Characterization of Nascent and Secreted Thyroxine-binding Globulin in Cultured Human Hepatoma (Hep G2) Cells

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Thyroxine-binding globulin (TBG) synthesis by human hepatoma (Hep G2) cells was demonstrated by pulse labeling with [35S]methionine or [3H]mannose and subsequent immunoprecipitation in the medium or cell lysate. Secreted TBG was glycosylated and had the same apparent molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as TBG purified from human serum. Pulse-chase experiments failed to show any precursor form intracellularly. Treatment of cells with the amino acid analogs, canavanine and thialysine, did not cause secretion of large-molecular-weight moieties, in contrast to what was observed in the case of albumin. Thyroxine-binding activity, as assessed by [125I]thyroxine immunoprecipitation with anti-TBG serum, was detectable in the media of oocytes injected with RNA from Hep G2 cells. Translation of this RNA in rabbit reticulocyte lysate, followed by immunoprecipitation with anti-TBG serum, revealed a protein having the same electrophoretic mobility as deglycosylated TBG purified from human serum (M, > 45,000). Since deglycosylated TBG still contains 3% of its weight as carbohydrate, it appears that the translation product contains an additional fragment (signal peptide) of about 1,500 daltons. It is unlikely, however, that TBG is synthesized via a larger-molecular-weight precursor.

TBG is the major thyroid-hormone transport protein in human plasma. It is a glycoprotein with a M, of ~54,000 and a polypeptide-chain weight of 42,000 (2,3). Using isolated monkey hepatocytes, it was shown by immunodiffusion and immunoelectrophoresis that TBG is secreted by the liver (4). However, no evaluation of precursor forms of TBG in whole cells or in cell-free systems has been provided. Furthermore, many physicochemical properties of TBG remain to be clarified. For example, it is not known why the protein undergoes irreversible denaturation after mild acidification or at a low concentration of guanidinium chloride (5,6). One possible explanation for the irreversibility might reside in the presence, during biosynthesis, of a precursor form (pro-TBG) from which a polypeptide is cleaved before secretion.

RESULTS

Identification of TBG—Evidence that TBG is synthesized and secreted by human Hep G2 cells was provided by immunoprecipitation with anti-TBG serum of media from cells continuously labeled for 4 h with [35S]methionine. In 12.5% SDS-PAGE, a band of M, ~ 54,000 was observed (Fig. 1, lane 1) which co-migrated with [125I]-TBG (lane 4). The identity of TBG was confirmed by the finding that the addition of unlabeled TBG blocked the precipitation of biosynthetically labeled protein (lane 2). The higher-molecular-weight fainter band in lane 1 was not blocked by excess TBG and was not consistently present.

To assess whether TBG secreted by Hep G2 cells is glycosylated, cells were labeled for 4 h with [3H]mannose and analyzed by SDS-PAGE after immunoprecipitation with anti-TBG. As illustrated in Fig. 2, both intracellular and secreted TBG were glycosylated, as judged by the single prominent band of M, ~ 54,000. A second, less radioactive band was found in the medium. It had a M, > 100,000 and it is likely to be an aggregate (see below).

Search for a Precursor—To determine whether TBG is synthesized via a higher-molecular-weight precursor (pro-TBG), Hep G2 cells were labeled with [35S]methionine for 4 h in the presence of 2.8 mM L-canavanine, an analog of arginine which is incorporated into proteins and blocks the translation process.

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1 The abbreviations used are: TBG, thyroxine-binding globulin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; D-TBG, enzymatically deglycosylated TBG; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; PPO, 2,5-diphenyloxazole; Tm, thyroxine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 Portions of this paper (including "Materials and Methods") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-0451, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Fig. 1. Immunoprecipitation of $^{35}$S-methionine-labeled TBG in the media from Hep G2 cells. Cells were labeled for 4 h with $^{35}$S-methionine (100 μCi/ml). The medium (500 μl) was immunoprecipitated with anti-TBG (10 μl) and protein A (150 μl) and subsequently analyzed by 12.5% SDS-PAGE and fluorography. Lane 1, immunoprecipitation in control cells; lane 2, immunoprecipitation in the presence of 10 μg of unlabeled TBG; lane 3, immunoprecipitation in cells grown in the presence of 2.8 mM L-canavanine; lane 4, $^{35}$S-labeled serum TBG. Arrows indicate the position of molecular weight standards.

