Ligand selectivity of 105 kDa and 130 kDa lipoprotein-binding proteins in vascular-smooth-muscle-cell membranes is unique

Valery N. BOCHKOV*, Vsevolod A. TKACHUK*, Maria P. PHILIPPOVA*, Dimitri V. STAMBOLSKY*, Fritz R. BÜHLER* and Thérèse J. RESINK†‡

*Laboratory of Molecular Endocrinology, Institute of Experimental Cardiology, Cardiology Research Center, Moscow, Russia, and †Department of Research, Basel University Hospital, CH 4031 Basel, Switzerland

Using ligand blotting techniques, with low-density lipoprotein (LDL) as ligand, we have previously described the existence of atypical lipoprotein-binding proteins (105 kDa and 130 kDa) in membranes from human aortic medial tissue. The present study demonstrates that these proteins are also present in membranes from cultured human (aortic and mesenteric) and rat (aortic) vascular smooth-muscle cells (VSMCs). To assess the relationship of 105 and 130 kDa lipoprotein-binding proteins to known lipoprotein receptors, ligand binding specificity was studied. We tested effects of substances known to antagonize ligand binding to either the LDL [apolipoprotein B,E (apo B,E)] receptor (dextran sulphate, heparin, pentosan polysulphate, protamine, spermine, histone), the scavenger receptor (dextran sulphate, fucoidin), the very-low-density-lipoprotein (VLDL) receptor [receptor-associated protein (RAP)], or LDL receptor-related

protein (RAP, α_2 -macroglobulin, lipoprotein lipase, exotoxin-A). None of these substances, with the exception of dextran sulphate, influenced binding of LDL to either 105 or 130 kDa proteins. Sodium oleate or oleic acid, known stimuli for the lipoprotein binding activity of the lipolysis-stimulated receptor, were also without effect. LDL binding to 105 and 130 kDa proteins was inhibited by anti-LDL (apo B) antibodies. LDL and VLDL bound to 105 and 130 kDa proteins with similar affinities ($\approx 50 \mu\text{g/ml}$). The unique ligand selectivity of 105 and 130 kDa proteins supports the existence of a novel lipoprotein-binding protein that is distinct from all other currently identified LDL receptor family members. The similar ligand selectivity of 105 and 130 kDa proteins suggests that they may represent variant forms of an atypical lipoprotein-binding protein.

INTRODUCTION

Specific endocytosis of low-density lipoproteins (LDLs) is mediated by the apolipoprotein B,E (apo B,E) receptor [1]. This receptor has been extensively characterized, both structurally and functionally, and its role in both normal metabolism of lipoproteins and the development of pathological conditions such as hypercholesterolaemia and premature atherosclerosis is well established [1–3]. Another lipoprotein-endocytosing receptor, the scavenger receptor, does not recognize native LDL, but is capable of binding anionized and oxidized LDL [4] as well as some other polyanionic compounds [5]. Unlike the apo B,E receptor, expression of the scavenger receptor is not down-regulated by an excess of cellular cholesterol [6]. This has important pathophysiological consequences since unlimited endocytosis of oxidized lipoproteins by macrophages within the vessel wall produces the highly lipid-laden foam cells characteristic of atheromatous lesions [7].

It is now evident that the apo B,E receptor is a member of a family of structurally related proteins, known as the LDL receptor family. In mammalian species the family currently includes four members: (1) the apo B,E receptor, the prototype of the family [8]; (2) the LDL receptor-related protein (LRP) [9], which is identical to the α_2 -macroglobulin (α_2 -M) receptor and therefore often referred to as LRP/ α_2 -M receptor [10]; (3) the very-low-density-lipoprotein (VLDL) receptor [11,12]; and (4) Heymann's nephritis antigen gp330/megalin [13]. Two other LDL receptor homologues are present in lower species, namely an avian vitellogenin receptor [14] and a protein of nematode

Caenorhabditis elegans [15]. Sequence homologies between LDL receptor family members include: (a) multiple copies of cysteine-rich ligand-binding repeats; (b) cysteine-rich repeats of epidermal growth factor (EGF) precursor; (c) the tetrapeptide sequence tyrosine-tryptophan-threonine-aspartic acid; (d) a single membrane-spanning region; and (e) at least one copy of an asparagine-proline-Xaa-tyrosine sequence within the cytoplasmic tail that directs clustering of the receptors in coated pits [4]. Key elements for ligand recognition are the ≈ 40 -amino-acid-long imperfect repeats containing six cysteine residues arranged in a 'class A motif' [9,16]. Each family member possesses a specific number of class A repeats as well as a specific number and arrangement of EGF-precursor repeats. This structural variation evidently explains the differing ligand specificities of the LDL receptor family members, in spite of their sequence homologies.

The apo B,E receptor selectively recognizes apo B- and apo E-containing lipoproteins [1]. LRP binds apo E-enriched β -VLDL [17], as well as several non-lipoprotein molecules that include activated α_2 -M [10], lipoprotein lipase (LPL) [18], lactoferrin [19], plasminogen activators and their complexes with inhibitors [19,20] and *Pseudomonas aeruginosa* exotoxin A (exo-A) [21]. gp330/megalin exhibits essentially the same selectivity as LRP, the only exception being non-recognition of α_2 -M [4,19]. The VLDL receptor binds the triacylglycerol-rich, apo E-containing lipoproteins VLDL, β -VLDL and intermediate-density lipoproteins (IDLs), but does not recognize LDL [11,12,22]. A unique ligand which interacts with several members of the family is the so-called receptor-associated protein (RAP) [23]. This protein inhibits binding of ligands to gp330/megalin and LRP

Abbreviations used: apo B,E, apolipoprotein B,E; EGF, epidermal growth factor; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; RAP, receptor-associated protein; LRP, LDL receptor-related protein; exo-A, exotoxin A; LPL, lipoprotein lipase; α_2 -M, α_2 -macroglobulin; PPS, pentosan polysulphate; VSMC, vascular smooth-muscle cell.

