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Solubilization of apolipoprotein B and its specific binding by the cellular receptor for low density lipoprotein

(fibroblast/lymphocyte/hyper-β-lipoproteinemia/LDL degradation/apolipoprotein B–bovine serum albumin complex)

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ABSTRACT **Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) bind specifically to a receptor on fibroblasts, and it has been postulated that the apoprotein of LDL (apo B) confers the specificity of cellular binding. This hypothesis has been tested in the present study with a water-soluble apo B–bovine serum albumin complex. The binding of ^{125}I-labeled apo B to cultured fibroblasts was temperature-dependent. Specific binding ranged between 183 and 859 ng/mg of cell protein at a concentration of 5 μg/ml; at 37°, 750–2199 ng/mg was bound and internalized. The binding of apo B greatly exceeded the amount of ^{125}I-labeled LDL bound at 4° and 37° in the same experiment. Fibroblasts from a subject homozygous for hyper-β-lipoproteinemia showed minimal binding of ^{125}I-labeled LDL, consistent with the absence of the cellular LDL receptor. Such cells also had depressed binding of ^{125}I-labeled apo B.**

Lymphocytes grown in lipoprotein-deficient medium demonstrated specific binding of LDL; however, freshly isolated lymphocytes did not show such binding. The binding of ^{125}I-labeled apo B to lymphocytes paralleled the binding of ^{125}I-labeled LDL. Unlabeled LDL and apo B–albumin complex both competitively inhibited the binding of ^{125}I-labeled apo B and ^{125}I-labeled LDL to fibroblasts. When labeled LDL was incubated with fibroblasts for 6 hr at 37°, it underwent cellular internalization and degradation, as measured by the release of ^{125}I-labeled fragments into the medium. This degradation was inhibited by unlabeled apo B. Conversely, ^{125}I-labeled apo B also was internalized and degraded by fibroblasts, and this process was inhibited by LDL. These findings demonstrate that apo B binds specifically to the LDL receptor and that the cellular binding of LDL is determined by this apoprotein.

The structure of apo B, the apoprotein of low density lipoprotein (LDL), remains a subject of controversy (1, 2). The water insolubility of this protein is certainly the major reason for the slow progress in elucidating its structure, and attempts at solubilization with detergents and dissociating agents (3, 4) or by chemical modification (5, 6) have yielded conflicting data with regard to its size and other physical properties.

apo B is an important protein because it is a component of VLDL and is the major, if not the only, protein in LDL. It has been suggested that the binding of LDL and VLDL to receptors on human fibroblasts involves apo B (7). The insolubility of the protein has precluded testing this hypothesis; however, an understanding of this cellular receptor–lipoprotein interaction is of utmost biological importance.

We report here our method for solubilizing delipidated apo B and describe its binding properties with the plasma membrane receptor of human fibroblasts.*

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METHODS

LDL was isolated from plasma in the presence of 0.02% sodium azide and 0.01% Merthiolate by differential density preparative ultracentrifugation as described (9). The apoprotein homogeneity of the isolated LDL was routinely monitored by sodium dodecyl sulfate/acrylamide gel electrophoresis (10). The protein concentration of LDL was determined by the method of Lowry *et al.* (11), and radioiodination was performed by a modification (12) of the iodine monochloride technique of MacFarlane (13). Urea was prepared free of cyanate by passage over a Rexyn I-300 (Fisher) column, the conductivity of the solution being less than 2 μmho (14). Lipoprotein-deficient serum was prepared by removal of the lipoprotein from human serum by preparative ultracentrifugation at a solution density of 1.20 g/ml adjusted by the addition of KBr. The serum was dialyzed against an isotonic Tris buffer (15) and sterilized by filtration.

