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APOLIPOPROTEIN B IS A CALCIUM BINDING PROTEIN

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SUMMARY: Human hepatocarcinoma Hep G2 cells were grown in culture medium containing [$^{45}Ca^{2+}$]. The secreted lipoproteins of d < 1.063 g/ml and d 1.063-1.21 g/ml were isolated from the culture media and analyzed by 3.3% and 7% SDS-polyacrylamide gel electrophoresis. Radioactivity profiles of [^{45}Ca] from the gels showed that the peak of radioactivity corresponded to the apolipoprotein B band. The molar ratio of the incorporated [$^{45}Ca^{2+}$] and apolipoprotein B was close to unity. No radioactivity was found associated with any other secreted apolipoproteins. To confirm these findings, apolipoprotein B-containing lipoproteins were precipitated with anti-apolipoprotein B and high density lipoproteins were precipitated with anti-apolipoprotein A-I. Only the former precipitate was radioactive. These results suggest that apolipoprotein B is a calcium binding protein.

Apolipoprotein B (ApoB*), the major protein moiety of triglyceride-rich and low density lipoproteins (LDL), is probably one of the most important apolipoproteins of the plasma lipoprotein system. Its functions as a lipid carrier and a ligand for LDL receptor and subsequent regulation of cellular cholesterol synthesis have been well documented (1). However, other possible functions of ApoB remain to be explored.

During the course of studying ApoB we have observed that ApoB behaved differently in the presence and absence of EDTA, particularly in aged LDL preparations. ApoB fragmented in aged LDL in the absence but aggregated in the presence of EDTA (Singh and Lee, unpublished results). These observations suggested

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*The abbreviations used are: ApoB, apolipoprotein B; ApoA-I, apolipoprotein A-I; LDL, low density lipoproteins (d 1.006-1.063 g/ml); HDL, high density lipoproteins (d 1.063-1.21 g/ml); SDS-PAGE, polyacrylamide gel electrophoresis containing sodium dodecyl sulfate; DATD, N,N'-diallyltartardiamide; Bis, N,N'-methylenebisacrylamide.

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that a divalent cation may be associated with ApoB. To test the hypothesis that ApoB may be a calcium binding protein, we studied quantitatively the incorporation of [$^{45}Ca^{2+}$] into ApoB- and ApoA-I-containing lipoproteins synthesized and secreted by cultured human hepatocarcinoma cell line Hep G2.

MATERIALS AND METHODS

Cell culture. Human hepatocarcinoma Hep G2 cells were plated in 100 mm Falcon Petri dishes in 15 ml of modified minimum essential medium (Grand Island Biological Co., Grand Island, NY) containing 10% (v/v) fetal calf serum (Hazleton Research Products, Inc., Denver, PA) and grown at 37°C as described previously (2,3). After 4 days, when dishes were near confluence, the maintenance medium was removed and the monolayers were washed three times with $CaCl_2$-free minimum essential medium and incubated in the same medium. Synthesis and secretion of ApoB-containing lipoproteins by Hep G2 cells were tested at various Ca^{2+} concentrations ranging from 0 to 1.8 mM to establish the optimal concentration of radioactive Ca^{2+}. Based on this test, six plates received 13 ml each of medium which had no $CaCl_2$ but contained 0.035 mM [^{45}Ca]Cl_2 (Amersham Corp., Arlington Heights, IL) at 24 µCi/ml. To six control plates, 13 ml of medium containing the same concentration of unlabeled $CaCl_2$ was added and cells were incubated for 20 h.

Isolation of lipoproteins. The media from each group of cells were pooled. Lipoproteins of d < 1.063 g/ml and high density lipoproteins (HDL) (d 1.063-1.21 g/ml) were isolated by sequential ultracentrifugation (4). The lipoproteins were dialyzed against 0.02 M phosphate-buffered or Tris-buffered saline, pH 7.2, at 4°C and concentrated with sucrose. In a separate experiment, to examine the molecular form(s) of ApoB produced by Hep G2 cells under usual culture condition, lipoproteins of d < 1.063 g/ml isolated from culture medium containing 1.8 mM $CaCl_2$ were dialyzed under N_2 at 4°C against saline containing preservatives: 500 units/ml penicillin-G, 50 µg/ml streptomycin sulfate, 0.1% EDTA, 0.13% ε-amino caproic acid and 0.05% reduced glutathione, pH 7.0 (5), and concentrated under N_2 with Amicon membrane cone before analyses.

