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# Isolation and Characterization of Four Heparin-Binding Cyanogen Bromide Peptides of Human Plasma Apolipoprotein B†

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ABSTRACT: Apolipoprotein B-100 (apoB-100) is the major protein constituent of human plasma low-density lipoproteins (LDL). On the basis of its amino acid sequence [Chen, S.-H., Yang, C.-Y., Chen, P.-F., Setzer, D., Tanimura, M., Li, W.-H., Gotto, A. M., Jr., & Chan, L. (1986) *J. Biol. Chem. 261*, 12918–12921], apo B-100 is one of the largest monomeric proteins known with a calculated molecular weight of 512 937. Heparin binds to the LDL surface by interacting with positively charged amino acid residues of apoB-100, forming soluble complexes in the absence of divalent metals and insoluble complexes in their presence. The purpose of this study was to isolate and characterize the heparin-binding domain(s) of apoB-100. Human plasma LDL were fragmented with cyanogen bromide (CNBr). After delipidation and reduction–carboxymethylation, the CNBr peptides were fractionated by sequential chromatography on DEAE-Sephacel, Mono S, and high reactive heparin (HRH) AffiGel-10; HRH was purified by chromatography of crude bovine lung heparin on LDL AffiGel-10. Heparin-binding peptides were further purified by reverse-phase high-performance liquid chromatography. Heparin-binding activity was monitored by a dot-blot assay with $^{125}$I-HRH. The amino-terminal sequences of four CNBr heparin-binding peptides (CNBr-I–IV) were determined. CNBr-I–IV correspond to residues 2016–2151, 3109–3240, 3308–3394, and 3570–3719, respectively, of the amino acid sequence of apoB-100. Each CNBr peptide contains a domain(s) of basic amino acid residues which we suggest accounts for their heparin-binding activity. CNBr-I is located near the middle of the apoB-100 sequence whereas CNBr-II, -III, and -IV are clustered near the carboxyl-terminal end of the protein. The proposed heparin-binding domains of CNBr-II (residues 3150–3157) and -IV (residues 3670–3677) show structural homology to the reported heparin-binding region of human vitronectin; CNBr-III (residues 3361–3368) shows structural homology to the known receptor/heparin-binding region of apolipoprotein E (residues 144–151). We suggest that one or more of these heparin-binding domains possibly corresponds to the receptor-binding region(s) of LDL as heparin is known to displace LDL from its membrane receptor.

Plasma low-density lipoproteins (LDL)[1] are the major carriers of cholesterol in the circulation. LDL are isolated in the density range 1.019–1.063 g/mL and represent a distribution of particles differing in size, hydrated density, and lipid composition (Crouse et al., 1985; Fisher, 1983; Krauss & Burke, 1982; Shen et al., 1981). LDL contain one major protein termed apolipoprotein B-100 (Kane et al., 1980) with a calculated molecular weight of 512 937 based on the complete amino acid sequence reported by Chen et al. (1986). In normal man, very low density lipoproteins (VLDL) represent the major apoB-100-containing precursor lipoproteins of plasma LDL. LDL are removed from plasma by either receptor-dependent (Goldstein & Brown, 1984; Brown & Goldstein, 1986: Dietschy, 1984) or receptor-independent processes (Spady et al., 1986); the liver is the major tissue site for LDL clearance.

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[1] Abbreviations: apoB-100, apolipoprotein B-100; VLDL, very low density lipoprotein(s); LDL, low-density lipoprotein(s); GAG, glycosaminoglycans; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRH, high reactive heparin; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; CNBr, cyanogen bromide; PTH, phenylthiohydantoin; HBD, heparin-binding domain.

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Interest in understanding the structure and metabolism of LDL has been stimulated by recent reports that the plasma concentration of LDL–cholesterol is highly positively correlated to the incidence and severity of coronary heart disease and that lowering LDL reduces risk (The Lipid Research Clinics Program, 1984).

Alterations in the rates of either synthesis or catabolism of LDL may give rise to elevated levels of plasma LDL–cholesterol and increased risk of disease. However, these changes do not explain why LDL accumulates in the arterial wall during the atherosclerotic process. Gofman et al. (1950) were the first to suggest that a particular LDL subfraction which is larger and has a lighter density than normal LDL is correlated with coronary heart disease. Since this initial observation, a number of reports [see Rudel et al. (1986) for a review] have appeared suggesting that certain LDL subpopulations are more atherogenic than others. It is also known that LDL bind to the extracellular matrix, such as glycosaminoglycans, proteoglycans, and collagen, forming insoluble complexes in the presence of $Ca^{2+}$ (Camejo et al., 1980; Camejo, 1982; Radhakrishnamurthy et al., 1982). Because of the potential importance of the interaction of LDL and the extracellular matrix in the pathogenesis of atherosclerosis, we have identified four heparin-binding peptides of apoB-100 and have shown that these peptides contain specific domains which may account for their binding to glycosaminoglycans.

## Materials and Methods

*Materials.* DEAE-Sephacel and Mono S (cation-exchange resin) were purchased from Pharmacia. Nitrocellulose paper (0.45 μm) and AffiGel-10 were obtained from Bio-Rad. *N*-Dansylaziridine was from Pierce. Bovine lung heparin was a generous gift from Hepar Industries (Franklin, OH). $Na^{125}I$ (17 Ci/mg) was from New England Nuclear. HPLC solvents were obtained from Burdick & Jackson. Water was purified by a Milli-Q system (Millipore Corp.).

