Binding of a Synthetic Apolipoprotein B-100 Peptide and Peptide Analogues to Chondroitin 6-Sulfate: Effects of the Lipid Environment†

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ABSTRACT: The association of apolipoprotein B-100 (apoB-100) containing lipoproteins, low-density lipoproteins (LDL), very low density lipoproteins (VLDL) and lipoprotein(a) [Lp(a)] with chondroitin sulfate-rich proteoglycans (CSPG) of the arterial intima appears to contribute significantly to lipoprotein deposition during atherogenesis. Using frontal elution analysis and competition experiments, we have previously suggested that the apoB-100 segment RLTRKRGLK (3359–3367) is a mediator of the association between LDL and arterial CSPG. Here, with direct binding measurements and fluorescence titrations, we evaluated the effect of the lipid environment on the affinity of the above apoB-100 segment for chondrotin 6-sulfate (C6S). We synthesized a secondary model peptide with hydrophobic tails which allowed its binding to lipid vesicles and lipoproteins (VVWRLTRKRGLKVVV). When associated with lipid vesicles, this peptide showed a higher affinity (K_D = 3.9 μM) for C6S than the free peptide (K_D = 18.7 μM). However, the affinity was still lower than that of LDL (K_D = 0.21 μM). The increase in affinity for the peptide after association with lipid vesicles indicates that the secondary structure induced by its association with lipid vesicles is a significant modulator of the affinity for glycosaminoglycans. When bound to LDL and VLDL subfractions, VVWRLTRKRGLKVVV increased the affinity of the lipoproteins for C6S. The results suggest that, with the proper secondary structure induced by the lipid environment, the segment RLTRKRGLK of apoB-100 is an important determinant of the association of LDL and VLDL with glycosaminoglycans but that probably other basic segments contribute to this interaction.

The subendothelial space of the blood vessel wall is composed mainly of collagens, elastin, and proteoglycans (PG). Formation of soluble and insoluble complexes between apoB-containing lipoproteins and sulfated proteoglycans of the arterial intima appears partially responsible for the extracellular lipoprotein accumulation during atherogenesis (Wight, 1989; Berenson et al., 1988; Camejo, 1982; Srinivasan et al., 1986; Frank & Fogelman, 1989). Low-density lipoprotein (LDL) forms soluble complexes with arterial proteoglycans at physiological salt concentration and pH and insoluble aggregates at low salt concentrations (Camejo et al., 1991). In earlier studies we have identified apoB as the component of the lipoprotein that mediates this binding (Camejo et al., 1988) and chondroitin 6-sulfate (C6S) as the major glycosaminoglycan (GAG) component responsible for the interaction (Camejo et al., 1983). The probable binding regions on apoB-100 appear to be peptides containing five positively charged amino acids (arginine or lysine) within a segment of 9–15 amino acids (Hirose et al., 1987; Weisgraber & Rall, 1987). ApoB segment 3359–3367 fits these requirements (Olsson et al., 1991). One weakness of our earlier results using competition experiments with the synthetic apoB-100 segments is that the peptides show affinities in the micromolar range. On the other hand, the dissociation constant for the association of LDL with arterial chondroitin 6-sulfate rich proteoglycans (CSPG) has been reported to be in the nanomolar range (Camejo et al., 1988). This led us to explore whether the affinity of the peptides for C6S could be increased by incorporation into a lipid interface. Previously we had focused our studies on defining the minimal requirements for an apoB segment in water solution interacting with CSPG. ApoB segment 3359–3367 was found to be the shortest and most potent inhibitor of LDL/C6S interaction and is here investigated further. The interstitital glycine within this sequence indicates that an amphiphatic helical conformation is probably not required, since glycine is rarely found in helices (McKnight, 1991). Neither this peptide nor a longer version of it, apoB segment 3359–3377, spontaneously associated with LDL. Using the minimal sequence of a synthetic peptide, we made the peptides lipophilic by introducing hydrophobic tails and including a tryptophan, thus facilitating evaluation of lipid binding.

We also used VLDL subfractions to look into how C6S binding could be induced in lipoproteins of different size and composition by incorporation of new C6S binding sites. Our data suggest that the affinity of apoB segment 3359–3367 for C6S increases when the peptide is incorporated into a lipid membrane. It also suggests that native LDL binding to C6S may be a cooperative process and that apoB segment 3359–3367 is only one (probably the one with the highest affinity) of several binding sites.

MATERIALS AND METHODS

Lipoproteins. Very low density lipoprotein, VLDL (supernatant of 1.019 g/mL) and low-density lipoprotein, LDL (1.019–1.063 g/mL) were isolated with differential ultracentrifugation from fresh human plasma containing 1 mg/mL Na_2EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF), and 2 mM NaN_3, using KBr solutions with the same additions (Havel & Eder, 1955). For the binding experiments, lipoproteins were used that had been stored at 4 °C in KBr and with 1 mg/mL EDTA for up to 14 days.

