Identification of Apo B-100 Segments Mediating the Interaction of Low Density Lipoproteins with Arterial Proteoglycans

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The interactions of low density lipoprotein (LDL) and apolipoprotein (apo) B-100 segments with chondroitin-6-SO₄ rich arterial proteoglycans aggregate (CSPG) were studied by using quantitative frontal elution affinity chromatography. The affinity of the agarose-CSPG was higher for LDL than for very low density lipoprotein, and high density lipoprotein was not bound. LDL from different individuals had dissociation coefficients (Kd) from 28 to 179 nM. Experiments with tryptic hydrolysates of apo B suggested that the capacity of LDL to bind with CSPG resides in the protein. Nine apo B-100 hydrophilic peptides, 12 to 26 amino acids long, were selected, and three were found to interact with the agarose-bound CSPG: apo B P-1 (LRKHKLIVSMY-RELLKDSLKEA, residues 4230 to 4254), apo B P-2 (RLTRKQGLKLATLKSNNK, residues 3359 to 3377), and apo B P-11 (RQVSHAKEKLTALTKK, residues 2106 to 2121). These peptides competed with LDL for binding to the agarose-bound and soluble CSPG; apo B P-2 was the most effective. This correlates with Kd values: 63, 86, and 82 μM for apo B P-2, P-1, and P-11, respectively. The peptides shared an excess of positive-charged side chains. Apo B P-2 belongs to the lys- and arg-rich, LDL-receptor domain. Apo E also binds to the agarose-proteoglycan. The results suggest that apo B regions with sequences and charge distributions analogous to those of residues 3359 to 3377, 4230 to 4254, and 2106 to 2121 are among those responsible for the interaction of LDL with intima-media CSPG.

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Accumulation of apo B-containing lipoproteins in atherosclerotic lesions is observed in human and experimental animal models. These lipoproteins appear to be the main source of the lipids that accumulate extra- and intracellularly during atherogenesis. Immunohistochemical studies have shown a close spatial correlation between the apo B antigen and the extracellular matrix acidic glycosaminoglycans (GAG). Furthermore, complexes of apo B lipoproteins with GAG have been isolated from atherosclerotic lesions in rabbits and in humans. The finding that arterial GAG ("acidic mucopolysaccharides") can form complexes in vitro with apo B lipoproteins suggested that specific proteoglycans (PG) may be responsible for some of the extracellular accumulation of apo B lipoproteins. However, several of the studies of GAG interaction with lipoproteins have been carried out with nonarterial GAG obtained with hydrolytic procedures that require high Ca²⁺ concentrations for complex formation. Using nondissociating conditions, we found that it is possible to isolate, from rabbit and human arterial intima-media, chondroitin-6-SO₄-rich proteoglycan aggregates (CSPG) that form specific soluble and insoluble complexes with apo B lipoproteins at physiological pH and Ca²⁺ concentrations. The formation of such complexes induces changes in the organization of the low density lipoprotein (LDL) and appears to depend strongly on the balance of charges on the lipoprotein surface. The extracellular interactions of apo B lipoproteins in the arterial intima-media may be regulators of lipoprotein accumulation during atherogenesis. It is, therefore, desirable to characterize some of the molecular parameters responsible for such interactions. Studies on the interaction of LDL and heparin indicate that specific sequences of apo B-100 mediate this association. Here we report quantitative experiments indicating that sequences of apo B, which are rich in positively charged side chains and are probably located on the lipoprotein surface, are some of the structures responsible for the association of apo B lipoproteins with human arterial PG.