Fig. 2. Immunoprecipitation of $^3$H-mannose-labeled TBG. Hep G2 cells were labeled for 4 h with $^3$H-mannose (200 μCi/ml). Medium and cell lysate were subjected to 7.5% SDS-PAGE, after which the gels were sliced into 1-mm sections and the radioactivity measured in each slice. Arrows indicate the position of molecular weight standards. ●—●, medium; ○—○, cell lysate.

cleavage of propeptides (9–12). As shown in Fig. 1 (lane 3), TBG secreted in the presence of L-canavanine had the same apparent $M_s$ as TBG secreted by control cells and $^{125}$I-TBG. A similar lack of effect on the size of secreted TBG was observed when thiylsine, an analog of lysine, was used instead of L-canavanine (data not shown). As reported by Redman et al. (9), we also found that L-canavanine produced a shift in the electrophoretic mobility of albumin (data not shown).

To study further the biosynthesis of TBG, cells were pulse labeled with $^{35}$S-methionine for 10 min and then chased for 2 h after the addition of 20,000-fold excess of unlabeled methionine. A progressive decrease of intracellular radioactivity precipitated by anti-TBG (Fig. 3, left panels) and a progressive accumulation in the medium (Fig. 3, right panels) were observed. At any chase time, only one protein was immunoprecipitated within the cells, with an apparent $M_s$ of $\approx$54,000, equal to that of secreted TBG. The apparently identical $M_s$ of intracellular and secreted TBG, and the absence of a larger $M_s$ intracellular protein, suggest that the pro-TBG is unlikely to be present at any stage of TBG biosynthesis.

After a 2-h chase, another protein was precipitated from

Fig. 3. $^{35}$S-Methionine pulse-chase experiment. Hep G2 cells were pulse labeled for 10 min with $^{35}$S-methionine (100 μCi/ml) and then chased for 2 h after addition of a 20,000-fold excess of unlabeled methionine. Medium and cell lysates were immunoprecipitated with anti-TBG serum (10 μl) and analyzed by 7.5% SDS-PAGE. Gels were sliced into 1-mm sections and the radioactivity measured in each slice. Arrows indicate the position of molecular weight standards.
The data in Table I also show the dependence of TBG secretion on time and on RNA concentration. It has been shown in our laboratory that TBG represented only a minor cell-free translation product of total liver mRNA, since the radioactivity precipitated with anti-TBG serum and analyzed by SDS-PAGE disproportionate to the actual difference in M, of -45,000 and coincided with [14C]ovalbumin of unlabeled TBG. A major question in this study was whether or not TBG is synthesized via a larger precursor (pro-TBG) containing an additional peptide fragment that is cleaved prior to secretion by the hepatoma cells. During chase, there was a progressive accumulation of TBG in the medium and a concomitant decrease in the 35S-labeled intracellular protein. Nonetheless, secreted and intracellular TBG had apparently identical molecular weights in SDS-PAGE, with no evidence of a precursor form. These results, however, have to be interpreted with caution, since the additional peptide of a putative precursor might be too small to be detected by this technique. In proalbumin, for example, it is composed of only six amino acids (19, 20).

A different approach to detect a possible precursor is to use amino acid analogs. Cleavage of many propeptides, both hormones and other secretory proteins, occurs at pairs of basic amino acids (Arg-Lys, Lys-Arg, or Arg-Arg) (21, 22). The substitution of arginine by its analog canavanine, or of lysine by thialysine, alone or in combination, has been reported to block the cleavage of propeptides, leading to the secretion of unprocessed prohormones or proproteins (9-12). In particular, in the case of albumin, secretion of canavanine-containing proalbumin by Hep G2 cells is associated with a lower mobility in SDS-PAGE disproportionate to the actual difference in M, between proalbumin and albumin, and probably related to structural changes in the analog-containing protein (9). We confirmed this finding in the case of albumin, whereas treat-
ment with canavanine or thialysine did not lead to a modification of the electrophoretic mobility of TBG, providing further evidence against the existence of a pro-TBG. As pointed out above, it is possible that the techniques employed in this study are not sensitive enough to detect a small pro-piece. Since a partial NH2-terminal amino acid sequence of TBG has been reported (23), microsequencing of canavanine-containing TBG could make it possible to ascertain or rule out the existence of a precursor of secreted TBG.