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Immediately before use, the lipoproteins were equilibrated with the desired solution using PD-10 columns (Pharmacia, Uppsala, Sweden). The LDL was repeatedly checked with nondenaturing electrophoresis using 2/16 PAA agarose–acrylamide gels obtained from Pharmacia, according to Krauss and Burke (1982) with the difference that LDL was prestained with 5% (v/v) of 0.65% (w/v) Sudan black in ethylene glycol. The integrity of the low density lipoprotein apoB-100 was checked in the Phastsystem (Pharmacia, Uppsala, Sweden) using denaturing gradient SDS–polyacrylamide gels from Pharmacia. Neither LDL nor apoB-100 showed signs of aggregation or fragmentation after storage in KBr and EDTA for 14 days. No detectable difference in lipid oxidation was found between freshly prepared LDL and the same LDL stored for 14 days in KBr and EDTA when thiobarbituric acid reaction was used as a measurement of oxidation (Yagi, 1976). In order to check diene formation as another index of oxidation, absorption at 232 nm was measured on 2-propanol extracts of LDL. No difference was found between freshly prepared LDL and the same LDL stored for 14 days in KBr and EDTA. The number of LDL particles was calculated as the molar concentration of apoB-100 from LDL protein determinations assuming that all the protein was apoB-100.

VLDL was subfractionated by cumulative rate centrifugation as described by Lindgren et al. (1972) essentially as applied by Redgrave and Carlson (1979). In this procedure particles with diameters >75 nm (S_f > 400, fraction A), 50–75 nm (S_f 175–400, fraction B), 37–50 nm (S_f 100–175, fraction C), and 20–37 nm (S_f 20–100, fraction D) are floated to the top of the tube. The calculated centrifugal field–time requirements for these flotations at 20 °C were 4.5×10^6, 17.5×10^6, 31.2×10^6, and 152×10^6 g·min. VLDL was adjusted to density 1.10 g/mL by addition of solid KBr and a 4-mL sample was transferred to a cellulose nitrate centrifuge tube. Three milliliters of d 1.065, 3 mL of d 1.02, and 3 mL of d 1.006 g/mL KBr solutions were layered above the sample. Ultracentrifugation was carried out in an SW 40 Ti rotor at 20 °C. In the SW 40 rotor, after correction for acceleration and deceleration and previous runs, centrifugation was for 43 min at 28 300 rpm (fraction A), then for 67 min at 40 000 rpm (fraction B), then for 71 min at 40 000 rpm (fraction C), and finally for 18 h at 37 000 rpm (fraction D). Times are given from switch-on to switch-off of drive power. Maximum acceleration was used, and the brake was not used in a Beckman XL-90 ultracentrifuge (Palo Alto, CA). Each fraction (1 mL) was carefully aspirated from the top of the tube, and salt solution of 1.006 g/mL density was used to refill the tube before the next run, except before the last run, when the tube was refilled with 1.019 g/mL salt solution. The diameter of the lipoprotein particles was determined by direct measurements of negatively stained preparations and electron microscopy. The molecular weights of the lipoprotein particles were calculated from their composition as described in Redgrave and Carlson (1979). The relative content of apoB and apoE in the VLDL subfractions and LDL was estimated by separating the proteins on a 4–15% gradient conventional SDS–polyacrylamide gel, staining for protein with Coomassie brilliant blue, and scanning the gel with an Ultroscan XL densitometer (LKB, Bromma, Sweden), assuming that the staining is proportional to relative protein content. ApoB content is given as the sum of the bands corresponding to apoB-100 and apoB-48.

Peptide Synthesis and Purification. Synthetic apoB segments and model peptides were synthesized by solid-phase procedures with the fluorenylmethoxycarbonyl (FMOC) method (Sheppard, 1986). The peptides contained C6S-binding regions of 9 amino acids (RLTRKRGLK) and 13 amino acids (SVKAQYKKNKHRH) in the center and two hydrophobic tails made of valine and tryptophan. The final sequences were VVWRLTRKRGLKVVV and VVWSVKAQYKKNKHRHVVV, respectively. The tryptophan was included in the sequence to facilitate measurements of the peptide (tryptophan fluorescence). A third nonbinding peptide with hydrophobic tails was also synthesized with the sequence VVWPDFDVDLGTILRVNDESTEVVV. The peptides were synthesized on preloaded FMOC-protected amino acid ester pepsyn KA resin using a Milligen 9050 peptide synthesizer (Bedford, MA) and FMOC-protected amino acid esters purchased from Milligen following a standard synthesis protocol. Peptides were decoupled from the resin by treatment with trifluoroacetic acid (TFA) (Milligen) in the presence of 2% phenol, 2% ethanedithiol, and 2% anisole (Merck, Darmstadt, Germany) as scavengers. In this procedure the arginine-protecting methoxytrimethylbenzenesulfonyl (Mtr) group was removed and its interaction with the indole ring of tryptophan was avoided. After reduction, ether precipitation, and lyophilization, the peptides were dissolved in 60% (v/v) acetonitrile in water and purified by preparative, reverse-phase HPLC on a 25- × 1-cm column packed with LiChrosorb (7 μm) RP-18 (Merck) and eluted with linear water–acetonitrile gradients containing 0.1% (v/v) TFA. The final purity of the peptides was at least 95% as assessed by analytical reverse-phase HPLC on a 12.5- × 0.4-cm C18 column [LiChrospher (5 μm) 100 RP-18, Merck), using the following conditions: solvent system A, 0.1% TFA (v/v) in water; solvent system B, 0.1% TFA (v/v) in 80% (v/v) acetonitrile (Merck) in water; gradient 0–50% in 35 min; flow rate 1.5 mL/min; detection at 214 nm. The sequences of the peptides VVWRLTRKRGLKVVV and VVWPDFDVDLGTILRVNDESTEVVV were confirmed by automated protein sequencing (Edman degradation) using an Applied Biosystems 477A protein sequencer (Foster City, CA).

