and 17, which react with the same epitope or with a cluster of epitopes,15 react in this region of apo B. Their epitopes are expressed on both LDL apo B and soluble apo B, and have been found to be generally unaffected by lipid.15,17 It will be of interest to delineate more precisely at the amino acid level the position of this antigenic determinant which resides in a portion of apo B that does not require lipid for its native conformation. Monoclonal antibodies 1, 3, 10, and 14 compete with one another15 and react at a site also located within 48 kDa of residue 259 (Figure 9). Their epitopes are also expressed on both LDL and soluble apo B and may be analogous to that for Mab 1D1 in that they do not have a specific requirement for lipid. In addition, this lack of effect of lipid on the above-mentioned epitopes does extend to the three other epitopes that have been mapped to T4. Monoclonal antibodies 12, 9, and 11 share one common property: the react strongly with LDL adsorbed to polystyrene, but their determinants are poorly expressed, if at all, on LDL in solution.15 Furthermore, the reactivity of these Mabs decreases when soluble apo B is incorporated into a lipid structure. Therefore these epitopes for Mabs 9, 11, and 12 would appear to exist also independently of the presence of lipids. In summary, the
five epitopes that have been mapped to T4 on the N-terminal end of apo B are expressed on soluble apo B and do not require apo B association with lipids. This may indicate that apo B conformation in this region of the molecule, which is common to B-100 and B-48, is more affected by intrachain bonding, probably including disulfide bridges, consistent with the locally high number of cysteines in T4,21 than by protein-lipid interaction.

A minimum of four distinct epitopes which have been assigned to fragment T3 react with 2D8, 3F5, 4G3 and with the group of Mabs 4, 5, 6, and 8 (Figure 10). While 2D8 is found on both B-100 and B-48, 3F5, 4G3 as well as Mabs 4, 5, 6, and 8 are found on B-100 only.2,15 The epitope for 2D8 has also been shown to be located within 68 kDa of the peptide 1303–1325 (Figure 6). Therefore 2D8 is found toward the N-terminal end of T3, while the epitopes for Mabs 4, 5, 6, and 8 and for 3F5 and 4G3 are located more toward the C-terminal of T3 (Figure 10). As one moves downstream from the N-terminus of T3, the determinants found on this fragment are characterized by a gradual change in their conformational requirement for lipids. Indeed, the recovery of immunoreactivity of apo B-liposome recombinants relative to LDL is maximal (75%) with 2D8,17 intermediate (22%) with Mabs 4, 5, 6, and 8,15 and absent with 3F5 and 4G3.17 Concomitantly, the recovery of apo B immunoreactivity upon recombination with microemulsion is better than with liposome recombinants, but also decreases gradually from 90% for 2D817 to 90% for Mabs 4, 5, 6, and 815 to 41% for 3F5, to 31% for 4G3, and reaches only 14% for 5E1117 which is located on T2. Hence it would appear that the degree of apo B structural organization that depends on interaction with lipids increases progressively from the N-terminus to the C-terminus of T3 and changes from an interaction with surface amphipathic lipids, cholesterol, and phosphatidylcholine, to one with hydrophobic core lipids such as cholesteryl oleate. It is notable that this progression in lipid dependency is manifested in the immunoreactivity of Mabs prepared against both native LDL and delipidated, resolubilized apo B.

The epitopes corresponding to Mabs that inhibit the binding of LDL apo B to its receptor have been found on both T3 and T2 (Figure 10). Those for antibodies 3F5 and 4G3 are contained on a 65-kDa fragment which is at the C-terminus of T3 and comprises the sequence of the basic region composed of residues 3120–3156.11 Epitopes for antibodies 3A8, 5E11, 4F6, and 6A8 are present on a 61-kDa fragment which is on the N-terminus of T2 and comprises the sequence of the basic region consisting of residues 3352–3371.11 Thus it would appear that both the epitopes for Mabs that can block binding to the receptor and the two basic regions that have been proposed as the receptor binding sites could be distributed over a rather large segment of apo B on both sides of the T3-T2 cleavage site (Figure 11). The presence of cysteine residues

![Diagram](http://atvb.ahajournals.org/)

**Figure 9.** Details of the mapping of epitopes on the amino-terminal region of apo B-100. The hatched boxes on the linear representation of the apo B sequence indicate the position of the partial synthetic peptides used in these studies. The numbers below the linear sequence identify the residues of the synthetic peptides and the T4/T3 cleavage site relative to the N-terminus. The alpha-numerical notation above the linear sequence identifies the epitope location. The epitope clusters recognized by groups of competing Mabs are linked by a vertical bracket. The horizontal brackets represent the probable location of the apo B tryptic fragments that cross-react with the indicated Mab and antisera to synthetic peptides.