Quantitation of apolipoproteins B and A-I. Concentrations of apolipoproteins B and A-I in the lipoproteins of d < 1.063 g/ml and HDL, respectively, isolated from Hep G2 cell culture media, were determined by electroimmunoassay according to the methods described previously (6,7).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The lipoproteins of d < 1.063 g/ml and HDL were analyzed by 3.3% and 7% polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), respectively, by the method of Fairbanks et al. (8) as modified previously (9). When solubilization of polyacrylamide gel was required for measurement of radioactivity incorporated into the apolipoproteins, N,N'-diallyltartardiamide (DATD) (Bio-Rad Laboratory, Richmond, CA) was substituted for N,N'-methylenebisacrylamide (Bis) as the cross-linking agent on an equal molar basis (5). About 200-250 µl of lipoproteins with known amount of apolipoprotein B or A-I were applied onto the gel in duplicate: one for staining and one for cutting and counting. For measurement of radioactivity, the gel was cut into 3 mm segments from top to bottom and each segment was dissolved in 0.2 ml of 2.0% periodic acid solution.

Measurement of [^{45}Ca]-radioactivity. [^{45}Ca] radioactivity was measured in a liquid scintillation counter (Beckman 7800, Irvine, CA). Standard solution containing known amount of radioactivity of [^{45}Ca]Cl_2 was used to make serial dilutions for constructing an efficiency curve to convert cpm into dpm. Window setting at 0-670 channels was chosen based on a pre-scan of a [^{45}Ca]Cl_2 solution containing 100,000 dpm. The aqueous solution was mixed with 5 ml of liquid

scintillation cocktail (Ready Solv™ MP, Beckman, Fullerton, CA). The dpm values were converted to μCi and then to nmol of [$^{45}Ca^{2+}$] based on the specific activity of [^{45}Ca] on the day of counting.

Immunoprecipitation of ApoB-containing lipoproteins and ApoA-I-containing lipoproteins. The immunoprecipitation of ApoB-containing lipoproteins from the d < 1.063 g/ml fraction was carried out by a modification of a previously described procedure (10). A 0.20 ml sample of [^{45}Ca]-containing d < 1.063 g/ml lipoproteins was mixed with unlabeled carrier lipoprotein B containing 80 μg protein (11) and 0.12 ml IgG fraction of a polyclonal antiserum to human ApoB resulting in the precipitation of labeled antigen. The reaction mixture was incubated for 2 h at 4°C and the precipitated antigen-antibody complex was collected by low-speed centrifugation for 10 min. The supernatant fraction was removed, the precipitate was washed with 0.02 M Tris, pH 7.2, and then redissolved in a small volume of 0.1 N NaOH and counted for radioactivity. HDL containing [^{45}Ca]-radioactivity was immunoprecipitated with a polyclonal antiserum to ApoA-I by a similar procedure.

RESULTS AND DISCUSSION

Synthesis of ApoB by Hep G2 cells under regular Ca^{2+} concentration. Under regular Ca^{2+} concentration (1.8 mM) in culture medium, the secreted lipoproteins of d < 1.063 g/ml gave positive immunoreaction against antibodies to ApoB, showing a rocket by electroimmunoassay. When applied onto 3.3% SDS-PAGE, a single band with M_r 360,000 was observed which had a mobility identical to that of ApoB present in human plasma LDL (Fig. 1), suggesting the synthesis and secretion of ApoB (B-100)-containing lipoproteins by Hep G2 cells.