Phospholipid Vesicle Preparation. Small unilamellar vesicles (SUV) were prepared by ultrasonification as described by New (1990) with egg yolk phosphatidylcholine (type III-E, Sigma, St Louis, MO) in a buffer containing 50 mM NaCl and 5 mM Hepes (Flow Laboratories, Irvine, Scotland), pH 7.2. A MSE Soniprep 150 (MSE Scientific Instruments, Crawley, England) sonifier was used. Gel-filtration chromatography was performed using Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in a 1.6- X 38-cm column with a flow rate of 15 mL/h at room temperature. The column was preequilibrated with 5 mM Hepes, pH 7.2, with 50 mM NaCl. The liposome preparation was applied in a volume of about 4 mL, and 1-mL fractions were collected. Fractions 40–60, corresponding to the elution profile of LDL, were pooled, the density was adjusted to 1.25 g/mL by addition of solid KBr, and the liposome-containing solution was centrifuged at 195000g (SW 40 rotor, no brake) for 2 h. The top 1 mL from each tube was carefully aspirated and collected as the SUV preparation. The phospholipid concentration in the SUV preparations was between 10 and 20 mM. The SUV were kept at 4 °C under nitrogen in the refrigerator for up to 14 days. Just before an experiment was performed, the SUV were equilibrated in 5 mM Hepes, pH 7.2, and 50 mM NaCl containing also 5 mM Ca^{2+} and 2 mM Mg^{2+}. This was to avoid fusion of the SUV upon long-time storage in buffer containing divalent cations (New, 1990). After storage in the refrigerator for 2 weeks under nitrogen in Hepes buffer

without divalent cations, the SUV still eluted within fractions 40–60.

Fluorescence Measurements. Fluorescence was measured with a Shimadzu RF-540 spectrofluorophotometer (Shimadzu, Kyoto, Japan). The excitation wavelength was 280 nm (bandwidth 5 nm), and the emission was read at 340 nm (bandwidth 5 nm).

SUV and LDL Titration Experiments. Peptide interaction with SUV and LDL was studied by monitoring the changes in the tryptophan fluorescence spectra of the peptides upon addition of SUV or LDL essentially according to De Kroon et al. (1990). Small aliquots of a concentrated (10 mM) SUV or (about 2.5 mg/mL protein) LDL suspensions were successively added to a solution of 3 μM peptide. After at least 10 min of equilibration with continuous shaking, a spectrum was recorded and the emission at 340 nm was read. For each amount of lipid added, a corrected tryptophan spectrum was obtained by subtraction of the appropriate vesicles blank. The fluorescence intensity readings were corrected (1) for the vesicle blank (scatter), (2) for the dilution caused by the SUV or LDL addition, and (3) for the inner-filter effect. The latter correction factor was determined in a parallel lipid titration of the free amino acid tryptophan, which does not interact with lipids in aqueous quenching experiments (London, 1986). A dissociation constant could be derived from the fluorescence titration curve according to Surewicz and Epand (1984) and Bashford et al. (1979) with the use of

$$F/F_0 - 1 = [(F/F_0)_b - 1] - \left[\frac{K_D(F/F_0 - 1)n}{m}\right] \quad (1)$$

where K_D is the dissociation constant of the lipid–peptide complex, m is the lipid concentration, and n is the number of lipid molecules constituting one peptide binding site; F/F_0 represents the relative change of fluorescence intensity, and $(F/F_0)_b$ is the maximum relative change of intensity attainable when all peptide is bound. The parameter K_D/n, which is obtained as the slope of a plot of $F/F_0 - 1$ vs $(F/F_0 - 1)/m$, provides a reliable criterion for comparing the lipid affinities; therefore, no further attempts were made to resolve K_D and n separately (Surewicz & Epand, 1984). The method and instrumentation probably gives an overestimation when measuring very small fluorescence differences very close to the resolution limit of the instrument. This is why one data point corresponding to the lowest SUV/peptide ratio was excluded in Figure 2, panel A. To estimate how many molecules of model peptides were incorporated into native LDL and modified LDL, the lipoproteins were incubated with the model peptides in a molar ratio of surface lipid to peptide (R_i) of about 100:1 for 1 h at 37 °C. The samples were passed through a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column, 20 × 1.6 cm, to remove unbound peptide, lipoprotein fractions were collected, and 100-μL aliquots were extracted with 1 mL of cold 2-propanol. The samples were kept at −20 °C overnight and centrifuged at 10 000 rpm for 10 min, and the supernatant was carefully decanted and measured for tryptophan fluorescence. After lipoprotein blank subtraction, the peptide concentration was calculated using model peptides in 2-propanol as standards. The peptide concentrations were correlated to the number of lipoprotein particles calculated from cholesterol determinations in the LDL + peptide gel filtrates.