‡ To whom correspondence should be addressed.

[18–20,24–26] and blocks VLDL binding to purified VLDL receptor [22]. An RAP- α apo B,E receptor interaction is questionable, having been reported as only very weak [27] or not significant [19,28]. The broad ligand specificity of LRP, VLDL receptor and gp330/megalin suggests that they may function as lipoprotein receptors, as well as participating in endocytotic uptake of a number of protein ligands such as protease/inhibitor complexes and certain toxins [4].

Recently, an unusual protein, potentially related to lipoprotein receptors, has been described in the mollusc *Lymnaea stagnalis* [29]. Its structure includes 12 of the cysteine-rich class A repeats characteristic of the LDL receptor family, but whereas all other described LDL receptor family members possess a single membrane-spanning domain, the mollusc protein possesses seven membrane-spanning domains [29]. These particular characteristics suggest the existence of a receptor protein which constitutes a hybrid between a classical lipoprotein-binding receptor and a signal-mediating G-protein-coupled receptor [29]. Lipoproteins are capable of stimulating second messenger systems in several mammalian cell types [30,31], and at least in endothelial and vascular smooth-muscle cells (VSMCs) this stimulation occurs in a G-protein-dependent manner [32–34]. Classical binding studies have indicated the existence of atypical lipoprotein-binding sites in platelets and VSMCs [34–36]. Furthermore, in VSMCs a close correspondence between the properties of ^{125}I -LDL binding and LDL-mediated signalling has been demonstrated [34,35]. Thus atypical hybrid lipoprotein receptors probably also exist in mammalian cells.

Ligand blotting techniques have revealed the presence of a major atypical lipoprotein-binding protein (molecular mass of 105 kDa) in membranes from aortic medial tissue [37]. Although this protein possesses some characteristics typical of recognized lipoprotein receptors [37], its identity is not known. In order to assess the relationship of the 105 kDa protein to known lipoprotein receptors and to shed light on possible mechanisms of LDL–105 kDa protein interactions, we have further characterized ligand binding specificity of lipoprotein-binding proteins in smooth-muscle cells using chemicals known to interfere with lipoprotein binding to currently identified lipoprotein-binding proteins.

MATERIALS AND METHODS

Preparation of lipoproteins

VLDL ($\rho < 1.006 \text{ g/ml}$) and LDL ($\rho 1.019\text{--}1.063 \text{ g/ml}$) were isolated from the plasma of healthy male humans using sequential buoyant density centrifugation techniques with use of sodium bromide for density adjustments [34,38].

Preparation of membranes from aortic tissue and cultured VSMCs

Thoracic aortae were obtained *post mortem* (autopsies performed within 1–5 h of death) from ten individuals (aged 15–55) who died from different accidental causes. Sarcolemmal membranes were isolated as previously detailed [37]. Confluent and quiescent cultures of rat aortic, human aortic or omental mesenteric VSMCs [31] were rinsed with PBS before being scraped into lysis buffer containing 2 mM EDTA, 20 mM Tris/HCl (pH 7.4), 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin and 1 mM PMSF. After sonication, membranes were collected by centrifugation of cell lysates at 100 000 g for 30 min at 4 °C. All membrane preparations were stored in aliquots at -70°C as suspensions in a buffer containing 250 mM sucrose, 1 mM PMSF and 20 mM 3-morpholino-2-hydroxypropanesulphonic acid (pH 7.4).

Antibodies

One goat and three rabbits were immunized with a mixture of LDL and Freund's adjuvant (complete for the first and incomplete for the following injections) according to standard procedures [39]. Affinity purification of anti-LDL antibodies was performed using a column prepared by immobilization of LDL on cyanogen bromide-activated Sepharose 4B. After application of serum and extensive washing, specific antibodies were eluted with 0.1 M glycine (pH 3.0), neutralized, dialysed against PBS and stored at -20°C . Fab fragments were prepared according to standard procedures [39] with minor modifications. Affinity-purified rabbit or goat anti-LDL antibodies (at 5 mg/ml) were incubated in 0.1 M sodium acetate buffer (pH 5.5), containing 50 mM cysteine and 1 mM EDTA, with papain-agarose (Sigma Chemical Co. St. Louis, MO, U.S.A.) at a 1:100 ratio of LDL protein to immobilized enzyme. The incubation was performed for 3 h (goat IgG) or 4 h (rabbit IgG) at 37 °C in a shaker. Incubation was terminated either by addition of iodoacetamide [39] or by centrifugation and discarding the pelleted immobilized enzyme. Completeness of digestion, as analysed by SDS/gel electrophoresis, was demonstrated by the lack of an IgG band in papain-treated samples even in heavily overloaded gels. The samples were dialysed against PBS and stored at -20°C . Purified rabbit IgG (reagent grade) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant human RAP, rabbit affinity-purified anti-RAP and a rabbit polyclonal antibody raised against a synthetic peptide ASVGHTYPAISVVSTDD-DLA, which represents the C-terminus of rabbit and human VLDL-receptors, were gifts from Dr. D. K. Strickland, American Red Cross, Washington, U.S.A.