Methods

Arterial Proteoglycans

Human aortas were obtained at necropsy from young individuals within 12 hours after accidental death and...
were placed immediately in a 4°C preserving solution containing 0.15 M NaCl, 5 mM Tris-HCl (pH 7.4), 0.5 mM Na-EDTA, 0.02% (wt/vol) NaN₃, 10 mM epsilon-amino-caproic acid, 0.1 mM CuSO₄, and 0.2 mM phenylmethyl-sulphonyl fluoride (PMSF). The material was transported to the laboratory and the intima-media of grossly normal regions or of those with only fatty streaks was stripped, placed again in the preserving solution, and stored at −80°C. To partially purify the PG, 10 g of the intima-media was minced into approximately 2 mm pieces; this was extracted with continuous stirring for 24 hours at 2°C to 4°C with 15 volumes of 4 M urea, 1 M NaCl, 10 mM EDTA, 10 M PMSF, 5 mM benzamidine-HCl, 10 mM epsilon aminocaproic acid, and 10 mM N-ethyl maleimide as enzyme inhibitors. This was adjusted to pH 4.5 with 50 mM Na-acetate. The suspension was centrifuged at 100,000 g for 60 minutes at 2°C, and the pellet was discarded. The supernatant was thrice dialyzed against 1 liter changes for 24 hours in distilled water containing the enzyme inhibitors. At this step, most of the proteins that were co-extracted with the PG precipitated and were eliminated by centrifugation at 100,000 g at 2°C for 1 hour. The supernatant was lyophilized, was dissolved in 4 ml of 4 M urea, 1 M NaCl, and the enzyme inhibitors, was buffered to pH 4.5 with 50 mM Na-acetate, and was loaded in a Sepharose 2B column (Pharmacia Fine Chemicals, Uppsala, Sweden) that was 98 cm long and 1.5 cm in diameter. The solution was equilibrated in the same buffer. The elution was performed at 10 ml/hour. The hexuronate-containing material (Kav between 0.35 and 0.45) that contained the LDL complexing activity was collected and dialyzed thrice against 1 liter changes for 24 hours in distilled water and the solution was then lyophilized. This material, labeled PG₁, was stored at −20°C until used. In the experiments to be described, we used three PG₁ preparations, each from one individual aorta. They contained PG aggregates with 52% to 60% chondroitin-6-SO₄, 28% to 20% chondroitin-4-SO₄, and less than 20% of hyaluronic acid and dermatan sulfate, as indicated by high-performance, liquid chromatography of the unsaturated disaccharides produced by treatment with chondroitinases ABC and AC. The GAG/protein ratio was between 6:4 and 7:3 (for characterization methods, see reference 12). The dermatan and heparan-rich PG eluted mostly after Kav 0.45 together with most of the remaining contaminating proteins and showed little LDL-complexing capacity. We decided to use this stage of purification of the PG₁ aggregates because further fractionation procedures, as CsCl - Gd-HCl centrifugation and gel exclusion chromatography in dissociating conditions, led to up to 60% reduction on LDL-complexing activity, and because PG₁ in intima-media probably exists as aggregates that are the only LDL-insolubilizing macromolecules in the extracts.12,14 Covalent Binding of Arterial PG₁ to Agarose Lyophilized PG₁ containing approximately 1 mg of hexurionate, was dissolved in 5 ml of 0.1 M NaHCO₃ (pH 8.0) and this was coupled to 3 ml of Affi-Gel 10 following the manufacturer’s instructions (Bio-Rad, Richmond, CA). The gel bound 100 to 200 µg of hexurionate/ml. The unbound material was eluted with 5 mM Tris-HCl (pH 7.2), 4 M CaCl₂, 2 mM MgCl₂, 0.2 mM PMSF, and 0.2 mM Na₂SO₄ (buffer A) made in 1 M NaCl. Then the agarose-PG₁ was washed extensively in buffer A and stored at 2°C to 4°C. Lipoproteins Very low density lipoprotein (VLDL) (1.006 to 1.019 g/ml), LDL (1.019 to 1.063 g/ml), and high density lipoproteins (HDL) (1.063 to 1.21 g/ml) were isolated from fresh human plasma containing 1 mg/ml Na₂-EDTA, 2 mM PMSF, and 2 mM NaN₃ by using KBr solutions with the same additions and differential ultracentrifugation.16 For the binding experiments, lipoproteins that had been stored at 4°C in KBr were equilibrated with the desired solutions by using PD-10 columns (Pharmacia). Apo E was prepared from the VLDL fraction of type IIb subjects.19 Partial tryptic hydrolysates of LDL were obtained by incubating 45 to 50 mg of the lipoprotein with 5 units of agarose-bound enzyme (Sigma, St. Louis, MO) for 12 hours at 25°C. After the incubation, lipid-free and lipid-bound peptides were separated on a Sephadex G-100 (F) column.

Table 1. Sequence of Synthetic Apo B Peptides Used for Binding Experiments with Agarose-Arterial Proteoglycan Columns

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Apo B* segment</th>
<th>Sequence</th>
<th>Charge</th>
<th>Hydrophilicity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B P-1</td>
<td>4230 to 4254</td>
<td>LRRKLIDVSMYRELLKDLŠKEA</td>
<td>+3</td>
<td>3.4</td>
</tr>
<tr>
<td>Apo B P-2</td>
<td>3359 to 3377</td>
<td>RLRTRKRLKLATLSLSNK</td>
<td>+6</td>
<td>7.3</td>
</tr>
<tr>
<td>Apo B P-4</td>
<td>3987 to 4003</td>
<td>DEDDDFSWNYYSQPS</td>
<td>−4</td>
<td>20.5</td>
</tr>
<tr>
<td>Apo B P-5</td>
<td>4094 to 4105</td>
<td>RKIDDVRFQK</td>
<td>0</td>
<td>12.9</td>
</tr>
<tr>
<td>Apo B P-6</td>
<td>3488 to 3513</td>
<td>SEANTLYNSKSTRSSVVLQGSKIDD</td>
<td>+1</td>
<td>29.9</td>
</tr>
<tr>
<td>Apo B P-7</td>
<td>455 to 481</td>
<td>EDYLIRVIGINGMTMEQLPELKS</td>
<td>−2</td>
<td>11.4</td>
</tr>
<tr>
<td>Apo B P-8</td>
<td>671 to 696</td>
<td>EPTLEALFGKQSVGDFSINKALYIVWN</td>
<td>−1</td>
<td>4.2</td>
</tr>
<tr>
<td>Apo B P-9</td>
<td>1041 to 1059</td>
<td>PDFVDLGTLRWNDEST</td>
<td>−5</td>
<td>15.5</td>
</tr>
<tr>
<td>Apo B P-11</td>
<td>206 to 212</td>
<td>RQVSHALEKLTTK</td>
<td>+5</td>
<td>18.7</td>
</tr>
<tr>
<td>Apo E (141-150)‡</td>
<td>141 to 150</td>
<td>LRLKLRKLLR</td>
<td>+6</td>
<td>−8.1</td>
</tr>
</tbody>
</table>