Binding Studies. Chondroitin sulfate-derivatized agarose beads were prepared as described by Hunter (1987) and Dawes (1988). Chondroitin sulfate, type C, (Seikagaku Kogyo Co., Tokyo, Japan) was coupled to Affi-Gel 10 (Bio-Rad, Richmond, CA) following the instructions of the manufacturers. A blank gel was made in parallel with the C6S Affi-Gel, following the same procedure except that no C6S was added. The amount of C6S attached to a preparation of agarose beads was determined as described by Hunter (1987) to be 9 μg of C6S/mg (dry wt) of gel, using glucuronic acid as standard. A suspension was made containing one-third (volume) C6S Affi-Gel or blank Affi-Gel and two-thirds 5 mM Hepes buffer, pH 7.2, with 5 mM Ca^{2+}, 2 mM Mg^{2+}, and 50 mM NaCl. To run binding isotherms, 200 μL of the suspension was carefully withdrawn and added to plastic tubes. During sampling the gel suspension was continuously stirred. Peptides or SUV or LDL were added in different concentrations and buffer was added to a final total volume of 0.5 mL. The tubes were then shaken for 30 min. The gel was allowed to settle for 30 min and a sample was carefully withdrawn from the supernatant. In experiments involving LDL or VLDL subfractions, cholesterol concentrations in the supernatant was determined. In experiments without lipoproteins, 1 mL of 2-propanol was added to the sample and tryptophan fluorescence was used to calculate the peptide concentration. From the data, Scatchard plots were constructed and dissociation constants were calculated. In experiments with modified (blocked) LDL and VLDL subfractions, 0.05% bovine serum albumin (BSA) was included in the assay to reduce unspecific binding to the agarose beads and the binding experiments were conducted in the same Hepes buffer as above but with 20 mM NaCl instead of 50 mM NaCl. In these experiments another batch of C6S Affi-gel with a slightly different capacity to bind LDL was also used, since we ran out of the first batch. The lower salt concentration was used to increase somewhat the binding in the second gel batch used, thus giving similar binding constants with the two gel preparations. In experiments involving preincubation of lipoproteins or SUV with peptides, the lipoprotein or SUV were incubated with the peptide in a molar ratio of surface lipid to peptide (R_i) of about 100:1 for 1 h at 37 °C. The samples were passed through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column, 20 × 1 cm, to remove unbound peptide prior to use in binding experiments.

Modifications of Lipoproteins. The positive charges on the arginine residues in the lipoproteins were blocked by reaction with 1,2-cyclohexanedione (Mahley et al., 1977). The samples were equilibrated in Hepes buffer using gel-filtration with PD-10 columns and used directly in binding studies or were preincubated with the peptides, passed through a Sephadex G-25 column (20 × 1 cm), and subsequently used for binding experiments.

Conformation Studies. Circular dichroism (CD) spectra were recorded at room temperature using a Jasco J-720 recording spectropolarimeter (Tokyo, Japan); cells with a path length of 1 mm were used. The peptide concentration was 100 μM and the SUV (phosphatidylcholine) concentration was 0.46 mM. The CD measurements were performed in the same buffer that was used in the experiments (5 mM Hepes, 50 mM NaCl, 5 mM Ca^{2+}, and 2 mM Mg^{2+}) and were corrected for buffer absorption and lipid blank.

Analytical Methods. Protein concentration was estimated by the method of Lowry et al. (1951), utilizing the modification of Markwell et al. (1978), which incorporates 1% SDS in both bovine serum albumin standard and samples. Cholesterol was estimated by an enzymatic microassay (Auerbach et al., 1990). Phospholipid, triglyceride and cholesteryl ester, and free cholesterol concentration in Table I was determined using

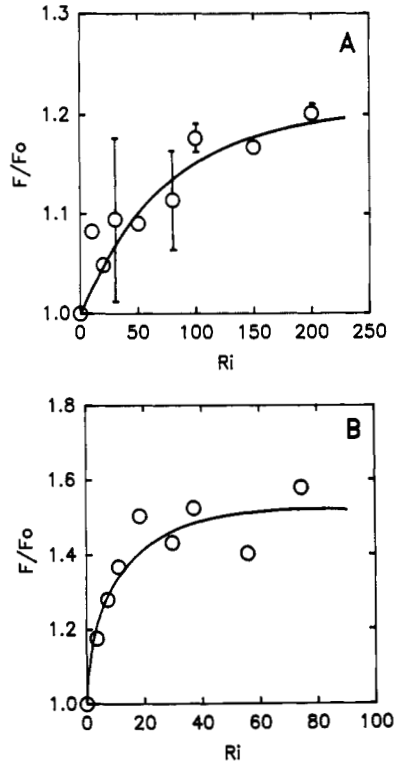

FIGURE 1: Tryptophan fluorescence emission intensities at 340 nm related to the intensity in the absence of lipid (F/F_0) as a function of the molar ratio, R_i, of (panel A) the phosphatidylcholine of SUV to peptide VVWRLTRKRGLKVVV and (panel B) the surface lipids of LDL (free cholesterol and phospholipids) to peptide VVWRLTRKRGLKVVV. The data in panel A are averages of two separate measurements; standard deviation is shown as vertical bars. The data in panel B are from a single measurement.