*Preparation of CNBr Peptides of ApoB.* LDL were isolated from plasma as described by Cardin et al. (1984b). LDL (5 mg of protein/mL) in 70% formic acid were incubated with CNBr (500 molar excess relative to methionine) for 48 h at room temperature in the dark with continuous stirring. After incubation, LDL were dialyzed extensively against 10 mM Tris-HCl, pH 8.0. To delipidate LDL, 45 mL of acetone/ethanol (1:1) was added to 5 mL of LDL solution in a 50-mL screwcap conical tube. The solutions were mixed and placed at −20 °C for 1 h; the protein precipitates were collected by centrifugation. The extraction procedure was repeated 5 times, and then the protein precipitates were washed with diethyl ether. The pellets were partially dried with ultrapure nitrogen and then solubilized in 6 M guanidine hydrochloride/10 mM Tris-HCl, pH 8.6, to give approximately 3 mg of protein/mL. Residual ether in the sample was evaporated by a gentle nitrogen stream. β-Mercaptoethanol was then added to a final concentration of 100 mM. After incubation with stirring for 16 h at room temperature in the dark, a 1.2 molar excess (relative to β-mercaptoethanol) of iodoacetic acid was added, and the pH of the solution was maintained at 8.6 with 2 M NaOH. After incubation for 5 h in the dark, the CNBr peptides were dialyzed (3500 molecular weight cutoff) extensively against 10 mM Tris-HCl, pH 8.0, and the heparin-binding peptides were purified as described below.

*Isolation of Heparin-Binding CNBr Peptides.* The CNBr peptides in 10 mM Tris-HCl, pH 8.0, were adjusted to 6 M urea by the addition of solid urea. The CNBr peptides (5 mg/mL) were applied to a column (2.6 × 40 cm) of DEAE-Sephacel equilibrated with 10 mM Tris-HCl, pH 8.0, and 6 M urea (buffer A). After the sample entered the column, the resin was washed with buffer A until the absorbance at 280 nm was <0.05. The column was then eluted sequentially with buffer A containing 0.15 M NaCl followed by 2 M NaCl. Chromatography was performed at room temperature with a flow rate of 20 mL/h. Fractions were monitored by the absorbance at 280 nm.

The unbound fraction from the DEAE-Sephacel column was dialyzed (3500 molecular weight cutoff) extensively against 10 mM ammonium bicarbonate and lyophilized. The peptides were dissolved in 30 mM HEPES, pH 8.0, and 6 M urea (buffer B) to give 0.5 mg of protein/mL and incubated at 30 °C for 1 h with continuous stirring. After filtration through a 0.22-μm filter (Magna Nylon 66 Fisher) and degassing, the peptide solution (20 mL, 2.0 mg/mL) was pumped onto a column (1 × 10 cm) of Mono S connected to a FPLC system (Pharmacia) at room temperature. The column was washed with buffer B until the absorbance at 280 nm decreased to <0.01, and then the peptides were eluted with buffer B containing 0.2 M NaCl. The flow rate was 2 mL/h, and 3-mL fractions were collected. Fractions were assessed for heparin binding as described below. The peptides that eluted with 0.2 M NaCl were pooled, dialyzed (3500 molecular weight cutoff) extensively against 10 mM ammonium bicarbonate, and lyophilized.

High reactive heparin (HRH) was isolated from crude bovine lung heparin as described previously (Cardin et al., 1984a,d). An affinity column of HRH coupled to AffiGel-10 was prepared as follows. HRH (100 mg of uronic acid) was dissolved in 20 mL of 0.1 M 3-(*N*-morpholino)propanesulfonic acid, pH 7.5, and 80 mM $CaCl_2$ and added to 25 mL of AffiGel-10. After incubation overnight at 4 °C with continuous rocking, the remaining reactive sites of AffiGel-10 were blocked with the addition of 2.5 mL of 1 M ethanolamine. HRH AffiGel-10 was washed with 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 1 mM EDTA, and 0.01% sodium azide and then equilibrated in 80 mM HEPES, pH 7.4, and 2 M urea (buffer C). The Mono S bound fraction was dissolved in 80 mM HEPES, pH 7.4, and 6 M urea to yield a protein concentration of 2 mg/mL. After incubation at 37 °C for 1 h, the solution was diluted with 80 mM HEPES, pH 7.4, to give a final urea concentration of 2 M. The sample was applied immediately to HRH AffiGel-10, and the column was washed with buffer C. The heparin-binding peptides were eluted with buffer C containing 1 M NaCl. The column was then washed with 80 mM HEPES, pH 7.4, and 0.1% SDS. The flow rate was 6 mL/h.

The bound fraction from HRH AffiGel-10 was pumped directly onto a 4.6 × 250 mm analytical C-18 (5-μm particle size/300-Å pore size) reverse-phase VYDAC protein–peptide column (The Separations Group) by use of a solvent-select valve connected to the solvent "A" pump. A Waters HPLC system was used. A flow rate of 1 mL/min of solvent "A" was allowed to flow through the system until the absorbance due to nonretained components (salts, urea) returned to base line. The heparin-binding peptides were then eluted with the ternary gradient program shown in the inset of Figure 7.