Ligand binding

Ligand binding procedures have been fully described and validated previously [37]. SDS/8 % -PAGE was performed under non-reducing conditions, and 100 μg of protein was loaded per slot. A molecular mass standard mixture (#SDS-6H; Sigma Chemical Co., St. Louis, MO, U.S.A.), consisting of carbonic anhydrase (29 kDa), egg albumin (45 kDa), phosphorylase *b* (97 kDa), β -galactosidase (116 kDa) and myosin (205 kDa), was used for calibration. Proteins were electrophoretically transferred on to nitrocellulose membranes, detected by Ponceau S staining, and blots were destained in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris/HCl, pH 7.4). Unless otherwise stated TBS containing 5 % (w/v) delipidated milk (TBSM: Rapilait milk, Zurich, Switzerland) was used in all preblocking, incubation and washing procedures. Blots were preincubated for 1 h in TBSM, and then incubated for 2 h with the selected substance(s) (specified in the Results section and legends to Figures) in TBSM. After repeated washes ($5 \times 5 \text{ min}$) with TBSM, blots were incubated for 2 h with the appropriate primary antibodies (either rabbit or goat anti-LDL, or rabbit anti-RAP), washed again and then incubated for 2 h with secondary ^{125}I -labelled antibodies [either donkey anti-(rabbit IgG) or rabbit anti-(goat IgG)]. After incubation with secondary antibodies, blots were repeatedly washed with TBSM, dried and autoradiographed. Following localization of protein-bound ligands, radiolabelled bands (molecular masses 130 kDa and 105 kDa) were excised and counted in a gamma-counter. To control for residual non-specific binding to nitrocellulose, background sections of nitrocellulose of the same size as the ligand-binding bands were also excised from each strip and counted. The mean value of background radioactivity was subtracted from that radioactivity associated with the 130 kDa and 105 kDa bands, and resulting values taken to represent specific binding. Both densitometric

analysis of autoradiographs (results not shown) and individual radioisotopic quantification of 105 kDa and 130 kDa proteins revealed that the two proteins behaved identically in all different experimental protocols, with the exception of experiments with RAP and anti-(VLDL receptor) antibodies (see Figure 1B and appropriate text in Results section). Values in Figures (and referred to in the text as specific lipoprotein binding to 105/130 kDa proteins) represent the sums of the individual c.p.m. values obtained for lipoprotein specifically bound to 105 kDa and 130 kDa proteins. Ligand binding activity of the individual proteins is evident in autoradiographs. Two independent experiments with each of the different protocols were performed on at least three of the four different membrane preparations (specified in Figure legends). Matching autoradiographic and quantitative results were obtained for each of the experiments.

Protein determinations

Protein concentrations were determined using the Lowry method with BSA as standard. Concentrations of affinity-purified antibodies and Fab fragments were determined spectrophotometrically assuming $\epsilon^{1\%}_{280}$ 14.

RESULTS

Characterization of LDL- and RAP-binding proteins in cultured VSMCs

We have previously demonstrated [37] that human aortic medial membranes contain a major LDL-binding protein of molecular mass 105 kDa and a minor LDL-binding protein of 130 kDa (observation confirmed for this study in Figure 1A). In the present study we observed that 105 kDa and 130 kDa LDL-binding proteins were also present in membranes of cultured VSMCs isolated from human aorta (Figure 1B) and human omental mesenteric vessels (see Figure 3), although the relative amounts of the two lipoprotein-binding proteins in both cell isolates was consistently opposite to those observed in aortic medial membranes (compare Figures 1A and 1B). In ligand (LDL) blots of membranes from cultured rat aortic VSMCs, the 130 kDa band was also more prominent than the 105 kDa band, and an additional LDL-binding band with a molecular mass of 115 kDa was also present (Figure 1C). Figure 1(C) illustrates the dose-dependent binding of LDL to 105, 115 and 130 kDa proteins in rat aortic VSMC membranes. The dose-dependent binding of LDL to 105 and 130 kDa proteins in human aortic VSMC membranes is illustrated in Figure 2. The estimated affinity ($\approx 40 \mu\text{g/ml}$) for LDL-binding to VSMC membrane proteins (Figures 1 and 2) is comparable with that previously observed for LDL binding to aortic medial membrane proteins [37].

RAP is known to interact with several members of the LDL receptor family, namely VLDL receptor, LRP and gp330/megalin [18–20,22,24–27]. To investigate whether the 105/130 kDa LDL-binding proteins could also bind RAP, nitrocellulose blots of membrane proteins were sequentially incubated with RAP (3 $\mu\text{g/ml}$), rabbit affinity-purified anti-RAP antibodies and ^{125}I -labelled donkey anti-(rabbit IgG) antibodies. We found that membranes from cultured VSMCs, but not from aortic medial tissue, contained a major RAP-binding protein of 105 kDa and two other minor RAP-binding proteins with molecular masses above 200 kDa (Figure 1B). Since the electrophoretic mobility of the most prominent RAP-binding band (105 kDa) is close to that expected for the VLDL receptor [22,40], the 105 kDa protein in VSMC membranes may be the VLDL receptor. This proposal is supported by immunoblotting experiments in which

Figure 1 Lipoprotein-binding proteins in smooth-muscle cell membranes

Membrane proteins (100 μg of protein/slot) from human aortic medial tissue (A) and from cultured human aortic (B) and rat aortic (C) VSMCs were electrophoresed under non-reducing conditions in SDS/8%-polyacrylamide gels, and transferred to nitrocellulose. After blocking of non-specific binding, blots of membranes from aortic medial tissue and human aortic VSMCs were examined for LDL-binding proteins (using 20 $\mu\text{g/ml}$ LDL as ligand and anti-LDL antibodies as primary antibodies), RAP-binding proteins (using 3 $\mu\text{g/ml}$ RAP as ligand and anti-RAP antibodies as primary antibodies) and VLDL-receptor immunoreactivity [using anti-(VLDL receptor) antibodies]. Blots of membranes from rat aortic VSMCs were examined for the dose-dependent binding of LDL (0–100 $\mu\text{g/ml}$ LDL as ligand and anti-LDL antibodies as primary antibodies). ^{125}I -labelled secondary antibodies were used to identify LDL-, RAP- and VLDL-binding proteins and representative autoradiographs are presented. Methodological details for ligand binding and immunoblotting are described in the Materials and methods section and in the Results section.

the 105 kDa band was observed to interact with antibodies generated against the N-terminal peptide of the human VLDL receptor (Figure 1B). The identity of the high-molecular-mass RAP-binding proteins of cultured VSMCs was not further studied.

