Conversion of Rat Pre-proalbumin to Proalbumin in Vitro by Ascites Membranes

DEMONSTRATION BY NH₂-TERMINAL SEQUENCE ANALYSIS*

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Rat liver poly(A)-containing RNA was translated in an ascites cell-free system. Labeled protein precipitable by antibody directed against rat serum albumin was identified as pre-proalbumin based on its size and partial NH₂-terminal sequence. However, when an ascites membrane fraction was added to the translation reaction, the albumin antibody-precipitable material was smaller than pre-proalbumin. Partial NH₂-terminal sequence analysis of this protein revealed that it was proalbumin. Conversion of pre-proalbumin to proalbumin by the ascites membrane fraction was complete and precise—i.e. no serum albumin was observed. Reconstitution in vitro of the processing of pre-proalbumin to its stable intracellular form, proalbumin, provides a method for studying the initial proteolytic event involved in secretion of rat serum albumin.

Since these mRNAs code for pre-proteins and since smaller forms of the proteins (the pro form or the serum form) are found in vesicles of the RER, proteolytic conversion must occur. Since significant amounts of intact pre-proteins have not been demonstrated in vivo, this conversion must either occur during translation of the growing nascent chain, or within seconds after its completion (16-22).

This “processing” of the pre-proteins has recently been studied by adding membrane fractions derived from RER to in vitro translation reactions (16-19). These experiments demonstrated that the growing nascent chains of pre-proteins were proteolytically processed by the membrane fractions to smaller proteins. Birken et al. (17) determined by NH₂-terminal sequence analysis that pre-hPL had been converted to hPL by an ascites membrane fraction. Subsequent reports revealed that pre-growth hormone, pre-prolactin, and preproinsulin were appropriately converted to their stable intracellular forms by RER membranes during in vitro translation of their mRNAs (18, 19).

The early events in the synthesis and secretion of some proteins and hormones, including rat serum albumin, thus probably include 1) initiation of translation of the peptides; 2) binding of the growing nascent chains to RER membranes; 3) proteolytic removal of the amino acids of the prepeptide; and 4) vectorial discharge of the completed peptide across the membrane into the RER vesicular space (7, 23, 24).

In order to study these early events in the secretory process, we coupled translation of albumin mRNA to cleavage by an ascites membrane fraction. The results showed by NH₂-terminal sequence analysis that in vitro conversion of pre-proalbumin to proalbumin had occurred. The cleaved product contained the hexapeptide pro sequence intact on the NH₂ terminus of rat serum albumin. Moreover, no detectable conversion to rat serum albumin was observed.

Experimental Procedures

⁻³¹[H]Leucine (57 Ci/mmol), [³¹]H]arginine (23 Ci/mmol), and [⁵⁵]S]-methionine (550 Ci/mmol) were purchased from New England Nuclear.

RNase-free sucrose and heparin were obtained from Schwarz/Mann; oligo(dT)-cellulose (type 2-T) from Collaborative Research; antisera from Cappel Laboratories; and rat serum albumin (Fraction V) from Miles Laboratories. All other biochemicals were from Sigma.

Poly(A)-containing RNA from rat liver and ascites ribosomes and ribosome-free supernatant were prepared as previously described (6, 16).

An ascites membrane fraction (16, 17) was isolated by layering a preincubated 30,000 X g (av) supernatant of ascites cell lysate over 1 M sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM sucrose in Buffer A (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 5 mM su
magnesium acetate, 7 mM 2-mercaptoethanol) and centrifugation at 200,000 x g (av) at 4°C for 5 h. The cloudy layer banding at the top of the sucrose was removed, diluted with Buffer A, and collected by centrifugation (100,000 x g (av) for 1 h). The “membrane” pellet was then dissolved in Buffer A to a final concentration of 40 mg/ml

Fractionated ascites translation assays contained (final concentrations): 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.6), 105 mM KCl, 2.8 mM Mg(acetate), 2 mM dithiothreitol, 1 mM ATP, 0.4 mM GTP, 10 mM creatine phosphate, 160 μg/ml of creatine kinase, 50 μg/ml unlabeled amino acids, 40 μg/ml of rat liver poly(A)-containing RNA, 110 μg/ml of ascites ribosomes, and 300 μM (L.7 mg of protein/ml) of ascites ribosome-free supernatant (6, 16, 17). Concentrations of added labeled amino acids were 8 μM [3H]leucine, 12 μM [3H]arginine, or 0.6 μM [35S]methionine. The ascites membrane fraction was added to some translation assays to achieve a final concentration of 2 mg/ml.

