Monoclonal antibodies to human apolipoprotein AI: probing the putative receptor binding domain of apolipoprotein AI

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We have used four monoclonal antibodies (MAbs) specific for human apolipoprotein (apo) AI, designated AI-1, AI-3, AI-4.1 and AI-4.2, to study the interaction between high-density lipoprotein HDL₃ and rat liver plasma membranes. MAbs AI-1 and AI-3 recognize epitopes within residues 28–47 and 140–147 respectively of apoAI [Allan, Tetaz and Fidge (1991) *J. Lipid Res.* 32, 595–601]. Two previously unreported MAbs, AI-4.1 and AI-4.2, were raised against purified CNBr fragment 4 (CF4) of apoAI, the C-terminal region. Using e.l.i.s.a. and immunoblotting techniques, we have demonstrated that all four MAbs recognize distinct epitopes within apoAI. Epitope mapping studies using endoproteinase cleavage peptides of CF4 showed that AI-4.1 binds to an epitope within residues 223–233, which is

poorly exposed on apoAI molecules associated with lipid. Fab fragments derived from MAb AI-4.2 inhibited the binding of ¹²⁵I-labelled HDL₃ to rat liver plasma membranes, whereas Fab fragments from AI-4.1, AI-3 and AI-1 had little or no effect. In ligand blotting studies with purified CNBr fragments of apoAI and using apoAI-specific antibodies for detection, CF4 showed the highest capacity to recognize two HDL-binding proteins previously identified in rat liver plasma membranes. We propose that the specific interaction between HDL and liver plasma membranes is largely mediated through a binding domain in the C-terminus of apoAI, which is consistent with the involvement of specific receptors for the apolipoprotein moiety of HDL.

INTRODUCTION

Apolipoprotein AI (apoAI) is the major protein constituent of high-density lipoprotein (HDL) and represents the major apolipoprotein of normal human plasma. The structure and function of apoAI have been studied extensively, particularly with regard to understanding its role in lipid transport and metabolism. Its ability to activate lecithin:cholesterol acyltransferase has been well established [1]. In addition, several studies have also implicated a role for apoAI as a lipoprotein ligand capable of recognizing specific HDL cellular binding sites [2–10].

Human HDL appears to bind to specific sites which are distinct from those of the low-density lipoprotein receptor (apoB/E) and the apoE receptor [10–14], and which are present in a variety of cell types including adipocytes [5,6], endothelial cells [14], macrophages [4,9,15], steroidogenic tissue [7,13,16] and cells of the liver [2,8,11] and intestine [12,16]. Despite these findings, the precise mechanisms responsible for the specific interactions between HDL and cells have not been determined. Many studies have focused on the identity of the HDL₃ moiety which serves as a ligand for cellular binding sites. The use of artificial apolipoprotein/phospholipid complexes in lipoprotein binding studies has implicated the involvement of apoAI in the cellular binding of HDL [2,3,7], while immunological approaches using specific antibodies directed towards different apolipoproteins also suggest that apoAI is a potential HDL ligand [3–5,8]. Furthermore, several laboratories have isolated apoAI and HDL-binding proteins which may serve as the HDL receptor system [17–20].

To gain more information about the mechanisms involved in the cellular recognition of apoAI, we have focused attention on the putative receptor-binding domains of apoAI. In order to probe this binding site in more detail we have prepared four monoclonal antibodies (MAbs) which recognize different regions

of apoAI, and have compared their ability to inhibit the interaction of HDL₃ with rat liver plasma membranes. The present study indicates that the binding of HDL₃ to hepatic membranes is mediated by the C-terminal region of apoAI. In addition, the findings from ligand blotting studies, in which antibodies were used to detect the recognition of isolated apoAI CNBr fragments by HDL-binding proteins derived from rat livers [17,20], support the proposal that a domain in the C-terminal portion of apoAI mediates the binding of HDL to specific receptors [21].

MATERIALS AND METHODS

Isolation of apoAI, apoAII and HDL₃

Human HDL (*d* 1.063–1.210) and HDL₃ (*d* 1.125–1.210) were obtained from human plasma (Red Cross) by ultracentrifugation as previously described [22]. ApoAI and apoAII were isolated from HDL treated with 6 M guanidine/HCl [23] by ultracentrifugation at *d* 1.210 for 20 h at 360 000 *g* and 4 °C. The infranate containing apoAI dissociated from HDL was dialysed against 5 mM ammonium acetate (pH 6.8), lyophilized, dissolved in urea buffer (6 M urea, 0.02 M Tris, pH 8.0) and subjected to chromatography on DEAE-Sephacel (Pharmacia) as described previously [24]. ApoAII was isolated from the supernatant following ethanol/ether (3:1, v/v) delipidation and chromatography on DEAE-Sephacel in 6 M urea as described previously [25]. The purified apolipoproteins, verified by chromatography on SDS/10–15 % polyacrylamide gradient gels (Phastgel system, Pharmacia) stained with Coomassie Brilliant Blue R-250, were stored lyophilized at –20 °C.