*References 24, 25, and 26.
†The average hydrophilicity of the segment was calculated as part of the intact apolipoprotein using Parker, Guo, and Hodges algorithm (reference 22).
‡Region of apo E recognized by the apo B,E receptor.
The lipid-bound apo B-100 fragments in the $V_o$ were delipidated with hexane/isopropanol (3:2 vol/vol); the precipitated peptides were collected by centrifugation, were dissolved in buffered 6 M urea, and were equilibrated with buffer A by using PD-10 columns (Pharmacia) before the binding experiments.

**Synthetic Apo B-100 Peptides**

Based on the recently established apo B-100 consensus sequence, regions of relatively high hydrophilicity with different side-chain charge distributions were selected and synthesized by solid-phase procedures by using Ferring AB (Malmo, Sweden). The peptides were purified to more than 95% homogeneity by high-performance, liquid chromatography on reverse-phase Microbondapack C-18 columns (Waters, Inst., Milford, MA) by using gradients of water or water-NH$_3$OH and acetonitrile-0.1% trifluoroacetic acid. After lyophilization, the peptides were stored dry at -20°C. The sequence of the peptides was confirmed by sequential N-terminal analysis and is presented in Table 1.

**Evaluation of Binding Parameters**

The macroscopic dissociation coefficient ($K_d$) and the maximal binding capacity (Bt) were evaluated by using frontal elution affinity chromatography as developed by Kasai et al.$^{27}$ This is a near-to-equilibrium procedure that permits measurements of binding parameters without assumptions about the nature and structure of the ligands involved. The method is based on the evaluation of elution profiles of the soluble ligand A that is passed at constant concentration $[A]_o$ through a column packed with an inert material to which the ligand B, the arterial PG, in these experiments, is covalently immobilized. Once the experimental values ($V$) are obtained from profiles like those in Figure 1, the $K_d$ and Bt are calculated using the equations developed by Kasai et al., which relate the elution volumes, in binding ($V$) and nonbinding conditions ($V_o$), to the macroscopic dissociation constant ($K_d$):

\[
\frac{1}{[A]_o} (V - V_o) = (K_d/Bt)(1/\frac{1}{[A]_o} + 1/Bt)
\]

Where $V$ is the elution volume of ligand A in the presence of a concentration ($l_o$) of the inhibitor, and $V_m$ is the elution volume at maximal dilution, calculated from the equation:

\[
V_m = V_o + Bt/K_d
\]
amount of ligand bound at concentration \( [A]_0 \) of the free ligand, after corrections for baseline displacement or changes in flow. Figure 1 presents the experimental profiles for this type of measurement using a continuous 226 nm absorbance-monitoring system for the soluble ligand LDL. The lower panel of Figure 2 presents the Scatchard plot obtained with this last procedure on the same runs used for measurements by frontal elution affinity chromatography (top panel, in Figure 2). Most of the experiments were carried out using as a base buffer: 5 mM Tris-HCl (pH 7.2) containing 4 mM CaCl
_2_, 0.2 mM NaN
_3_, 0.2 mM PMSF, and 25 mM NaCl. The columns with the agaro-se-PG, gel had volumes of 0.5 and 1.0 ml and were designed to minimize dead volume. The flow was 0.1 to 0.2 ml/min, adjusted with a 2152 LKB controller and a 2150 LKB pump (LKB, Bromma). The elution profiles were followed by several methods, depending upon the nature of the experiment and the soluble ligands. On-line absorbance monitoring at 206 and 226 nm (LKB Uvicord 2158) was used for most of the experiments with pure apo B peptides and LDL. To measure very low peptide concentrations, the collected fractions were reacted with Fluorescamine and the fluorescence was evaluated. To runs with low lipoprotein concentrations, an intrinsic fluorescence of 350 nm after 280 nm excitation was used. To quantify LDL in the presence of other proteins or peptides, cholesterol was measured with the method of Bowman and Wolf except that excitation at 450 nm and fluorescence at 530 nm was used instead of photometry. With this procedure, cholesterol concentrations as low as 10 ng/ml could be detected in the collected fractions. An electromunoassay was used to measure apo B in the presence of other lipoproteins. Zonal elution experiments were also used to evaluate the affinity of lipoproteins and peptides for the PG. An agaro-se-PG column (1 ml) was equilibrated with buffer A without NaCl, and 25 to 50 μg of lipoprotein, apolipoprotein, or the peptides in the same buffer were bound to it. The column was washed with five volumes of buffer A, after which a linear gradient of 0 to 250 mM NaCl in buffer A was used to elute the bound apolipoprotein, lipoprotein, or peptide with a flow of 0.2 ml/min.