Preparation of apo B. Prior to delipidation, LDL was dialyzed against 0.2 M NH_4HCO_3/0.02% NaN_3. The solution was then made 6 M in guanidine·HCl and precooled to 4° prior to four gentle extractions with cold diethyl ether/ethanol (3:1), followed by two extractions with cold diethyl ether.† The excess ether was removed by a jet of nitrogen while the protein solution was kept in ice. The delipidated apo B was dialyzed at 4° against two exchanges of 6 M guanidine·HCl/0.2 M NH_4HCO_3/0.02% NaN_3 followed by dialysis against a similar solution but also containing 6 M urea. Thereafter, the guanidine was omitted and the urea concentration decreased to 2 M stepwise (apo B precipitates if the urea concentration is decreased further). When delipidation is performed carefully, apo B remains fully soluble and there is no measurable loss of protein into the ether extracts. The total lipid content of apo B was measured as described by Bragdon (16) and the phospholipid content, by the method of Baginski and Zak (17).

The apo B solution was concentrated on an Amicon UM 10 membrane and was prepared for tissue culture studies by addition of 5 mg of bovine serum albumin (Sigma: lyophilized and crystallized) per mg of apo B. The concentration of apo B was then adjusted to 1 mg/ml. The solution was dialyzed against water. The protein remained soluble if the pH was maintained above 7.0; the solubility decreased when the ionic strength was increased.

Abbreviations: apo B, the apolipoprotein of LDL; LDL, plasma low density lipoprotein; ^{125}I-LDL, ^{125}I-labeled LDL; ^{125}I-apo B, ^{125}I-labeled apo B; LDM, lipoprotein-deficient medium.

* A preliminary report of these findings has been published (8).

† Because apo B must be kept in a mildly alkaline solution during delipidation, after addition of the guanidine·HCl to the LDL it may be necessary to adjust the pH to 8 with NaOH.

Viscosity measurements were performed at 25° by using a capillary viscometer with an outflow time of greater than 200 sec. Measurements were performed in 0.2 M NH_4HCO_3/0.2% NaN_3 containing either 6 M guanidine·HCl or 2 M urea.

Binding Experiments. Normal human fibroblasts, purchased from American Type Culture Collection, Rockville, MD, were maintained in culture and were used in the 13–16th passages. The protocol and assay for specific binding and uptake of ^{125}I-labeled LDL (^{125}I-LDL) were as described by Brown and Goldstein (7, 18). Cells were seeded into 35 × 10 mm Falcon plastic dishes at a density of 2.0×10^5 cells per plate and were grown to confluency. Eighteen hours before the experiment, the medium on each plate was replaced by 2 ml of lipoprotein-deficient medium. In the experiments, cells were incubated with 1 ml of medium containing the indicated concentrations of ^{125}I-LDL or ^{125}I-labeled apo B (^{125}I-apo B)–bovine serum albumin complex at either 4° or 37°. Unlabeled LDL or apo B–albumin complex was added as indicated in the legends to the figures.

The cells were then washed at 4° three times with 2 ml of an isotonic Tris buffer (15) containing bovine serum albumin (2 mg/ml) and three additional times with the Tris buffer alone. The cells were removed from each plate with 1.0 ml of 0.1 M NaOH and their radioactivity was determined in a gamma counter. Aliquots (0.2 ml) were then taken for protein determination. Binding was expressed as ng of ^{125}I-LDL or ^{125}I-apo B bound per mg of cell protein. Specific binding was calculated as the difference between the amounts bound in the absence and presence of excess unlabeled LDL.

Homozygous mutant fibroblasts (GM-2000 cells) lacking the LDL receptor were obtained from The Human Genetic Cell Repository, Camden, NJ and were maintained in culture as described for normal cells. The GM-2000 cells used in binding experiments were in the fifth to eighth passages (7).

In experiments determining the degradation of ^{125}I-LDL or ^{125}I-apo B, the cell monolayers were prepared as in the binding studies. The cells were incubated for 6 hr with the indicated concentrations of labeled LDL or labeled apo B–albumin. The medium was then drawn off, the protein was precipitated with trichloroacetic acid, and free radioiodide was removed by chloroform extraction after addition of KI and oxidation with H_2O_2 (7). Radioactivity in aliquots of the aqueous phase was assayed and, expressed as cpm/mg of cell protein, represented ^{125}I-labeled peptide degradation products of ^{125}I-apo B. As a control for nonspecific proteolysis by plasma proteases, ^{125}I-LDL or ^{125}I-apo B was incubated in tissue culture dishes with the medium used in these experiments but in the absence of cells.

