Characterization of the Human Plasma Binding Protein for Vitamin D and Its Metabolites Synthesized by the Human Hepatoma-derived Cell Line, Hep 3B*

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Screening of three human hepatoma-derived cell lines revealed the presence of an immunologically similar plasma binding protein for vitamin D and its metabolites in media from Hep 3B cells. Approximately 3% of protein synthesized and secreted by these cells was immunoprecipitated by specific antiserum to the D-binding protein. Medium content of the protein increased over 11 days following cell seeding, and negligible amounts of [125I]-labeled binding protein added to cultures were degraded over 48 h. The hepatoma-derived binding protein was indistinguishable from plasma binding protein or reference pure protein in gel filtration, sucrose gradient ultracentrifugation, and polyacrylamide gel electrophoresis systems. The Hep 3B cell product was found to bind mole/mole with monomeric actin, and bind vitamin D sterols with an affinity and specificity characteristic of the human plasma binding protein. The findings argue strongly for the identity of the Hep 3B cell product and the human plasma protein. The continuous availability of the Hep 3B cell line provides a reasonable model for investigations of biosynthesis and release of this important plasma protein.

The human plasma binding protein for vitamin D and its metabolites is a major constituent of the α-globulins (1–3) and is recognized to be a circulating transporter of anti-rachitic sterols (4, 5). Initially isolated as a group-specific component (Gc globulin) (6, 7), DBP presumably carries out critical function(s) since its deletion is thought to be a lethal mutation (8). In addition to its sterol-carrier function, DBP has recently been shown to interact avidly with a component of the cytoskeleton, actin (9–12). Utilizing Gc globulin antisera in analyses of radioamino acid incorporation into protein manufactured by liver segments in vitro, evidence for the hepatic origin of DBP was obtained (13). Further, measurements of the plasma concentration of DBP in various subjects has led to the observations of lowered DBP levels in hepatic diseases (5, 14, 15, 17) and increased levels during estrogen therapy and pregnancy (4, 5, 15). Vitamin D deficiency and excess, however, were not associated with changes in the circulating concentrations of DBP (5, 15, 18), nor were other disorders of mineral homeostasis (5, 15, 17).

Since DBP could have bifunctional roles, and its production clearly matches its brisk plasma clearance in man (19) and in the rabbit (20), additional information about its biosynthesis and secretory regulation would be of interest. Of several human hepatoma-derived cell lines established in culture, some of these retain parenchymal cell morphology and synthesize and secrete major plasma proteins (21–25). The Hep 3B line, for example, synthesizes at least 37 different plasma proteins, as well as the hepatitis B virus surface antigen (22–25). The continuous availability of this line affords a reasonable tool for the investigation of plasma protein biosynthesis. We now report our investigations of the characterization of the human DBP biosynthesized and secreted by the Hep 3B cells.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were used as reagent grade or best grade available. Sephadex G-25, G-200, DEAE-Sephadex, dextran 20, and blue dextran gels were purchased from Pharmacia Inc. Ovalbumin (grade III), lactoperoxidase, low electroendosmosis agarose (Type III), bovine serum albumin (Fraction IV powder), trypsin (bovine pancreas, Type III), and bovine γ-globulin were obtained from Sigma. 25-Hydroxy[26,27-3H]cholecalciferol (specific activity 11.7 Ci/mmol) was purchased from Amersham Corp. and purified on high pressure liquid chromatography columns (26) prior to use. Carrier-free [3H] was purchased from Amersham Corp. Unlabeled sterols were gifts from Dr. John Babcock of The Upjohn Co. and Dr. Milan Uskokovic of Hoffmann-LaRoche, and their quantitation was derived from their extinction at 264 nm in absolute ethanol. [35S]Methionine (600 Ci/mmol) was purchased from New England Nuclear. Rabbit skeletal muscle G-actin was prepared from the acetone powder as previously described (27).

