Purification of an apolipoprotein A binding protein from mouse adipose cells

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A protein recognizing apolipoproteins A_I, A_{II} and A_{IV} was purified from cultured mouse adipose cells of the Ob17MT18 clonal line. Apolipoprotein A binding sites were solubilized in the presence of proteinase inhibitors using the non-denaturing detergent CHAPS. Chromatography of the soluble extract on DEAE-Trisacryl was followed by immunoaffinity chromatography of the complex apolipoprotein A_I-binding proteins on anti-(apolipoprotein A_I) coupled to Sepharose 4B and then by h.p.l.c. on an RP-Select B column. A 1400-fold purification over the starting crude homogenate was achieved. The purified material contained two proteins that were both able to bind apolipoproteins A_I, A_{II} and A_{IV}, but not low-density lipoprotein. Glycopeptidase F treatment showed the existence of a single protein bearing either N-linked high-mannose or complex oligosaccharide chains. The purified material showed an apparent molecular mass of 80 ± 9 kDa by h.p.l.c. on a TSKG 3000 SW column. Rabbit polyclonal antibodies directed against the purified material revealed two protein bands of 80 and 92 kDa after SDS/PAGE under reducing conditions and immunoblotting. These bands were undetectable in growing Ob17PY cells previously shown not to bind the various apolipoproteins A and not to undergo cholesterol efflux, whereas they were conspicuous in growth-arrested Ob17PY cells which have recovered these properties.

INTRODUCTION

Plasma levels of high-density lipoproteins (HDL) are inversely correlated with the incidence of coronary heart disease [1]. HDL are believed to be involved both in the flux of cholesterol into cells of steroidogenic tissues and in the efflux of cholesterol from cells of other peripheral tissues for excretion by the liver [2]. Among peripheral tissues, adipose tissue has long been known for its ability to accumulate, store and, when needed, mobilize a large pool of unesterified cholesterol in both humans and rodents [3,4]. We have shown previously that cultured adipose Ob1771 cells (a subclone of parental Ob17 cells established from mouse epididymal fat pads) have specific receptor sites for apolipoprotein-E-free HDL, and that apolipoproteins A_I and A_{II} (apo A_I and A_{II}) [5], as well as apo A_{IV} [6], are the ligands for these sites. The saturable sites recognizing apolipoproteins are present in intact cells and crude membranes and show in both cases similar binding properties [5]. Moreover, following accumulation of cellular unesterified cholesterol by exposure of Ob1771 adipose cells to low-density lipoproteins (LDL), the addition of HDL₃, apo-A_I-containing liposomes [5] or apo-A_{IV}-containing liposomes [2] promotes cholesterol efflux. In contrast, apo-A_{II}-containing liposomes do not [5]. These results suggest that apo A_{II} seems to behave as a typical 'antagonist' and are in agreement with our report showing that lipoprotein particles containing apo A_I (LpA_I particles) but not lipoprotein particles containing apo A_I and apo A_{II} (LpA_I:A_{II} particles) promote cholesterol efflux from cultured adipose Ob1771 cells [6]. In agreement with the role postulated for apo A_I, receptor sites recognizing this apolipoprotein are detectable by cross-linking experiments at the cell surface of Ob1771 cells [7]. The critical role of these receptor sites in cholesterol efflux was strongly suggested by experiments using parental Ob17 cells after transformation by the complete

early region of polyoma virus (Ob17PY cells) [8]. Thus growing Ob17PY cells showed levels of LDL and transferrin receptor sites similar to those of Ob1771 cells, whereas no binding of HDL₃, apo A_I or apo A_{II} was observed [7]. After thymidine block, growth-arrested Ob17PY cells recovered the ability to bind these ligands within a few hours, and this recovery was prevented by actinomycin D or cycloheximide. Following cholesterol accumulation in the presence of LDL cholesterol, subsequent exposure to HDL₃ or apo-A_I-containing liposomes promoted cholesterol efflux from growth-arrested Ob17PY cells as well as from growing and growth-arrested Ob1771 cells [7]. In this study, we report the purification from Ob17MT18 cells of two proteins of 80 and 92 kDa that are able to bind apo A_I, A_{II} and A_{IV}, but not LDL. After glycopeptidase F treatment a single protein was obtained. In immunoblot experiments, both glycoproteins were undetectable in growing Ob17PY cells but became detectable in growth-arrested Ob17PY cells.