**Competition Experiments In Solution**

To investigate the effect of the apo B-100 peptides on the interaction between LDL and soluble arterial PG, and heparin, we used competition experiments. The optimal ratio of PG, and heparin to LDL that will give a maximal precipitate was established for the system. A ratio of 4 μg of hexonate to 0.1 mg LDL protein (0.13 mg total cholesterol) in 1 ml of buffer A, 25 mM in NaCl gave the maximum amount of precipitated cholesterol precipitation (100%). LDL (50 μl) was added to the solution of PG, or heparin in 1.5 ml conical centrifuge tubes. After 1 hour at 20 ± 2°C, the tubes were centrifuged for 3 minutes at 10,000 g, the supernatant was discarded, the pellets were washed with buffer A, and they were centrifuged again. The pellets were suspended in 0.1 ml of 0.1 M Tris (pH 10), and cholesterol was extracted and measured. A similar procedure was followed for exploration of the inhibitory effect of apo B-100 peptides. A 50-fold molar excess of each one was added to PG, and the heparin solutions before adding the LDL, and the samples were similarly processed to measure the amount of LDL cholesterol insolubilized.

**Results**

**Binding Parameters from Frontal Affinity Chromatography**

This procedure appears especially well-suited for the type of interaction studied here, since it provides a near-equilibrium condition in which ligands of different molecular size, such as the lipoproteins and the apo B synthetic peptides, can be tested in the same system. Furthermore, the technique allows unequivocal direct measurements of the different ligands. Figure 2 presents the evaluation of Kd for the association between the same preparation of LDL and agarose-bound arterial PG, by using the analysis of the frontal elution profiles as proposed by Kasai et al. 27

### Table 2. Interaction of Different Lipoproteins with Agarose-Arterial Proteoglycan

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Kd (nM)</th>
<th>Bt (nMol × 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL-LS</td>
<td>299</td>
<td>37</td>
</tr>
<tr>
<td>VLDL-MH</td>
<td>330</td>
<td>43</td>
</tr>
<tr>
<td>VLDL-GC</td>
<td>273</td>
<td>30</td>
</tr>
<tr>
<td>HDL-LS</td>
<td>no binding</td>
<td>no binding</td>
</tr>
<tr>
<td>HDL-MH</td>
<td>no binding</td>
<td>no binding</td>
</tr>
<tr>
<td>HDL-GC</td>
<td>no binding</td>
<td>no binding</td>
</tr>
<tr>
<td>LDL-LS</td>
<td>40</td>
<td>69</td>
</tr>
<tr>
<td>LDL-MH</td>
<td>78</td>
<td>81</td>
</tr>
<tr>
<td>LDL-GC</td>
<td>35</td>
<td>83</td>
</tr>
<tr>
<td>LDL-RH</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>LDL-RS</td>
<td>103</td>
<td>84</td>
</tr>
<tr>
<td>LDL-CT</td>
<td>179</td>
<td>143</td>
</tr>
<tr>
<td>LDL-AM</td>
<td>28</td>
<td>83</td>
</tr>
<tr>
<td>LDL-RT(5)</td>
<td>105 ± 10</td>
<td>110 ± 24</td>
</tr>
</tbody>
</table>

The lipoproteins were tested against the same batch of agarose-arterial PG. The molarity of the lipoproteins was calculated approximately by using the protein content of the particles and assuming 2.5 and 7.5 × 10^10 daltons as the approximate molecular weights for LDL and VLDL, respectively. Kd = dissociation coefficient; Bt = binding capacity; VLDL = very low density lipoprotein; HDL = high density lipoprotein; LDL = low density lipoprotein.
or the measurement of the bound LDL in terms of the parameter $r$ and a Scatchard plot. The $K_d$ values were similar.