Cell Culture and Media Collection—Three human cell lines of hepatoma origin were evaluated for their ability to synthesize DBP. The Hep G2, Hep 3B, and PRL/PPF/5 cell lines were grown to confluence in T-75 flasks (Falcon) as previously described (24). In addition, human fetal lung fibroblasts (WI-38) and Hep 3L lung fibroblasts were cultured similarly. All cells were grown in Eagle's minimal essential medium (Auto Pow, Flow Laboratories Inc.) with 10% fetal bovine serum, and passaged by exposure to trypsin/EDTA (0.25%; 0.1 m) and pipetting. For some experiments, cells were washed and incubated in serum-free medium. In other studies, cells were maintained in methionine-free medium prior to analyses of [35S] methionine incorporation into proteins.

Purified Proteins and Antiserum—Human plasma DBP was purified from human plasma or Cohn Fraction IV (Armour) as previously reported (1). Monospecific antiserum was raised in New Zealand rabbits as described (15), and an immune γ-globulin fraction was prepared by (NH)2SO4 precipitation, dialysis, and DEAE-Sephadex chromatography. DBP was iodinated by the lactoperoxidase tech-
The medium was analyzed for HDBP by radioimmunoassay (15). Pure human plasma DBP, human DBP (W) and medium concentrate were incubated in the NaCl/P04 serum, and reference human DBP to bind 25-hydroxy[\textsuperscript{3}H]cholecalciferol was estimated. The material was centrifuged at 500 g, washed three times in the cold NaCl/PO\textsubscript{4} buffer. Precipitates were dissolved in NaOH for scintillation counting or were solubilized in 1% SDS, 1% \beta-mercaptoethanol.

**Methods**

**Sucrose Gradient Ultracentrifugation**—Linear 4.8-ml (5-20%, w/v) sucrose gradients were prepared in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride. Samples were layered in 250-µl volumes and centrifuged at 40,000 rpm in a Beckman Ultracentrifuge in an SW 50.1 rotor. Fractions were collected through a bottom puncture into counting vials or glass test tubes. In the actin-binding experiments, gradients were prepared in 0.01 M Tris buffer, pH 7.4, containing 0.5 mM ATP, 0.2 mM CaCl\textsubscript{2}, 5 mM \beta-mercaptoethanol.

**Polyacrylamide Gel Electrophoresis**—Analytical and preparative electrophoreses were carried out in 7% polyacrylamide tube gels in the absence or presence of 0.1% SDS, as previously described (9,12). The R\textsubscript{f} values were calculated as fractions of the migration of bromophenol blue tracking dye. Gels were sliced into 2-mm segments and dissolved in 33% hydroxide peroxide for scintillation counting or stained with Coomassie blue.

**DBP Radioimmunoassay and Immunoprecipitation**—DBP radioimmunoassay was performed as previously reported (15). Immunoprecipitation of the cell culture medium’s radiolabeled products was carried out by the addition of anti-DBP \gamma-globulin and 2 pg of reference bovine DBP to 100 μl of medium. After overnight incubation at 4 °C, the material was centrifuged at 500 g, and the precipitates were washed three times in the cold NaCl/PO\textsubscript{4} buffer. Precipitates were solubilized in NaOH for scintillation counting or were solubilized in 1% SDS, 1% \beta-mercaptoethanol prior to SDS-polyacrylamide gel electrophoresis (12).

**Gel Filtration**—Specific details of procedures are provided for representative examples in the legends to the figures.

**Sterol Binding Analyses**—The ability of cell medium, human serum, and reference human DBP to bind 25-hydroxy[\textsuperscript{3}H]cholecalciferol was estimated by incubating these materials in the NaCl/PO\textsubscript{4} buffer at 0 °C for 1 h and removing “free” sterol with dextran-coated charcoal as previously reported (28). Specific binding was appraised by analyzing aliquots of the charcoal supernatant for radioactivity.

**Other Methods**—Total protein concentrations were estimated by the method of Bradford (29). Protein incorporation of radiolabeled methionine was analyzed by precipitation of medium products in cold 10% trichloroacetic acid. Scintillation spectrometry was carried out in a Packard Tri Carb 460CD liquid scintillation spectrometer. \textsuperscript{125}I was analyzed in a Micromedic 4/600 γ-spectrometer.