Distinction between VLDL receptor and VSMC LDL-binding proteins

The VLDL receptor is known to bind triacylglycerol-rich, apo E-containing lipoproteins (VLDL, β -VLDL and IDL) but not LDL [11,12,22]. Thus to further investigate the identity of the 105 kDa protein as the VLDL receptor, the dose-dependent binding of LDL and VLDL to VSMC membranes was compared. We observed that VLDL also bound specifically to 105 kDa and 130 kDa proteins, and with an affinity ($\approx 50 \mu\text{g/ml}$) comparable with that for LDL binding (Figure 2). Furthermore, the estimated affinity with which VLDL binds to 105/130 kDa proteins in membranes from aortae (Figure 2) and VSMCs (results not shown) is at least one order of magnitude lower than that expected ($\approx 1 \mu\text{g/ml}$) for binding of VLDL to the VLDL receptor [41]. These results indicate that 105/130 kDa LDL-binding proteins and the VLDL receptor are distinct. Although not experimentally examined, the apparently lower B_{max} value of

Figure 2 Dose-dependent binding of VLDL and LDL to 105/130 kDa proteins in human aortic membranes

Blots of membrane proteins from human aortic VSMCs were incubated with the indicated concentrations of either LDL (●) or VLDL (▲). After autoradiographic visualization of LDL and VLDL binding to 105/130 kDa proteins, specific binding was quantified. Specific binding of LDL and VLDL is expressed as the percentage of maximum specific LDL binding (arbitrarily 100% at 100 $\mu\text{g/ml}$). The Figure shows matching autoradiographic and quantitative data from a single representative experiment. Experiments performed on membranes from human aortic medial tissue, human mesenteric and rat aortic VSMCs yielded dose-profiles (not shown) similar to those presented in this Figure. The experiment was performed on each of the different membrane preparations twice.

VLDL as compared with that of LDL may reflect either differing immunodetection sensitivities of the two types of lipoproteins or a steric hindrance of binding of larger VLDL particles.

However, it is particularly noteworthy (Figure 1) that expression of RAP-binding proteins is (i) different between membranes from aortic media and cultured VSMCs, and (ii) distinct from expression of LDL-binding activity. In VSMC membranes, major RAP-binding activity and VLDL receptor immunoreactivity were evident in the 105 kDa band, whereas LDL bound predominantly to the 130 kDa band (Figure 1B). In aortic medial membranes the 105 kDa protein exhibits major LDL-binding activity but undetectable RAP-binding activity and VLDL receptor immunoreactivity (Figure 1A). It is possible therefore, that the 105 kDa band in membranes from VSMCs contains more than one protein, namely the VLDL (RAP-binding) receptor and an LDL-binding protein which does not bind RAP. Nevertheless, since the dose-dependent requirements for VLDL binding to the 105 kDa band ($K_d \approx 40 \mu\text{g/ml}$, this study) is not consistent with VLDL binding to VLDL receptor ($\approx 1 \mu\text{g/ml}$, [41]), we infer that VLDL binding to 105 kDa protein in membranes from VSMCs is almost entirely mediated by the novel LDL-binding protein, rather than by the VLDL receptor.

Effect of LRP ligands

The apparent molecular mass (500–600 kDa) of LRP, an apo E-binding protein [17], is much greater than that of the LDL-binding proteins in smooth-muscle cell membranes. Nevertheless, since LRP is also an RAP-binding protein [20,26,27], we considered whether 105/130 kDa proteins in smooth-muscle cell membranes might be variant forms or proteolytic fragments of LRP. To exclude this possibility we investigated the ability of LDL-binding proteins of smooth-muscle cells to bind other proteins known to interact with LRP, such as α_2 -M, RAP, LPL and *Pseudomonas* exo-A [4]. To this end, we performed competition experiments, in which LDL was used at a concentration (20 $\mu\text{g/ml}$) below its approximated K_d (40 $\mu\text{g/ml}$) and competitors were added at concentrations vastly in excess of their reported K_d values for binding to LRP [18,21,42]. We found

Figure 3 Effect of LRP-ligands on LDL binding to 105/130 kDa proteins

Blots of membrane proteins from human mesenteric and rat aortic VSMC cultures were incubated in the presence of LDL (20 $\mu\text{g/ml}$) either without or with inclusion of RAP (3 $\mu\text{g/ml}$), exo-A (Ex-A, 10 $\mu\text{g/ml}$), LPL (10 $\mu\text{g/ml}$) and activated α_2 -M (α -MG, 10 $\mu\text{g/ml}$). (α_2 -M was activated by incubation with 200 mM methylamine for 1 h at room temperature with subsequent extensive dialysis against PBS [41]). Specific binding to 105/130 kDa proteins was quantified. The Figure presents quantitative data for human mesenteric VSMCs (A), and autoradiograms are from human mesenteric VSMCs (A) and rat aortic VSMCs (B). Representative data from three individual experiments are shown. The tested LRP ligands also did not influence LDL binding to 105/130 kDa proteins in membranes from human aortic medial tissue and human aortic VSMCs (results not shown). The experiment was performed on each of the different membrane preparations twice.