The products of translation were purified by indirect immunoprecipitation with rabbit anti-rat serum albumin antibody and goat-anti-rabbit IgG as described (4, 6). For larger scale isolation and purification of cell-free products, indirect immunoprecipitation, preparative SDS-polyacrylamide gel electrophoresis, and acetone/HCl precipitation were employed as previously outlined (4, 6).

Partial sequences of the purified, immunoprecipitated, cell-free products were determined by automated Edman degradation. Method of degradation and analysis of radioactivity found per cycle have been described (4, 6). Repetitive yields of unlabeled amino acids derived from carrier rat serum albumin in all sequencer runs were 94%. Calculation of “per cent theoretical yields” for radioactive peaks found following similar analysis of assays without added RNA.

RESULTS

When added to an in vitro ascites cell-free translation system, the ascites membrane fraction employed in these experiments correctly converted pre-hPL to its mature, serum form, hPL (16, 17). We, therefore, tested the ability of this in vitro system to translate rat liver mRNA. Addition of rat liver RNA to the ascites translation system resulted in a 2.5- to 14-fold increase in the amount of radioactivity incorporated into protein (Table I). Although the membrane fraction partially inhibited the translation of rat liver RNA, stimulation over minus RNA blanks was still 1.9- to 10.3-fold. However, recovery of labeled protein precipitable by rat serum albumin antibody was only 0.2% of the total labeled protein. These data indicated that this translation system was much less efficient in making this large peptide than the wheat germ translation system which we have previously used (4, 6).

We isolated the [35S]methionine-labeled, rat serum albumin antibody-precipitable material from translation assays performed in the presence (Fig. 1C) and absence (Fig. 1B) of the ascites membrane fraction. The major immunoprecipitable product synthesized in the absence of membranes migrated somewhat slower than unlabeled rat serum albumin run in the same slot. This band migrated identically with pre-proalbumin synthesized in wheat germ and reticulocyte lysates (not shown). However, when membranes were added to the translation system, the rat serum albumin antibody-precipitable material migrated more rapidly than pre-proalbumin. These results were confirmed when [3H]leucine-labeled, immunoprecipitable material was analyzed by SDS-gel electrophoresis and slicing and counting of the gels (data not shown). The difference in mobility between material from translations in the absence and presence of membranes was 1 to 2 mm, which indicated a change in molecular weight of about 2,000. These results suggested the membrane fraction cleaved pre-proalbumin to a smaller form.

In order to prove that pre-proalbumin was the initial product of translation of rat liver albumin mRNA in this system, we purified the [3H]leucine-labeled protein precipitated by rat serum albumin antibody (see “Experimental Procedures”). The translation assay contained no added membranes. Following automated Edman degradation of this sample, peaks of 3H were found at cycles 7, 8, 9, and 10 (Fig. 2A). This tetra-leucine sequence at these positions occurred in pre-proalbumin synthesized in wheat germ and reticulocyte lysates (6 and Fig. 3). These results indicated that albumin mRNA directed the synthesis of pre-proalbumin in this fractionated ascites translation system if no membranes were added.

| Table I |
| Translation of liver mRNA in fractionated ascites cell-free system |

<table>
<thead>
<tr>
<th>Amino acid incorporated</th>
<th>Protein dpm/5 µl x 10^2</th>
<th>Fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-[3H]Arg</td>
<td>-RNA</td>
<td>+RNA^a</td>
</tr>
<tr>
<td>Minus membranes</td>
<td>10.1</td>
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</tr>
<tr>
<td>Plus membranes</td>
<td>14.4</td>
<td>27.5</td>
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<tr>
<td>L-[4,5-3H]Leu</td>
<td>-RNA</td>
<td>+RNA^a</td>
</tr>
<tr>
<td>Minus membranes</td>
<td>6.2</td>
<td>86.3</td>
</tr>
<tr>
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<tr>
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<td>-RNA</td>
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</tr>
<tr>
<td>Plus membranes</td>
<td>15.0</td>
<td>54.0</td>
</tr>
</tbody>
</table>

^a Following indirect immunoprecipitation and analysis by SDS-polyacrylamide gel electrophoresis. 0.2% of the total protein radioactivity was found in a band of molecular weight 60,000 to 70,000 in assays containing added RNA. No such peak of radioactivity was found following similar analysis of assays without added RNA.