Lipoprotein binding studies were performed with peripheral blood lymphocytes isolated by a modification of the method of Boyum (19). Whole venous blood was layered over a solution of 6% Ficoll 400 (Pharmacia, Uppsala, Sweden) and 12.5% Hypaque (diatrizoate sodium) and then centrifuged at 500 × *g* for 40 min. Recovery of the mononuclear cell fraction and the maintenance of these cells in lipoprotein-deficient medium were as described by Ho *et al.* (20). The binding of ^{125}I-LDL and ^{125}I-apo B to lymphocytes was assayed at specified times from 0 to 72 hr, as described by Ho *et al.* (20, 21).

RESULTS

Fig. 1 shows the appearance of isolated LDL when electrophoresed on 5% acrylamide gels in the presence of sodium dodecyl sulfate. After delipidation, the apo B showed a similar pattern on electrophoresis, with only a large molecular weight protein revealed. Lipid analyses of the apo B revealed less than 2% by weight as total lipid and less than 1% as phospholipid.

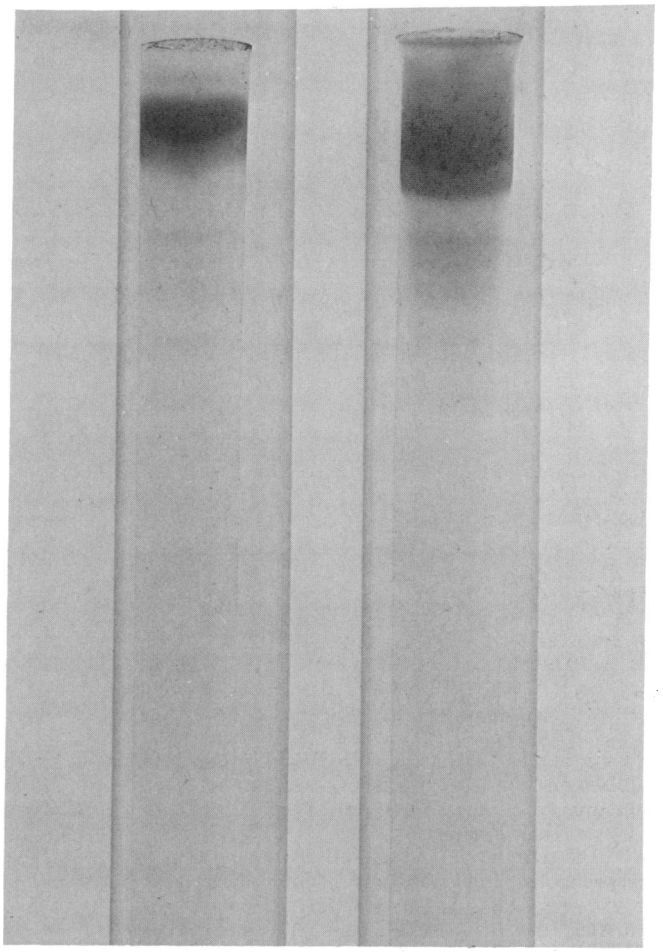

FIG. 1. Electrophoresis of LDL (*Left*) and apo B (*Right*) on 5% acrylamide gels in the presence of 0.1% sodium dodecyl sulfate at pH 7.2. LDL was isolated by ultracentrifugation; apo B was prepared by delipidation in the presence of 6 M guanidine. Protein concentration, approximately 40 μg per gel.

Viscosity measurements in 6 M guanidine indicated an extremely large molecular domain;‡ however, when the solvent was changed to 2 M urea, the intrinsic viscosity of apo B was 14 cm^3/g, indicating that the protein had assumed a more compact conformation.