EXPERIMENTAL

Materials

Sephadex G-25 and CNBr-activated Sepharose were purchased from Pharmacia France S.A. (Bois d'Arcy, France). The anion-exchanger DEAE-Trisacryl was purchased from IBF (Villeneuve-la-Garenne, France). Phenylmethanesulphonyl fluoride (PMSF), BSA (fatty-acid-free) and glycopeptidase F were products of Boehringer (Mannheim, Germany). All other products, including microcarrier beads (Cytodex I), were purchased from Sigma Chimie (Le Verpillière, France). Homogeneous human apo A_I and A_{II} were prepared according to Mezdoor *et al.* [9] and apo A_{IV} according to Steinmetz *et al.* [10]. The preparation of human LDL ($d = 1.006-1.063$) was carried out according to Havel *et al.* [11]; it contained no detectable apolipoprotein other than apo B.

Abbreviations used: HDL, high-density lipoproteins; LDL, low-density lipoproteins; PMSF, phenylmethanesulphonyl fluoride; DMPC, 1- α -dimyristoyl phosphatidylcholine; apo A_I, apolipoprotein A_I; apo A_{II}, apolipoprotein A_{II}; apo A_{IV}, apolipoprotein A_{IV}.

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Cell culture

Ob17MT18 cells were obtained after transformation by the middle-T-only gene of polyoma virus [8]. They were used for purification of receptor sites of apo A since they showed a 3-fold enrichment of the latter compared with parental Ob17 cells. Ob17MT18 cells were grown on 180 μ m Cytodex I microcarrier beads. Ob17PY cells were grown in 100 mm-diam. dishes (Falcon) to favour firm attachment; under the conditions used, both actively growing cells and cells which had been growth-arrested by thymidine block [7] were still dispersed and did not show any intercellular contacts.

Cells were cultured in the presence of Dulbecco's modified Eagle's medium supplemented with 3% fetal bovine serum, 35 μ M-biotin, 17 μ M-pantothenate and antibiotics as previously described [5]. This medium was changed every other day. At 18–24 h after the last medium change, cells were rinsed twice at 4 °C with buffer A (phosphate-buffered saline, pH 7.4, containing 140 mM-NaCl, 3 mM-KCl, 8 mM- Na_2HPO_4 and 1.5 mM- KH_2PO_4). Ob17MT18 cells were detached from microcarrier beads after treatment for 30 min with ice-cold buffer B (10 mM-Tris/HCl buffer containing 300 mM-sucrose, 1 mM-iodoacetamide, 1 mM-1,10-phenanthroline, 1 μ M-pepstatin and 1 mM-PMSF. After sonication for 2 min using a Branson sonifier at a setting of 50 W, detached cells were filtered through a 100 μ m pore size nylon screen and then used as starting material for purification. Both growing and growth-arrested Ob17PY cells were obtained after rinsing with buffer A and scraping with buffer B.

Membrane preparation and solubilization of binding proteins

Cell homogenates were prepared using a Dounce homogenizer (10 strokes with pestle A and 20 strokes with pestle B) and ice-cold buffer B. A final protein concentration of about 2 mg of protein/ml was obtained (measured by the Bio-Rad procedure, with BSA as a standard). All operations were carried out at 4 °C. After addition of EDTA (1 mM final concentration), the post-nuclear supernatant was obtained by a centrifugation at 900 *g* for 10 min. After centrifugation of this supernatant at 105 000 *g* for 60 min, the membrane pellet was resuspended and solubilized (2 mg of protein/ml) in buffer C sterilized by filtration through a 0.45 μ m pore size filter. Buffer C contained 50 mM-Tris/HCl buffer, pH 7.4, 2 mM- CaCl_2 , 50 mM-NaCl, 0.1 mM-PMSF, 1 mM-iodoacetamide, 1 mM-1,10-phenanthroline, 1 μ M-pepstatin, 30% glycerol and 30 mM-CHAPS. The suspension was sonicated twice for 30 s at a setting of 50 W. After 16 h, the undissolved material was removed by centrifugation at 105 000 *g* for 60 min. The CHAPS-solubilized extract were used immediately for purification as described below.