**Interaction between Agarose-PG, and Lipoproteins**

VLDL, LDL, and HDL were prepared from normolipidemic subjects and were compared for their capacity to interact with the same agarose-PG. Figure 3 presents the elution profiles, indicating the affinity order for lipoproteins of the same subject. Table 2 lists the measured $K_d$ values for the lipoprotein classes from three subjects and for seven different LDL preparations. To compare the different lipoproteins in the same units, the binding parameters are expressed on a molar basis, assuming $2.5 \times 10^5$ and $7.5 \times 10^5$ as average molecular weights for LDL and VLDL from normolipidemic subjects. The concentration was evaluated by protein determinations on the purified lipoproteins. Table 2 also presents an evaluation of the variability of $K_d$ and $B_l$ measurements on the same LDL-agarose-PG system. LDL was consecutively analyzed on the same column five times within a 15-day period. The standard deviation indicates a 15% to 20% variation for the system.

All the LDL measured had lower $K_d$ than VLDL; it is clear that LDL from each individual had its own specific affinity for the same agarose-PG preparation.

The possible biological significance of the interactions of apo B lipoproteins with arterial PG is related to their occurrence in conditions close to those that may exist in tissue. To explore whether the apo B lipoproteins are recognized by the agarose-PG in the presence of other plasma components, 20-fold diluted total plasma was passed through the agarose arterial-PG column. The plasma selected contained 180 mg/dl LDL cholesterol, 55 mg/dl HDL cholesterol, and 10 mg/dl VLDL cholesterol. Therefore, the elution profile, followed as cholesterol and apo B protein (Figure 4) is mostly a function of the interaction of LDL with arterial PG. The measured $K_d$ for this apo B in the presence of the other plasma components was 130 nM, compared to 80 nM for the isolated LDL from the same subject (LDL-RH, Table 2). We conclude that other plasma components do not block the association of LDL with PG.

The presence of nonspecific interaction with the agarose (Affi-Gel 10) was tested by passing LDL through a column prepared in the same way as the PG-agarose, but without PG. The elution profile in binding and nonbinding conditions was similar, indicating no interaction. In another experiment, a column of agarose-PG was incubated for 1 hour with chondroitinase AC and, once the enzyme had been eluted, the LDL binding capacity of the column was restested. The chondroitinase treatment increased the $K_d$ threefold. These results indicate that the elution profiles observed for binding conditions require intact PG in PG column. The remaining binding after chondroitinase AC treatment was probably due to GAG that were not completely digested under the conditions used and which remained covalently attached to the agarose.

**Effect of Ionic Environment on Association of PG, with LDL**

The interaction between LDL and arterial PG appears to be initially Coulombic and should be sensitive to the ionic environment. We performed three types of experiments to study this phenomena: 1) the effect of NaCl on $K_d$, 2) an evaluation of the NaCl concentration required to dissociate the lipoproteins from the agarose-PG column, and 3) measurements of the binding capacity of the agarose-PG gel at different NaCl concentrations and with two buffers. In all cases, the concentration of Ca++ and Mg++ was maintained at 4 mM and 2 mM, respectively, and the pH was 7.2. The relation between the NaCl concentration and $K_d$ for the association of LDL with agarose-PG was described by the exponential function in $Y = 4.3 \times 10^{-0.04}$, ($r=0.98$) for the range 10 to 150 mM NaCl. Figure 5 presents this exponential relation in terms of ln of bound LDL per ml of agarose-PG gel for 5 mM phosphate buffer and for 5 mM Tris-HCl. Figure 6 shows the results obtained in the zonal elution experiments by using a NaCl gradient on buffer A (see Methods). We emphasize that, although NaCl concentration has a strongly depressing effect in the association, this is still measurable in the physiological range of Na++, Ca++, and Mg++. 

![Figure 4](http://atvb.ahajournals.org/)

*Figure 4.* Elution profiles for apo B lipoproteins in diluted human plasma from an agarose-arterial PG column. Conditions as in previous figures. Plasma (50 µl) was diluted to 2 ml with buffer A or with buffer A and 0.5 NaCl and was passed through the column.

![Figure 5](http://atvb.ahajournals.org/)

*Figure 5.* Effect of NaCl concentration on the amount of LDL bound to a 0.5 ml agarose-arterial PG column in 5 mM buffer phosphate or 5 mM buffer Tris-HCl. The buffers contained 4 mM CaCl₂ and 2 mM MgCl₂.
was collected the partially digested apo B-100 that remained with the agarose-arterial PG. To evaluate the part played by apo B-100 regions, whereas the released fragments did not. In competition experiments with a fivefold excess of peptide to LDL concentrations. If the Ca\(^{++}\) or Mg\(^{++}\) is raised to 8 mM and 4 mM, respectively, the interaction is measurable above 160 mM NaCl, especially in Tris buffer.