Translation in a rabbit reticulocyte lysate of RNA extracted from Hep G2 cells represented a further approach to determine the size of the apoprotein. So far, translation in a cell-free system of TBG mRNA from normal human liver has been only preliminarily reported by Seo et al (3). Although translation of TBG mRNA is complicated by the fact that TBG, and hence its mRNA, represents only a small fraction of total liver mRNA or proteins secreted by the liver, immunoprecipitation of the in vitro translation product by anti-TBG serum showed a single band which had the same mobility as D-TBG. Since it has been demonstrated that D-TBG still contains ~3% of its weight in carbohydrate (3), it is conceivable that the translation product contains an additional fragment of 1500 daltons or more, since the presence of some carbohydrates in D-TBG might be responsible for a lower mobility in SDS-PAGE (25). This is in keeping with the presence of a signal peptide in the translation product, as is found in most membrane and secretory proteins (26, 27).

Xenopus oocytes have been extensively used to study the translation of foreign messenger RNAs, since they faithfully reproduce not only the biosynthesis of proteins encoded by the message injected, but also the correct modification, processing, and secretion of the translation product (28). The stabilization and repeated translation of the injected mRNA allows an amplification of the process, which is particularly advantageous in working with low abundance messengers, such as those encoding TBG. The fact that we detected TBG in the medium of oocytes injected with Hep G2 RNA is of some significance in further studies on the elucidation of post-translational processing and secretion of this protein. The identification of TBG mRNA by cell-free translation and oocyte microinjection will facilitate the preparation of molecular hybridization probes for extending these studies to the regulation of expression of gene(s) coding for TBG.

In conclusion, our study provides convincing evidence that Hep G2 cells synthesize TBG, which is identical to circulating TBG in immunological and ligand-binding properties. In a cell-free translation system, TBG mRNA directs the synthesis of a product that appears to be larger than circulating TBG apoprotein by at least 1500 daltons. Since direct evidence for the existence of a large pro-TBG is lacking, the irreversibility of TBG denaturation (5, 6) is probably due to phenomena other than the presence of another peptide piece in a putative pro-TBG, such as the addition of the carbohydrate units to the nascent polypeptide chain. Preliminary data from our laboratory seem to indicate that enzymatic deglycosylation of TBG does not affect the metastability of the protein (29).

In the accompanying paper, we evaluate the role of carbohydrates in TBG secretion and the kinetics of TBG secretion in the presence of tunicamycin and menonisin.

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REFERENCES

24. See Footnote 3.

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Characterization of Nascent and Secreted Phenylthiocarbamyl Globulin (TBG) in Cultured Human Hepatoma (Hep G2) Cells.

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MATERIALS AND METHODS

Induction of TBG and TB6

The purified free secreted human plasma (13609) and TB6 were prepared by sequential chromatography on Sephadex G-200 and DEAE-cellulose. TBG was purified via the immunoprecipitation method and separated from 25% myoglobin (Steffens 1971), as described in detail elsewhere (15).

Cell Culture

Human hepatoma cells (Hep G2) (16) were kindly provided by Dr. K. Ericsson, Winter Institute, Philadelphia, PA. Cells were grown to confluency in T-75 or T-25 plastic flasks in Eagle's Minimum Essential Medium with Earle's balanced salt solution supplemented with 2% fetal calf serum, 10 mM Hepes buffer, 2% newborn calf serum, and 40 μg/ml neomycin sulfate, at 37°C under 5% CO₂ in air.