CNBr cleavage of apoAI

CNBr fragments were prepared using modifications [24] of the

Abbreviations used: Apo, apolipoprotein; CF, CNBr fragment; DMPC, dimyristoylphosphatidylcholine; HDL, high-density lipoprotein; IEF, isoelectric focusing; MAb, monoclonal antibody; RP-h.p.l.c., reversed-phase h.p.l.c.; TFA, trifluoroacetic acid.

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method described by Baker et al. [26]. Briefly, apoAI was dissolved in 70% (v/v) trifluoroacetic acid (TFA) with a 500-fold molar excess of CNBr. The reaction mixture was incubated for 24 h at 25 °C, in the dark, under N₂. CNBr-treated samples were then lyophilized and redissolved in 6 M urea and 0.05 M sodium citrate, pH 3.8. Digest fragments were separated by gel filtration through a Sephadex G-50 (Pharmacia) column (1.5 cm × 200 cm) equilibrated in the urea buffer. The appropriate fractions were pooled and desalted by reversed-phase (RP)-h.p.l.c. Samples were loaded on to an RP-300 column (20 µm particle size; 10 mm × 100 mm; Brownlee) equilibrated with 0.1% TFA. Fragments were eluted with a linear gradient of 0–60% acetonitrile at a flow rate of 2 ml/min. CNBr fragments 1 (CF1) and 4 (CF4) required additional purification by DEAE-Sephacel chromatography, as previously described [26]. The purity of the fragments was investigated by amino acid composition, isoelectric focusing (IEF) and immunoblotting as described below.

Endoproteinase cleavage of CF4

The endoproteinases Asp-N (cat no. 1054-589), Arg-C (cat no. 269-590), Glu-C (cat no. 791-156) and Lys-C (cat no. 476-986) were purchased from Boehringer Mannheim (Australia) and used according to the recommendations of the manufacturer. Purified CF4 was digested with Asp-N, Arg-C, Glu-C and Lys-C at enzyme/protein ratios of 1:200, 1:20, 1:20 and 1:20 (w/w) respectively, using procedures similar to those described elsewhere [27]. Complete digestion of CF4 was achieved using endoproteinase concentrations falling within the ranges recommended by the manufacturer. Peptides from Asp-N digestion of CF4 were analysed and purified by RP-h.p.l.c. as previously described [27]. Purified CF4 was also digested with trypsin [50:1 (w/w); Tos-Phe-CH₂Cl-treated; Sigma] in 0.1% M NH₄HCO₃ for 16 h at 37 °C. Complete digestion of CF4 was verified by RP-h.p.l.c. using the same procedures as described above.

MAb production

Production and characterization of the MAbs designated AI-1 and AI-3 has been previously described [28,29]. Two additional anti-apoAI MAbs, designated AI-4.1 and AI-4.2, have since been obtained from one fusion using standard hybridoma fusion protocols as previously published [28], following immunization of female Balb/c mice with purified CF4. The two MAbs were detected by e.l.i.s.a. (described below) using purified CF4 as antigen and sheep anti-mouse γ -chain-specific horseradish peroxidase (Bio-Rad) diluted 1:1000. All hybridomas were cloned at least twice by limiting dilution. Ascites fluids containing the antibodies were obtained from Balb/c mice which had been pristane-primed and injected intraperitoneally with 5 × 10⁷ hybridoma cells.

Purification of MAbs and preparation of Fab fragments

MAB heavy and light chain classes were determined using a mouse monoclonal antibody isotyping kit (MISOTEST-CSL; Australia). Monoclonal IgGs were purified from ascites fluid by affinity chromatography on Protein A–Sepharose-4B as described by Pharmacia. The antibodies were eluted with 0.1 M glycine/HCl, pH 2.8, and neutralized immediately with 1 M Tris buffer, pH 8.0. Isolated IgG was subjected to papain digestion according to the procedures of Gorini et al. [30]. Fab fragments were removed from undigested IgG and Fc fragments by passage on Protein A–Sepharose-4B. The purity and reactivity of the

isolated Fab fragments were assessed by SDS/10–15%-polyacrylamide gradient gel electrophoresis (Phastgel system, Pharmacia) and e.l.i.s.a. respectively.