*Heparin-Binding Assays.* For radiolabeling, HRH was first coupled with fluoresceinamine (Glabe et al., 1983) and then iodinated with Iodo-Gen (Pierce) as described by Smith and Knauer (1987); $^{125}I$-HRH was reisolated on LDL AffiGel-10. For ligand blotting after SDS–PAGE on 3–20% polyacrylamide gels, proteins were electrophoretically transferred to nitrocellulose paper in 25 mM Tris-HCl, pH 8.6, 192 mM glycine, and 20% methanol at 4 °C (Cardin et al., 1984d).

Table I: Isolation of Heparin-Binding CNBr Peptides of ApoB-100

| purification step | protein (mg) | heparin-binding act. (μg of uronic acid/ mg of protein) |
|---|---|---|
| LDL | 1400 | 2.0 |
| CNBr peptides | 1250 | 4.4 |
| DEAE-Sephacel | | |
| unbound peptides | 100 | 17.6 |
| bound peptides | 750 | <1.0 |
| Mono S bound peptides | 30 | 23.0 |
| HRH AffiGel-10 bound peptides | 10 | 22.0 |

Optimal time for electrotransfer was 10 h at a constant current of 200 mA. After being blotted, the paper was washed with 50 mM HEPES, pH 7.4, 100 mM NaCl, and 2 mM $CaCl_2$ (incubation buffer); the buffer was changed several times, and nitrocellulose paper was incubated overnight with $^{125}$I-HRH (20 dpm/ng of uronic acid) in the incubation buffer. The paper was then washed 4 times (1 min each) with incubation buffer, and the nitrocellulose was air-dried on filter paper. The paper was placed in an X-ray cassette (Cronex HI-PLUS, Du Pont), overlaid with X-ray film (XAR-2, Kodak), and stored at −70 °C before development. For the heparin dot-blot assay, samples were spotted onto nitrocellulose paper in a Bio-Dot apparatus as described previously (Hirose et al., 1986). Each spot was then washed 3 times with 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl. The paper was subjected to the same procedure as the ligand-blotting assay. To quantitate heparin-binding activity after film development, each spot was excised, and radioactivities were determined with a γ counter.

*Amino Acid Sequence Analysis.* Automated Edman degradations were performed on a Model 470A protein–peptide sequencer (Applied Biosystems, Inc.) with reagents, instructions, and standard programs supplied by the manufacturer. The phenylthiohydantoin (PTH)-derivatized amino acids were analyzed at each cycle on a Model 120 PTH analyzer (Applied Biosystems, Inc.) directly on-line with the sequencer.

*Other Methods.* Analytical polyacrylamide gel electrophoresis in SDS and urea was performed as described previously (Cardin et al., 1982). In some experiments, peptides (nonalkylated) were reacted with the fluorescent dye *N*-dansylaziridine (Scouten et al., 1974) and electrophoresed on preparative gels (Cardin et al., 1986b), and the peptide bands were visualized under UV light. The peptides were then electroeluted from the gel into 0.05 M *N*-ethylmorpholine acetate, pH 8.5, as described by Bhown and Bennett (1983), in preparation for sequence analysis. Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. Uronic acid was determined by the method of Bitter and Muir (1962).

## Results

*Isolation of Heparin-Binding Peptides.* To isolate heparin-binding peptides from apoB, LDL were fragmented at methionine residues with CNBr followed by delipidation and reduction–carboxymethylation. The CNBr peptides were then fractionated by ion-exchange, heparin affinity, and high-performance liquid chromatography. A summary of the purification scheme is shown in Figure 1. Table I gives the recovery of protein and the heparin-binding specific activity at each step of the purification procedure.

A number of preliminary experiments were required to maximize peptide yields. The delipidation of LDL prior to digestion with CNBr resulted in incomplete cleavage and aggregation of the peptides. The initial step in the purification procedure was chromatography of the total CNBr peptides

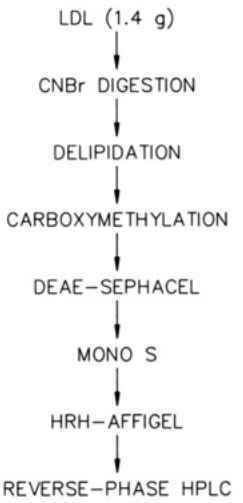


FIGURE 1: Purification scheme for the isolation of the heparin-binding domains of apoB-100.

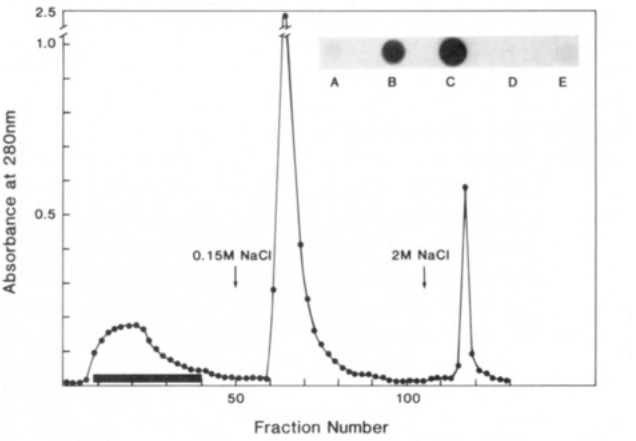


FIGURE 2: Chromatography on DEAE-Sephacel of the CNBr peptides of reduced–carboxymethylated apoB-100. LDL (1.4 g of protein) were fragmented with CNBr, delipidated, reduced–carboxymethylated, and fractionated on DEAE-Sephacel as described under Materials and Methods. The unbound fraction was pooled (solid bar), dialyzed against 10 mM ammonium bicarbonate, and lyophilized. The inset shows the radioautograph of a dot-blot assay for heparin-binding activity. Peptides (20 μg) were applied to nitrocellulose and incubated with $^{125}$I-HRH ($10^5$ dpm, 20 dpm/ng of uronic acid) as described under Materials and Methods. (A) LDL; (B) CNBr-digested LDL; (C) DEAE-Sephacel unbound fractions; (D) peptides eluting with 0.15 M NaCl; (E) peptides eluting with 2 M NaCl.