Figure 4 Effects of polyanions on LDL binding to 105/130 kDa proteins

Blots of membrane proteins from human mesenteric VSMCs (A) and rat aortic VSMCs (B) were incubated in the presence of LDL (20 $\mu\text{g/ml}$) either without or with inclusion of PPS (○), heparin (●), fucoidin (○) or dextran sulphate (●) at concentrations indicated in the Figure. After autoradiographic visualization of LDL-binding proteins, specific binding to 105/130 kDa proteins was quantified. The Figure shows matching autoradiographic and quantitative data from individual representative experiments. In experiments performed on membranes from human aortic medial tissue and human aortic VSMCs (results not shown), dextran sulphate was also found to be the only polyanion which inhibited LDL binding to 105/130 kDa proteins. The experiment was performed on each of the different membrane preparations twice.

that none of the tested LRP ligands significantly influenced LDL binding to 105/130 kDa proteins (Figure 3). Partial inhibition of LDL binding by LPL was observed in one experiment but

Figure 5 Effects of polycations on LDL binding to 105/130 kDa proteins

Blots of membrane proteins from rat aortic VSMCs (A) and human aortic VSMCs (B) were incubated in the presence of LDL (20 μ g/ml), without or with inclusion of protamine ($\square$), histone H1S ($\blacktriangle$), putrescine ($\bullet$) or spermine ($\circ$) at concentrations indicated in the Figure. LDL-binding proteins were visualized by autoradiography, and specific binding to 105/130 kDa proteins was quantified. The Figure shows matching autoradiographic and quantitative data from individual representative experiments. The tested polycations also did not influence LDL binding to 105/130 kDa proteins in membranes from human aortic medial tissue (results not shown). Experiments were performed on each of the different membrane preparations twice.

analysis of data from four separate experiments indicated that this effect was not statistically significant. Since binding of ligands to VLDL receptor is known to be inhibited by RAP [22], the presently observed inability of RAP to inhibit LDL binding to 105/130 kDa indicates that the VLDL receptor, which may co-migrate with the 105 kDa protein (see above text relating to Figure 1B), does not contribute to the binding of LDL to this protein. This conclusion is in agreement with the reported inability of VLDL receptor to bind LDL [11,12,22].

Effects of polyanions and polycations

We performed further inhibitor studies using ligands known to block interaction of lipoproteins with the apo B,E- and scavenger-receptors. The polyanions heparin, pentosan polysulphate (PPS) and dextran sulphate form complexes with positively charged residues of apo B and thereby inhibit binding of LDL to the apo B,E receptor [43]. Fucoidin is capable of blocking interaction of acetyl-LDL with scavenger receptor, and does so by binding to the receptor rather than to the ligand [5]. The data in Figure 4 demonstrate that of the above-mentioned polyanions, only dextran sulphate exerted inhibitory activity on LDL binding to 105/130 kDa proteins (Figure 4). Concentrations of dextran sulphate required for half-maximal and maximal inhibition were $\approx$ 1 mg/ml and 5 mg/ml, respectively.

In order to investigate the role of positive protein charge in interactions between LDL and 105/130 kDa proteins, we tested whether binding of LDL could be blocked by polycationic substances, some of which are known to inhibit LDL-apo B,E-receptor interactions [44]. Our data indicate that the binding of

Figure 6 Anti-LDL antibodies block LDL binding to 105/130 kDa proteins

Blots of human aortic VSMC membrane proteins were incubated in the presence of: (A) LDL (20 μ g/ml) without or with inclusion of Fab fragments prepared from rabbit anti-LDL immunoglobulins ($\bullet$) and rabbit non-immune serum ($\circ$); (B) the indicated concentrations of LDL without ($\triangle$) or with ($\blacktriangle$) inclusion of 200 μ g/ml goat anti-LDL antibodies. After autoradiographic visualization of LDL-binding proteins, specific binding to 105/130 kDa proteins was quantified. The Figure shows matching autoradiographic and quantitative data from individual representative experiments. Experiments performed on membranes from human aortic medial tissue yielded qualitatively comparable results (not shown) to those presented in this Figure. Experiments were performed on each of the different membrane preparations twice.

LDL to 105/130 kDa proteins is not influenced by a 50- to 500-fold excess of either basic proteins (protamine or histone) (Figure 5A) or low-molecular-mass polycations (spermine or putrescine) (Figure 5B).

Effects of anti-LDL antibodies

We examined the effects of anti-LDL antibodies on LDL binding to 105/130 kDa proteins in human aortic VSMC membranes using Fab fragments prepared from goat or rabbit anti-LDL immunoglobulins which were affinity-purified on columns with immobilized LDL. Both rabbit anti-LDL (Figure 6A) and goat anti-LDL (Figure 6B) effectively blocked specific binding of LDL, whereas non-immune rabbit Fab (Figure 6A) was without effect. The effect of anti-LDL could be overcome at high concentrations of LDL, this being in agreement with an expected competitive mechanism of inhibition (Figure 6B). In three additional experiments (results not shown) using goat anti-LDL Fab preparations and membranes from both human aortic and mesenteric VSMC isolates, half-maximal inhibition of binding to 105/130 kDa proteins was consistently obtained at Fab concentrations approx. 25-fold molar in excess of LDL.

Effects of non-esterified fatty acid

The lipolysis-stimulated receptor (LSR) is a receptor whose lipoprotein-binding activity is stimulated by non-esterified fatty acids [45]. In order to test whether LDL-binding activity of 105/130 kDa proteins might be regulated in the same way, oleic acid or sodium oleate was included with blots during both preincubation of blots and incubation with LDL. Since non-esterified fatty acids can bind to albumin and other proteins, the effects of oleic acid or sodium oleate were tested in three types of blocking/incubation solution: 5% non-fat milk, 0.2% albumin

(fraction IV) and 0.2 % fatty acid-free albumin. Both free acid or salt were added from stock solutions in isopropanol [36] to give final concentrations ranging from 3 μ M to 3 mM. Neither oleic acid nor sodium oleate was observed to have any effect on LDL binding (results not shown), a finding that was true for all incubation conditions.

