Communication

Biosynthesis of Plasma Retinol-binding Protein in Liver as a Larger Molecular Weight Precursor*

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A study was performed to identify the first translated product of messenger RNA for retinol-binding protein (RBP), the specific plasma transport protein for vitamin A. Poly(A)* RNA was isolated from rat liver and translated in the rabbit reticulocyte in vitro protein-synthesizing system. RBP was identified and separated from other translated products by immunoprecipitation with specific rabbit anti-rat RBP antiserum. Sodium dodecyl sulfate-polycrylamide gel electrophoresis and fluorography of the immunoprecipitate consistently revealed one major product which migrated more slowly than purified rat serum RBP. The protein (preRBP) had an approximate molecular weight of 24,000. When dog pancreas microsomal membranes were cotranslationally present, the newly synthesized preRBP was processed to a protein which migrated coincidentally with purified rat serum RBP, approximately 20,500 daltons. These results indicate that RBP is synthesized as a larger molecular weight precursor (preRBP) which is rapidly processed by the removal of a peptide of approximately 3,500 daltons to the size of the final RBP molecule that circulates in the plasma.

Retinol-binding protein is a single polypeptide chain with a molecular weight of 20,006-21,000 (see Refs. 1 and 2 for recent reviews). This protein functions as the plasma transport protein for retinol and serves to mobilize vitamin A from its stores in the liver and to deliver it to its peripheral sites of action. RBP* has a single binding site for one molecule of retinol and is present in plasma mainly as the RBP-retinol complex (holo-RBP) (3, 4). In addition, RBP interacts strongly with plasma prealbumin, and normally circulates as a 1:1 molar RBP::prealbumin complex.

A great deal is now known about the chemical structure, metabolism, and biological roles of RBP (1, 2). RBP is synthesized in the liver (5, 6) and is secreted into the plasma largely as the RBP-retinol complex; the rates of these processes are normally highly controlled. Only a limited amount of information is, however, available about the molecular mechanisms involved in RBP synthesis and secretion, and its regulation.

A number of secretory proteins of eukaryotic and prokaryotic origin have been demonstrated to be synthesized initially as larger molecular weight precursors (see Ref. 7 for review). These preproteins contain an NH2-terminal extension usually ranging in size from 15 to 30 amino acid residues. For secreted proteins, the NH2-terminal signal or leader sequence is usually clipped cotranslationally by a microsomal protease, and the protein is translocated into the cisternae of the endoplasmic reticulum and eventually secreted (7-9). Plasma proteins secreted by the liver which have been shown to be synthesized initially as preproteins include albumin (10), transferrin (11), prothrombin (12), and cockerel very low density apolipoprotein II (13). We now report evidence that RBP is synthesized as the larger molecular weight preRBP, which is rapidly processed by the removal of a polypeptide of about 3500 daltons to the size of the final, secreted RBP molecule. These events thus emerge as possible sites of regulatory control of RBP synthesis and secretion.

EXPERIMENTAL PROCEDURES

RNA Isolation—Total RNA was isolated from the livers of 200- to 250-g male Holtzman rats by the guanidine-HCl method of Chirgwin et al. (14). Poly(A)* RNA was obtained by subjecting the total liver RNA to oligo(dT)-cellulose affinity chromatography as described by Pickett et al. (15). RNA was quantitated utilizing the extinction coefficient of 1 A260 unit/50 μg of RNA. All poly(A)*RNA samples had a 280/290 absorbance ratio of approximately 2.0.

Cell-free Protein Synthesis—The rat liver poly(A)* RNA was translated for 60 min at 28 °C in the microsomal nuclease-treated rabbit reticulocyte lysate system (16), the components of which were purchased from Bethesda Research Laboratories. The reaction mixture, with a final volume of 120 μl, contained 40 μl of reticulocytes, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 10 mM creatine phosphate, 48 mM K-acetate, 1.2 mM MgCl2, 50 PM of each of 19 amino acids (without methionine), 80 μCi of [35S]methionine (New England Nuclear; specific activity, >1000 Ci/mmol) and 4 to 6 μg of poly(A)*RNA. Protein synthesis was determined by quantitation of the amount of [35S]methionine incorporated into trichloroacetic acid-precipitable protein in 2-81 samples of the reaction mixture as described by Mans and Novelli (17). The dog pancreas microsomal membranes used in some of the experiments were a generous gift of Dr. Philip Feigelson, Columbia University.