FIG. 1. Analysis by SDS-polyacrylamide gel electrophoresis and autoradiography of rat serum albumin antibody immunoprecipitates of ascites translation assays. [35S]Methionine was used as the amino acid precursor in 120-µl ascites translation assays (see “Experimental Procedures”) performed with (A) no added rat liver RNA, but with ascites membranes; (B) rat liver poly(A)-containing RNA; and (C) rat liver RNA and ascites membranes. The radioactivity in protein following 3 h of incubation was (A) 0.4 x 10^6 dpm; (B) 2.5 x 10^6 dpm; and (C) 1.2 x 10^6 dpm. The samples were subjected to indirect immunoprecipitation and the immunoprecipitates analyzed on 7.5% SDS-polyacrylamide gels with 4 µg of rat serum albumin added to each. Dots on either side of gel lanes identify position of unlabeled rat serum albumin. The dried gels were examined by autoradiography following exposure for 3 days.
In Vitro Conversion of Pre-proalbumin to Proalbumin

The sequence shown is that determined in protein obtained following translation in the wheat germ system (4, 6). The sequence of proalbumin begins at cycle 19 of pre-proalbumin. The sequence of serum albumin begins at cycle 25 of pre-proalbumin and at residue 7 of proalbumin. Circled numbers above the sequence indicate the positions of residues confirmed by data in Fig. 2, A and B to occur in pre-proalbumin. Circled numbers below sequence indicate cycles at which arginine residues were found (Fig. 2C) in the cleaved product, i.e. proalbumin. Note that arginine residues at cycles 19, 23, and 24 of proalbumin correspond to arginines found at cycles 1, 5, and 6 of proalbumin and that arginine at cycle 16 of proalbumin corresponds with the arginine known to occur at position 10 in the NH2-terminal sequence of serum albumin.

Fig. 2. Sequential, automated Edman degradation of rat serum albumin antibody-immunoprecipitable material from various ascites translations. Reactions were performed in 1.5 ml volume with (A) [3H]leucine and rat liver RNA; (B) [3H]arginine and RNA; and (C) [3H]arginine, RNA, and ascites membranes. Following purification (see “Experimental Procedures”), the samples were separately sequenced for 26 cycles by automated Edman degradation. Disintegrations per min subjected to sequencing was (A) 35,800, (B) 11,000, and (C) 11,800. “Per cents of theoretical yield” (Refs. 4 and 6 for this calculation) for peaks at various cycles were: (A) cycle 7 (59%), cycle 8 (89%), cycle 9 (83%), and cycle 10 (64%); (B) cycle 1 (240%), cycle 19 (25%), cycle 23 (29%), and cycle 24 (58%); and (C) cycle 1 (336%), cycle 5 (64%), cycle 6 (57%) and cycle 16 (81%). Per cent recoveries (solid lines) in all figures were graphed such that 0% equalled the background radioactivity of the sequencer run and 100% equalled “100% theoretical yield” (4, 6).

[3H]Arginine was then incorporated into protein in a translation assay which contained no added membranes. Edman degradation of the purified, rat serum albumin antibody-precipitable material from a translation assay performed in the presence of the ascites membrane fraction. Following automated Edman degradation of this sample, peaks of [3H]were found at cycles 1, 5, 6, and 16 (Fig. 2C). The large radioactive peak at cycle 1 contained more counts than expected. As suggested above, this peak probably included contaminating [3H]arginine. However, the amount of radioactivity found at cycle 1 in this sequencer run was twice as much as was found at cycle 1 in the Edman degradation of the same amount of [3H]arginine labeled pre-proalbumin (compare Figs. 2C and 2B). Therefore, [3H]arginine label at cycle 1 in this sequencer run (Fig. 2C) also included radioactivity derived from an arginine at the NH2 terminus of this peptide. Similar results were obtained in a second experiment, i.e. peaks of [3H]arginine were found at cycles 1, 5, 6, and 16 following Edman degradation. The known NH2-terminal sequence of rat liver proalbumin (Fig. 3) contains arginine residues at positions 1, 5, 6, and 16 (3–5, 25). Since proalbumin contains a hexapeptide extension on the NH2 terminus of serum albumin, the arginine residue at position 16 of proalbumin corresponds to the arginine at position 10 of the NH2-terminal sequence of serum albumin (3, 25). The partial sequence data shown in Fig. 2C thus demonstrated that the ascites membrane fraction converted pre-proalbumin to proalbumin during translation of albumin mRNA in this in vitro system. Since no radioactivity over background was found at cycles 19, 23, and 24, the conversion was complete, i.e. all pre-proalbumin was correctly cleaved to proalbumin. Moreover, since no peak of [3H]arginine was found at cycle 10, the data indicated that no detectable serum albumin was formed during this in vitro cleavage reaction. (The methods used would have detected conversion of 10% or more of pre-proalbumin to serum albumin.)