**Role of Apo B Peptides**

Apo B lipoproteins contain many surface lipids and protein components that may be involved in the interaction with the agarose-arterial PG. To evaluate the part played by apo B, LDL was partially degraded by treatment with immobilized trypsin. The resulting product was separated into two peaks by gel exclusion chromatography. In the Vo of the different peptides and the Ki was calculated as described in Methods. In all the experiments, the same batch of agarose-PG and LDL preparation was used. Table 3 lists these values for different apo B peptides tested against the same LDL and agarose-arterial PG column. It is clear that apo B P-2 is the more efficient competitor. The synthetic peptides were also examined for their direct binding capacity to the same agarose-PG column. Figure 6 shows the elution profiles for the same concentration of each apo B peptide. The affinity order is apo B P-2 > apo B P-1 > apo B P-11; the peptides apo B P-4, 5, 6, 7, 8, and 9 had no measurable interaction. These results correlate well with those of the competition experiments. Table 3 lists the Kd values obtained using frontal elution affinity chromatography and several peptide concentrations, indicating that apo B P-2 has the lowest Kd and also the highest maximal binding capacity (Bt) for the agarose-arterial PG. Gradient elution experiments indicate that the association of apo B P-2 with arterial-PG is measurable at physiological ionic strength, being the peptide eluted with a range of NaCl concentrations similar to that required to elute the LDL (Figure 6). In the same figure, the zonal elution profile of apo E is presented showing that it also binds to the agarose-arterial PG.

**Interactions In Solution**

The binding of PG, to agarose may lead to alterations in recognition regions for LDL and the competition specificity of the apo B-100 peptides. To explore this, competition experiments of the peptides with the PG,–LDL and heparin-LDL interactions in solution were carried out. The results presented in Table 4 indicate that, as with the affinity column, apo B P-1, P-2, and P-11 were the only active peptides; P-2 and P-1 were the more efficient inhibitors with both PG, and heparin. The data suggest that similar structures are recognized by PG, in solution or when immobilized on agarose. The results with heparin indicate that PG, and heparin share structural recognition.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Ki (µM)</th>
<th>Kd (µM)</th>
<th>Bt (mMol)</th>
<th>Kd LDL (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo B P-1</td>
<td>2.3</td>
<td>86</td>
<td>96</td>
<td>413</td>
</tr>
<tr>
<td>Apo B P-2</td>
<td>4.5</td>
<td>63</td>
<td>151</td>
<td>800</td>
</tr>
<tr>
<td>Apo B P-11</td>
<td>3.2</td>
<td>82</td>
<td>67</td>
<td>510</td>
</tr>
<tr>
<td>Control LDL</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Apo B peptides 4, 5, 6, 7, 8, and 9</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>95 to 110</td>
</tr>
</tbody>
</table>

For measurement of direct interaction, the peptides or the LDL were passed through the agarose-PG column at different concentrations, and the V values and Kd were evaluated as described in the Methods section. For the competition experiments, the same concentration of LDL (160 nM) was passed through the column in the presence of 5 µM of the different peptides and the Ki was calculated as described in Methods. In all the experiments, the same batch of agarose-PG and LDL preparation was used.

*Dissociation coefficients for agarose-PG/LDL interaction in the presence of the apo B-100 synthetic peptides.
specificities toward LDL and the apo B-100 segments in the conditions tested.

Discussion

The association of apo B lipoproteins with the extracellular matrix and their subsequent handling by the tissue appear to play an important role in atherosclerosis.\(^1,2,6,8,15,32\) The nature of the interaction between LDL and acidic complex carbohydrates has been the subject of intensive research. We know that complex formation is initiated by the Coulombic attraction of the charged carboxyl and sulfate groups of the polysaccharides and positively charged regions of the lipoprotein.\(^33,34\) Studies with a chondroitin-6-SO\(_4\)-rich PG aggregate isolated from human intima-media indicate that the specificity and affinity of the association depend on the intact structure of the PG and are finely modulated by the balance of charges and lipid composition of the apo B lipoproteins.\(^11-14,25\) However, direct evidence of the part played by specific regions of the apo B has been unavailable. The recent reports establishing the sequence of human apo B-100 have made possible direct testing of the involvement of selected peptide regions in the interactions of apo B lipoproteins with cells and extracellular mesenchyme.\(^20,24,25,26\)

With the presence of apo B-100 and its many regions of hydrophobic and hydrophilic character, the availability of positively charged areas on the surface of LDL can result from at least three possible structural arrangements. First, hydrophilic sequences with clusters of lysine (K), arginine (R), and histidines (H), with no aspartic (D) or glutamic (E) interdispersed; second, a high density of positive side chains from noncontiguous segments that reside in the same area of the particle surface; and third, a combination of the first and second. Our experiments were designed to explore the first possibility. The nine peptides tested were from hydrophilic regions of apo B-100, covered a wide combination of charged side chains (+6 to −4), and were from regions that were, with the possible exception of apo B P-1, accessible to trypsin (4230 to 4254).\(^25\)