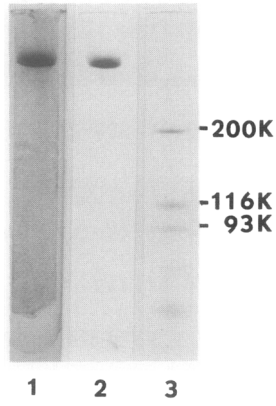

Fig. 1. 3.3% SDS-PAGE analysis of lipoproteins of d < 1.063 g/ml from culture medium of cell line Hep G2. Gel 1. Approximately 15 μg ApoB was applied as lipoproteins of d < 1.063 g/ml isolated from culture medium of Hep G2 cells grown in 1.8 mM Ca^{2+}, dialyzed against saline containing preservatives under N_2 at 4°C and concentrated with Amicon membrane cone under N_2 at 4°C (see Methods for details). Gel 2. Approximately 10 μg ApoB was applied as LDL of d 1.032-1.043 g/ml isolated from normal human plasma and dialyzed under the same condition as above. Gel 3. Calibration protein standards, the top three bands are: myosin (M_r 200,000); β-galactosidase (M_r 116,500); and phosphorylase B (M_r 93,000). All gels were cross-linked with Bis and the sample and running buffers contained EDTA.

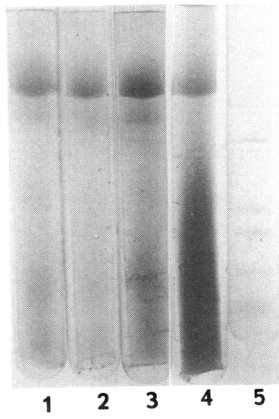

Fig. 2. 3.3% SDS-PAGE analyses of lipoproteins of d < 1.063 g/ml from culture media of Hep G2 cells incubated in various concentrations of Ca^{2+}. Lipoproteins of d < 1.063 g/ml isolated from cell culture media containing Ca^{2+} concentration: 0 mM (gel 1), 0.05 mM (gel 2), 0.1 mM (gel 3) and 0.5 mM (gel 4). The lipoproteins were dialyzed against 0.02 M Tris-buffered saline (pH 7.2) at 4°C and concentrated with sucrose before analyses. Calibration protein standards (gel 5) were the same as in Fig. 1. All gels were cross-linked with Bis but the sample and running buffers contained no EDTA.

Synthesis of ApoB by Hep G2 cells at lower Ca^{2+} concentrations. Synthesis of ApoB-containing lipoproteins by Hep G2 cells was tested at lower Ca^{2+} concentrations in culture medium including 0, 0.05, 0.1 and 0.5 mM. After isolation of the lipoproteins of d < 1.063 g/ml from the culture media, dialyses against Tris-buffered saline (pH 7.2) and concentration with sucrose, all preparations showed the presence of ApoB band on 3.3% SDS-PAGE (Fig. 2). Since neither EDTA nor other preservatives were used during isolation and dialyses of the lipoproteins, fragments with faster mobility (B-74-like) were observed in some of the preparations as shown in gels 1 and 3 of Fig. 2.

Incorporation of [$^{45}Ca^{2+}$] into newly synthesized ApoB. The newly synthesized and secreted lipoproteins of d < 1.063 g/ml by Hep G2 cells grown in the presence of 0.035 mM [$^{45}Ca^{2+}$] or in the presence of an equal concentration of unlabeled Ca^{2+} were characterized by 3.3% SDS-PAGE. Figs. 3 and 4 show the radioactivity profiles of [$^{45}Ca^{2+}$] along the 3.3% gels for d < 1.063 g/ml lipoproteins from two separate experiments. As shown in Fig. 3, the peak of [$^{45}Ca^{2+}$] radioactivity corresponded to the ApoB band. Although the presence of apolipoproteins C-II, C-III and E in d < 1.063 g/ml lipoproteins is known (12),