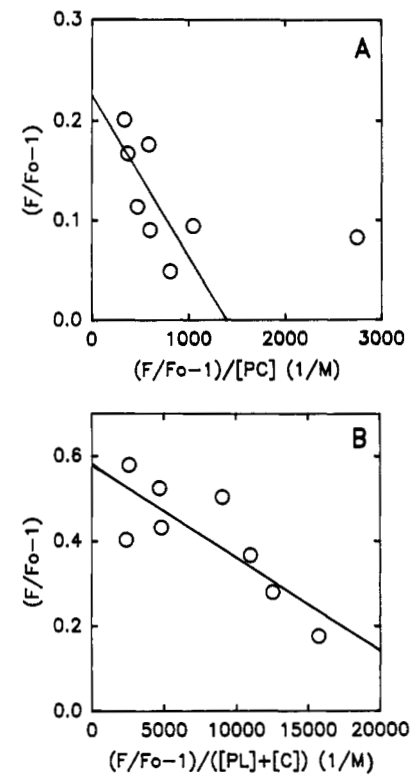

FIGURE 2: Determination of apparent affinity parameters, K_Dn. Plotting the data from Figure 1 in this way gives for panel A K_Dn = 0.15 mM and for panel B K_Dn = 0.022 mM. In panel A are shown average values from Figure 1. One data point, corresponding to the lowest SUV/peptide ratio in Figure 1, was excluded in the calculation of K_Dn.

a combination of thin-layer chromatography and flame ionization detection on an Iatroscan TH-10 MK2 (Newman-Howells, Llandwrtyd Wells, Wales, U.K.) (Ackman, 1981).

RESULTS

We have published data on competition experiments between apoB-100 peptide segments and LDL for arterial chondroitin sulfate-rich proteoglycans (Camejo et al., 1988) and for chondroitin sulfate (Olsson et al., 1991). Here we present data on the effect of incorporating apoB model peptide segments into SUV and LDL particles on the interaction with C6S. In addition we explored the possibility of an arginine-blocked LDL and VLDL regaining their C6S-binding properties by incorporation of the model peptides. The VLDL was subfractionated into density (size) classes and the relation between structural characteristics of the subclasses and their affinity for C6S was also studied. Apolipoprotein B-100 segment 3359–3367, RLTRKRGLK, could not be attached to lipid vesicles (not shown). This is probably because of its high hydrophilicity. Therefore we constructed a model peptide with the apoB-derived peptide in the middle and hydrophobic tails consisting of valine-valine-tryptophan (VVW) in the N-terminal end and valine-valine-valine (VVV) in the C-terminal end. The selection of tryptophan was in order to be able to follow fluorescence from the peptides for evaluation of lipid binding and concentration measurements. Valine was chosen because its hydrophobicity would make the peptides prefer a lipid environment and because valine is ill-suited for "leuzine zipper"-like dimerization of the peptides (Landschulz et al., 1988). The peptide with hydrophobic ends (VVWRLTRKRGLKVVV) appeared to have similar affinity for C6S as the original peptide (RLTRKRGLK). Both of them were eluted at the same NaCl concentration (0.11 M) when the peptide was loaded at low salt concentration (20 mM) and then a linear salt gradient was applied to the same affinity column which contained immobilized C6S (not shown). These experiments were performed in 5 mM Hepes buffer, pH 7.2, containing 5 mM Ca^{2+} and 2 mM Mg^{2+}.

In order to get an indication of the affinity for lipid vesicles, changes in the intrinsic fluorescent properties of tryptophan of the peptides were monitored upon titration with egg yolk phosphatidylcholine SUV or LDL (Figure 1). The peptide VVWRLTRKRGLKVVV had a much greater affinity for LDL (phospholipids and free cholesterol) as compared to the SUV phosphatidylcholine. Quantitative determination of the apparent affinity parameter, K_Dn, is shown in Figure 2.

Effect of Incorporation of the Peptide into SUV and LDL. The C6S-binding segment of apoB with hydrophobic tails remained associated with SUV and LDL even after gel filtration. Scatchard plots (Figure 3)of the soluble peptide or the peptide in association with SUV indicates that (a) binding to lipid interface increases the affinity of the peptide for C6S and (b) the peptide confers C6S-binding capacity to the SUV.

The peptide with hydrophobic tails also bound to LDL. This caused a 2-fold increase in the affinity of the lipoprotein for GAG (K_D = 0.21 μM vs K_D = 0.099 μM, Figure 4). Blocking of the exposed arginine groups of LDL with 1,2-cyclohexanedione almost completely eliminated the binding to C6S (Figure 5). To see if the putative C6S binding sites of the LDL particle could be regained by incorporation of synthetic binding sies, the 1,2-cyclohexanedione-treated LDL was incubated with peptide VVWRLTRKRGLKVVV. This regained the C6S affinity but not the total binding. To test

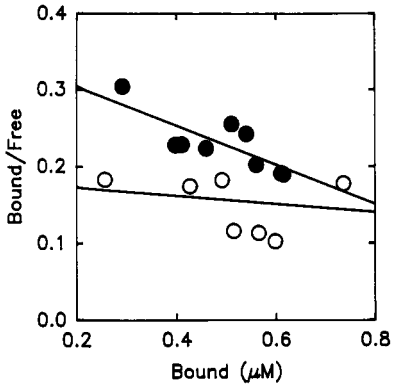

FIGURE 3: Comparison of affinities for C6S between free and membrane-immobilized peptide VVWRLTRKRGLKVVV. Free peptide (open symbols) gives a K_D of 18.7 μM. Peptide immobilized into a SUV membrane (closed symbols) gives a K_D of 3.9 μM. Each value was calculated from the average of measured duplicates.