on DEAE-Sephacel in the presence of 6 M urea (Figure 2). Since it was previously shown (Iverius, 1972; Mahley et al., 1979; Vijayagopal et al., 1981; Noel et al., 1981) that chemical modification of lysine and arginine residues of LDL abolishes heparin-binding activity, we assumed that the domain(s) had a basic characteristic and would not bind to an anion-exchange resin. Chromatography on DEAE-Sephacel also permitted the use of 6 M urea, an absolute necessity for complete solubilization of the peptides. Approximately 7% of the peptides applied to DEAE-Sephacel were unretained by the resin. Figure 2 (inset) shows that only the unbound fraction from DEAE-Sephacel had heparin-binding activity. The unbound fraction was next applied to a cation-exchange (Mono S) resin equilibrated with 6 M urea. Figure 3 shows the elution profile and heparin-binding activity of each fraction. Approximately 30% of the peptides applied to the column were retained by the cation-exchange column; heparin-binding activity was present in the bound fraction.

The Mono S bound fraction was next applied to a column of HRH AffiGel-10; the HRH utilized was purified against

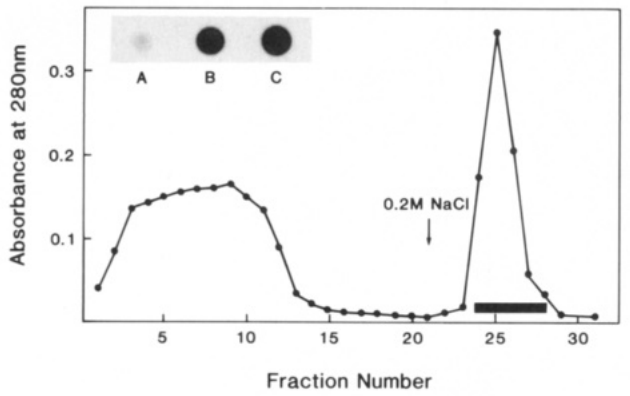


FIGURE 3: Chromatography of DEAE-Sephacel unbound fraction on Mono S. The lyophilized unbound fraction (50 mg) shown in Figure 2 was dissolved in 30 mM HEPES, pH 8.0, and 6 M urea and fractionated on Mono S as described under Materials and Methods. The peptides that eluted with 0.2 M NaCl were pooled (solid bar), dialyzed, and lyophilized. The inset shows the radioautography of a dot-blot assay for heparin-binding activity: (A) unbound fraction; (B) fraction 25; (C) fraction 28. Experimental conditions for the assay were the same as in Figure 2.

the surface determinants of LDL. The purpose of using HRH as opposed to total crude heparin was to isolate only those heparin-binding peptides that were exposed on LDL. Preliminary experiments also indicated that nonspecific binding of the CNBr peptides was less when the affinity column was prepared with LDL-specific heparin. Since the lyophilized Mono S bound fraction was difficult to solubilize in 2 M urea, the peptides were first dissolved in 6 M urea and then diluted just prior to application of the sample to the affinity column. The peptides that bound to HRH AffiGel-10 were eluted with 1 M NaCl (Figure 4, upper panel); elution with a gradient of NaCl did not improve the separation of the heparin-binding peptides and resulted in lower yields. Approximately 40% of the peptides applied to the affinity column were eluted with NaCl. A small amount (<10%) of the peptides was recovered with 0.1% SDS. As is shown in Figure 4 (inset), heparin-binding activity was present mainly in the NaCl-eluted fraction. The small amount of heparin-binding peptides that did not bind on the initial chromatography was recovered by recycling the unbound fractions through the same column (Figure 4, lower panel).

*Characterization of Heparin-Binding Peptides.* SDS–PAGE of the CNBr peptides at each step in the purification procedure is shown in Figure 5. The molecular weight distribution of CNBr peptides both before and after delipidation was nearly identical (lanes A and B), suggesting that no peptides were lost during the delipidation step. On the basis of the amino acid sequence of the mature apoprotein (Chen et al., 1986), 79 CNBr peptides are expected. However, the SDS–PAGE profile indicates about 25 peptides. The fewer peptides found than anticipated is partly explained by their molecular weights. Approximately 34 of the predicted peptides are less than 3K and are likely not observed on the gel. The remaining peptides have similar molecular weights, the majority being between 14K and 20K. Therefore, these peptides will not all be resolved. The peptide(s) that bound to HRH AffiGel-10 consisted of a major component(s) of 20K (Figure 5, lane F). When various peptide fractions were analyzed by ligand-blot analysis, one major peptide of 20K accounted for the binding of $^{125}$I-HRH as visualized on radioautographs (Figure 6). However, amino-terminal sequence analysis of this peptide band that was electroeluted from gels of different preparations showed multiple sequences suggesting several comigrating peptides. To further fractionate these peptides,