The experiments presented here can be considered as extensions of the ones carried out by Iverius\(^36\) who investigated the interactions of LDL and nonarterial GAG attached to agarose. Our results with a human arterial lipoprotein-complexing PG and the frontal affinity chromatography developed by Kasai et al.\(^27\) allowed a quantitative evaluation of the interactions, since this is based on near-equilibrium conditions adjustable to the widely different molecular species studies. The results indicate that some of the structures responsible for the arterial PG\(_1\)/apo B lipoproteins association reside in segments of apo B-100 that are probably located on the surface of the lipoprotein particles. However, the interaction also appears to be controlled by other structural features of the lipoprotein particle. The two- to eight-fold difference between the Kd values of LDL and VLDL, each containing one apo B-100 molecule, indicates that other molecules in VLDL can reduce the affinity for the arterial PG\(_1\). This may be due to localized differences between LDL and VLDL in the surface accessibility of the apo B-100 or to a general effect, such as reduction in the Coulombic-dependent concentration around the charged surfaces of the agarose-PG induced by the lower net charge of the VLDL at physiological pH.\(^37\) The results with LDL from different individuals (Table 2) are related to previous findings, which indicate that there are individuals with high- and low-reacting LDL for the arterial chondroitin-6-SO\(_4\)-rich PG probably associated with small differences in lipid composition and isoelectric point of the lipoproteins.\(^13,14\)

The potential relevance of this finding to differences in accumulation rates of LDL in the intima-media during atherogenesis merits a more extensive study. For the experiments here reported, we used a chondroitin-6-SO\(_4\)-rich PG aggregate, in which its chondroitin sulfate moiety appears to be the most active LDL-binding component.\(^11,12\) Recently Steele et al.\(^38,40\) used dissociating conditions to isolate and characterize an LDL-complexing chondroitin sulfate monomer from pigeon aortas that distinguishes LDL from atherosclerotic-resistant and atherosclerotic-prone birds. Probably a similar PG monomer is responsible for the LDL-complexing ability of our preparations, but this should be explored with human arterial PG prepared.

### Table 4. Competition of Apo B-100 Peptides with Insolubilization of LDL by Soluble Arterial PG, and Heparin

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<th>Apo B peptide</th>
<th>PG(_1)-LDL % Inhibition</th>
<th>Heparin-LDL % Inhibition</th>
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<tr>
<td>Apo B P-1</td>
<td>81, 90</td>
<td>70, 75*</td>
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<tr>
<td>Apo B P-2</td>
<td>85, 86</td>
<td>72, 78</td>
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<tr>
<td>Apo B P-11</td>
<td>25, 32</td>
<td>20, 27</td>
</tr>
<tr>
<td>Apo B P, 4, 5, 6, 7, 8, and 9</td>
<td>0 0</td>
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The results are expressed as µg cholesterol precipitated in the presence of the apo B peptides divided by the amount precipitated in the absence of the peptides x 100 subtracted from 100.

*The values are from two experiments run in duplicate using the same PG\(_1\), heparin, and LDL preparation.
under conditions similar to those described by Steele et al. We cannot disregard the contribution of small amounts of PG other than chondroitin sulfates in our experiments, but this should not diminish their biological significance since probably within the arterial mesenchyma, the LDL interacts with a mixture of complex PG.

The apo B-100 is a protein that has internal repeats with partial homologies. Therefore, several segments on the surface of the lipoproteins may be involved in the same class of interaction, such as receptor binding or, in this case, association with arterial PG. In most of the LDL preparations studied, the evaluation of the binding isotherms indicated a single class of binding species. However, in LDL preparations from two individuals, we detected irregularities in the elution profiles and non-linearity of the Scatchard plots that indicate more than one population of binding species. Our results do not allow conclusions about secondary and tertiary structure modulation of the actual binding sites induced by the lipoprotein interactions in the LDL particle. However, the binding regions for the interaction of two large macromolecules, such as apo B-100 lipoproteins and agarose-PG, should be located within segments that have a high probability of residing on the surface of the lipoprotein particle. The results from the competition and direct interaction experiments with the nine synthetic apo B peptides of relative high hydrophilicity suggest that this condition, plus a high density of lysine and arginine side chains, are part of the structural characteristics recognized by the agarose-arterial PG. A search along the 4536 residues of apo B with the procedure of Parker, et al. for detecting possible surface segments showed that, besides apo B P-2 (3359 to 3377), apo B P-1 (4230 to 4254), and apo B P-11 (2106 to 2121), the segments 17 to 26, 603 to 615, 891 to 907, 1259 to 1277, 2888 to 2909, 2935 to 2942, and 3147 to 3157 share the properties of being both hydrophilic and having three or more positive charges within less than 15 residues and may therefore also contribute to the binding of the intact LDL.