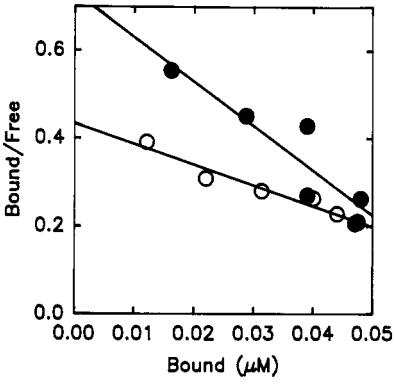

FIGURE 4: Comparison of affinities for C6S between native LDL and LDL that had been preincubated with peptide VVWRLTRKRGLKVVV. Native LDL (open symbols) gives a K_D of 0.21 μM. The preincubated LDL (closed symbols) gives K_D = 0.099 μM. Each value was calculated from the average of measured duplicates.

if the binding could be restored with another putative binding site, 1,2-cyclohexanedione-treated LDL was incubated with the apoB-derived model peptide, VVWSVKAQYKKNKHRHVVV [corresponding to apoB segment 3145–3157, known to interact with the C6S/LDL association (Olsson et al., 1991), plus hydrophobic tails]. Very little C6S binding could be regained this way. As a control we incubated the 1,2-cyclohexanedione-treated LDL with the peptide VVWPDFDVDLGTILRVNDESTEVVV [corresponding to apoB segment 1041–1059, known not to interact with the C6S/LDL association (Olsson et al., 1991), plus hydrophobic tails]. Incubation with this peptide did not change the binding from that of 1,2-cyclohexanedione-treated (blocked) LDL itself (Figure 5). Under the conditions used, native LDL bound about 5 peptide VVWRLTRKRGLKVVV molecules per LDL particle and the 1,2-cyclohexanedione-treated LDL bound 12 peptide VVWRLTRKRGLKVVV molecules per LDL particle, 6 peptide VVWSVKAQYKKNKHRHVVV molecules per LDL particle, and 3 peptide VVWPDFDVDLGTILRVNDESTEVVV molecules per LDL particle. The lack of GAG binding in the blocked LDL preincubated with the peptide corresponding to apoB segment 3145–3157 was unexpected. This peptide 3145–3157 was the second most efficient apoB segment found in interacting with the LDL/C6S association in previous experiments. However, in competition experiments used in earlier studies (Olsson et al., 1991) apoB segment 3145–3157 (IC_{50} = 33.6 μM) was some orders of magnitude less efficient in inhibiting LDL/C6S precipitation as compared to apoB segment 3359–3367 (IC_{50} = 7.9 μM). For these reasons a lower regain of C6S binding could perhaps be expected for the blocked LDL preincubated with the peptide 3145–3157 as compared to that preincubated with apoB segment 3359–3367, and perhaps in this assay the potential increase in binding was too low to overcome the overall increase in negative charge of the lipoprotein particle that should be an effect of blocking the positively charged arginines on the surface of the LDL.

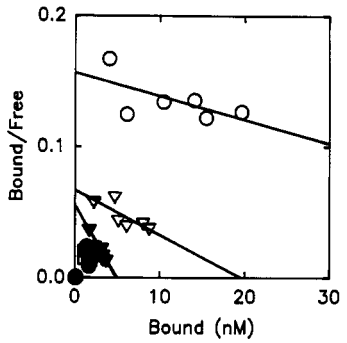

FIGURE 5: Effect of LDL modification. The binding of native LDL (open circles) to C6S (K_D = 0.58 μM) was abolished after treatment with 1,2-cyclohexanedione (closed circles). Incubating 1,2-cyclohexanedione-treated LDL with the peptide VVWRLTRKRGLKVVV (open triangles) restored the C6S affinity (K_D = 0.30 μM) but not the total binding. Analogous treatment and incubation with peptide VVWSVKAQYKKNKHRHVVV (closed triangles) restored C6S affinity and little total binding, whereas peptide VVWPDFDVDLGTILRVNDESTEVVV (open squares) did not regain any binding.

Table I: Composition of VLDL Subfractions and LDL

	VLDL subfraction			
	B	C	D	LDL
triglyceride (%)[a]	66.2	59.0	44.3	9.0
cholesteryl ester (%)	5.0	8.8	19.5	46.1
free cholesterol (%)	3.5	4.3	5.4	7.6
phospholipid (%)	12.8	13.6	16.3	17.0
protein (%)	12.5	14.2	14.5	20.1
% of which apoB	60	69	89	100
% of which apoE	7	5	4	
diameter (nm)	57	34	26	20
molecular weight (g/mol)	58.7×10^6	12.2×10^6	5.3×10^6	2.6×10^6

[a] Percentages are given by weight.

Incorporation of C6S-Binding Peptides into VLDL Subclasses. When VLDL is subfractionated by cumulative rate centrifugation according to Redgrave and Carlson (1979) a cascade of particles, different in lipid composition, was obtained (Table I). We incubated 1,2-cyclohexanedione-blocked VLDL subfractions with peptide VVWRLTRKRGLKVVV and measured their binding to C6S (Figure 6). Fraction A is composed mainly of very large triglyceride-rich chylomicron remnants and was not used. VLDL subfraction B treated with 1,2-cyclohexanedione and incubated with the peptide showed very little total C6S binding. Fractions C and D represent two populations with differing binding parameters. The calculated number (B_t) of modified VLDL subfraction particles that C6S appeared to have capacity to bind was only a few percent (fraction C about 5%, fraction D a little more than 6%) of that of native LDL (89 nM). The affinity of fraction D for C6S was higher than that of fraction C, suggesting that differences in lipid composition and size are of significance for the binding. With 9 μg of C6S/mg (dry wt) of gel, if we estimate that 1 g of gel gives about 3 mL of swollen gel, this gives us with the assay conditions used here (200 μL of gel suspension) about 200 μg of C6S, equal to (molecular weight about 60 000) 3.3 nmol of C6S molecules