E.l.i.s.a.

The reactivities of MAbs from hybridoma culture supernatants, ascites fluids, or following affinity chromatography were determined by e.l.i.s.a. Purified apoAI, apoA-II, apoAI–dimyristoylphosphatidylcholine (DMPC), CF4 or HDL₃ were diluted to 10–50 µg/ml in 0.05 M carbonate buffer, pH 9.6, and added to 96-well plates (Immulon II, Dynatech) at 100 µl/well. After an overnight incubation at 4 °C, the wells of plates coated with apoAI, apoA-II or CF4 were washed three times with PBS containing 0.05% Tween 20, followed by the addition of 100 µl of the serially diluted (1:2) anti-apoAI MAB sample or test antisera. After 1 h at 37 °C, the wells were washed as before, then incubated with 100 µl of horseradish peroxidase conjugated to sheep anti-mouse immunoglobulin (Silenus Laboratories, Australia), diluted 1:2000 in PBS containing 0.05% Tween 20. The wells were then washed, and the colour which developed after adding 100 µl/well of a substrate solution {0.1% 2',2'-azino-bis[3-ethyl-benzthiazoline sulphonic acid] (ABTS), 0.02% H₂O₂, 0.1 M citrate, pH 4.0} for 30 min was then quantified using a Titertek Multiscan (Flow Laboratories Inc.) with a filter setting of 414 nm. The same procedure was followed for wells coated with apoAI–DMPC or HDL₃, except that 0.05% Tween 20 was replaced with 0.5% casein to avoid the reported inhibitory effects of Tween 20 on antibody–lipoprotein interactions [31,32]. Results were expressed as % (B/B_0), where B is the absorbance of the diluted antibody expressed as a percentage of the maximum absorbance (B_0) obtained for each antibody. The concentration of MAB (ng) which gave 50% of the maximal response (50% B_0) was used as a relative measure of antibody titre.

IEF and immunoblotting

IEF was performed on 8 cm × 7 cm 7.5%-polyacrylamide slab gels (0.75 mm) in a Mini Protean II electrophoresis chamber (Bio-Rad). Gel solutions contained 6 M ultrapure urea (Bethesda Research Laboratories) and 5% ampholine (pH 3.5–9.5; Pharmacia–LKB). Following prefocusing for 20 min at 100 V, protein samples dissolved in urea containing 10% ampholine were loaded (20–30 µg/well) and focused for 30 min at 200 V, then for 2 h at 300 V. At the completion of IEF the gels were removed, and the protein transferred to nitrocellulose sheets (0.1 µm pore size; Bio-Rad) by diffusion blotting at 37 °C over 1 h. After transfer, the sheets were treated overnight at 4 °C in a solution of 1% casein in 0.1 M Tris buffer, pH 7.4, followed by 0.1 M Tris buffer, pH 8.8, containing 0.5% Tween 20 for 1 h at room temperature. The nitrocellulose sheets were then incubated with each purified MAB for 2 h at room temperature. Unbound antibody was removed by three washes in PBS containing 0.05% Tween 20. The sheets were further incubated for 1 h at room temperature with a 1:500 dilution of affinity-purified horseradish peroxidase conjugated to sheep anti-mouse immunoglobulin (Silenus Laboratories). After a second washing, specifically bound antibody was visualized by development with 0.5 mg/ml 4-chloro-1-naphthol (Sigma) in 0.1 M phosphate buffer, pH 6.0, containing 0.05% H₂O₂.

Competitive e.l.i.s.a. for epitope mapping

96-well plates were coated with 10 µg/ml apoAI as described above, then washed three times with PBS containing 0.05%

Tween 20. Duplicate wells received 50 μ l of serially diluted (1:2) endoproteinase digest mixture or purified peptide, followed by 50 μ l of either AI-4.1 or AI-4.2. The MAbs were diluted to achieve absorbance readings of 50–60 % of maximal absorbance (in the absence of competing antigen), as predetermined by e.l.i.s.a. titrations. Following incubation for 1 h at room temperature, the wells were washed three times with PBS/Tween 20; specific binding of the MAbs was then detected with horseradish peroxidase conjugated to sheep anti-mouse immunoglobulin, as previously described.