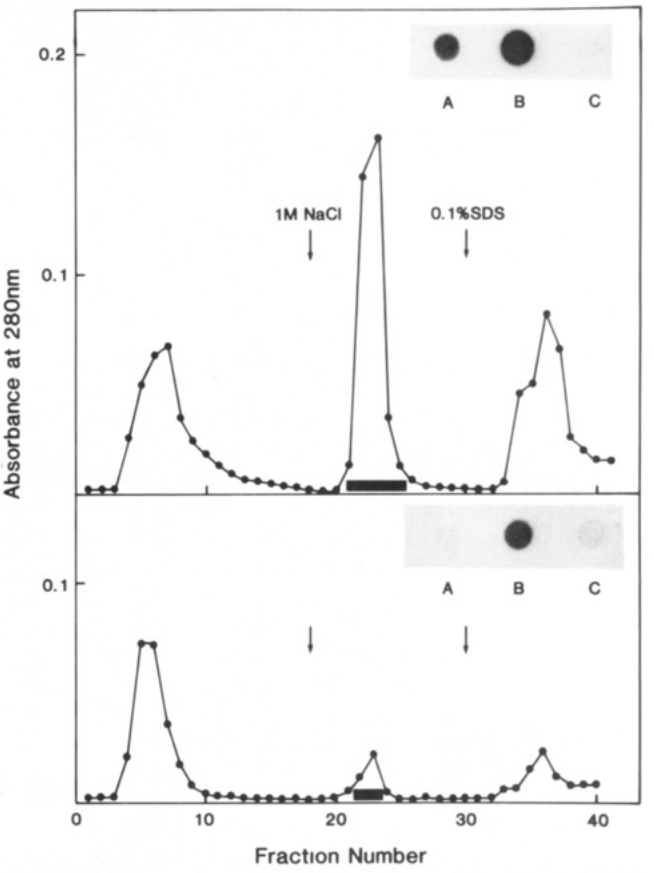


FIGURE 4: Chromatography of Mono S bound fraction on HRH AffiGel-10. (Upper panel) Peptides (15 mg) that eluted from Mono S with 0.2 M NaCl (Figure 3) were applied to HRH AffiGel-10 as described under Materials and Methods. The peptides were eluted with 80 mM HEPES, pH 7.4, and 2 M urea containing 1 M NaCl and then with 80 mM HEPES, pH 7.48 and 0.1% SDS. (Lower panel) Rechromatography of the HRH AffiGel-10 unbound fraction. The insets show the radioautographs of dot-blot assays for heparin-binding activity, (A) Unbound fractions; (B) peptides eluting with 1 M NaCl; (C) peptides eluting with 0.1% SDS.

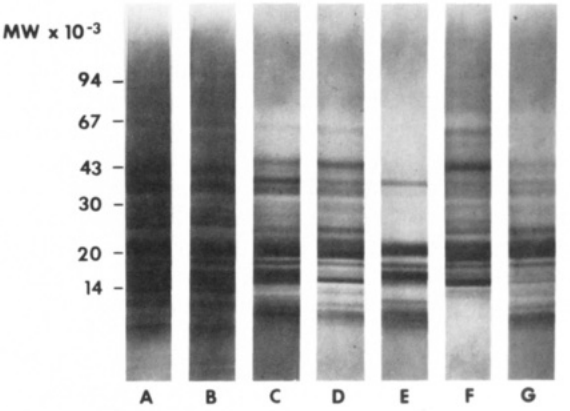


FIGURE 5: Polyacrylamide gel electrophoresis of CNBr peptides of apoB-100. (A) CNBr peptides (100 μg) of LDL; (B) CNBr peptides (100 μg) of delipidated LDL; (C) DEAE-Sephacel unbound CNBr peptides (100 μg); (D) Mono S bound CNBr peptides (50 μg); (E) Mono S unbound CNBr peptides (50 μg); (F) HRH AffiGel-10 bound CNBr peptides (50 μg); (G) HRH AffiGel-10 unbound CNBr peptides (50 μg).

the HRH-bound fraction was next applied to a C-18 reverse-phase column (Figure 7). The HPLC elution profile gave a complicated pattern that is partially explained by the two forms of methionine (Pucci et al., 1985). Each fraction showed heparin-binding activity (Figure 7, bottom inset) and similar molecular weights as determined by SDS–PAGE

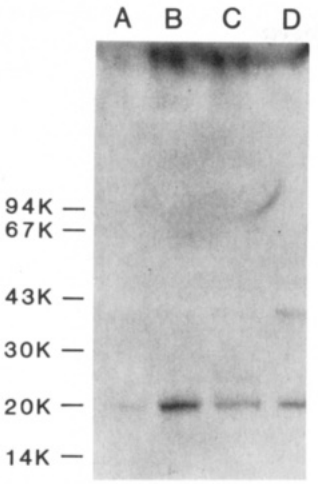


FIGURE 6: Visualization of heparin-binding peptides by ligand-blot analysis. Peptides were subjected to SDS–PAGE on 3–20% polyacrylamide gels and then electrophoretically transferred to nitrocellulose as described under Materials and Methods. The blot was incubated with $^{125}$I-HRH and developed by radioautography. (A) CNBr peptides (100 $\mu$g) of delipidated LDL; (B) DEAE-Sephacel unbound CNBr peptides (50 $\mu$g); (C) Mono S bound CNBr peptides (20 $\mu$g); (D) HRH AffiGel-10 bound CNBr peptides (20 $\mu$g).