Although soluble in 2 M urea/0.2 M NH_4HCO_3 up to a concentration of 3 mg/ml, apo B precipitates upon further decrease of the urea concentration. The addition of bovine serum albumin renders the apo B soluble in water at an apo B concentration of 1 mg/ml or less, provided that the pH is maintained above 7.0. The solubility of the apo B–albumin complex is decreased upon the addition of salts.

When normal fibroblasts were incubated for 2 hr in medium containing increasing concentrations of ^{125}I-LDL at 37° or 4°, temperature-dependent high-affinity binding of LDL could be measured, as initially reported by Brown and Goldstein Brown (18) (Fig. 2 *C* and *D*). On repeated experiments, the magnitude of LDL binding, at a concentration of 5 μg/ml, varied from 136 to 370 ng/mg of cell protein at 37° and from 41 to 268 ng/mg at 4°.

Under similar conditions the temperature-dependent binding of ^{125}I-apo B was also demonstrated, and specific binding of apo

‡ Viscosity data of apo B in 6 M guanidine are difficult to interpret because of partial aggregation of the protein in this solvent (J. A. Reynolds, personal communication).

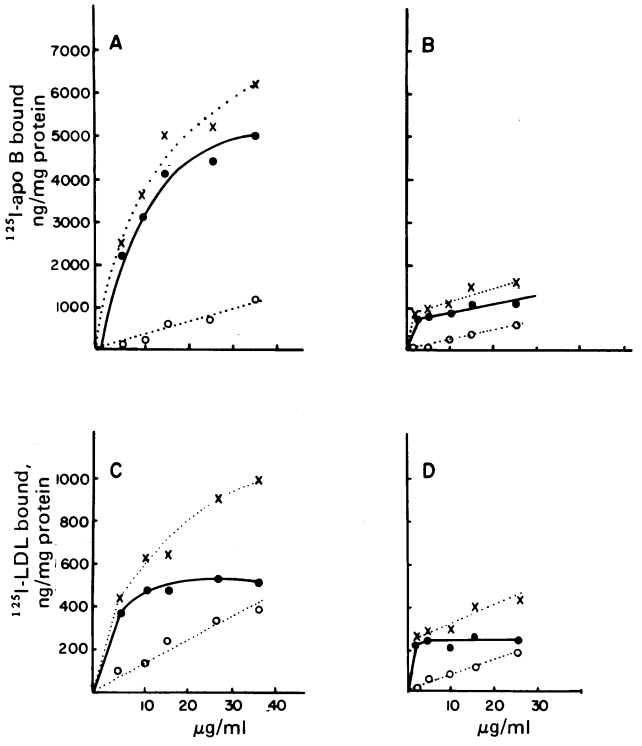

FIG. 2. ^{125}I-LDL and ^{125}I-apo B binding to fibroblasts at 37° and 4°. Normal human fibroblasts were incubated in 1 ml of medium containing increasing concentrations of ^{125}I-apo B–albumin complex 1:5 (wt/wt), at 37° (*A*) or 4° (*B*) or in medium with ^{125}I-LDL at 37° (*C*) or 4° (*D*). Specific binding (●) was calculated as the difference between total binding (×) and binding in the presence of native LDL at 250 μg/ml (○). At 37°, the amount bound includes that protein internalized by the cells. Each point represents a single determination. There was 88 ± 6 μg of cell protein per dish. The specific activities of the ^{125}I-LDL and ^{125}I-apo B were 94 and 51 cpm/ng.

B to the LDL receptor was indicated (Fig. 2 *A* and *B*). For apo B, the magnitude of binding, also at a concentration of 4 μg/ml, ranged from 750 to 2199 ng/mg of cell protein at 37° and from 183 to 859 ng/mg at 4°.

The binding and uptake of ^{125}I-apo B were time-dependent at 37°, but at 4° the magnitude of binding remained constant over 4 hr (Fig. 3).