Apolipoprotein–phospholipid complexes

DMPC (Calbiochem) was dissolved in chloroform, dried under N_2 (g) and resuspended to approx. 20 mg/ml in 50 mM Tris/HCl and 100 mM NaCl, pH 7.4. The aqueous dispersion was sonicated as previously described [33] and centrifuged at 100 000 *g* for 1 h. ApoAI or CF4 was then incubated with the DMPC liposomes at ratios of 1:4 (w/w, protein:lipid) in 50 mM Tris/HCl and 100 mM NaCl, pH 7.4, for 20–24 h at 23 °C [21]. The ratio of 1:4 ensures that no uncomplexed apoAI remains after incubation [34], as verified by electrophoresis on native 4–30 % polyacrylamide gradient gels. The recombinant particles were stored at 4 °C for up to 3 weeks.

Rat liver plasma membranes

Rat liver plasma membranes were prepared using minor modifications [19] of the method described by Fleischer and Kervina [35]. Plasma membranes obtained from this procedure were quickly frozen in liquid N_2 and stored at –80 °C. Glycoproteins were isolated from rat liver plasma membranes as previously described [20] by chromatography on DEAE-Sephacel (Pharmacia) and wheat-germ-lectin–Sepharose 6MB (Pharmacia).

Binding studies

Binding assays were performed using duplicate tubes containing 200 μ g of plasma membrane protein, 0.2 μ g of 125 I-labelled HDL₃ and various amounts of purified Fab fragments in a final volume of 200 μ l. Incubations were performed in buffer containing 100 mM NaCl, 50 mM Tris/HCl, 0.01 % EDTA and 0.1 % casein. Different concentrations of Fab fragments were pre-incubated with the radioiodinated lipoproteins for 3 h at 37 °C in 1.5 ml microfuge tubes (Eppendorf). Rat liver plasma membranes were then added and the tubes incubated for 4 h at 37 °C. Duplicate tubes were incubated with 100-fold excess HDL₃ to assess non-specific binding, and tubes containing no plasma membrane were included as 'no-membrane' controls. After incubation, 170 μ l of the incubation mixture was transferred to a vacuum filter manifold fitted with Whatman GF/C glass fibre filters (Whatman Inc.) which had been presoaked for 3 h in 0.1 % casein. Under vacuum, the membranes were washed with 6 $\times$ 1 ml of incubation buffer, then transferred to tubes for counting of radioactivity. Non-specific binding to the GF/C filters (measured in the absence of plasma membranes) represented approx. 5–8 % of the total counts bound to the filters. Results were expressed as a percentage of the total 125 I-HDL₃ bound in the absence of antibody.

Ligand blotting

Glycoprotein samples isolated from rat liver plasma membranes by wheatgerm lectin chromatography [20] were diluted 2:1 in

buffer containing 10 % SDS and 25 % glycerol, loaded (50 μ g/gel) across SDS/8 %-polyacrylamide gels (80 mm $\times$ 70 mm $\times$ 0.75 mm) containing 0.1 % SDS and electrophoretically separated [36]. The separated proteins were then electrophoretically transferred [37] on to nitrocellulose membranes (0.45 μ m pore size) which were then cut into strips and incubated with a blocking buffer containing 50 mM Tris/HCl, 100 mM NaCl and 3 % (w/v) skim milk (pH 7.4) for 1 h at room temperature. For competition studies, separate strips were then preincubated for 1 h in the same buffer containing purified competing ligands (100–200 μ g/ml). After 3 $\times$ 5 min washes in buffer containing 50 mM Tris/HCl, 100 mM NaCl and 1 % (w/v) skim milk, the strips were incubated for 1 h in blocking buffer containing 1–20 μ g/ml apoAI or apoAI CNBr fragments. Following 3 $\times$ 5 min washes, the strips were then incubated for 1 h either with affinity-purified rabbit anti-apoAI IgG [3] or with MAb AI-3, diluted 1:200 in 1 % skim milk buffer. The anti-apoAI IgG, which recognized all four CNBr fragments by e.l.i.s.a. (results not shown), was diluted to achieve equivalent immunoreactivities for each fragment. Specific binding was then detected using either goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (diluted 1:1000; Bio-Rad), or sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (diluted 1:1000; Silenus), followed by a substrate solution containing 0.5 mg/ml 4-chloro-1-naphthol and 0.05 % H_2O_2 .