Apolipoprotein labelling and binding assays

The procedure of Bilheimer *et al.* [12] was used for the radioiodination of LDL: the specific radioactivity varied between 150 and 500 c.p.m./ng of protein. Binding of LDL was measured according to Schneider *et al.* [13]; non-specific binding, determined in the presence of a 100-fold excess of unlabelled LDL, ranged between 25 and 40%. Radioiodination of apo A_1 , A_{II} and A_{IV} was carried out using ^{125}I as described by Sinn *et al.* [14]; the specific radioactivities ranged between 1000 and 5000 c.p.m./ng of protein. Complexes containing apolipoproteins and L- α -dimyristoyl phosphatidylcholine (DMPC) were prepared by the cholate dialysis procedure as described for apo- A_1 -containing liposomes by Chen & Albers [15] (molar ratio of phosphatidylcholine/protein = 150:1). The Stokes radius was estimated to be 100 ± 10 Å (10 ± 1 nm). Binding of labelled apolipoprotein to intact cells (35 mm dishes) or to the various fractions obtained during purification was performed for 2 h at 4 °C in buffer C as

previously described [7], except that the incubation volume was 0.1 ml. Labelled apolipoproteins were either used directly for binding (procedure 1) or complexes containing apolipoproteins and DMPC were first prepared (procedure 2, see below). Non-specific binding was determined in parallel experiments by measuring the amount of bound radioactivity when incubations were carried out in the presence of a 100-fold excess of the corresponding unlabelled apolipoprotein-DMPC complexes. Values of non-specific binding varied from 30 to 40% for apo A_{I} , from 20 to 30% for apo A_{II} and from 15 to 20% for apo A_{IV} . The ratios of bound to free ^{125}I -apolipoprotein-DMPC complexes were plotted against bound complexes according to the method of Scatchard [16]. Two procedures, both at 4 °C, were used for binding assays: in procedure 1, the separation of bound from unbound labelled ligand was achieved by gel filtration. Duplicate aliquots (50 μ l) of the incubation medium were loaded on 5 ml columns of Sephadex G-50 equilibrated with 50 mM-Tris/HCl buffer, pH 7.4, containing 30 mM-CHAPS. Elution was performed with 2 ml of the same buffer. The radioactivity associated with proteins in the void volume was counted directly in a gamma counter [17]. In procedure 2, the separation of bound from unbound labelled ligand was achieved by ion-exchange chromatography. Duplicate aliquots (50 μ l) were applied on 0.1 ml columns of DEAE-Trisacryl equilibrated with 150 mM-NaCl. Under these conditions, unbound apos A_{I} , A_{II} and A_{IV} did not bind to the resin, whereas receptor proteins with bound ligand remained on the column and were only eluted at higher salt concentrations (see Fig. 1). Therefore, after washing the column five times with 1 ml of 150 mM-NaCl, receptors bound to labelled ligand were eluted with 1 ml of 300 mM-NaCl. The radioactivity was then determined as described above. The specific activities were expressed in pmol of apo A bound/mg of protein. When control experiments were performed with procedure 2 to determine whether fatty-acid-free BSA binds to labelled apo A_1 (see Results), elution was performed at 500 mM-NaCl.

DEAE-Trisacryl chromatography

This was performed at 4 °C at a flow rate of 0.5 ml/min. The CHAPS-solubilized material (~100 ml) was applied to a column of DEAE-Trisacryl (1.5 cm $\times$ 15 cm) equilibrated in buffer C. The column was then washed with 1 litre of buffer C. Binding activity was eluted with a 60 ml linear gradient of 50–350 mM-NaCl in buffer C. For each fraction (3 ml), the binding of apo- A_1 -DMPC complexes (procedure 1) and protein content were determined. The fractions containing the binding activity were combined (5–7 fractions) and used subsequently.

Immunoaffinity chromatography

Preparation of the immunoaffinity column. The IgG fraction from rabbit serum directed against human apo A_1 was first prepared. Then, specific polyclonal antibodies against apo A_1 were isolated by passing the IgG fraction over a CNBr-activated Sepharose 4B column to which apo A_1 had been previously coupled; once purified, the specific antibodies (100 mg) were coupled to a CNBr-activated Sepharose 4B column (5 mg/g of gel) [18,19]. After antibody coupling, the column was equilibrated with phosphate-buffered saline, pH 7.4.

Immunoaffinity chromatography procedure. Apo- A_1 -DMPC complexes were incubated for 2 h at 4 °C with the combined fractions containing the binding activity (final apolipoprotein concentration 3.6 μ M). The mixture was then gently mixed with 20 ml of the immunoaffinity gel for 30 min at 37 °C. The gel was then cooled to 4 °C, promptly collected by filtration through a glass filter and washed within 5–10 min with 1 litre of ice-cold

Table 1. Purification of the apolipoprotein A binding proteins from mouse adipose cells

Step	Protein (mg)	¹²⁵ I-Apo A _I binding activity (pmol/mg)	Purification (-fold)	Recovery (%)
1. Cell homogenate	3200	8.2	1.0	100
2. Solubilized membrane	200	128.5	15.6	97.8
3. CHAPS extract	82	300	36.5	93.5
4. Eluate from DEAE-Trisacryl	12.1	1568	190.9	72.0
5. Eluate from affinity column	0.7	9564	1164.4	25.5
6. Eluate from h.p.l.c. (peak 2)*	—	11 536	1407	—
Theoretical value†	—	11 230	1400	—

* Protein content was determined by h.p.l.c. using a calibration curve obtained with BSA as standard.