**RESULTS**

Analyses of media from Hep 3B, Hep G2, PLC/PRF/5, and Hep 3L cells revealed that only the Hep 3B cells were producing a material which was immunologically similar to human DBP (Fig. 1). Media from cultures of the other cell lines, as well as medium containing 10% bovine serum and no cells, failed to displace \textsuperscript{125}I-DBP from the antiserum. These findings persisted after a 3-5-fold concentration of the media on a Minicon (Amicon Corp.) filtration apparatus. In other experiments, concentrated Hep 3B medium formed rocket immunoprecipitates when electrophoresed against anti-DBP antiserum (30).

Evidence for vigorous DBP production was observed in aliquots of media from cultures of the Hep 3B cells 3 days after plating (Fig. 2). Continuing production was seen for 11 days, or 4 days after cells reached confluency. Since there was no information available concerning the integrity of secreted DBP in Hep 3B cultures, we estimated the survivorship of \textsuperscript{125}I-DBP after its addition to flasks containing Hep 3B or Hep 3L cells, or no cells. Table I indicates that SDS-polyacrylamide gel electrophoresis and immunoprecipitation were very similar to native human DBP.

**TABLE I**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>2 h</th>
<th>8 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cells</td>
<td>99</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>Hep 3B</td>
<td>96</td>
<td>91</td>
<td>90</td>
</tr>
<tr>
<td>Hep 3L</td>
<td>94</td>
<td>89</td>
<td>87</td>
</tr>
</tbody>
</table>
rlylamine gel electrophoresis analysis of such incubation media reveals very little degradation of the protein over 48 h. Degraded material was of smaller molecular weight and was not further characterized (data not shown).

When analyzed after 1 h of labeling of the Hep 3B cells, approximately 3% of the protein-incorporated amino acid was immunoprecipitated by anti-DBP antiserum (Table II). In order to correct for nonspecific radioactivity trapped by the immunoprecipitation technique, Hep 3L cell media were concurrently analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis separation of the dissolved precipitate. Since non-DBP incorporation of radioactivity in the apparent rate of synthesis and stability of secreted DBP are medium and the media were collected 48 h later. After ultra-

carrier added with blue dextran (BD), bovine serum albumin (BSA), and cyanocobalamin for outer volume (Vo).

FIG. 3. Sephadex G-200 gel filtration of Hep 3B medium and human serum. 1.0 ml of serum-free Hep 3B medium (O—O) or 1.0 ml of human serum in 1.0 ml of NaCl/PO₄ buffer (C—C) was incubated with 2.5 pmol of 250H[3H]D₃ for 1 h at 0 °C prior to application onto a column (1.5 × 60 cm) of Sephadex G-200 slurred in the same buffer. Ascending filtration was carried out at 4 °C at a flow rate of 16 ml/h, with 1-ml fractions collected. Calibrations were carried out with blue dextran (BD), bovine serum albumin (BSA), and cyanocobalamin for outer volume (Vo).

FIG. 4. Sucrose gradient ultracentrifugation analyses of Hep 3B medium before and after incubation with G-actin. A, sucrose density gradient ultracentrifugation of Hep 3B medium and pure human DBP. 0.3 ml of serum-free, 2-fold concentrated Hep 3B medium or 0.3 ml of NaCl/PO₄ buffer containing 1 µg of pure human DBP was incubated with 5 pmol of 250H[3H]D₃ for 1 h at 0 °C prior to layering onto linear 4.8-ml sucrose (20-5%) gradients. After ultracentrifugation as described under "Methods," bottom puncture eluates were collected. The sedimentation of bovine serum albumin is indicated by the arrow. B, bottom, and T, top, of gradient. B, sucrose density (20-5%) ultracentrifugation of Hep 3B medium and Hep 3B medium plus G-actin. 0.3 ml of serum-free Hep 3B medium containing 60 ng (~1.0 pmol) of DBP (radioimmunoassay) was dialyzed in the 0.01 M Tris buffer. Half was incubated for 1 h at 4 °C with 25 ng (0.6 pmol) of monomeric actin (C—O) and half with the Tris buffer alone (C—O) for 1 h at 4 °C. Each incubation fluid was layered and analyzed as described in A.

