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CELL-FREE TRANSLATION OF APOCYTOCHROME b_5 MESSENGER RNA AND ANALYSIS OF THE PEPTIDE PRODUCT

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SUMMARY

Using a reticulocyte lysate system, total rat liver poly A+ RNA was translated *in vitro* and the apocytochrome b_5 product examined by immunochemical methods. Demonstrably specific antisera to cytochrome b_5 was produced in sheep against whole purified apoprotein isolated from rat liver microsomes. The results show that apocytochrome b_5 is translated as a protein that is not appreciably different in size from that isolated from membranes.

INTRODUCTION

Cytochrome b_5, an integral protein of the endoplasmic reticulum (ER), is composed of a hydrophilic domain and a hydrophobic segment on the carboxyl end that anchors the apoprotein to the membrane (1). Other ER proteins such as NADH- and NADPH-cytochrome *c* reductase have a similar two-domain structure (2,3). Recent studies have shown that proteins in general which cross membranes to get to their destination, whether they are secretory (4) or mitochondrial (5), are synthesized in a precursor form having a hydrophobic sequence at or near the N-terminus that may be subsequently removed proteolytically from the protein. The G-coat protein of vesicular stomatitus virus is an additional well studied example (6,7). We wanted to know if an ER protein such as apocytochrome b_5 is also made as a large precursor consistent with the presence of a non-anchoring N-terminal hydrophobic sequence.

EXPERIMENTAL METHODS

Purification of cytochrome b_5 and production of antisera: Cytochrome b_5 was purified from rat liver microsomes according to Ozols (8). Approximately 3 mg in Freund's complete adjuvent was injected into a sheep; a second injection of 3 mg was administered 4 weeks later. Blood was collected one

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week after the booster and processed for antisera. Immunoelectrophoresis was performed according to Crowle (10) using an ethyelenediamineacetic acid buffer system containing 1.5% Triton X-100. Microsomes were solubilized by stirring overnight with 1.5% Triton X-100 at 5°C; the supernatant was collected after centrifugation at 100,000 xg for 1 hour. Immunoelectrophoretic plates were incubated for 2 days at 4°C before analysis. The equivalence point of the sheep antisera, determined by radial immunodiffusion with serial dilutions of antisera, was 10 µg of cytochrome b_5 precipitated by 0.1 ml of sheep antisera.

Isolation of liver mRNA and cell-free translation: Rat liver mRNA was isolated according to Naguchi et al. (10). Reticulocyte lysates were prepared according to Hunt and Jackson (11) using phenylhydrazine to induce anemia. Lysates were treated with micrococcal nuclease (12). Twelve micrograms of rat liver polyA+ RNA was incubated in ½ ml of lysate with 25 µCi of (^{3}H)-leucine for one hour at 30°C. After translation, the lysate was diluted to one ml with phosphate-buffered saline, and Triton X-100 and sodium deoxycholate were added to final concentrations of 1% and 0.5%, respectively. After centrifugation at 10,000 xg for 10 minutes to clarify the sample, 0.1 ml of antisera was added to the supernatant. The mixture was incubated at room temperature for one-half hour, and then 6 µg of carrier protein was added and stored for 2 days at 4°. The immunoprecipitate was then pelleted and washed once with PBS containing 1% Triton X-100 and 0.5% deoxycholate. The pellet was resuspended in buffer and pelleted through 1.0 M sucrose at 10,000 x g for 10 minutes. The whole tube was then frozen. The tip of the frozen tube was cut off and the thawed contents solubilized in 90 µl of SDS-solubilization buffer (13). The protein was run on Laemmli gels containing 12.5% acrylamide (14). Gels were frozen, sliced into 2 mm discs and solubilized in scintillation vials containing ½ ml of NCS:H_2O (9:1) overnight at 40°C. Ten ml of toluene scintillation fluid was added and the vials counted. Total incorporation into protein was determined by TCA-precipitation of 5 µl lysate and collection on GF/C filters which were then dissolved in 1 ml of NCS:H_2O (9:1) and counted in a toluene fluor.