† Calculated for a 92 kDa protein with one apo A_I binding site.

buffer A. After suspension in the minimum volume of the same buffer (20 ml), the gel was poured into a 1.5 cm × 30 cm column. Elution was performed by addition 40 ml of 3 M-KSCN. This dissociating agent was immediately removed by chromatography on a column of Sepharose G-25 (1.5 cm × 20 cm) which was equilibrated with buffer A and connected to the exit of the immunoaffinity column by plastic tubing (0.1 cm × 10 cm). The eluate (~ 50 ml) was immediately placed on a 1 ml column of DEAE-Trisacryl equilibrated with 150 mM-NaCl. After rinsing the column with 100 ml of 150 mM-NaCl, the binding activity was eluted with 2 ml of 300 mM-NaCl. A 1.5 ml sample was diluted 2-fold with buffer D (50 mM-Tris/HCl buffer, pH 7, containing 30 mM-CHAPS, 0.1 mM-PMSF, 1 mM-iodoacetamide, 1 mM-1,10-phenanthroline and 1 μM-pepstatin); this sample was used for molecular sieving and glycopeptidase F digestion as described below. The remaining undiluted sample (0.5 ml) was used directly for h.p.l.c. on an RP-Select B column.

H.p.l.c. on an RP-Select B column

H.p.l.c. (Waters Associates) was carried out using an RP-Select B (5 μm; 0.75 cm × 25 cm) column (Merck-Clévenot, France). A sample of 0.5 ml from immunoaffinity chromatography was injected and the elution was performed at a flow rate of 1 ml/min with a linear gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid. The effluent was monitored at 280 nm and 1 ml fractions were collected and stored at –20 °C until assayed. The various peaks were quantified by integration using a PU6003 diode array detector (Philips Scientific).

H.p.l.c. on a TSKG 3000 SW column

For molecular sieving, a TSKG 3000 SW (1.25 cm × 60 cm) column (LKB, Stockholm, Sweden) was used. Protein obtained by immunoaffinity chromatography, concentration on DEAE-Trisacryl column and dilution with buffer D was injected (2 ml). Elution was performed at a flow rate of 0.3 ml/min with buffer D. The effluent was monitored at 254 nm and 1 ml fractions were collected and stored at –20 °C until assayed.

Gel electrophoresis

Proteins were analysed by PAGE under reducing conditions according to Laemmli [19] with 10% separating gels in the presence of SDS.

Digestion with glycopeptidase F

A sample of 0.5 ml of binding protein in buffer D from immunoaffinity chromatography was treated for 2 h at 37 °C in the presence of 10 m units of glycopeptidase F/ml. After

digestion, the mixture was directly analysed by h.p.l.c. on an RP-Select B column, as described above.

Antibody preparation

Polyclonal antibodies were raised in a rabbit according to Sigel *et al.* [20] against a purified preparation obtained by immunoaffinity chromatography. Briefly, a solution (20 μg in 50 μl of 300 mM-NaCl) of the protein mixture (see Fig. 2) was emulsified with an equal volume of Freund's complete adjuvant and injected into the left popliteal lymph node. After 3 weeks, 40 μg in incomplete adjuvant was injected subcutaneously at four sites in the back; 1 week later, the same procedure was used. After a further 1 week, the rabbit was bled weekly (20 ml of blood) for 3 weeks without booster injections. All antisera were filtered through 0.45 μm pore size filters and stored at –20 °C. The first antiserum preparation was routinely used at 1:500 dilution for immunoblot experiments carried out according to Towbin *et al.* [21].