FIG. 5. 7% polyacrylamide gel electrophoresis separations of Hep 3B medium and human serum. 0.1 ml of serum-free Hep 3B medium containing 24 ng (0.4 pmol) of DBP (radioimmunoassay) or 0.1 ml of NaCl/PO₄ containing 0.1 µl of human serum (40 ng of DBP) was incubated with 0.2 pmol of 250H[3H]D₃ at 0 °C for 1 h prior to analyses. 1-mm gel segments were cut, solubilized, and assayed for tritium content. Bromphenol blue (BPB) served as the anodal tracking dye (arrow).
The sterol binding by DBP synthesized by Hep 3B cells was analyzed in the absence and presence of competing unlabeled sterols. As shown in Fig. 6, Hep 3B medium contained material which displayed high affinity toward 25-hydroxycholecalciferol and 24,25-dihydroxycholecalciferol. When graphically corrected for high affinity binding sites (31), a dissociation constant of \( k_d \) was found (data not shown).

Since both sterols without 25-hydroxyl groups, at the concentrations tested, did not compete for 25-hydroxycholecalciferol binding sites, the binding by Hep 3B protein closely resembles the binding specificity characteristic of human plasma DBP (1).

SDS-polyacrylamide gel electrophoresis of anti-DBP immunoprecipitates from the medium, following 1 h of exposure of Hep 3B cells to \([^{35}S]methylene\), revealed a single region of radioactivity which was consistent with the known molecular weight of human DBP (Fig. 7). This migration was identical with that of reference DBP detected by staining. Similar results (not shown) were obtained by adding anti-DBP \( \gamma \)-globulin in the absence of carrier DBP, and adsorbing the immune complex onto Staphylococcus Cowan I bearing protein A prior to solubilization for the electrophoretic analyses.

**DISCUSSION**

The present results argue strongly for the identical nature of human plasma DBP and the DBP-synthesized and secreted by a human hepatoma-derived cell line, Hep 3B, in culture. In addition to immunological similarity, if not identity, the Hep 3B protein had behavior which was indistinguishable from reference pure or human plasma DBP in gel filtration, sucrose gradient ultracentrifugation, and polyacrylamide gel electrophoresis systems. The Hep 3B protein also bound to monomeric actin to form the characteristic complex recognized for DBP and this cytoskeletal protein (11, 12). Finally, the hepatoma-derived DBP product possessed the sterol binding features displayed by human plasma DBP (1, 32). These findings appear to confirm the hepatic origin of DBP.

Although DBP is a major plasma constituent, little is currently known about the regulation of its biosynthesis and release. The availability of vitamin D sterols is not thought to condition the synthesis or release of the protein (5, 15), but no direct information is available on this point. Since the liver is the site for vitamin D 25-hydroxylation (33), it is possible that sterol entry or metabolism might affect or be affected by DBP biosynthesis and/or release from liver cells. Of the circulating vitamin D sterols, the dominant form is the hepatic metabolite, 25-hydroxyvitamin D (33), a sterol to which DBP displays high affinity.

The function(s) of DBP is also incompletely understood. The likely roles of the protein in the aqueous solubilization, transport, and protection of vitamin D sterols have been mentioned (1, 15, 34). Since sterol delivery to perfused organs (35) and cells (16) in vitro is apparently impaired by the presence of DBP, several investigators consider its function to be extracellular (15, 34). There is, however, some limited evidence for its cellular access (9, 10). The finding of high affinity and selective binding of actin by DBP (11, 12), however, has generated speculations about another role for DBP as an actin scavenger (11).

The Hep 3B cell line appears to expend reasonable energy toward synthesizing and secreting DBP, as has been shown for other transport proteins (23, 25) and coagulation factors (23). Vitamin D-25-hydroxylase activity has not been studied in these cells. Further characterization of cellular DBP products, such as precursor forms, is currently planned. The continuous availability of this line appears to provide a reasonable model for investigations of the biosynthesis and secretion of this interesting, high affinity, high capacity, and rapidly turned-over plasma protein.

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