DISCUSSION

Variant forms of lipoprotein-binding proteins

We have previously demonstrated that membranes from aortic medial tissue contain atypical lipoprotein-binding proteins, namely a major 105 kDa LDL-binding protein and a minor 130 kDa LDL-binding protein [37]. The present study demonstrates that lipoprotein-binding proteins with molecular masses of 105 and 130 kDa are also present in membranes from cultured human (aortic and mesenteric) and rat (aortic) VSMCs. The relative LDL-binding activity of the two proteins in VSMC membranes (major in 130 kDa and minor in 105 kDa) was opposite to that in membranes from aortic medial smooth-muscle cells. We have not yet determined whether this difference in LDL-binding activity is an artefact of cell culture. Nevertheless, the presently tested properties of 105 kDa and 130 kDa proteins in smooth-muscle cell membranes from both medial tissue and cultured cells are apparently identical, the only exception being expression of RAP-binding activity and VLDL-receptor immunoreactivity within the 105 kDa protein band of membranes from cultured VSMCs. A striking similarity of properties suggests that 105 kDa and 130 kDa proteins may represent variant forms of the same protein.

Mechanisms of binding of LDL to lipoprotein-binding proteins

A major objective of the present study was to study the ligand binding properties of lipoprotein-binding proteins of VSMCs in order to shed light on mechanisms of lipoprotein recognition. Previous studies on apo B,E and scavenger receptors have revealed that at least two factors are important for lipoprotein binding. The first factor relates to ionic interactions. Modification of positive charges of lysine or arginine residues of apo B or apo E, as well as chemical modifications that confer extra negative charge on apo B, block LDL binding to the apo B,E receptor [43]. Such modifications render LDL recognizable to the scavenger receptor [5]. Lipoprotein binding to apo B,E receptor is inhibited by negatively and positively charged molecules, such as dextran sulphate, heparin, protamine and others [43,44], while binding to scavenger receptor is sensitive to dextran sulphate, fucoidin and other polyanions [5]. Nevertheless, net charge in itself is not sufficient for high-affinity binding. The second, and equally important, factor concerns spatial distribution of charges. This is well illustrated by the fact that in spite of the net negative charge of apo B and apo E, only clusters of basic residues are recognized, evidently by complementary clusters of negatively charged amino acids within the C-termini of cysteine-rich ligand-binding repeats of apo B,E receptor [1].

The role of charge distribution is even more dramatic in the case of the scavenger receptor which is known to recognize only specific polyanions. For example, in spite of similar chemical structures, polyinosinic acid binds to the receptor while polyadenylic acid does not [5]. Specificity of recognition by the scavenger receptor is also evident for sulphated glycosaminoglycans, whereby dextran sulphate blocks the scavenger receptor while heparin does not [5,46]. The basis for such selectivity is thought to be a distribution of clusters of positively charged

residues and conserved negatively charged residues in the collagen-like receptor domain [4]. It is clear that the surface charge of the ligand should be complementary to that of the receptor ligand-binding domain. However, while ionic interactions play an important role in mechanisms of molecular recognition by lipoprotein receptors, mere charge on ligands is an insufficient determinant, albeit a necessary prerequisite, for high-affinity binding.

We have found that binding of LDL to the lipoprotein-binding proteins of VSMCs is insensitive to a variety of polyanions and polycations, the only exception being the polyanion dextran sulphate. Inhibition by the latter may either reflect a genuine ligand selectivity, similar to that described for the scavenger receptor [5], or may be due to formation of insoluble LDL-dextran sulphate complexes [46]. In any case, the lack of inhibition by several other oppositely charged compounds rules out simple ionic interaction as the sole mechanism of binding of LDL to 105/130 kDa lipoprotein-binding proteins. This conclusion is in agreement with our previous observation that the binding is only weakly inhibited by high ionic strength [37], a finding that also suggests binding is not due to hydrophobic interactions, these being favoured in the presence of high salt. Therefore, as for molecular recognition of lipoproteins by other receptors, interaction between LDL and 105/130 kDa lipoprotein-binding proteins involves mechanisms more complex than pure electrostatic or hydrophobic interactions.

All presently known ligands of the LDL receptor family members are lipoproteins or proteins. In the case of lipoproteins, and as suggested by the distinct lipoprotein specificity of each of the family members, the apoprotein rather than the lipid moiety is recognized. The important role of apoprotein in recognition of lipoproteins by 105/130 kDa lipoprotein-binding proteins is supported by their distinctive lipoprotein selectivity, namely a 100-fold greater affinity for both LDL and VLDL as compared with apo E-free high-density lipoprotein (HDL) (this study and [37]). This selectivity is not due to the lower size and therefore lower lipid content of HDL particles, since VLDL, which has a higher amount of lipids per apo B molecule (versus LDL), bound to 105/130 kDa proteins with the same affinity as LDL. These results rather indicate that smooth-muscle-cell lipoprotein-binding proteins preferentially recognize apo B-containing lipoproteins. In agreement with this interpretation are the results demonstrating inhibition of LDL binding to 105/130 kDa proteins by anti-LDL antibodies. We assume that apo B is the main, if not the only, immunogen in the LDL particle and thus the inhibitory actions of anti-LDL most likely reflect a masking of apo B rather than some lipid moiety of the lipoprotein. While we surmise that 105/130 kDa proteins recognize protein rather than lipid constituents of LDL particles, spatial arrangement of the whole lipoprotein particles might also be relevant.