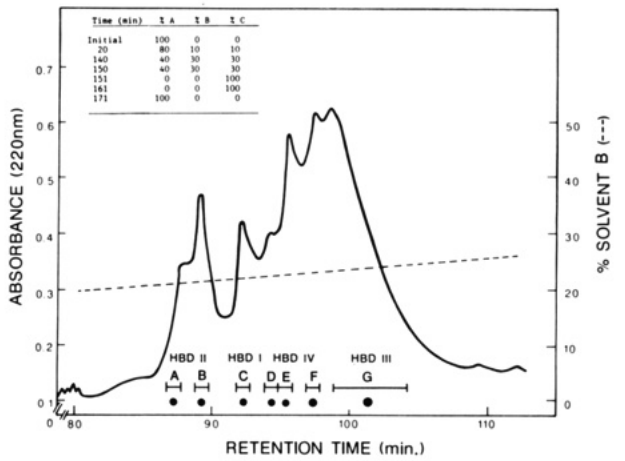


FIGURE 7: Chromatography of HRH AffiGel-10 bound peptides on HPLC. Peptides obtained from HRH AffiGel-10 (5 mg) in 80 mM HEPES, pH 7.4, 2 M urea, and 1 M NaCl were pumped directly onto a VYDAC C-18 reverse-phase column. Peptides were eluted at 27 °C by a linear ternary gradient consisting of 0.1% TFA/$H_2O$ (solvent A), 0.1% TFA/methanol (solvent B), and 0.1% TFA/propanol (solvent C; see top inset for gradient program). Fractions A–G were pooled as indicated and assessed for heparin-binding activity by dot-blot analysis (bottom inset).

(Figure 8) but different amino-terminal sequences indicating unique peptides of similar size. As shown below, sequence analysis revealed four major peptides and several minor ones resulting from incomplete cleavage at methionine by CNBr. Initially, each fraction of the HPLC was subjected to amino acid sequence analysis. Those fractions yielding a major sequence were rechromatographed by HPLC as indicated in Figure 7 to give a purified peptide for final sequence analysis.

Table II gives the amino-terminal sequence of the first 20 amino acids of 4 major CNBr heparin-binding peptides. The sequence determination for each peptide was performed several times on at least three different preparations. One additional peptide of overlapping sequences from surrounding fragments was found in low yield and results from incomplete cleavage of a Met–Arg bond at the amino-terminal end of CNBr-II (Chen et al., 1986). The amino-terminal sequences of CNBr-I–IV align with the predicted sequence of methionine-containing peptides of the cDNA and the protein sequence of

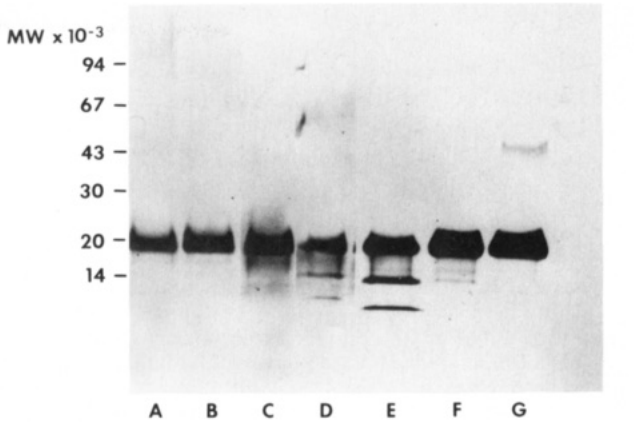


FIGURE 8: Polyacrylamide gel electrophoresis of heparin-binding CNBr peptides of apoB-100 isolated by HPLC. Fractions A–G (Figure 7) were subjected to SDS–PAGE.

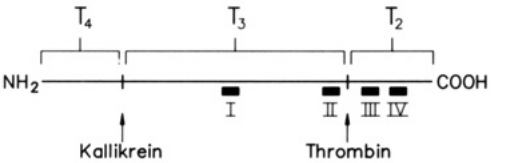


FIGURE 9: Sequence organization of CNBr-I–IV in the primary structure of apoB-100. The major sites of kallikrein and thrombin cleavage are shown. These cleavages generate three major domain structures of apoB-100 termed fragments $T_2$, $T_3$, and $T_4$. For further details, see Cardin et al. (1984c, 1986b).

Table II: Amino-Terminal Sequences of Four Heparin-Binding CNBr Peptides of ApoB-100

| cycle | CNBr-I | CNBr-II | CNBr-III | CNBr-IV |
|---|---|---|---|---|
| 1 | R (2016)[a] | R (3109) | G (3308) | S (3570) |
| 2 | D | L | *c* | A |
| 3 | A | P | I | L |
| 4 | V | Y | T | V |
| 5 | E | T | Y | Q |
| 6 | K | I | D | V |
| 7 | P | I | F | H |
| 8 | Q | T | S | A |
| 9 | E | T | F | S |
| 10 | F | P | K | Q |
| 11 | T | P | S | P |
| 12 | I | L | S | S |
| 13 | V | K | V | S |
| 14 | A | D | I | F |
| 15 | F | F | T | H |
| 16 | V | S | L | D |
| 17 | K | L | N | F |
| 18 | Y | *b* | T | P |
| 19 | D | E | N | D |
| 20 | K | K | A | L |

[a] The numbers in parentheses correspond to the amino acid sequence of Chen et al. (1986). [b] Trp residue in the cloned sequence of Knott et al. (1985) and Chen et al. (1986). [c] Asn with putative carbohydrate attachment in the cloned sequence of Knott et al. (1985) and Chen et al. (1986).

apoB-100 reported by Chen et al. (1986).