Other procedures

HDL₃ was radiolabelled with 125 I as described previously [38], using the McFarlane procedure [39]. The specific radioactivity of 125 I-HDL₃ ranged between 280 and 400 c.p.m./ng respectively. Amino acid analysis of CNBr fragments of apoAI and endoproteinase peptides of CF4 were performed using a Model 6300 Beckman Amino Acid Analyser equipped with a Model 3390 Hewlett Packard integrator (Hewlett Packard, Waldbronn, Germany). Samples were hydrolysed in sealed evacuated hydrolysis tubes for 22 h at 110 °C. Protein concentrations were measured according to Lowry et al. [40], using BSA as a standard; peptide concentrations were determined by amino acid analysis. Phospholipid was measured on a COBAS-Bio (Roche) using a phospholipid enzyme kit purchased from Boehringer Mannheim (Penzburg, Germany).

RESULTS

The MAbs AI-1 and AI-3, recognizing residues 28–47 and 140–147 of apoAI respectively, have been previously described [28,29]. Two additional MAbs directed towards the C-terminal

Table 1 Characterization of human apoAI-specific MAbs

Titre values express the amount of purified MAb (ng) required to produce 50 % of the maximal response (50 % B_0); values in parentheses represent the relative titres as a proportion of the titre obtained for AI-3. 'Identified epitopes' refers to peptides which are recognized by the MAbs.

MAb	Ig heavy chain	Immuno-gen	Relative titres for apoAI by e.l.i.s.a. (ng)	Immunoblotting ApoAI CNBr fragment	Identified epitopes
AI-1	IgG ₁	ApoAI	8.1 (0.6)	CF1	AI-(27–48)
AI-3	IgG ₁	ApoAI	14.0 (1.0)	CF3	AI-(140–147)
AI-4.1	IgG ₁	CF4	182.3 (13.0)	CF4	–
AI-4.2	IgG _{2b}	CF4	39.7 (2.8)	CF4	AI-(223–233)

Figure 1 Binding curves of MABs AI-4.1 (a) and AI-4.2 (b) as determined by e.i.s.a.

ApoAI (●), apoAII (△), apoAI-DMPC (□) or HDL₃ (▲) were adsorbed to 96-well plates, followed by the addition of serially diluted MABs (1:2, from 80 and 17 µg/ml IgG respectively), as described in the Materials and methods section. Results are expressed as antibody bound per well (*B*) as a percentage of maximum binding (*B*₀).

Figure 2 Epitope mapping of MABs by immunoblotting

CNBr fragments of apoAI were separated by IEF on urea/polyacrylamide gels (pH range 3.5–9.5), passively blotted on to nitrocellulose and incubated with the anti-apoAI MABs as described in the Materials and methods section. Lanes: 1, whole apoAI CNBr digest; 2, CF1; 3, CF3; 4, CF4.

region of apoAI, as described below, have since been generated. These MABs, denoted AI-4.1 and AI-4.2, were identified as belonging to the IgG₁ and IgG_{2b} subclasses respectively, with both containing κ light chains (Table 1).

Reactivity of the anti-apoAI MABs

Titration curves of the diluted anti-apoAI MABs, determined by e.i.s.a., showed that AI-4.1 and AI-4.2 had significantly lower titres for apoAI than did AI-1 and AI-3 (summarized in Table 1), possibly reflecting the fact that both of the former antibodies were raised to a chemically derived subfragment of apoAI. When expressed as a proportion of the titre obtained for AI-3, the titres of AI-1, AI-3 and AI-4.2 for apoAI (0.6, 1.0 and 2.8 respectively) were similar to those obtained for apoAI-DMPC (0.5, 1.0 and 2.1). AI-4.1 shows weak reactivity for apoAI-DMPC, and little or no reactivity for HDL₃ adsorbed to e.i.s.a. plate wells (Figure 1a), suggesting that it binds an epitope which has limited

Table 2 Ability of MABs AI-4.1 and AI-4.2 to recognize whole endoprotease digests of CF1 and CF3 respectively

Inhibition, denoted by +, refers to samples that inhibited MAB binding to apoAI, as determined by competitive e.i.s.a.

Competing antigen*	Inhibition of AI-4.1	Competing antigen†	Inhibition of AI-4.2
CF4	+	CF4-DMPC	+
CF4 (Asp-N)	+	CF4 (Asp-N)	—
CF4 (Arg-C)	—	CF4 (Arg-C)	—
CF4 (Glu-C)	—	CF4 (Glu-C)	—
CF4 (Lys-C)	+	CF4 (Lys-C)	—

* Endoprotease digests are indicated in parentheses.

† Endoprotease digests used alone, or following incubation with DMPC liposomes.

Figure 3 Epitope mapping of MAB AI-4.1 by competitive e.i.s.a.