The inhibition of cellular binding of ^{125}I-LDL and of ^{125}I-apo B by unlabeled LDL and apo B was also measured (Fig. 4). The results demonstrate that the cellular binding of both LDL and apo B was inhibited by either LDL or apo B, thus providing evidence for binding to the same cellular site. These experiments were performed at 37°, and hence both cellular binding and uptake of lipoprotein were measured (22). A 50% decrease of ^{125}I-LDL binding and uptake occurred at an LDL protein concentration of 18 μg/ml in the medium, whereas a concentration of approximately 30 μg/ml of apo B was required to produce the same effect. The cellular binding of ^{125}I-apo B also was inhibited more effectively by LDL than by the apo B–albumin complex. In these experiments bovine serum albumin alone did not inhibit either ^{125}I-LDL or ^{125}I-apo B binding to fibroblasts.

In order to demonstrate that binding of apo B is by the LDL receptor, studies were performed with fibroblasts from subjects homozygous for the disease familial hyper-β-lipoproteinemia. The cells of these individuals lack the high affinity LDL receptor (7). As shown in Fig. 5, these cells had decreased binding of both LDL and apo B.

Cultured lymphocytes also demonstrate a receptor for LDL

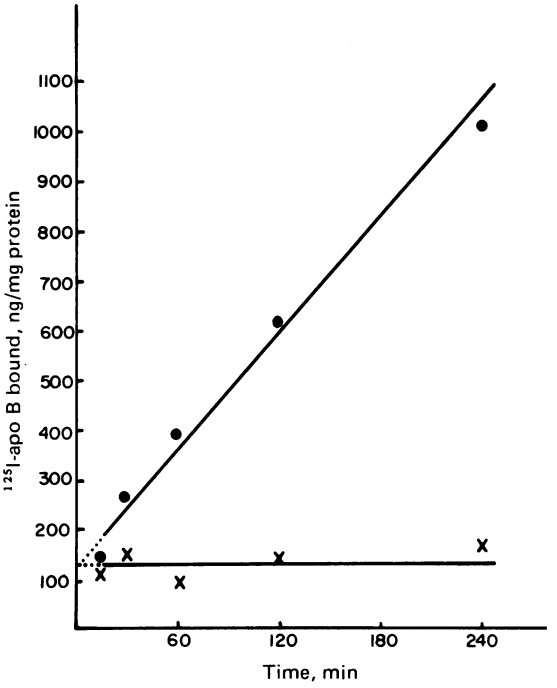

FIG. 3. Time-dependent specific binding of ^{125}I-apo B to fibroblasts at two temperatures. Cells were incubated in medium containing ^{125}I-apo B at 2.5 μg/ml for the indicated periods of time. Values were determined as the difference between total binding and binding in the presence of unlabeled LDL at 250 μg/ml. Each value represents duplicate determinations. ×, Incubation at 4°; ●, incubation at 37°.

after a period of incubation in LDL-deficient medium; however, freshly harvested lymphocyte binds LDL only minimally (20, 21). This system provided a further opportunity to determine whether apo B and LDL have similar cellular binding properties. As shown in Fig. 6, the binding of both substances by freshly harvested lymphocytes was low and increased upon prior incubation of the lymphocytes in LDL-deficient medium.