RESULTS

Solubilization of the apo A binding sites and properties after DEAE-Trisacryl chromatography

The first critical step in the purification of the binding proteins was the near-complete solubilization of crude membranes from Ob17MT18 cells by the zwitterionic detergent CHAPS (see Table 1). In comparison, octylglucoside, which was used for the solubilization of the apo B,E (LDL) receptor [13], proved to be less efficient than CHAPS. The solubilized binding proteins were stable for at least a few hours at 37 °C and for up to 2 weeks at 4 °C in buffer C; they were completely inactivated by trypsin treatment (0.2 mg/ml for 1 h at 37 °C). The second step in the purification of the solubilized binding proteins was anion-exchange chromatography of CHAPS extract on a DEAE-Trisacryl column. Approx. 40% of the proteins remained bound to the column. Elution was then carried out with a linear gradient of 50–350 mM-NaCl (Fig. 1a). More than 70% of the apo A_I binding activity was eluted as a single peak at 230 mM-NaCl, whereas the apo B,E receptor activity was eluted earlier as a single peak at 180 mM-NaCl. This step resulted in a 5-fold increase in the binding activity when using apo A_I as the ligand. At this stage the purified fraction was also able to bind ¹²⁵I-apo A_{II} (results not shown). The binding characteristics of the purified fraction from the DEAE-Trisacryl column are shown in Fig. 1(b) and are compared with those of intact cells. The association and dissociation curves obtained at 4 °C were similar in both cases and were similar to those obtained with intact Ob1771 cells [5]; equilibrium was reached within 2 h. Addition of unlabelled

Fig. 1. DEAE-Trisacryl chromatography of CHAPS-solubilized binding proteins

(a) Chromatography on DEAE-Trisacryl column was performed as described in the Experimental section. The fraction containing the maximal LDL-binding activity is indicated by an arrow. The pattern of elution is representative of fifteen independent experiments performed on different series of Ob17MT18 cells. (b) Kinetics of association (■, ●) and dissociation (□, ○) at 4 °C of labelled apo A_I-DMPC complexes in the presence of intact cells (■, □) or in the presence of the pooled fractions (●, ○) obtained from DEAE-Trisacryl chromatography (fractions 7–12). A concentration of 1.8 μM-¹²⁵I-apo A_I was used for association and a concentration of 36 μM unlabelled apo A_I was used for dissociation. Non-specific binding was measured in the presence of intact cells (◆) or in the presence of the pooled fractions (◇) as described in the Experimental section. The curves are representative of two independent series of experiments. (c) Isotherms of binding of labelled apo A_I-DMPC complexes to intact cells (grown in 16 mm dishes) (■) and to the pooled fractions (●) obtained after DEAE-Trisacryl chromatography. Binding was measured after 2 h at 4 °C as described in the Experimental section, using procedure 2 in the case of the pooled fractions. The Scatchard plots are representative of two independent experiments.

A_I-DMPC complexes led within 90 min to a complete displacement of bound radioactive ligand. As shown in Fig. 1(c), the binding of ¹²⁵I-apo A_I to the purified fraction was saturable, with a single class of binding sites with a K_d of about 1.8 μM, similar to the K_d value of 1 μM previously determined in intact cells [5].

Immunoaffinity chromatography

Since the dissociation rate of the ligand from the binding proteins was relatively low, we took advantage of this observation for a subsequent purification step by immunoaffinity chromatography. Apo A_I-DMPC complexes were first bound to the purified fraction until equilibrium was reached (2 h at 4 °C). The ligand-receptor complexes were then recognized by polyclonal antibodies directed against apo A_I and coupled to Sepharose 4B. This process was fast enough for recognition of the complex mediated by means of apo A_I, despite the fact that some dissociation of the binding protein(s) was also occurring. Binding proteins that remained bound specifically to the affinity column were eluted with 3 M-KSCN (immediately removed by chromatography on Sephadex G-25).

At that stage, to gain some insight into the apparent molecular mass of the binding proteins purified by immunoaffinity chromatography, gel filtration was performed by h.p.l.c. using a TSKG 3000 SW column. The elution profile is shown in Fig. 2. Portions of the collected fractions were assayed for binding of labelled apos A_I, A_{II} and A_{IV}. Calibration of the column with protein standards allowed us to estimate a molecular mass of 80 ± 9 kDa for the binding protein. The curves in Fig. 2 indicate that the elution profiles for the binding activities of apos A_I, A_{II}

Fig. 2. Gel filtration on a TSKG 3000 SW column of the apo A binding activity (purified by immunoaffinity chromatography)

Protein (350 μg) from the fraction purified by immunoaffinity chromatography was put on to a TSKG 3000 SW (1.25 cm × 60 cm) column. The elution was performed as described in the Experimental section and 1 ml fractions were collected. Binding assays were performed with 0.05 ml of each fraction using procedure 2 for the separation of bound and unbound labelled ligands in the presence of 3.6 μM-apo A_I (■), 1 μM-apo A_{II} (◆) or 1 μM-apo A_{IV} (□).

and A_{IV} were similar, and that no separation of the various binding activities could be achieved under these conditions. The low binding activity eluted with the void volume was probably due to some protein aggregation.