Analytical methods and materials: Protein determinations were done according to Lowry et al. (15) using bovine serum albumin as the standard. Cytochrome b_5 concentrations were measured according to Omura and Sato (16) using an Aminco DW-2 spectrophotometer. All glassware was acid-washed and solutions were autoclaved or prepared with autoclaved water. (^{3}H)-leucine (140 Ci/mmole), proteinase K and NCS were purchased from New England Nuclear or Boehringer Mannheim.

RESULTS

The detergent-solubilized cytochrome b_5 was homogeneous on SDS-acrylamide gels and had an apparent molecular weight of 17,000. Tryptic digest analysis has also confirmed its purity and identity (D. Peterson, unpublished results). It had a specific content of 20-23 nmoles/mg protein, identical to that reported by West and Lu who used a similar isolation technique (17).

The specificity of the b_5-antisera was demonstrated in 3 ways. First, it inhibited the NADH-cytochrome c reductase activity of isolated microsomal membranes by about 60%, whereas NADPH-cytochrome c reductase activity in the

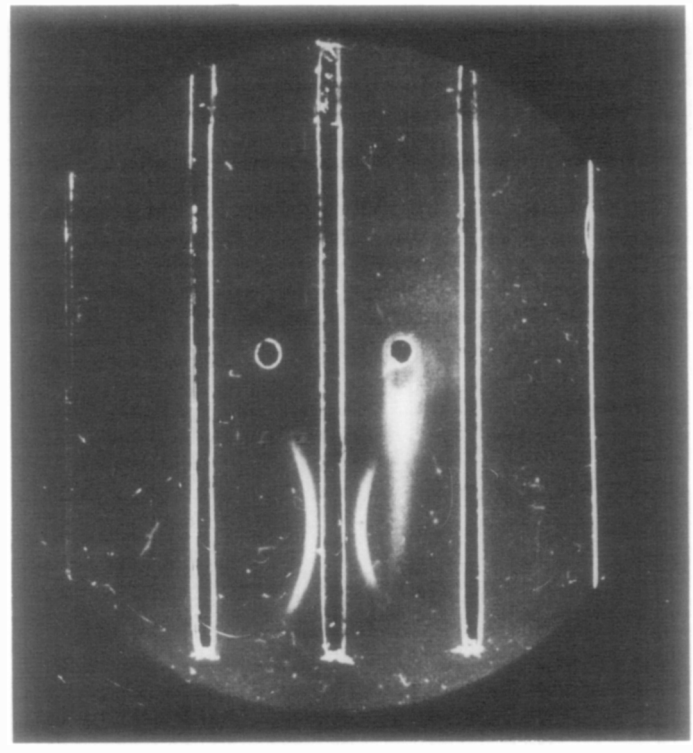

Figure 1. Immunoelectrophoresis of triton-solubilized microsomes and purified cytochrome b_5. Electrophoresis was performed as described in Methods. The Ig fraction was put into the trough and allowed to react for 2 days before photographing. Solubilized microsomes are on the right, purified protein on the left.

same membranes was unaffected (viz. 18). In these experiments, increasing amounts of antisera were mixed with a constant concentration of liver microsomes and the reductase activity determined. Neither microsomal enzymatic activity was affected by preimmune sera. Second, when detergent-solubilized microsomes were immunoelectrophoresed with b_5-antisera a single antigen band corresponding to that of purified cytochrome b_5 was obtained (Fig. 1). No cytochrome b_5 antigen bands were detected when preimmune sera was used. Thirdly, Ouchterlony double diffusion in 1% agarose containing 1½% Triton X-100 confirmed that the antisera reacted with a single component in the solubilized microsomes and showed identity with the purified protein.

In our hands the reticulocyte lysate system incorporated approximately 25-30,000 cpm of (^{3}H)-leucine per 5 µl lysate from rat liver polyA+ RNA.