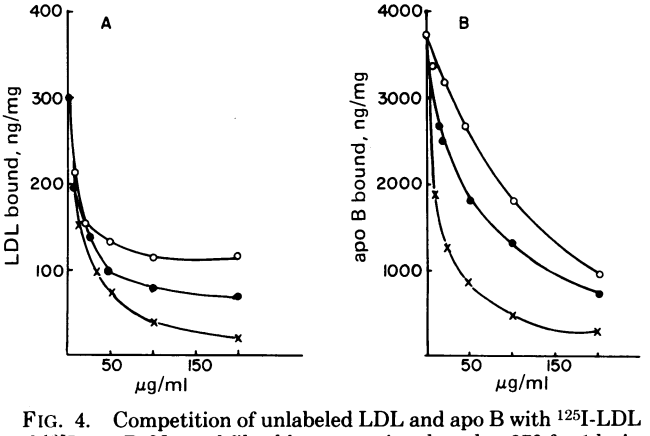

FIG. 4. Competition of unlabeled LDL and apo B with ^{125}I-LDL and ^{125}I-apo B. Normal fibroblasts were incubated at 37° for 1 hr in medium containing ^{125}I-LDL at 15 μg/ml (specific activity, 17 cpm/ng) (*A*) plus various concentrations of unlabeled LDL (●) or unlabeled apo B–albumin, 1:5, (wt/wt) (○). Each point represents the average of duplicate determinations. (*B*), The cells were incubated for 2 hr with medium containing ^{125}I-apo B–albumin at 15 μg/ml (specific activity, 10 cpm/ng) and the indicated concentrations of unlabeled LDL (●) or apo B–albumin (○). In both studies, the average of cell protein per plate was 85 ± 15 μg. ×, Theoretical curve for the inhibition of competitive binding by the addition of unlabeled LDL (*A*) or unlabeled apo B (*B*).

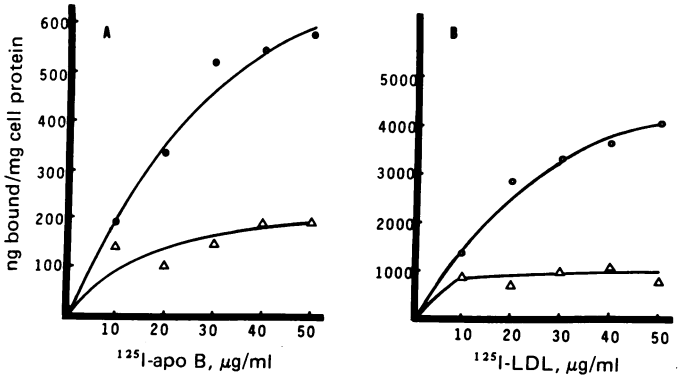

FIG. 5. Binding of LDL and apo B to normal fibroblasts (○) and to fibroblasts grown from a subject with familial hyper-β-lipoproteinemia (△). Cells were incubated for 2 hr at 37° in medium containing increasing concentrations of ^{125}I-LDL (*A*) or ^{125}I-apo B–albumin (*B*).

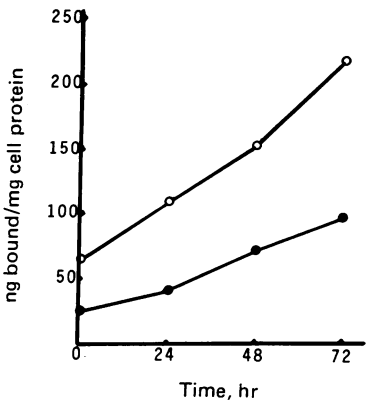

FIG. 6. Binding of ^{125}I-LDL (●) and ^{125}I-Apo B (○) to normal human peripheral blood lymphocytes that had been incubated in LDL-free medium for the indicated length of time and exposed to ^{125}I-LDL or ^{125}I-apo B–albumin at 25 μg/ml in the presence or absence of native LDL at 250 μg/ml for 30 min at 4°. Specific binding was calculated as the difference between total binding and binding in the presence of excess native LDL.

The binding of ^{125}I-LDL to the plasma membrane receptor is the first step in a sequence of cellular processes involving the internalization of the lipoprotein, its degradation with release into the medium of radioiodinated peptides, and the modulation of the cellular metabolism of cholesterol (7, 23). When ^{125}I-LDL was incubated at 37° for 6 hr at concentrations of 10–100 μg/ml in the absence or in the presence of either unlabeled LDL or apo B, the degradation of ^{125}I-LDL was inhibited by either LDL or apo B as measured by the release into the medium of soluble peptides (Fig. 7*A*). Thus, in the presence of excess apo B, the binding and therefore the internalization and degradation of ^{125}I-LDL were inhibited.