Competition curves of MAB AI-4.1 binding to apoAI in the presence of Asp-N digest products of CF4. 96-well plates were coated with increasing amounts of the purified peptides (▼, undigested CF4; ●, AI-(157–167); ○, AI-(168–178) and AI-(179–204); ▲, AI-(213–222); ▽, AI-(223–233); □, AI-(234–243)), followed by the addition of MAB AI-4.1. Bound antibody was detected by e.i.s.a. as described in the Materials and methods section.

expression on apoAI in a lipid environment. In contrast, AI-4.2 shows similar reactivity towards apoAI, apoAI-DMPC and HDL₃ (Figure 1b) demonstrating that the two antibodies recognize different regions of the C-terminal portion of the apoAI molecule.

Epitope mapping of the MABs

The regions of apoAI recognized by AI-4.1 and AI-4.2 were initially determined by immunoblotting of apoAI CNBr digest fragments following separation of IEF CNBr cleavage of apoAI produces four fragments [24,26] corresponding to CF1 (residues 1–86), CF2 (residues 87–112), CF3 (residues 113–148) and the C-terminal region, CF4 (residues 149–243). The identities of the purified fragments were established by amino acid compositions, which closely agreed with expected values. When subjected to IEF, the basic isoforms of CF4 barely enter the top (cathode region) of the gel, whereas the more acidic isoforms of CF1 and CF3 migrate towards the bottom (anode) region, as previously reported [41,42]. The epitopes of AI-1 and AI-3 have been previously mapped using peptides generated from endoprotease cleavage of CF1 and CF3 respectively, by means of a competitive

Figure 4 Effect of anti-apoAI MABs on ^{125}I -HDL₃ binding to rat liver plasma membranes

^{125}I -labelled HDL₃ (1 µg/ml) was preincubated with increasing concentrations of Fab fragments derived from AI-1 (○), AI-3 (▲), AI-4.1 (△) or AI-4.2 (●) for 3 h at 37 °C, and added to liver plasma membranes for 4 h binding studies at 37 °C. Results are expressed as antibody bound (B) as a percentage of the total ^{125}I -labelled HDL₃ bound to the liver membranes in the absence of antibody (B_0).

e.l.i.s.a. [29]. Immunoblotting of the separated fragments confirmed that AI-1 and AI-3 recognized CF1 and CF3 respectively (Figure 2), whereas the MABs AI-4.1 and AI-4.2 produced similar immunoblot patterns, quite distinct from those obtained using AI-1 or AI-3, which show that their epitopes are localized within CF4. To further localize the epitopes for AI-4.1 and AI-4.2, CF4 was cleaved with endoproteases to generate smaller peptides for use in a competitive e.l.i.s.a., as described in the Materials and methods section. For MAB AI-4.1, digest mixtures of CF4 cleaved by Asp-N or Lys-C retained their ability to recognize the antibody (Table 2). Peptides from Asp-N digestion of CF4 were purified [27] and compared for their abilities to inhibit AI-4.1 binding to apoAI. Only one peptide, AI-(223–233), reduced the binding in a similar manner to that of intact CF4 (Figure 3). In contrast, MAB AI-4.2 was unable to recognize free CF4 in solution, presumably due to the high level of self-association of CF4 [26] resulting in hidden epitopes. MAB AI-4.2 could effectively recognize CF4 incorporated into DMPC liposomes, and epitope mapping was performed using CF4 endoproteinase digest products alone or following incubation with DMPC. However, no digest mixture inhibited the binding of AI-4.2 to immobilized apoA-I, suggesting that none of the four CF4 digest mixtures retained the epitope for AI-4.2 (see Discussion).

Binding studies using the anti-apoAI MABs

To determine the effects of the antibodies on the binding of ^{125}I -labelled HDL₃ to rat liver plasma membranes, the radiolabelled lipoproteins were preincubated with each of the MABs prior to the addition of membranes. To minimize the possibility of steric inhibition, resulting from the use of whole IgG molecules, Fab fragments were prepared from the anti-apoAI MABs. The use of whole IgG molecules also resulted in enhanced levels of HDL₃ binding to liver plasma membranes, presumably due to the formation of HDL-antibody aggregates. Of the four Fab fragments used, only AI-4.2 significantly inhibited the binding of HDL₃ (Figure 4), with the other MABs having little or no effect.