Immunoprecipitation—At the end of the 60-min incubation period, SDS was added to the translation mixture to a final concentration of 2%, the mixture was then boiled for 3 min, and diluted 1:10 with a solution containing 2.5% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, pH 7.4, and 6 mM EDTA. Samples were then first treated with 100 μl of a Protein-A Sepharose (Pharmacia) suspension plus 20 μl of nonimmune rabbit serum for 1.5 h, to reduce nonspecific binding (18). The Protein-A Sepharose was removed by centrifugation and 100 μl of fresh Protein-A Sepharose suspension and 20 μl of specific rabbit anti-rat RBP antiserum (4) were then added. The samples were allowed to incubate overnight at 4 °C with constant mixing. After incubation and formation of antigen-antibody (RBP-antibody) complexes bound to Protein-A Sepharose, the Protein-A Sepharose was collected on plastic filter discs (Isolab Inc., Akron, OH) followed by extensive washing with a solution containing 0.1 M Tris-HCl, pH 9.0, 0.5 M LiCl, and 1% β-mercaptoethanol. The antigen-antibody complexes were released from the Protein-A Sepharose by incubation for
for 15 min in SDS sample buffer (5% SDS, 5.0 mM Tris-HCl, pH 6.8, 2% β-mercaptoethanol, 20% sucrose, and 0.05% bromphenol blue). After elution from the Protein-A Sepharose, the immunoprecipitated products were subjected to SDS-12.5% polyacrylamide slab gel electrophoresis (19). Gels were fixed, stained, soaked in ENHANCE (New England Nuclear) and radioactive products were visualized by fluorography (20).

The purified rat RBP used in these experiments was isolated from rat serum in the manner described previously from this laboratory (21).

In Vivo Synthesized [35S]RBP—250- to 300-g male Holtzmann rats were injected intraperitoneally with 500 μCi/100 g of body weight of (35S)methionine (New England Nuclear; specific activity, 1200 Ci/mmol). Twelve min after injection (22), the livers were removed and homogenized in 3 volumes of 10 mM Na-phosphate, pH 7.4, 125 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate and 10−4 M phenylmethylsulfonyl fluoride. Samples were centrifuged for 2 h at 100,000 g.

Translation products were subjected to SDS-12.5% polyacrylamide slab gel electrophoresis (20). After elution from the Protein-A Sepharose, the immunoprecipitated bands were visualized by fluorography (20).

Addition of the poly(A)+RNA to the mRNA-dependent rabbit reticulocyte lysate-translation system resulted in a 6- to 10-fold stimulation in the incorporation of (35S)methionine into trichloroacetic acid-precipitable protein. The rate of protein synthesis was linear with time up to 60 min and was concentration-dependent for mRNA up to 50 μg of poly(A)+RNA added per ml of reaction mixture.

Immunoreactive RBP was separated from other mRNA-directed translation products by precipitation with a specific rabbit anti-rat RBP antiserum. Fig. 1 (Lane B) shows that one major protein was immunoprecipitated by the anti-rat RBP serum. This protein consistently migrated more slowly than RBP isolated from rat serum (Fig. 1, position indicated by the arrow marked RBP). Thus, the immunoreactive product translated from liver mRNA was distinctly larger than serum RBP itself. In four separate experiments, the molecular weight estimate of the translated product was 24,000 ± 700 (mean ± SD), whereas that of pure RBP was 20,700 ± 300. These findings suggest that RBP is first synthesized as a larger molecular weight precursor (preRBP). As can be seen in Fig. 1 (Lane A), this preRBP was not detected when the translation products were immunoprecipitated with nonimmune rabbit serum (Lane A) or rabbit anti-rat RBP antiserum (Lane B). Liver poly(A)+RNA was also translated in the rabbit reticulocyte lysate protein-synthesizing system with the co-translational (Lane C) or posttranslational (Lane D) addition of six A530 units of dog pancreas microsomal membranes/ml of reaction mixture and immunoprecipitated with rabbit anti-rat RBP antiserum. Lane E shows the migration of in vivo synthesized [35S]RBP immunoprecipitated from rat liver homogenates. Immunoprecipitated products were analyzed by SDS-12.5% polyacrylamide slab gel electrophoresis and fluorography. Migration positions of purified serum RBP and of 14C-labeled molecular weight standards are indicated by the arrows. The standards used were lactoglobulin (18,367), carbonic anhydrase (30,000), ovalbumin (46,000), albumin (69,000), and phosphofructokinase (92,500).