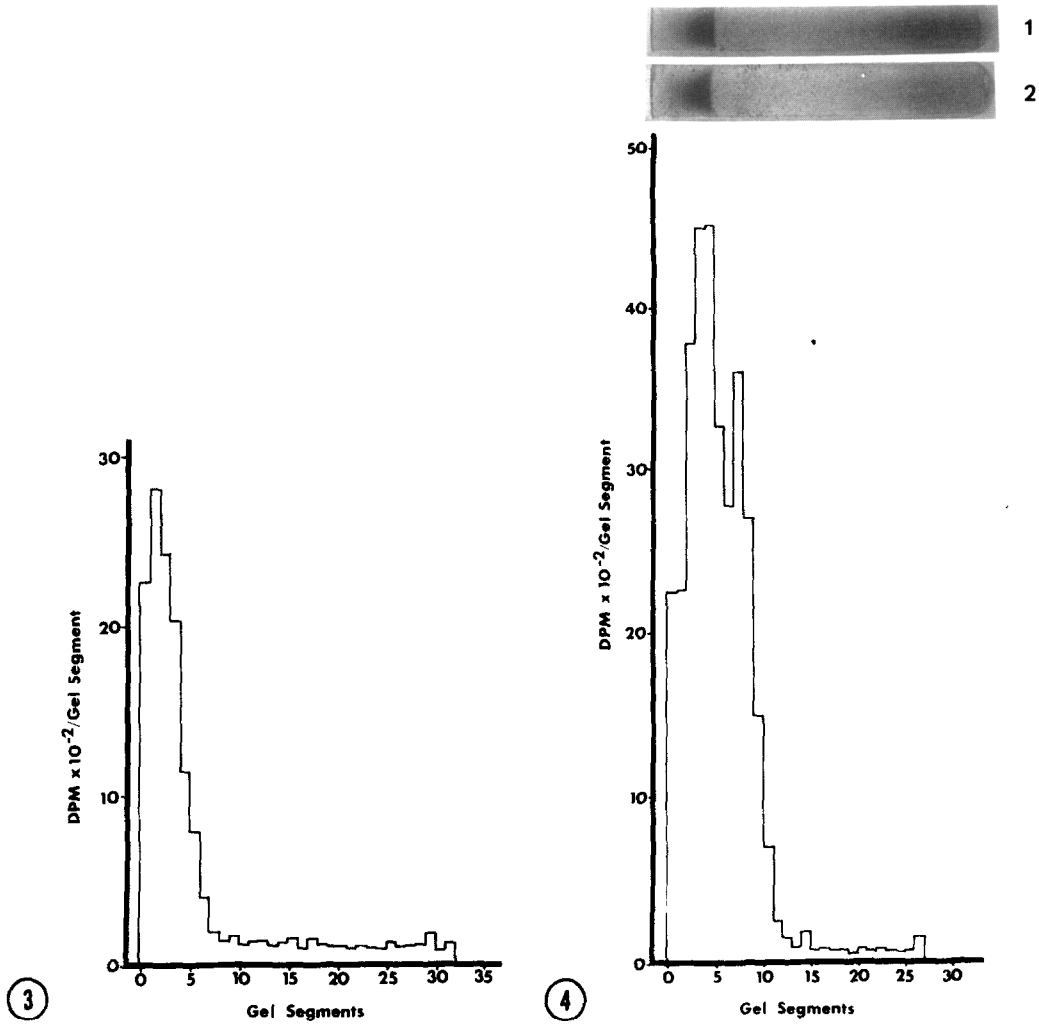

Fig. 3. Radioactivity profile of [^{45}Ca] for lipoproteins of d < 1.063 g/ml (Experiment 1) on 3.3% SDS-PAGE. The lipoproteins containing 1.2 µg ApoB synthesized and secreted by cell line Hep G2 grown in culture medium containing 0.035 mM [$^{45}Ca^{2+}$] were applied onto 3.3% SDS-PAGE. The gels were cross-linked with DATD and electrophoresis carried out in the absence of EDTA. At the completion of electrophoresis, the gel was cut into 3 mm segments from top to bottom and the radioactivity of [^{45}Ca] measured as described in Methods.