Ligand blotting studies using CNBr fragments of apoAI

Recent ligand blotting studies using ^{125}I -labelled CNBr fragments demonstrated that only CF4 specifically recognized two HDL-

Figure 5 Binding of purified CNBr fragments of apoAI to HDL-binding proteins by ligand blot analysis

Solubilized glycoproteins from rat liver plasma membranes (50 µg/gel) were fractionated on SDS/8% polyacrylamide gels, and transferred to nitrocellulose membranes. Nitrocellulose strips were then separately incubated with: lane A, HDL₃; B, apoAI; C, CF1; D, CF2 + 3; or E, CF4 (10 µg/ml). Specific binding was detected using anti-apoAI IgG diluted as described in the Materials and methods section. Molecular mass standards (kDa) are indicated.

Figure 6 Binding of apoAI to HDL-binding proteins in the presence of the CNBr fragments of apoAI determined by ligand blot analysis

Glycoproteins isolated from rat liver plasma membranes were electrophoretically separated and transferred to nitrocellulose as described in the Materials and methods section. Nitrocellulose strips were then separately incubated with: lane A, buffer alone; B, 100 µg/ml CF1; C, 100 µg/ml CF2 + 3; D, 100 µg/ml CF4 or E, 100 µg/ml CF2 + 3 for 1 h at room temperature. The MAB AI-3 (diluted 1:200), which recognizes an epitope localized to residues 140–147 [29], was then used to detect specific binding of 2 µg/ml apoAI as described in the Materials and methods section (except for lane E).

binding proteins, HB₁ and HB₂, isolated from rat liver membranes [21]. To determine whether the unlabelled fragments showed similar specificities, the binding of purified CF1 (residues 1–86), CF2 + 3 (residues 87–148) and CF4 (residues 149–243) to both HDL-binding proteins was determined using anti-apoAI IgG, which recognizes all four CNBr fragments of apoAI. As shown in Figure 5, CF4 showed the highest capacity to bind to the two HDL-binding proteins. In contrast to previous findings, CF1 also showed detectable levels of binding to HB₁ and HB₂. Furthermore, CF1 and CF4 were both found to inhibit the binding of apoAI to HB₁ and HB₂, although CF4 showed a greater capacity to reduce the binding when added at similar concentrations (Figure 6). The discrepancy between the present studies and those reported by Morrison et al. [21] possibly reflects the increased level of sensitivity of the immunochemical versus radiolabelling detection methods [20]. The radioiodination of CF1 did not compromise its receptor-binding activity, since CF1 labelled with ^{125}I as previously described [21] was clearly recognized by the HDL-binding proteins using antibody detection (results not shown); bound ^{125}I -labelled CF1 on the

Figure 7 Binding of trypsin-treated CF4 to HDL-binding proteins

The binding of apoAI to HDL-binding proteins in the presence of undigested CF4, or trypsin-treated CF4, was determined by ligand blot analysis. Nitrocellulose strips were then separately incubated with: lanes A and B, buffer alone; C, 200 μ g/ml CF4; D, 200 μ g/ml trypsin-treated CF4; or E, trypsin alone, at a similar concentration to that used in the CF4 digest mixture, for 1 h at room temperature. ApoAI (2 μ g/ml) was then added to each strip, except strip A, and incubated for a further 1 h at room temperature. Specific binding of apoAI was then detected using the MAb AI-3, diluted 1:200 as described in the Materials and methods section.

identical blot was barely detectable following development on high performance autoradiography film (Hyperfilm-MP; Amersham). Recent studies have shown that the high-affinity binding of HDL₃ to fibroblasts or hepatic membranes includes a trypsin-sensitive component which presumably reflects the involvement of apolipoproteins [43,44]. CF4, which clearly showed the highest capacity to bind to the liver membrane HDL-binding proteins, was digested with trypsin to determine whether its binding to HB₁ and HB₂ could be inhibited by proteolysis. As shown in Figure 7, trypsin-treated CF4 was unable to compete for the binding of apoAI to either HB₁ or HB₂.

DISCUSSION

ApoAI has been consistently implicated as a specific ligand for HDL-binding sites [2–10]. To identify the region of apoAI which may mediate this specific interaction, we have used anti-(human apoAI) MAbs to study the binding of HDL₃ to rat liver plasma membranes, which are thought to contain HDL receptors [2,19]. The MAb AI-4.2, directed towards the C-terminal portion of apoAI, inhibited the binding of HDL₃ to the liver membranes. In addition, ligand blotting studies have shown that the HDL-binding proteins HB₁ and HB₂, derived from rat liver plasma membranes, specifically recognize CF4, the C-terminal end of apoAI. The present findings are consistent with the proposal that the specific interaction between HDL and cellular receptor sites involves a binding domain located towards the C-terminus of apoAI.