## DISCUSSION

*Localization of ApoB-100 Heparin-Binding Domains.* Four unique CNBr heparin-binding peptides were isolated, and their locations in apoB-100 were determined by comparing their amino-terminal sequences (see Table II) with the total sequence for human apoB-100 (Chen et al., 1986). Figure 9 shows the locations of these peptides relative to the points of apoB-100 cleavage in LDL by kallikrein and thrombin (Cardin et al., 1984c, 1986b). CNBr-I corresponds to residues

| PEPTIDE DOMAIN | SEQUENCE | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HBD $I_{a\ 2081-2088}$ | Val | Arg | Lys | Tyr | Arg | Ala | Ala | Leu |
| HBD $I_{b\ 2119-2126}$ | Thr | Lys | Lys | Tyr | Arg | Ile | Thr | Glu |
| HBD II $_{3150-3157}$ | Tyr | Lys | Lys | Asn | Lys | His | Arg | His |
| HBD III $_{3361-3368}$ | Thr | Arg | Lys | Arg | Gly | Leu | Lys | Leu |
| HBD IV $_{3670-3677}$ | Gly | Arg | Arg | Gln | His | Leu | Arg | Val |
| $ApoE_{144-151}$ | Leu | Arg | Lys | Arg | Leu | Leu | Arg | Asp |

FIGURE 10: Proposed heparin-binding domains of CNBr-I–IV of apoB-100. The numbering of amino acid residues for the apoB peptides is based on the cloned cDNA sequence reported by Chen et al. (1986). The heparin-binding region of apoE (Cardin et al., 1986a; Weisgraber et al., 1986) is shown for comparison.

2016–2151 of mature apoB-100 and is positioned near the middle of domain $T_3$. CNBr-II and -III correspond to residues 3109–3240 and 3308–3394, respectively. These peptides, residing in domains $T_3$ and $T_2$, immediately flank the major site of thrombin cleavage. CNBr-IV is defined by residues 3570–3719 and is located in $T_2$, carboxy terminal to CNBr-III. As can be seen, CNBr peptides II, III, and IV are clustered about the $T_3/T_2$ region. On the basis of several lines of evidence, we suggest that these heparin-binding domains are exposed on the LDL surface. Chen et al. (1986) have suggested that the region 3050–3450, which includes CNBr-II and -III, is N-glycosylated and may represent surface antigenic determinants. Hydropathy calculations (Chen et al., 1986) predict that these regions contain extensive hydrophilic domains. Forgez et al. (1986) sequenced seven apoB-100 peptides liberated from the LDL surface by trypsin. These peptides were localized near the thrombin cleavage site on LDL, showing the surface accessibility of the $T_2$ and $T_3$ regions. Two of the tryptic peptides (2091–2106 and 3218–3242) are in CNBr-I and -II, and two others (3265–3275 and 3828–3841) are 30–100 residues from CNBr-III and -IV; only one tryptic peptide was liberated from the amino terminus. These findings are further consistent with CNBr-I–IV binding to HRH, the heparin fraction specific for the surface determinants of LDL. Finally, as described below, HBD-II, -III, and -IV closely resemble heparin-binding sequences of other proteins.

*Heparin-Binding Domains.* The isolated peptides (CNBr-I–IV) contain domains, designated HBD-I–IV, of basic amino acid residues that we suggest account for their heparin-binding activity (see Figure 10). CNBr-I contains two short sequences (Arg-Lys-Tyr-Arg and Lys-Lys-Tyr-Arg) which potentially bind heparin. HBD-II and -IV are strikingly similar in their distribution of basic residues about an Asn (HBD-II) or Gln (HBD-IV) residue. These sequences are almost identical in nature to the GAG binding domain (residues 347–353) of human vitronectin (Suzuki et al., 1985), i.e., Lys-Lys-*Gln*-Arg-Phe-Arg-His.

HBD-III shows high sequence homology with residues 144–151 of apoE. The minimal heparin-binding sequence of apoE resides within residues 142–169 (Cardin et al., 1986a; Weisgraber et al., 1986); removal of residues 144–147 (Leu-Arg-Lys-Arg) from synthetic peptide 144–169 abolishes heparin-binding activity (Cardin et al., 1986a). In addition, residues 140–150 of apoE contain the receptor-binding domain (Innerarity et al., 1983; Weisgraber et al., 1983). Knott et al. (1985) suggested that the homology of apoE residues 140–150 with apoB-100 residues 276–286 of their cloned sequence [i.e., residues 3357–3367 of the Chen et al. (1986) sequence and our HBD-III] indicated a potential function of this region in heparin and receptor binding. If HBD-III also functions as the receptor-binding domain, then the binding of heparin to HBD-III might account for the ability of heparin to displace LDL from its membrane receptor (Goldstein et al., 1976). However, it must also be considered that the binding of heparin to other sites such as HBD-II–IV may induce the release of LDL from its receptor.

The importance of basic amino acid residues in heparin binding is supported by reports that chemical modification of the lysine or arginine residues in apoB or apoE prevents complex formation (Iverius, 1972; Mahley et al., 1979; Vijayagopal et al., 1981; Noel et al., 1981; Cardin et al., 1987). In addition, we have verified that a synthetic peptide containing the HBD-III domain avidly binds HRH (data not shown). Together, these findings support the notion that HBD-I–IV are primarily responsible for the observed heparin-binding activity of the isolated peptides.