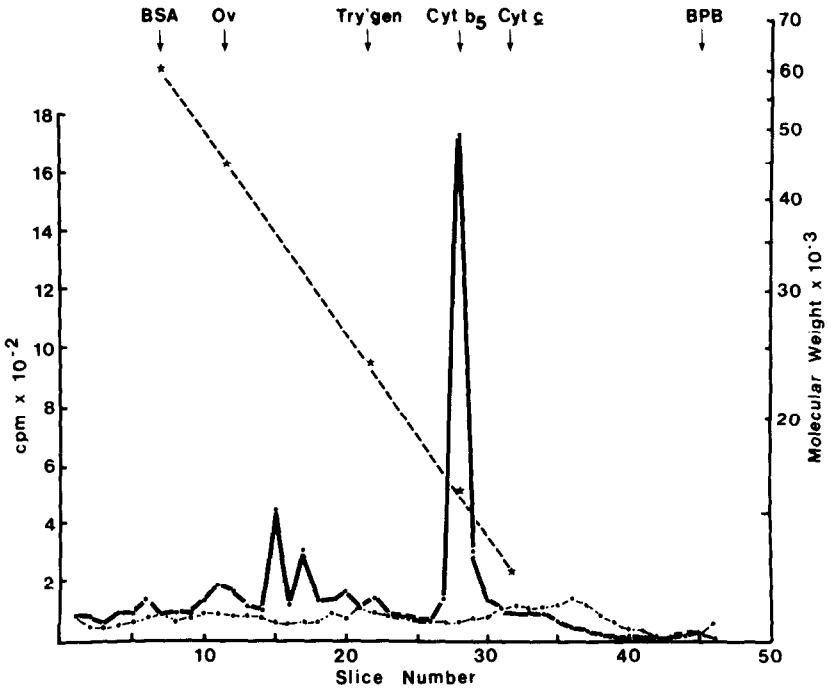

Figure 2. SDS-polyacrylamide gel electrophoresis of immunoprecipitate. .____. Immunoprecipitated product using immune sera. o____o Immunoprecipitated product using preimmune sera. Molecular weight standards: bovine serum albumin (67,000), ovalbumin (44,000), trypsinogen (24,000), cytochrome b5 (17,000), cytochrome c (13,400), bromphenol blue.

Lysates with no added RNA incorporated approximately 2,000 cpm. When proteins were labeled with (^{35}S)-methionine and subsequently run on SDS-polyacrylamide gels, a large number of bands were detected by autoradiography with molecular weights from 15,000 to over 70,000. After immunoprecipitation of incubated lysates with anti-cytochrome b_5 and presentation of the antigenic products on SDS-polyacrylamide gels a single band of radioactivity was detected (Fig. 2). This band had the same molecular weight as authentic cytochrome b_5 and was not seen when preimmune sera was used or when the immune sera was preincubated with excess amounts of purified cytochrome b_5. The radioactivity in the peak represented approximately 0.05% of the total radioactivity incorporated. As a further corroboration of the identity of the translated cytochrome b_5 apoprotein, the complete lysate mixture after incubation was passed through a 1 ml DEAE-cellulose column under conditions used to bind the hemoprotein

during its purification (8). The protein was then eluted with 0.25 M thiocyanate. Immunoprecipitation was then done using the eluted material and labelled cytochrome b_5 was detected on the SDS gels with the same molecular weight as the authentic protein.

DISCUSSION

The results demonstrate that in the reticulocyte lysate system apocytochrome b_5 is not synthesized as a significantly larger precursor. Endogenous processing of the translational product by lysate currently appears unlikely. The only known rabbit lysate protease that acts on newly-synthesized proteins is one that removes the N-terminal methionine (19). Glycosylation of the protein could also alter the apparent molecular weight on SDS gels. Sequence data, though, does not support the presence of any carbohydrate side chains in cytochrome b_5 (20). Also, the membrane fractions needed for glycosylation are not present in the reticulocyte system (21).

Since it appears that apocytochrome b_5 is not synthesized in significantly larger precusor form, there is the good possibility that it is made on free polysomes (4) and released into the cytosol where it would incorporate then into the membrane (22). Since the hydrophobic tail is only 30 residues long, it would not be exposed outside the large ribosomal subunit until the apoprotein was released from the ribosome (23).

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