Table 2. Binding activity ratios during purification of the apo A binding proteins

n.d., not determined.

Step	Bound apo A _I (pmol/mg)	Bound apo A _{II} (pmol/mg)	Bound apo A _{IV} (pmol/mg)	Apo A _I / apo A _{II}	Apo A _I / apo A _{IV}
1	8.2	3.9	n.d.	2.1	n.d.
2	128.5	69.0	n.d.	1.86	n.d.
3	300	157.9	322.6	1.90	0.93
4*	1568	681.7	1493	2.3	1.05
5	9564	4970	9860	1.92	0.97
6: peak 2*	11536	6104	11652	1.89	0.99
peak 3*	12250	5918	11779	2.07	1.04

* Protein content determined by h.p.l.c. using a calibration curve obtained with BSA as standard.

H.p.l.c.

The resolution and final purification was accomplished by h.p.l.c. on an RP-Select B column using a linear gradient of 0–100% acetonitrile. As shown in Fig. 3(a), seven minor bands and two major bands (peaks 2 and 3) were detected. The first major band had a shoulder (peak 1) which was identified by its elution time as BSA (Fig. 3b); this contaminant probably originated from the culture medium, since Ob17MT18 cells were grown in the presence of 3% fetal bovine serum. Binding assays were performed on the various peaks; the bulk of binding activity was confined to peaks 2 and 3. In each case, the binding of labelled apos A_I, A_{II} and A_{IV} was observed (see Table 2).

To exclude the possibility that the binding activity of peak 2 was due, at least in part, to BSA, various binding assays were performed using fatty-acid-free BSA. With both binding assays (10 µg of BSA per assay), as well as in competition experiments between BSA and solubilized membranes of Ob17MT18 cells for

¹²⁵I-apo-A_I-DMPC complexes, the binding activity of BSA was below the limits of detection.

Fractions 33 and 35, which contained the maximal binding activity of peaks 2 and 3 respectively were rechromatographed under the same conditions. The elution profiles in Figs. 3(c) and 3(d) show for each fraction a single peak with elution times identical with those in Fig. 3(a). These results indicate the homogeneity of the protein eluted as peak 3 and also that of the second half of peak 2, which was not contaminated by serum albumin.

Table 1 summarizes the purification data obtained in a representative experiment (one out of ten different experiments) carried out under optimal conditions; the final specific activity was very similar to the theoretical value calculated for a 92 kDa protein with one apo A_I binding site. No specific binding of LDL could be detected in the eluate from immunoaffinity chromatography or in the eluate from h.p.l.c.

As shown in Table 2, the stoichiometry of binding was identical for apos A_I and A_{IV}, but only 1 mol of apo A_{II} was bound per 2 mol of apo A_I or A_{IV}. The stoichiometry of binding to apos A_I, A_{II} and A_{IV} remained constant during purification, suggesting that a single activity towards these apolipoproteins was present in cell homogenates of Ob17MT18 cells. It is of interest to note that the stoichiometry of binding to apos A_I and A_{II} was also identical with that obtained for intact Ob17 cells (results not shown).

Together with our previous results on the affinity labelling of apo A_I binding sites in intact Ob1771 cells and derived crude membranes, which showed two protein bands [7], the results of Fig. 3 suggested the possibility that peaks 2 and 3 are two forms of the same binding protein with different degrees of glycosylation. Therefore deglycosylation experiments were undertaken.

Digestion with glycopeptidase F

Glycopeptidase F is known to remove both high-mannose and complex oligosaccharide side-chains. After treatment of the

Fig. 3. Reverse-phase h.p.l.c. separation of the apo A binding proteins before and after glycopeptidase F treatment

The chromatography was carried out using an RP-Select B (0.75 cm × 25 cm) column with a linear gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid. The patterns of elution are as follows: (a) fraction purified by immunoaffinity chromatography; (b) BSA; (c) sample of peak 2 from experiment (a); (d) sample of peak 3 from experiment (a); (e) fraction purified by immunoaffinity chromatography and treated with glycopeptidase F; (f) glycopeptidase F.

mixture of peaks 1, 2 and 3 with glycopeptidase F, the whole incubation medium was analysed by h.p.l.c. using the same RP-Select B column.