In like manner, the binding of ^{125}I-apo B to the receptor apparently was followed by the internalization and degradation of apo B, as indicated by the data in Fig. 7*B*. In the presence of excess unlabeled LDL, the binding and degradation of ^{125}I-apo B were similarly inhibited.

DISCUSSION

The delipidation of LDL may be accomplished with diethyl ether/ethanol, without precipitation of apo B, if the lipoprotein is solubilized in mildly alkaline 6 M guanidine, the solution is maintained at 4°, and vigorous agitation is avoided. apo B so solubilized is extensively unfolded in 6 M guanidine but assumes a compact configuration in 2 M urea. By permitting apo B to complex with bovine serum albumin, it is possible to maintain the apoprotein in solution in the absence of denaturing agents or detergents, and thus the biological properties of apo B may be studied in living systems.

As shown in Fig. 2, apo B binds to fibroblasts under conditions that are similar to those for the binding of LDL. At 4° the binding appears to be saturable; however, at 37° saturation is not reached even at an apo B concentration of 35 μg/ml, possibly as a result of the cellular internalization of apo B which occurs at this temperature (Fig. 7). The hypothesis that apo B binds specifically to the high-affinity cellular LDL receptor is supported by the competitive inhibition between LDL and apo B for cellular binding (Fig. 4).

However, it has recently been reported that LDL binds to glass beads (24); we also observed that ^{125}I-LDL and ^{125}I-apo B–albumin bind to empty Falcon culture dishes, with binding being suppressed in the presence of unlabeled LDL. Therefore, the interpretation of data on the specificity of LDL and apo B binding to normal fibroblasts was not definite.

To ensure that the phenomena under study were of biological consequence, the investigation of apo B binding with receptor-deficient mutant fibroblasts and with lymphocytes was undertaken, and in each instance LDL and apo B behaved similarly (Figs. 5 and 6). Finally, the demonstration of the inhibition of the cellular degradation of LDL by apo B and the converse experiment (Fig. 7) appear to establish the biological import of the cellular binding of apo B. These studies also demonstrate that cellular metabolism of apo B occurs when the apoprotein is presented to the cell either in the delipidated state or as native LDL.

Because apo B was maintained in solution as a complex with bovine serum albumin, it was important to determine whether the albumin participated in the cellular binding of apo B. There was no appreciable binding of the albumin to LDL. Furthermore, in these experiments it was shown that the albumin does not alter the binding of ^{125}I-LDL to fibroblasts, and the presence of excess albumin does not affect the cellular binding of the ^{125}I-apo B–albumin complex. Although ^{125}I–albumin does bind to fibroblasts, the amount of binding is low compared to ^{125}I-LDL or ^{125}I-apo B–albumin. Thus, the available evidence indicates that bovine serum albumin does not bind to the LDL receptor on fibroblasts or to the receptor binding domain of LDL.

Binding of apo B to fibroblasts is saturable at 4° (Fig. 2) and the amount bound, with different apo B preparations, ranges between 183 and 859 ng of apo B per mg of cell protein. By contrast, when apo B is presented to the cell as native LDL, the amount bound varies between 41 and 268 ng/mg of cell protein. Thus, when apo B is delipidated and complexed with bovine serum albumin, the magnitide of its binding to cells is increased approximately 4-fold. Presumably, the binding interaction is between a single cellular receptor site and the binding domain of a single apo B molecule. Native LDL is thought to contain two apo B subunits (25). Accordingly, when LDL binds to the receptor, two molecules of apo B become fixed to the cell. When delipidated apo B complexes with bovine serum albumin the average number of apo B molecules per aggregate may well be greater than two and, if so, when such a particulate aggregate binds to the cell, the apparent binding of apo B in the aggregate would exceed that of apo B in LDL. In any event, it would seem unlikely that the cellular binding of apo B at 4° is freely reversible because the apo B–albumin complex must be re-formed if resolubilization of apo B is to be achieved.