Comparison of 105/130 kDa lipoprotein-binding proteins with known types of lipoprotein receptors

The identity of 105/130 kDa proteins is not known, but our data suggest that these proteins are unique. We tested effects of all groups of substances previously shown to antagonize ligand binding to either the LDL (apo B,E) receptor (dextran sulphate, heparin, PPS, protamine, spermine, histone), the scavenger receptor (dextran sulphate, fucoidin), the VLDL receptor (RAP), or LRP (RAP, α_2 -M, LPL, exo-A). None of these substances, with the exception of dextran sulphate, exerted any effect on binding of LDL to 105/130 kDa proteins. Ligand blotting experiments with RAP as ligand also indicated that RAP does

not bind to 105/130 kDa proteins with high affinity. We did not find any effect of oleic acid, a known stimulus for the lipoprotein-binding activity of the lipolysis-stimulated receptor. Finally, no other lipoprotein-binding receptors have been shown to exist as two or three (in rat VSMCs) closely migrating forms which bind LDL with apparently identical properties. Thus we conclude that the 105/130 kDa lipoprotein-binding proteins of smooth-muscle cells differ from all currently recognized lipoprotein receptors.

While our data clearly demonstrate a unique ligand-binding profile of 105/130 kDa proteins, these proteins do share some binding properties with other members of the LDL receptor family. First, all members of the family, including apo B,E receptor [47], LRP [27], gp330/megalin [19], VLDL receptor [22], and chicken vitellogenin receptor [48] are capable of specific and high-affinity ligand binding on Western blots. This indicates that ligand-recognizing domains possess a structure which is stable to the strong denaturing conditions of SDS/gel electrophoresis. The physiological importance of high stability of the receptors is the necessity to tolerate exposure to extracellular medium and acidic environment of endosomes in the multiple cycles of endocytosis. The structural basis of such stability evidently resides in the presence of disulphide bonds. Each ligand-binding repeat contains six cysteines, and it is known that they cannot be labelled by [³H]iodoacetamide without prior reduction, suggesting that all of them are involved in disulphide linkages [1]. The important role of disulphide bonds in ligand recognition is evidenced by the fact that under reducing conditions the lipoprotein-binding activity of all LDL receptor family members [27,47,49,50], as well as the lipoprotein-binding proteins of smooth-muscle cells [37], is significantly inhibited. Secondly, the ligand-binding activity of all members of the LDL receptor family [17,27,41,47–49,51] as well as smooth-muscle-cell lipoprotein-binding proteins [37] depends on the presence of bivalent cations. This bivalent-cation-dependence is based on the Ca²⁺-binding ability of cysteine-rich ligand-binding repeats, which results in conformational changes [52]. Since the known LDL receptor family members [27,47–51] and the lipoprotein-binding proteins of smooth-muscle cells [37] display similar binding properties, namely a requirement for Ca²⁺ and intact disulphide bonds for full activity, it is tempting to speculate that they might also possess structural analogies.

In conclusion therefore, our results suggest that smooth-muscle cell membranes express variant forms (105/130 kDa) of a lipoprotein-binding protein, their unique ligand selectivity being indicative of an identity that is distinct from other LDL receptor family members. Lipoprotein recognition by 105/130 kDa proteins apparently involves apo B. Some similarities of binding properties suggest that the ligand-binding domains of 105/130 kDa lipoprotein-binding proteins may be structurally analogous to those of the LDL receptor family. Purification of the proteins is currently under way in order that we may proceed with detailed molecular and structural analyses.

This study was supported by the Swiss National Foundation (no. 32.41874.94), INTAS (93-3260), the Ciba Geigy Jubiläums Stiftung and the Helmut Horten Foundation.