We have characterized four different MAbs recognizing human apoAI with respect to their individual specificities and epitope locality within the apoAI molecule. The MAbs AI-1 and AI-3, recognizing the N-terminal (residues 28–47) and middle (residues 140–147) portions of apoAI respectively, have been previously described [28,29]. Two additional MAbs, AI-4.1 and AI-4.2, specific for the C-terminal end of apoAI, were obtained following hybridoma production using purified CF4 (Table 1). AI-4.1 binds weakly to apoAI-DMPC, and shows little or no reactivity towards HDL₃, demonstrating that the residues of apoAI recognized by AI-4.1 become obscured in a lipid environment. In contrast, AI-4.2 displays similar reactivities to apoAI, apoAI-DMPC and HDL₃, indicating that AI-4.2 binds an epitope which

is present on most apoAI molecules either associated or un-associated with lipid, similar to the epitopes of AI-1 and AI-3 [28]. Therefore the different reactivities of AI-4.1 and AI-4.2 towards apoAI-DMPC and HDL₃ (Figure 1) suggest that these MAbs are directed towards different epitopes within CF4.

More precise epitope mapping studies, using peptides obtained following endoprotease cleavage of CF4, demonstrated that AI-4.1 recognizes a region within peptide AI-(223–233) derived from Asp-N digestion of CF4 (Figure 3). Residues 222–233 form part of one of the predicted amphipathic α -helical structures on the apoAI molecule [45]. The lipid-binding properties of this region may obscure residues which are important for the AI-4.1 epitope, thus accounting for the inability of AI-4.1 to bind HDL₃. The epitope of MAb AI-4.2 could not be localized using similar approaches, since CF4 digested with Asp-N, Arg-C, Glu-C or Lys-C failed to bind MAb AI-4.2 (Table 2). It is possible that all four amino acid residues (Asp, Arg, Glu and Lys) are required for the integrity of the epitope, or alternatively the digest peptides may be unable to form a non-continuous epitope required by AI-4.2.

We have recently reported that Fab fragments derived from AI-1 and AI-3 have no effect on the binding of HDL₃ to liver plasma membranes, whereas the binding was inhibited in the presence of antibodies raised to two peptides corresponding to distinct regions in the C-terminus of apoAI [46]. In the present study, Fab fragments derived from AI-4.2, but not AI-4.1, were able to inhibit the binding of ¹²⁵I-labelled HDL₃ to liver plasma membranes, thus strengthening our proposal that a specific domain located in the C-terminal portion of apoAI participates in the interaction between HDL₃ and specific binding sites on hepatic membranes. This was confirmed in ligand blotting studies, where CF4 was shown to bind the HDL-binding proteins HB₁ and HB₂, derived from the rat liver plasma membranes (Figures 5 and 6). The comparative strength of association between the CNBr fragments and HB₁ and HB₂, detected by ligand blotting (Figure 5), parallels the predicted α -helical content of these proteins [45]. CF4, which contains the highest α -helical content, showed the strongest binding towards HB₁ and HB₂, whereas CF1, with less α -helical structure, was poorly recognized by the HDL-binding proteins, and CF2+3, which contains the least number of predicted α -helical repeats, was not recognized at all (Figure 6). Most of the HDL-binding proteins reported in the literature appear to recognize apoAI [18,19], apoAII [19] and apoAIV [47]. Although these proteins do not share a significant degree of sequence similarity, they all share characteristic α -helical regions (reviewed in [48]). Therefore the recognition of these apolipoproteins by the HDL-binding proteins may not only involve a specific amino acid sequence, but may also depend upon certain α -helical regions within these proteins. Whether or not the recognition of the A-type apolipoproteins by the HDL-binding proteins depends upon the optimal orientation of polar or non-polar residues, or requires a minimal length of α -helical structure, remains to be addressed.

In conclusion, the finding that MAb AI-4.2, raised to the C-terminal end of apoAI (residues 149–243), can inhibit the interaction between HDL₃ and liver plasma membranes supports our previous findings using anti-peptide antibodies, suggesting that a domain located in the C-terminal portion of apoAI mediates the binding of HDL₃ to binding sites in liver membranes. Taken together with the ligand blotting studies, in which CF4 (C-terminus) showed the highest capacity to bind the two HDL-binding proteins previously identified in rat liver plasma membranes, these findings support the identity of the C-terminus region of apoAI as at least one of the HDL receptor binding domains.

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