The elution pattern in Fig. 3(e) indicates the presence of four peaks. The first peak corresponds to glycopeptidase F, since the chromatography of the enzyme alone showed that glycopeptidase F was eluted with the same elution time under these conditions (Fig. 3f). Glycopeptidase F treatment led to a large decrease of peak 2 with a corresponding increase of peak 3, whereas the integrated value of peak 1, identified as the non-glycosylated serum albumin (Fig. 3b), remained unchanged after glycopeptidase F digestion. Altogether, these results indicate clearly that peak 2 is a glycoprotein whereas peak 3, the elution time of which remains unchanged after glycopeptidase F digestion, corresponds either to a non-glycosylated protein or to an O-glycosylated protein.

Immunoblot experiments

CHAPS extracts of Ob17MT18 cells, growing Ob17PY cells and growth-arrested Ob17PY cells were subjected to SDS/PAGE analysis under reducing conditions, electrophoretically transferred to nitrocellulose, and probed with the antibody preparation obtained after injection into rabbits of the mixture of peaks 1, 2 and 3. The blots were treated with peroxidase-conjugated goat anti-(rabbit IgG) and processed as described in the Experimental section.

The staining patterns of Fig. 4 demonstrate that the rabbit antibody preparation reacted with two major bands in CHAPS extracts of Ob17MT18 cells (lane a). These two bands, with apparent molecular masses of 80 and 92 kDa, were undetectable in a CHAPS extract of growing Ob17PY cells (lane c), which showed no binding activity towards apos A_I and A_{II} [7] or towards apo A_{IV} (results not shown). However, they became clearly detectable in CHAPS extracts of growth-arrested Ob17PY cells (lane e), which recovered the binding activity towards both apos A_I and A_{II} [7] and apo A_{IV} (results not shown). The bands of 80 and 92 kDa were not detected when using the non-immune control serum, whereas various bands of lower molecular mass, including one band at 66 kDa, were detected with both the

Fig. 4. Immunoblot of CHAPS extracts from Ob17MT18 cells, growing Ob17PY cells and growth-arrested Ob17PY cells

Protein samples from CHAPS extracts were resolved by SDS/PAGE and electrophoretically transferred to a nitrocellulose sheet. The nitrocellulose blot was reacted with rabbit antiserum (1:500 dilution) directed against the fraction purified by immunoaffinity chromatography (lanes a, c and e), or with rabbit non-immune serum (lanes b, d and f), and horseradish peroxidase-labelled goat anti-rabbit immunoserum. Lanes a and b, Ob17MT18 cells; lanes c and d, growing Ob17PY cells; lane e and f, growth-arrested Ob17PY cells.

immune and the non-immune sera (lane a compared with lane b, lane c compared with lane d, and lane e compared with lane f). It is unlikely that the antibody preparation was able to recognize BSA, as control dot-blot immunoassays using up to 20 μ g of this protein did not reveal any staining. A major protein band of 75–80 kDa was detected by immunoblot experiments after glycopeptidase F digestion of the mixture of peaks 1, 2 and 3 (results not shown). Under the conditions used, the reason why the bands were not discrete remains unclear, but could be due, at least in part, to various degrees of glycosylation of both proteins.

DISCUSSION

The results of our previous studies demonstrated that crude membranes of Ob1771 cells contain apo-E-free HDL-binding sites which are distinct from apo B,E (LDL) receptors. These sites were shown to recognize apos A_I and A_{II} [5] as well as apo A_{IV} , but not apo E [22], apo C_{II} or apo C_{III} (R. Barbaras, P. Puchois & G. Ailhaud, unpublished work). In the present study, we report the isolation and purification of a protein from Ob17MT18 cells able to recognize apos A_I , A_{II} and A_{IV} . It is of utmost importance to recall that these binding sites are present at the cell surface of intact cells, but more than 90% of apo A and apo B,E (LDL) binding sites were shown to be present intracellularly [7]. This situation is similar to that observed in skin fibroblasts where a large proportion of LDL-binding activity is also present within the cells (R. Barbaras, unpublished work). Therefore both cell-surface and intracellular binding sites were purified in the present study, but it must be recalled that the affinities of these binding sites for their ligands were very similar in intact Ob17 cells and derived crude membranes [5,7] and that the binding parameters were found to be very similar for intact Ob17MT18 cells and for the fraction purified from these cells by DEAE-Trisacryl chromatography (Figs. 1b and 1c). Thus it is assumed that cell-surface binding sites and intracellular binding sites are identical and that a receptor recognizing apo A has been purified. The critical step in the purification procedure was the solubilization of functional receptors. In this respect, the use of the zwitterionic detergent CHAPS proved to be very effective and compatible with subsequent chromatography on DEAE-Trisacryl column. The active fraction obtained through this purification step retained the binding characteristics of the starting material and thus could be used for immunoaffinity chromatography. The efficiency of the latter step is probably due to the fact that polyclonal antibodies are directed against several epitopes on apo A_I , including many that are not involved in recognition by receptor sites. This favours the association of the apo- A_I -binding protein complex with anti-(apo A_I) coupled to Sepharose 4B. Despite the fact that the rate of association of this complex with anti-(apo A_I) is unknown, it is obviously faster than the rate of dissociation of the binding proteins from apo A_I (Fig. 1b). Accordingly, a significant proportion of the binding proteins remains attached to the column by means of apo A_I , and an extensive purification results.