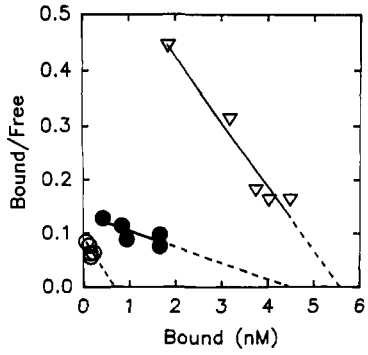

FIGURE 6: Chondroitin 6-sulfate binding of VLDL subfractions B (open circles), C (closed circles), and D (open triangles) after 1,2-cyclohexanedione treatment and incubation with peptide VVWRLTRKRGLKVVV.

accessible for interaction. So roughly 1–2 VLDL subfraction molecules (C and D) are bound per C6S molecule (B_t). The apoE content of the VLDL subfractions should be of no significance here since we pretreat the VLDL subfractions with 1,2-cyclohexanedione that blocks the positive charges of exposed arginines and both the suggested GAG binding segments of apoE contain multiple arginines (Cardin et al., 1991).

Conformation Studies. To investigate the conformation of the model peptide VVWRLTRKRGLKVVV in the presence and absence of lipid, CD recordings were made. The CD spectrum for the peptide VVWRLTRKRGLKVVV plus SUV was similar in the shape of the CD spectrum to that of the peptide alone in the far-UV region (195–260 nm), indicating no major conformation change in the peptide backbone (not shown). Both spectra had a minimum below 200 nm, which is characteristic of random structure. This is consistent with what Ono et al. (1990) found for short basic amphipathic β-structural peptides in aqueous solution and in neutral phospholipid bilayers. Ono et al. (1990) found, however, that these peptides were able to form an amphipathic β-structure in acidic phospholipid bilayer. Therefore, here the situation might be different in the lipoproteins but we have not overcome the methodological difficulties involved in these kinds of studies.

DISCUSSION

The data presented here indicate that it is possible to incorporate a C6S binding site into a lipid interface after modification of the active peptide by inclusion of hydrophobic tails. The peptide appears to remain surface-bound with the active site available for interaction with the glycosaminoglycan. Chondroitin sulfate-derivatized agarose beads, as previously used for studying cation binding (Hunter, 1987) and the affinities of glycosaminoglycans for antithrombin III and thrombin (Dawes, 1988), can also be used for studying binding of LDL to C6S. The system, as used here, depends on having a sensitive assay for measuring the concentration of ligand in the supernatant. However unspecific binding to the agarose beads can be a problem when using a system with a very low total binding (B_t), even if the avidity is high, and therefore such data should be interpreted cautiously.

Apolipoprotein B-100 containing lipoproteins, VLDL and LDL, bind to different sulfated glycosaminoglycans (e.g., heparin, dermatan sulfate, heparan sulfate, and chondroitin sulfate) with varying affinities in the presence of calcium (Iverius, 1972). Glycosaminoglycans, with the exception of hyaluronic acid, exist in the extracellular matrix linked to a core protein and occur also as cell-surface proteoglycans and integral components of basement membranes. We selected isolated C6S as a model to study the interaction with LDL because (1) C6S is the most abundant GAG in the human arterial wall (Camejo et al., 1980), (2) PG–LDL complexes extracted with saline from the aorta of cholesterol-fed rabbits contain C6S as the major glycosaminoglycan component (Srinivasan et al., 1982, 1984), and (3) after a hypercholesterolemic diet, lipoproteins accumulate in porcine aorta at sites where the relative amounts of aortic chondroitin sulfate increases (Hoff & Wagner, 1986). Finally, smooth muscle cell proliferation during atherogenesis is associated with increased PG synthesis and a shift toward increased relative amounts of chondroitin sulfate PG as compared to quiescent smooth muscle cells (Wight, 1989). A practical aspect that was considered is that proteoglycan aggregates obtained from tissue vary in composition. To overcome this added methodological complexity we used in these experiments commercially available, well-defined C6S.

In this study we explore the effect of incorporating properties of the C6S binding regions of apoB-100 (Camejo et al., 1988; Olsson et al., 1991) in a lipid environment. The inclusion of the peptide VVWRLTRKRGLKVVV into SUV particles increased its affinity for C6S approximately 5 times. The reason for this might be that the long, positively charged arginine and lysine groups are oriented toward the polar environment outside the SUV, thus increasing the regional positive charge density, facilitating the association to the negatively charged sulfate groups of C6S. It was also found possible to increase the affinity of native LDL for C6S by including more copies of a C6S binding region of apoB-100 into the surface of the LDL particle. In addition, if the binding of LDL to C6S was first blocked by treatment with 1,2-cyclohexanedione and then new binding sites were induced by incubation with the peptide VVWRLTRKRGLKVVV, the affinity of the modified LDL was partially restored. However, the total binding still was lower than that of native LDL (Figure 5). The total binding of such reconstituted LDL was about one-fourth that of native LDL. Two other model peptides, one with proteoglycan binding properties (3145–3157) and the other a nonbinding region (1041–1059), were also incorporated in cyclohexanedione-treated LDL. LDL with the apoB segment 3145–3157 bound C6S only to a limited degree, whereas LDL with the apoB segment 1041–1059 showed no binding, supporting the conclusion that this peptide is not involved in C6S binding.