RESULTS

Addition of the poly(A)+RNA to the mRNA-dependent rabbit reticulocyte lysate-translation system resulted in a 6- to 10-fold stimulation in the incorporation of (35S)methionine into trichloroacetic acid-precipitable protein. The rate of protein synthesis was linear with time up to 60 min and was concentration-dependent for mRNA up to 50 μg of poly(A)+RNA added per ml of reaction mixture.

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Further to establish that RBP is indeed first synthesized as a larger preprotein, the translation of the rat liver poly(A)+RNA was performed in the rabbit reticulocyte lysate system in the presence of dog pancreas microsomal membranes. When six A530 units/ml of lysate of dog pancreas microsomal membranes were added cotranslationally to the reticulocytes and the samples analyzed as previously described, a considerable amount of the preRBP was processed to a smaller protein (Fig. 1, Lane C). This protein migrated exactly to the same position on the gel as did purified rat serum RBP (Fig. 1, position indicated by the arrow), and as did immunoprecipitated in vivo synthesized [35S]RBP from rat liver homogenates (Fig. 1, Lane E). In several such experiments carried out, the extent of processing varied from about one-third to three-fourths of the preRBP. However, in the same experiment, when translation was carried out in the absence of microsomal membranes, the preRBP was the major immunoprecipitated band (Fig. 1, Lane B). Furthermore, posttranslational addition of six A530 units/ml of lysate of dog pancreas microsomal membranes did not process the preRBP to RBP (Fig. 1, Lane D).

DISCUSSION

Vitamin A mobilization from the liver is regulated by factors that control the rates of RBP synthesis and secretion. One factor which specifically regulates RBP secretion is the nutritional vitamin A status. Thus, studies in the rat have shown that retinol deficiency specifically blocks the hepatic secretion of RBP, which can then be stimulated rapidly by retinol repletion (5, 23, 24). This release of RBP is not blocked by inhibitors of protein synthesis. RBP in the liver is mainly found associated with microsomes, and is particularly enriched in the rough microsomal fraction (1). Recent studies suggest that the Golgi apparatus and secretory vesicles, and the microtubules are involved in the pathway of RBP secretion from the liver (24, 25). The subcellular site where retinol normally interacts and forms a complex with RBP is not known.

The experiments reported here were undertaken as part of a research program aimed at defining the molecular events involved in RBP synthesis and secretion, in order to subsequently identify and characterize sites and mechanisms of regulation. Our results show that immunoreactive RBP is initially synthesized from liver poly(A)+RNA as a product which migrates on gels more slowly than does pure rat serum RBP, and with an estimated molecular weight of 24,000. Similar results were also obtained (data not reported here in detail) with liver poly(A)+RNA isolated from vitamin A (retinol) deficient rats. Thus, RBP appears to resemble a number of other secretory proteins (7) in being synthesized initially as a larger molecular weight preprotein. In the case of RBP, however, it might be noted that the size of the prepropeptide in...
pre-RBP is rather large and at the upper limit of the size found for other secretory proteins (7, 26).

We next investigated the manner of processing of the larger molecular weight precursor to RBP. Two lines of evidence were obtained that suggest that pre-RBP is processed to a protein identical in size with serum RBP itself, presumably by translocation of the RBP across the endoplasmic reticulum bilayer (7–9). First, as indicated above, a smaller protein (of the size 8258 Biosynthetic Precursor of Retinol-binding Protein)

Several secretory proteins, including albumin (27), parathyroid hormone (28), and insulin (29) have been demonstrated to be synthesized initially as precursors which are rapidly processed cotranslationally to more long-lived proproteins. Most of the immunoreactive albumin present in liver microsomes consists, for example, of the larger protein, proalbumin (30, 31). Studies conducted with immunoreactive RBP solubilized and isolated from rat liver microsomes have, however, suggested that RBP is not present in the microsomes as a precursor (32). Second, posttranslational addition of the RBP across the endoplasmic reticulum of the pancreas microsomal membranes to the reticulocyte lysate resulted in the processing of a considerable proportion of the pre-RBP to a protein that migrated coincidentally with purified RBP. Several lines of evidence all suggest that a pro-RBP precursor, as compared to RBP isolated from serum, requires the obtaining

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