Hirose et al. isolated, from CNBr fragments of LDL, four peptides of 20 Kd, three of them from the C-terminal region of apo B-100 that binds to heparin through clusters of basic amino acids. Apo B P-2 (3359 to 3377) belongs to the heparin-binding segment CNBr-III (3308 to 3394), and apo B P-11 (2106 to 2121), to the segment CNBr-I (2016 to 2151). Weisgraber et al. isolated, from proteolytic digests of LDL, seven heparin-binding basic peptides. Two of them, residues 2016 to 2151 and 3356 to 3489, partially overlap with our synthetic peptides apo B P-2 (3359 to 3377) and apo B P-11 (2106 to 2121). Furthermore, the heparin binding sites C (875 to 932) and E (3134 to 3209) overlap with two of the above suggested as PG-binding regions (891 to 907 and 3147 to 3157). This suggests that the mechanism for LDL-GAG interactions might be similar for the arterial CSPG and heparin (Table 4), although binding to heparin could be tighter than to CSPG due to its higher density of sulphate residues. Experiments like those reported for the characterization of apo B-100-heparin binding regions should be carried out with the arterial CSPG and other PG to identify all the potential binding regions and to explore the existence of specificity for different GAG or PG. However, the identification of other PG-binding linear segments of apo B-100 does not exclude the possibility that the real binding areas may be formed in the intact LDL by contributions from peptides not necessarily linear neighbors.

The differences in affinity between the isolated apo B P-2, apo B P-1, and apo B P-11 and the fact that the pure amino acids, lysine and arginine, neither interact with PG nor compete with LDL indicate that not just charge and solubility in water control the interaction with the arterial PG. Apo B P-2 competes more effectively and has lower Kd and higher maximal binding than the other two peptides (Table 2). Therefore, its structure should be

Figure 8. Hypothetical helical circular projections of synthetic apo B-100 peptides. The projection (wheel) in A contains the total sequence of apo B P-2 (residues 3359 to 3377). B contains the first 19 amino acids of apo B-1 (residues 4230 to 4248). C represents apo B P-11 (residues 2106 to 2121).
closer to that of the segments responsible for the association with LDL. Application of Chou and Fasman’s empirical rules\(^1\) suggest that apo B P-1, 2, and 11 reside within alpha helix segments. Figure 8 presents the helical wheels\(^2\) of the hypothetical helices of apo B P-2 and the first 19 amino acids of apo B P-1 and apo B P-11. It should be noted that only in apo B P-2, are five possible charges located in the upper half of the helix and in close proximity since they reside between the first nine amino acids. Models built with the sequences of apo B P-2 suggest that this segment could exist as a right-handed alpha-helix in which the lysine and arginine positive side chains are separated by 10 to 15 Å. This is an appropriate distance for two-point interactions with a model at the same scale of the GalNAc (6-SO\(_4\))(beta->4)Glcuronol disaccharide unit of chondroitin-6-SO\(_4\), the most abundant GAG in PG. However, beta-sheet models with the apo B P-2 sequence also allow two-point interactions due to the high lysine and arginine content of the first nine amino acids. The sequences of apo B P-1 and apo B P-11 produce projections in which the positive charges are more evenly spread circularly and along the backbone of the chain. Experiments with shorter apo B segments and model peptides should be carried out to establish the more appropriate structure and the minimal recognition segment. Multiple binding sites in LDL, or the presence of a more favorable lipid-induced “frozen” conformation of the involved segments on intact LDL, may be the reason for the lower KD of the lipoprotein when compared with that of the synthetic peptides. Reconstitution studies with longer hydrophilic-hydrophobic segments with the appropriate charge distribution could clarify this point.

Although the KD measured in the nM to \(\mu\)M range and the free energy change involved in the interactions studied suggest specificity, these Coulombic interactions probably are caused by peptide sequences appropriate for general recognition of sulfated GAG or negatively charged proteins. The fact that apo B P-2 is part of the LDL receptor domain is probably the basis for the findings that indicate that sulfated GAG compete for the association of apo B/apo E-containing lipoproteins with the receptor.\(^3\) Trypsin has been shown to reduce the receptor binding of LDL without affecting heparin binding,\(^4\) and we found that apo B-100 tryptic peptides still interact with the agarose-PG\(_1\). This may be an indication that LDL-GAG interactions require smaller recognition domains than the ones for the LDL receptor. The residues 139 to 150 of apo E-3 have been identified by Weisgraber et al.\(^5\) as the heparin binding site. The helical wheel corresponding to the sequence leu-arg-lys-arg-leu-arg-leu (residues 141 to 150) of apo E is similar in charge distribution to that of residues 3359 to 3367 of apo B-100. This may explain the association of apo E with the arterial PG. However, the putative, second heparin-binding site of apo E (residues 192 to 215) does not display this arrangement, indicating that other sequences or combinations of non-neighboring regions can also bind GAG.

The importance of the chondroitin sulfates for focal atherogenic development has been underscored by recent findings about their presence in regions of apo B-

lipoprotein deposition in lesions.\(^6\) Further, complexing of LDL with heparin appears to inhibit their arterial deposition.\(^6\) If these phenomena are related to the interactions described here, the final establishment of the molecular events responsible will increase our knowledge of lipoprotein accumulation during atherogenesis.

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