Cell Labeling

After percutaneous staining in either spinachin, methionindine-free medium or mutant, low-glucose (10 mM) medium, cells were labeled with 1.0 × 10⁶ cpm (13609) or 1.0 × 10⁵ cpm (TB6), respectively. In continuous labeling experiments, incubation was terminated after 4 hours by chilling the flasks to 0°C. In pulse-chase experiments, after 10 min labeling (pulse), further labeling of protein was blocked by a 10×-addition of 20,000-fold excess unlabeled methionine for variable periods (chase). At the end of incubation, the medium was collected and centrifuged at 1,000 × g for 15 min to remove cell debris. The supernatant was added to 100 mM TrisPicolinate buffer, pH 7.4, containing 0.5% Triton X-100, 0.05% SDS, 25 mM NEM, 100 μM leupeptin, and 100 μM aprotenin, and proteins were centrifuged at 200,000 × g for 60 min at 4°C in a Beckman SW 50 rotor to remove cellular debris. Media and cell lysates were stored at -70°C until used.

In some experiments, Hep G2 cells were labeled with 1.0 × 10⁶ cpm (13609) for 4 hours in the presence or absence of 2.5 mM N-ethylmaleimide (NEM), an analog of glucose, which blocks the cleavage of phenylthiocarbamyl protein (15). Cells were preincubated overnight in methionine-free medium containing either 1-phenylalanine or leucine. At these concentrations, 1-phenylalanine only slightly affected total secreted and TB6 synthesis. In other experiments, 5 mM leucine was added to serum-free, leucine-free medium.

Trypsinization and Acidification

In vitro labeling of cells (13609) from a smooth muscle culture, 10 μl aliquots of cells or cell lysates were digested with 0.15 μl, 0.1% trypsin solution in 20 mM Tris-acetic acid, and incubated at room temperature for 30 min. After centrifugation for 2 min at 0.5 × g, the pellet was resuspended in 0.15 μl 0.1 M Tris and 0.15 M 20% trichloroacetic acid and washed. The pellet recovered after subsequent centrifugation was digested with 0.15 μl 0.1 M 20% Tris and the residue was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Cell-free translation of RNA

Total RNA (0.1 mg) was translated in vitro to explore tRNA-dependent protein synthesis in a rabbit reticulocyte lysate (New England Nuclear). The reaction mixture (20 μl) contained 100 mM Tris-Cl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM GTP, 20% glycerol, and 15% (v/v) cell-free protein synthesis was performed in a Parkhound T-cell lysate system (Pharmacia). Results were expressed as support of total radioactivity.

Identification of Anti-TBG serum

An antiserum specific for TBG was protected in female New Zealand rabbits using the TBG purified from human plasma. The serum was collected in an immunospecific (13611) and was used to prepare antiserum against TBG. Anti-TBG sera tested by immunoprecipitation with 20% anti-TBG sera were then examined for their ability to precipitate TBG from human plasma.

Immunoprecipitation with anti-TBG serum

An equal aliquot of the TBG serum was added to female New Zealand rabbits using the TBG-purified from human plasma. The serum was collected in an immunospecific (13611) and was used to prepare antiserum against TBG. Anti-TBG sera tested by immunoprecipitation with 20% anti-TBG sera were then examined for their ability to precipitate TBG from human plasma.

Identification of Anti-TB6 serum

An equal aliquot of the TB6 serum was added to female New Zealand rabbits using the TB6-purified from human plasma. The serum was collected in an immunospecific (13611) and was used to prepare antiserum against TB6. Anti-TB6 sera tested by immunoprecipitation with 20% anti-TB6 sera were then examined for their ability to precipitate TB6 from human plasma.

199-AB of Immunoprecipitated products

All precipitates were eluted from protein A and reduced by incubation with 20% 2-mercaptoethanol (13609) or 2-mercaptoethanol, 0.02 M EDTA, 0.01% acrylamide, 0.01% Triton X-100, 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.01% bovine serum albumin. After freezing samples at cryogenic temperatures at -196°C, the precipitates were collected and analyzed by 4% polyacrylamide gel electrophoresis (PAGE) with 0.1% SDS and 0.1% polyacrylamide gels resolving 0.5% (50% acrylamide) and 0.1% (15% polyacrylamide) proteins (13609). The remaining protein was digested with proteinase K for 1 hour at 37°C in 0.1% SDS. After digestion, the samples were then analyzed by SDS-PAGE.

Identification of 199-AB of Immunoprecipitated products

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