**Figure 10.** Summary of the theoretical linear map of the location of epitopes on apo B-100. The notations are the same as in Figure 9 with the exception of the uppermost horizontal bracket, which identifies the Mabs-recognized epitopes that can block LDL binding to the receptor. The dashed-line brackets link epitopes that have not been mapped in relation to one another.
on both sides of the cleavage point could also form disulfide bridges, which would bring the chain containing the two basic regions and the two groups of epitopes (3F5, 4G3 on T3 and 3A8, 5E11, 4F6, 6A8 on T2) into adjacent positions and increase the structural rigidity in this functionally important area of the molecule.

Finally, a minimum of four other distinct epitopes that react with Mabs 15, 20, 16, 2, 7, and 22 (but do not block binding to the LDL receptor) have been mapped to T2. Presumably, they are located downstream from the epitope of 4F6, 6A8 toward the C-terminal of apo B (Figure 10). The epitopes for Mabs 15 and 20 are expressed on soluble apo B and not on LDL, while those for Mabs 16, 2, 7, and 22 are equally reactive on soluble apo B and on LDL. It would therefore appear that the tertiary structure of the C-terminal portion of apo B is relatively independent of interaction with lipids.

Our results presented here and elsewhere are in apparent contradiction with those of others who have attempted to identify the epitopes recognized by certain of the same Mabs. Our results differ notably from those reported by Shoulders et al. who observed reactivity of 1D1 and 5E11 with fusion proteins which include sequences from the T2 region of apo B-100 (residues 3819–4271). In addition, Hosppatankar et al. have reported that Mabs 4G3 and 5E11 (identified at ABB-3 and ABB-5, respectively) reacted with an apo B fusion protein that included apo B-100 residues 458–1017. This would place the epitopes for these Mabs in T4 and in apo B-26. The reasons for the differences are not evident, but it is important to resolve these apparent discrepancies as the reported reactivity of 4G3 and 5E11 with the fusion protein from T4 has been used by others as evidence to implicate T4 in the recognition of apo B-100 by the LDL receptor.

In contrast, we have presented evidence that 4G3 and 5E11 react with epitopes near the T2/T3 junction. First, we reported that 4G3 and 5E11 react with apo B-74 and not apo B-26. Second, we have shown that antibodies 4G3 and 5E11 coupled to Sepharose can be used to separate apo B-48-VLDL (nonretained) from apo B-100-VLDL (retained), which is consistent with the notion that the two epitopes are contained within the region exclusive to apo B-100. Third, we demonstrated here that 4G3 and 5E11 react with T3 and T2, respectively, and that neither Mab reacts with T4. Fourth, we also showed here that 4G3 reacts at a site close to residues 3120–3156, while 5E11 reacts at a site close to 3352–3371. Fifth, we report elsewhere that 4G3 reacts with three apo B fusion proteins that include apo B-100 residues 2657–3286, 3029–3132, and 2488–3636, respectively. The latter protein also reacted with 5E11. Thus, based on five separate criteria, our results place the epitopes for 4G3 and 5E11 close to two defined sequences of apo B-100 that are located within 130 amino acids upstream and downstream respectively of the T2/T3 junction, and not within T4.

Recently an antiserum prepared against a synthetic peptide whose sequence was based on apo B-100 residues 3926 to 3947 of the apo B-100 primary structure was reported to cross-react with both apo B-100 and apo B-48. In contrast to our previous prediction that apo B-48 corresponds to an integral sequence of apo B-100 on the N-terminal side of the molecule, these authors suggest that apo B-48 is composed of disparate segments from the entirety of apo B-100 and which include apo B-100 residues 3926–3947. While this may be the case, our results using Mabs would nevertheless indicate that sequences from the N-terminal of apo B-100 predominate in apo B-48. Indeed, out of the nine epitopes that apo B-100 shares with apo B-48, eight are located in T4 and the ninth, which is the epitope for 2D8, is located in T3 near the T3/T4 junction (Figures 9 and 10). Furthermore, none of the 17 Mabs whose epitopes are downstream from that of 2D8 have been found to react with apo B-48.

In conclusion, the mapping of epitopes for Mabs that block the binding of LDL to its receptor to both sides of the T3-T2 cleavage site provides evidence for the role of this domain in receptor binding and corroborates the earlier identification of potential receptor-binding sequences. It is most significant that the median portion of apo B, represented by T3, contains epitopes that identify a protein structure which is progressively dependent upon interaction with a lipid phase containing a hydrophobic core of cholesteryl ester, because these epitopes are localized closer to the C-terminal end of T3. This structural dependence on interaction with hydrophobic lipids is also maintained in the N-terminal portion of T2 that bears the other epitopes close to the receptor-binding site, such as those for 3A8, 5E11, 4F6, and 6A8. In contrast, it would appear that both the N-terminal and C-terminal regions of apo B are characterized by three-dimensional structures that are relatively independent of lipid interaction.
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References

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Mapping of human apolipoprotein B antigenic determinants.
Y L Marcel, T L Innerarity, C Spilman, R W Mahley, A A Protter and R W Milne

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