The five heparin-binding domains identified in the present study are consistent with direct binding studies indicating five to seven sites for heparin contact on the LDL surface (Cardin et al., 1987). However, we can not exclude the possibility that additional heparin-binding peptides are present in apoB-100. Inspection of the complete sequence of apoB-100 (Chen et al., 1986) reveals a limited number of domains containing clustered basic residues that potentially bind heparin. With the exception of CNBr-I–IV, many of these domains are either flanked by or contain acidic residues which may cause heparin to bind with lower affinity, i.e., millimolar, and therefore were not detected in our assay. Moreover, it is possible that conformational or discontinuous heparin-binding domains (Barlow et al., 1986) formed by the native folding of the protein are destroyed by CNBr cleavage. Hospattankar et al. (1986) postulated 12 consensus sequences in the primary structure of apoB-100 for receptor and heparin binding. Only 2 of these 12 sequences correspond to peptides purified in this study, i.e., HBD-II and -III. Presently, what constitutes a heparin-binding domain in terms of a consensus structure is not well understood. Protter et al. (1986) suggested that residues 14–24 (Lys-Asp-Ala-Thr-Arg-Phe-Lys-His-Leu-Arg-Lys) of the amino-terminal end of apoB-100 might be a heparin-binding region. Although we did not isolate this peptide, we have found (unpublished results) that fragment $T_4$ (in addition to $T_2$ and $T_3$) binds $^{125}$I-HRH on ligand blots, indicating one or more heparin-binding domains in the amino-terminal B-26 fragment of apoB-100 (Cardin et al., 1984c, 1986b; Protter et al., 1986; Hardman et al., 1986).

*Relationship between Heparin- and Receptor-Binding Domains.* Several lines of evidence suggest a structural relationship between the binding domains of apoB and apoE for heparin and cell membrane receptors. (1) Heparin releases the lipoprotein from the fibroblasts' LDL receptor (Goldstein et al., 1976); (2) chemical modification of lysine and arginine residues abolishes both heparin and receptor binding (Mahley et al., 1979); (3) the presumed ligand-binding region of the LDL receptor (Yamamoto et al., 1984) contains negatively charged amino acids that may be considered similar to heparin from the standpoint of negative charge, the LDL-specific heparin being highly sulfated (unpublished observations); (4) $Ca^{2+}$ is required for receptor binding (Kovanen et al., 1979) and for the precipitation of heparin–LDL complexes (Burnstein & Scholnick, 1973); (5) the receptor-binding sequence of apoE is similar to that of HBD-III.

Although the above findings suggest similarities, recent reports indicate that the heparin- and receptor-binding domains

may be different. (1) Tikkanen and Schonfeld (1985) and Milne and Marcel (1982) reported that the binding of one monoclonal antibody to LDL blocks its binding to fibroblasts, suggesting that one epitope in the protein recognizes the receptor. The amount of LDL–monoclonal complex was quantitated by precipitation with heparin in the presence of $Mn^{2+}$ (Tikkanen & Schonfeld, 1985), indicating that a domain(s) other than the receptor-binding domain can also bind heparin. (2) Recent physical–chemical studies indicate multiple heparin contact regions on the LDL surface (Cardin et al., 1987). (3) Cardin et al. (1986c) have shown that trypsin treatment of LDL reduces receptor binding with little effect on heparin binding. (4) Moreover, reductive methylation of lysine residues on LDL, which conserves the positive charge, inhibits receptor binding but not heparin binding (Mahley et al., 1979). These studies support the hypothesis of heparin-binding sites in addition to the receptor-binding domain(s) on LDL.

*Relationship between Heparin Binding and Atherosclerosis.* The significance of heparin–LDL vs. receptor–LDL interactions in the accumulation of lipids and lipoproteins in the arterial wall is speculative. It is known that apoB is present in atherosclerotic tissue (Hoff et al., 1975) and that lipoproteins with properties nearly identical with plasma LDL have been isolated from human lesions (Hoff et al., 1979). Carew et al. (1984) have shown that approximately half of the LDL present in rabbit aorta is metabolized by a receptor-mediated mechanism. However, the total flux of LDL into arterial tissue appears to be receptor independent (Wiklund et al., 1985; Stender & Zilversmit, 1981). One possible explanation for the accumulation of LDL in atherosclerotic tissue is that the lipoprotein is trapped by its binding to the extracellular matrix, such as glycosaminoglycans, proteoglycans, or collagen, forming insoluble complexes in the presence of $Ca^{2+}$ (Hollander, 1976). It has also been suggested that a particular subpopulation(s) of LDL may be a more atherogenic lipoprotein (Rudel et al., 1986). Patton et al. (1983) detected a significant increase in a particular antigenic form of apoB present in plasma LDL of patients with coronary artery disease. Polymorphisms in the protein and DNA structure of apoB-100 have been detected by several investigators (Schumaker et al., 1984; Young et al., 1986; Tikkanen et al., 1986; Chan et al., 1985), and in two recent studies, mutations in LDL have been indicated in primary moderate hyperlipoproteinemia and atherosclerosis (Rapacz et al., 1986; Vega & Grundy, 1986). It is possible that certain LDL particles may be defective in receptor binding, possibly as a result of amino acid changes or lipid-induced conformational changes. It is tempting to speculate that particles defective in receptor binding still retain a high affinity for the arterial wall via interactions mediated through their GAG-binding domains. Whether arterial proteoglycans recognize the same heparin-binding domains on LDL as those identified in the present study remains to be determined.

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