Following h.p.l.c. a final purification of 1400-fold was obtained, with approx. 1 mol of apo A_I bound per mol of 92 kDa receptor. This step led to the separation of two peaks which showed very similar binding activities. It is striking to observe in Table 1 that the stoichiometry of binding of apo A_I , A_{II} and A_{IV} did not change throughout purification, suggesting that no other protein able to bind specifically to any of the three ligands was present in intact cells. It is also clear that the two receptor molecules separated by h.p.l.c. differ only by their degree of glycosylation. Glycopeptidase F digestion led to an increase in the elution time of peak 2, i.e. to an increase in its hydrophobicity, as expected from the release of hydrophilic complex

oligosaccharide chains. Peak 3 did not appear to be deglycosylated, as its elution time remained unchanged after glycopeptidase F digestion (Fig. 3e). Whether or not peak 3 represents a non-glycosylated form or an *O*-glycosylated form of the apo A receptor must await further investigation. In any event, the existence of a single receptor protein appears very likely. If so, the reason that more than 50% of these molecules escape *N*-glycosylation in the lumen of the endoplasmic reticulum remains unclear, but could be due either to the existence of two mRNA species or to a very short transit time within the lumen. Molecular mass determination by SDS/PAGE under reducing conditions and by gel filtration on TSKG 3000 SW under non-denaturing conditions also indicates that the binding activity was confined to a single polypeptide chain. The functional significance of glycosylation of the apo A receptor is unknown. However, the fact that apo A receptors not containing *N*-linked oligosaccharide chains still showed binding activity is reminiscent of similar observations made on LDL receptors synthesized in human fibroblasts in the presence of tunicamycin, which showed that their binding activity was unimpaired [23]. It has been reported that the molecular masses of the proteins that bind apos A_I and A_{II} in various tissues of different species range from 72 kDa [24] to 110–120 kDa [25,26]. Although we cannot exclude the possibility that some proteolysis still occurred under our conditions, it is equally possible that the differences in molecular mass observed by various investigators are due to differences in the degree of glycosylation of the receptor molecules.

Up to now, the question of involvement in cholesterol efflux of cell-surface binding sites has remained unsettled, as evidence in favour [7] and against [27] this possibility has been presented. Our previous studies had shown that no cholesterol efflux was taking place in growing Ob17PY cells which cannot bind HDL₃ and the various apos A [7]. However, cholesterol efflux recovered in growth-arrested Ob17PY cells at the same time as the binding properties for these various ligands reappeared [7]. Our present observation that the two protein bands of 80 and 92 kDa (detected by immunoblots of CHAPS extract from Ob17MT18 cells) were undetectable in growing Ob17PY cells, but became detectable in growth-arrested Ob17PY cells, favours a critical role for the apo A receptor in cholesterol efflux. We have previously shown that apos A_I and A_{IV} behaved as agonists and apo A_{II} as an antagonist of cholesterol efflux [5]. The observation that, both in intact cells and after receptor purification, approx. 1 mol of apo A_{II} is bound per 2 mol of apo A_I or apo A_{IV} (Table 2) remains unexplained. Among hypotheses to explain this difference, it is possible that the functional apo A receptor, required for cholesterol efflux but not for binding activity (Fig. 2), is a dimer of two single polypeptide chains. This dimeric structure would be able to recognize either 1 mol of the dimeric apo A_{II} or 2 mol of the monomeric apo A_I or A_{IV}. If so, we envision that the binding of 1 molecule of apo A_I (or A_{IV}), but not of apo A_{II}, might induce a conformational change, allowing the binding of a second molecule of apo A_I (or A_{IV}) and the formation of an activated receptor.

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