REFERENCES

- Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. and Schneider, W. J. (1985) *Annu. Rev. Cell. Biol.* **1**, 1–39
- Brown, M. S. and Goldstein, J. L. (1986) *Science (Washington D. C.)* **232**, 34–37
- Schneider, W. J. (1989) *Biochim. Biophys. Acta* **988**, 303–317
- Krieger, M. and Herz, J. (1994) *Annu. Rev. Biochem.* **63**, 601–637
- Brown, M. S., Basu, S. K., Falck, J. R., Ho, Y. K. and Goldstein, J. L. (1980) *J. Supramol. Struct.* **13**, 67–81
- Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333–337
- Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) *N. Engl. J. Med.* **320**, 915–924
- Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. and Russell, D. W. (1984) *Cell* **39**, 27–38
- Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H. and Stanley, K. K. (1988) *EMBO J.* **7**, 4119–4127
- Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M. and Argraves, W. S. (1990) *J. Biol. Chem.* **265**, 17401–17404
- Takahashi, S., Kawarabayashi, Y., Nakai, T., Sakai, J. and Yamamoto, T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9252–9256
- Sakai, J., Hoshino, A., Takahashi, S., Miura, Y., Ishii, H., Suzuki, H., Kawarabayashi, Y. and Yamamoto, T. (1994) *J. Biol. Chem.* **269**, 2173–2182
- Saito, A., Pietromonaco, S., Loo, A. K.-C. and Farquhar, M. G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9725–9729
- Barber, D. L., Sanders, E. J., Aebersold, R. and Schneider, W. J. (1991) *J. Biol. Chem.* **266**, 18761–18770
- Yochem, J. and Greenwald, I. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4572–4576
- Russell, D. W., Brown, M. S. and Goldstein, J. L. (1989) *J. Biol. Chem.* **264**, 21682–21688
- Kowal, R. C., Herz, J., Weisgraber, K. H., Mahley, R. W., Brown, M. S. and Goldstein, J. L. (1990) *J. Biol. Chem.* **265**, 10771–10779
- Chappell, D. A., Fry, G. L., Wanknitz, M. A., Iverius, P.-H., Williams, S. E. and Strickland, D. K. (1992) *J. Biol. Chem.* **267**, 25764–25767
- Willnow, T. E., Goldstein, J. L., Orth, K., Brown, M. S. and Herz, J. (1992) *J. Biol. Chem.* **267**, 26172–26180
- Bu, G., Williams, S., Strickland, D. K. and Schwartz, A. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7427–7431
- Kounnas, M. Z., Morris, R. E., Thompson, M. R., Fitzgerald, D. J., Strickland, D. K. and Saelinger, C. B. (1992) *J. Biol. Chem.* **267**, 12420–12423
- Batley, F. D., Gafvels, M. E., Fitzgerald, D. J., Argraves, W. S., Chappell, D. A., Strauss, J. F. and Strickland, D. K. (1994) *J. Biol. Chem.* **269**, 23268–23273
- Strickland, D. K., Ashcom, J. D., Williams, S., Batley, F., Behre, E., McTigue, K., Batley, J. F. and Argraves, W. S. (1991) *J. Biol. Chem.* **266**, 13364–13369
- Kounnas, M. Z., Argraves, W. S. and Strickland, D. K. (1992) *J. Biol. Chem.* **267**, 21162–21166
- Orlando, R. A., Kerjaschki, D., Kurihara, H., Biemesderfer, D. and Farquhar, M. G. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6698–6702
- Moestrup, S. K. and Gliemann, J. (1991) *J. Biol. Chem.* **266**, 14011–14017
- Mokuno, H., Brady, S., Kotite, L., Herz, J. and Havel, R. J. (1994) *J. Biol. Chem.* **269**, 13238–13243
- Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K. and Brown, M. S. (1991) *J. Biol. Chem.* **266**, 21232–21238
- Tensen, C. P., van Kesteren, E. R., Planta, R. J., Cox, K. J. A., Burke, J. F., van Heerikhuizen, H. and Vreugdenhil, E. A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4816–4820
- Block, L. H., Knorr, M., Vogt, E., Locher, R., Vetter, W., Groscurth, P., Qiao, B.-Y., Pometta, D., James, R., Regenass, M. and Pletscher, A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 885–889
- Scott-Burden, T., Resink, T. J., Hahn, A. W. A., Baur, U., Box, R. J. and Buhler, F. R. (1989) *J. Biol. Chem.* **264**, 12582–12589
- Smirnov, V. N., Voyno-Yasenetskaya, T. A., Antonov, A. S., Lukashev, M., Shirinsky, V. P., Tertov, V. V. and Tkachuk, V. A. (1988) *Ann. N. Y. Acad. Sci.* **598**, 167–181
- Resink, T. J., Rybin, V., Bernhardt, J., Orlov, S., Buhler, F. R. and Tkachuk, V. A. (1993) *J. Vasc. Res.* **30**, 169–180
- Bochkov, V. N., Tkachuk, V. A., Kuzmenko, Y. S., Borisova, Y. L., Buhler, F. R. and Resink, T. J. (1994) *Mol. Pharmacol.* **45**, 262–270
- Tkachuk, V. A., Kuzmenko, Y. S., Resink, T. J., Stambolsky, D. V. and Bochkov, V. N. (1994) *Mol. Pharmacol.* **46**, 1129–1137
- Pedreno, J., de Castellarnau, C., Collaré, C., Sanchez, J., Gomez-Gerique, J., Ordóñez-Llanos, J. and Gonzalez-Sastre, F. (1992) *Arteriosclerosis Thromb.* **12**, 1353–1362
- Kuzmenko, Y., Bochkov, V. N., Philippova, M. P., Tkachuk, V. A. and Resink, T. J. (1994) *Biochem. J.* **303**, 281–287
- Havel, R. J., Eder, H. A. and Bragdon, H. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
- Harlow, E. and Lane, D. (1988) in *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York
- Simonsen, A. C. W., Heegaard, C. W., Rasmussen, L. K., Ellgaard, L., Kjoller, L., Christensen, A., Elzerodt, M. and Andreasen, P. A. (1994) *FEBS Lett.* **354**, 279–283
- Friedman, G., Wernette-Hammond, M. E., Hui, D. Y., Mahley, R. W. and Innerarity, T. L. (1987) *J. Lipid Res.* **28**, 1482–1494
- Williams, S. E., Ashcom, J. D., Argraves, W. S. and Strickland, D. K. (1992) *J. Biol. Chem.* **267**, 9035–9040

- 43 Goldstein, J. L., Basu, S., Brunschede, G. Y. and Brown, M. S. (1976) *Cell* **7**, 85–95
- 44 Brown, M. S., Deuel, T. F., Basu, S. K. and Goldstein, J. L. (1978) *J. Supramol. Struct.* **8**, 223–234
- 45 Yen, F. T., Mann, C. J., Guermani, L. M., Hannouche, N. F., Hubert, N., Hornick, C. A., Bordeau, V. N., Agnani, G. and Bihain, B. E. (1994) *Biochemistry* **33**, 1172–1180
- 46 Iverius, P.-K. (1972) *J. Biol. Chem.* **247**, 2607–2613
- 47 Daniel, T. O., Schneider, W. J., Goldstein, J. L. and Brown, M. S. (1993) *J. Biol. Chem.* **258**, 4606–4611
- 48 George, R., Barber, D. L. and Schneider, W. J. (1987) *J. Biol. Chem.* **262**, 16838–16847
- 49 Moestrup, S. K., Holtet, T. L., Etzerodt, M., Thogersen, H. C., Nykjaer, A., Andreasen, P. A., Rasmussen, H. H., Sottrup-Jensen, L. and Gliemann, J. (1993) *J. Biol. Chem.* **268**, 13691–13696
- 50 Stifani, S., George, R. and Schneider, W. J. (1988) *Biochem. J.* **250**, 467–475
- 51 Lund, H., Takahashi, K., Hamilton, R. L. and Havel, R. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9318–9322
- 52 van Driel, I. R., Goldstein, J. L., Sudhof, T. C. and Brown, M. S. (1987) *J. Biol. Chem.* **262**, 17443–17449

Received 2 November 1995/11 March 1996; accepted 18 March 1996