In the system studied here, one important factor is the affinity of the model peptides for the lipid vesicles or lipoproteins (Figures 1 and 2). The apparent dissociation constants, K_Dn, reveal that the peptide VVWRLTRKRGLKVVV has an ~7-fold higher affinity for LDL than for the phosphatidylcholine of the SUV (K_Dn = 0.022 vs 0.15 mM). This suggests that for this interaction other factors such as packing and charge of the lipids in the lipoprotein surface determine the avidity in the association as recently demonstrated in a system of basic peptides and phospholipids (Mosior & McLaughlin, 1992).

The secondary structure of longer GAG binding segments of apoB (3345–3381) and apoE (129–167) in its interaction with heparin has been extensively studied (Cardin et al., 1989, 1991) using CD. ApoB segment 3359–3367 is actually included in the larger apoB fragment (3345–3381) studied by Cardin et al. (1989) and is also homologous to part (142–150) of the larger apoE segment (129–167) studied earlier. Cardin et al. (1989) found an increase in α-helix content of synthetic peptide segments of apoE (129–167) and apoB (3345–3381)

after addition of heparin. However, as mentioned in the introduction, the interstitial glycine within apoB segment 3359–3367, RKTRKRGLK, is intriguing and does not suggest α-helix in this short GAG-binding peptide (McKnight, 1991). In the homologous apoE segment 142–150, RKLRKRLLR, the glycine is exchanged for a leucine which will make the peptide much more prone to form helices. Our findings here do not necessary contradict the measurements done by Cardin et al. (1989) as the α-helicity could have been attributed to amino acids outside this GAG-binding region (apoB segment 3359–3367). Perhaps the α-helix is not an absolute requirement for interaction with GAG but rather the linear sequence and charge density. The increased stability of the peptide immobilized in a lipid bilayer certainly limits the degrees of freedom for the peptide and also results in an increased affinity for C6S (Figure 3). In the actual LDL particle the affinity for C6S can be regained after arginine blocking and reconstitution of binding by preincubation with the model peptide VVWRLTRKRGLKVVV (Figure 5). Why the B_t of native LDL was never reached remains unclear. One suggestion is that the peptide distributed unevenly among the LDL particles, leaving part of the charge-blocked LDL particles without any new binding sites. Another possibility is that additional binding sites are required to reach the B_t of native LDL.

To investigate further the effect of different lipid environments on the interaction between a C6S-binding apoB segment and the glycosaminoglycan, synthetic C6S-binding sites (peptide VVWRLTRKRGLKVVV) were incorporated into cyclohexanedione-treated VLDL subfractions. These experiments showed a higher total binding of the lipoproteins, the smaller the particles were (Figure 6, Table I). On the other hand, the C6S affinity constants of the modified VLDL subfractions were greater than those of native LDL but the total binding ranges were considerably smaller. Possible explanations for the increased affinity might be that the modified VLDL subfractions have a high capacity to hold many copies of the synthetic peptide in the membrane, thus increasing the regional binding site concentration on the lipoprotein surface. To explain the decreased total binding it may be relevant to consider that a C6S molecule with a molecular weight of 60 000 stretched out in a linear conformation has a total length of about 200 nm, containing 60–70 tetrasaccharide units [one tetrasaccharide unit possibly corresponds to a lipoprotein binding site (Olsson et al., 1991)]. In solution, or immobilized to agarose beads, the C6S might adopt any kind of tertiary structure like a globular conformation or a gel-like network. In its interaction with this structure it is imaginable that a large particle like the VLDL subfraction B with a diameter of 57 nm might by its own size block several binding sites on the C6S molecule and make them inaccessible for other lipoproteins (lattice model for binding).

The association between native VLDL and C6S is minor as compared to that between LDL and C6S (Camejo et al., 1988). The affinity for proteoglycans and glycosaminoglycans also varies between different LDL preparations and occasionally one LDL batch shows a dramatically greater affinity for C6S than the usual range (unpublished observation). Subpopulations of LDL with higher avidity for chondroitin sulfate PG are smaller and denser and have a lower ratio of surface polar lipid to core nonpolar components as compared to subpopulations with smaller avidity (Hurt-Camejo et al., 1990). This composition by physical necessity probably induces a higher overall exposure of the apoB-100 molecule on the surface of the LDL particle (Hurt-Camejo et al., 1990). Our laboratory has reported differences in affinity for arterial proteoglycans of LDL from different individuals (Camejo et al., 1989). Their differences may be caused by dissimilar content in the LDL range of subpopulations with more or less exposure of the apoB-100 GAG-binding regions.

Subjects with clinical manifestations of accelerated atherosclerosis show more frequently LDL with high affinity for arterial C6S-rich proteoglycans in vitro (Camejo, 1982; Lindén et al., 1989). Also, population and case control studies show that frequently coronary heart disease is associated with the presence of small cholesterol-rich LDL particles (Austin et al., 1988). These studies suggest the possibility of a relation between small size of LDL, increased exposure of GAG-binding apoB segments, and increased deposition in the intima of these particles during atherogenesis.

The results obtained suggest that the environment of the GAG-binding peptide segments controls their contribution to the association with the sulfated polysaccharides. However, the results presented here do not allow us to conclude that the apoB-100 segment 3359–3367 is the only GAG-binding site of VLDL and LDL. Most probably, other segments can act cooperatively in this association (Camejo et al., 1988; Olsson et al., 1991). Furthermore, in VLDL apoE with similar basic regions could contribute to GAG binding of this large particle.

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