Fig. 4. Radioactivity profile of [^{45}Ca] for lipoproteins of d < 1.063 g/ml (Experiment 2) on 3.3% SDS-PAGE. The lipoproteins containing 3.2 µg ApoB synthesized and secreted by cell line Hep G2 grown in culture medium containing [$^{45}Ca^{2+}$] were applied onto 3.3% SDS-PAGE. The gel system was the same as described in Fig. 3. The stained gels contained the same amount of ApoB from [$^{45}Ca^{2+}$]-labeled lipoproteins (gel 1) and from control experiment grown in the same concentration of unlabeled $CaCl_2$ (gel 2). Electrophoresis was carried out from left (cathode) to right of the gels.

there was no [$^{45}Ca^{2+}$]-radioactivity detected at the small molecular weight position in 3.3% gel. To confirm this observation, the samples were analyzed by 7% SDS-PAGE. Similarly, no radioisotope was found associated with any of

the small apolipoproteins. The results of a separate experiment (Fig. 4) showed that the major peak of [$^{45}Ca^{2+}$]-radioactivity overlapped with ApoB-100, and the minor peak corresponded to a position characteristic for intestinal ApoB (B-48) (9,13,14). Again, no radioactivity was found associated with any other secreted apolipoproteins. Since the mobility of ApoB was the same for lipoproteins produced in the presence of [$^{45}Ca^{2+}$] or unlabeled Ca^{2+} (Fig. 4), and since the well preserved ApoB-containing lipoproteins showed only a single ApoB-100 band (see Fig. 1), we presumed that the ApoB-48-like peak shown in Fig. 4 was derived from ApoB-100. In order to calculate the molar ratio of the incorporated [$^{45}Ca^{2+}$] and ApoB, 250,000 daltons was used as the molecular weight of ApoB (15). From the summation of radioactivity under ApoB peak and the specific activity of [$^{45}Ca^{2+}$] used, the incorporation of [$^{45}Ca^{2+}$] into ApoB was calculated to be 1.09 mol/mol for experiment 1 and 0.96 mol/mol for experiment 2. Thus, we conclude that each ApoB binds one mol of Ca^{2+}.

As negative controls, HDL from Hep G2 cells grown in [$^{45}Ca^{2+}$] were also analyzed by 7% SDS-PAGE. The gels were cut into segments and radioactivity measured. No radioactivity was associated with any of the gel segments, suggesting that the newly synthesized apolipoproteins A-I, A-II, C-II, C-III and E present in HDL (12) do not bind [$^{45}Ca^{2+}$]. These results suggest that among all known apolipoproteins synthesized and secreted by Hep G2 cells, ApoB is the only apolipoprotein which binds calcium.

Immunoprecipitation of apolipoprotein B-containing lipoproteins. To confirm that the above [$^{45}Ca^{2+}$] binding apolipoprotein is indeed ApoB, an aliquot of the lipoproteins of $d < 1.063$ g/ml was immunoprecipitated with antibodies to ApoB. The precipitate was washed eight times until the radioactivity in the supernate reached the background level of 36 cpm. The radioactivity associated with the ApoB precipitate was determined to be 7520 ± 456 dpm/ml ($n = 3$) of $d < 1.063$ g/ml lipoprotein fraction. The HDL was also tested for the presence of labeled ApoA-I-containing lipoproteins by the addition of antibodies to ApoA-I. The precipitated immuno-complex contained only background levels of radioactivity. These data confirm the findings that [$^{45}Ca^{2+}$] is incorporated into the secreted apolipoprotein B but not into A-I by Hep G2 cells.

To our knowledge, this is the first report demonstrating that ApoB is a calcium binding protein. This finding not only has some important structural implications but also suggests another specific function for ApoB. In addition to its major role as a lipid carrier, ApoB may also function as a vehicle for the transport of Ca^{2+} and its delivery into cells. The nature of amino acid residues involved in the calcium binding site and the question of its possible localization in the vicinity of the receptor binding site of ApoB remain to be explored.

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