The data points for the competitive inhibition for cellular

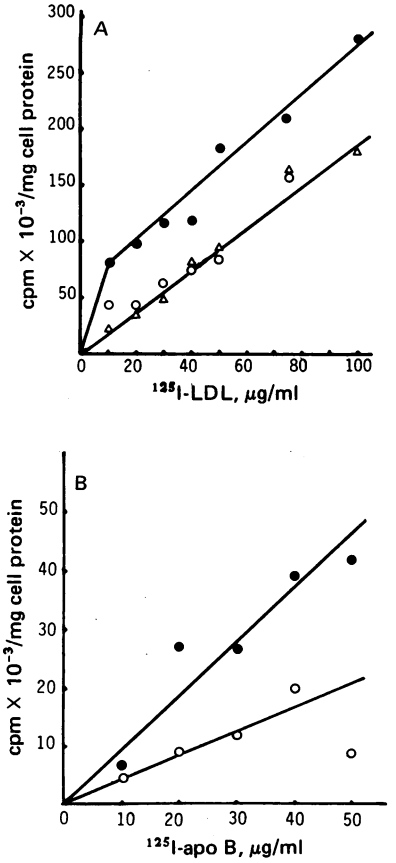

FIG. 7. Inhibition of cellular degradation of LDL and apo B by normal fibroblasts incubated at 37° for 6 hr. (*A*) Cells were incubated with increasing concentrations of ^{125}I-LDL (specific activity 13,300 dpm/μg) alone (●) or in the presence of unlabeled apo B (○) or unlabeled LDL (△) at 250 μg/ml. (*B*) Cells were incubated with increasing concentrations of ^{125}I-apo B-albumin (specific activity 9000 dpm/μg) alone (●) or in the presence of unlabeled LDL (○) at 250 μg/ml. The noniodide, trichloroacetic acid-soluble radioactivity, representing ^{125}I-labeled peptide degradation products released into the medium, was determined. Each point represents the average of duplicate determinations. These experiments were done with different cell populations.

binding of ^{125}I-LDL by LDL or ^{125}I-apo B by apo B (Fig. 4) do not fall on the expected theoretical curve. The explanation for this observation is not clear; however, it is certainly possible that an iodine atom is located strategically within the binding domain of the apoprotein so as to alter its binding affinity. In competition studies with ^{125}I-LDL and ^{125}I-apo B, LDL appears to compete more effectively than apo B for binding to the fibroblast receptor (Fig. 4). This conclusion, however, may well be spurious because the data are expressed as weight of apoprotein bound; but, as discussed previously, the extent of apo B aggregation is unknown and the number of molecules of apoprotein that became attached when a particle binds to the cell may well differ for LDL and for delipidated apo B.

In conclusion, it now appears that the cellular receptor for LDL recognizes apo B and that it is this apoprotein that confers specificity in the binding of LDL to the receptor.

Note Added in Proof. It has been shown that, as a consequence of the binding and internalization of LDL: (*i*) the number of cellular receptors for LDL decreases (26), and (*ii*) the cellular synthesis of cholesterol is suppressed (23). When fibroblasts were grown in lipoprotein-deficient medium (LDM) for 20 hr, the resulting increase in ^{125}I-LDL binding was suppressed by the addition of LDL (25 μg/ml) to the LDM as expected; however, the addition of apo B-albumin complex (25 μg/ml) to LDM failed to suppress ^{125}I-LDL binding. Similarly, fibroblasts grown for 20 hr in LDM show increased [^{14}C]-acetate incorporation into cholesterol which is profoundly suppressed by adding LDL (50 μg/ml) to LDM. The addition of up to 250 μg of apo B-albumin complex per ml to LDM did not suppress cholesterol synthesis.

Thus, apo B does not exert metabolic control on the number of cellular LDL receptors nor does it suppress cellular cholesterol synthesis. Rather, apo B appears to function as a transport protein for cholesterol and phospholipid and regulates the availability of these lipids to the cell by binding to a specific cellular receptor.

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