**DISCUSSION**

Previous reports demonstrated that the products of in vitro translations of mRNA's coding for numerous secretory proteins and hormones were larger than the serum proteins. These presumed precursors were designated pre-proteins (6–21). In all cases, the pre piece was an extension on the NH2
terminus of the protein, contained a high per cent of hydrophobic amino acids, and was 16 to 30 amino acids long. However, significant amounts of intact pre-proteins were not observed in vivo (22). Rather, the forms of these pre-proteins found in vesicles of RER were either serum proteins (i.e., growth hormone) or pre-proteins (proinsulin, proparathyroid hormone, proalbumin) intermediate in size between the pre forms and the serum forms of these proteins. From these results it was clear that rapid proteolytic removal of the amino acids of the pre piece must have occurred (18-24) in vivo.

In order to examine the molecular events involved in this processing of pre-proteins and as a preliminary step in the characterization of the protease(s) involved, in vitro reconstitution of translation of mRNAs and proteolytic processing was performed. Results of these experiments (16-24) indicated that (i) RER membranes stripped of ribosomes contained proteases which converted the pre forms to smaller forms. (ii) the smaller forms were sequestered inside the membranes and were resistant to attack by exogenously added trypsin, (iii) cleavage occurred during translation of the mRNAs when the nascent chain was about 60 residues long, and (iv) the conversion process was inhibited by detergent.

Several investigators then characterized the products of processing by NH₂-terminal sequence analysis of the smaller forms. Birken et al. (17) using a translation assay identical to the one reported here found that the ascites membrane fraction correctly and quantitatively converted pre-hPL to the serum form. It was subsequently demonstrated (18, 19) that dog pancreas or bovine pituitary RER membranes converted pre-growth hormone to two species of growth hormone, pro-lactin to prolactin and pre-insulin to proinsulin. In all cases, processing of the pre forms resulted in products with exactly the same NH₂-terminal sequence as the serum forms of these proteins.

In this report we provided evidence that rat pre-proalbumin is converted to proalbumin by the ascites membrane fraction during in vitro translation. This is the first demonstration of conversion of a pre-pro protein to the pro form containing the pre piece intact on the NH₂ terminus and is the first proven processing of a pre-protein for which the entire amino acid sequence of the pre piece is known. Since no serum albumin was found by sequence analysis of the cleaved product (Fig. 2C), a protease which would convert proalbumin to serum albumin was either absent from the membrane fraction or inactive under the conditions of the assay.

Several noteworthy points emerged from these in vitro processing studies. Different sources of protease(s) were employed—stripped dog pancreas RER membranes (18, 19, 24), bovine pituitary RER membranes (19), mouse ascites tumor cell membranes (17), and stripped liver RER membranes. Nonetheless, when the processed products were analyzed by NH₂-terminal sequence analysis, the cleavage proved to be precisely correct, producing polypeptides with NH₂-terminal sequences identical to the stable intracellular forms of the proteins. Although the membrane preparations were isolated from different species and tissues than the mRNAs being translated, the proteases were active against the peptide substrates (nascent pre-proteins) and removed exactly the correct number of amino acids. These results are perhaps surprising when the known NH₂-terminal sequences of the pre-proteins are compared (6, 9-21). Ascites membranes converted both nascent pre-hPL (17) and pre-proalbumin in the appropriate smaller form even though the two pre proteins differ in several respects (6, 17). (i) The lengths of the two pre pieces are different (25 versus 18 amino acids). (ii) The primary sequences of the pre pieces are not homologous and are especially different at the -COOH-terminal segments of the pre-pieces. (iii) The amino acid sequences around the final cleavage site are completely different. (iv) The final peptide bond which must be cleaved during processing is quite different (S-Val for pre-hPL versus Ser-Arg for pre-proalbumin). The NH₂-terminal sequences of the three pre-proteins correctly processed by dog pancreas membranes (18, 19) are also dissimilar. However, in spite of these differences, the fact that the same RER membranes correctly processed various nascent pre-proteins suggests that some common denominator must be recognized by the proteases such that only the pre piece is removed (21). Since the primary amino acid sequences are somewhat different, possibly the secondary and tertiary structures of the pre-protein NH₂ termini, a sequence somewhat distant from the final cleavage site or the interaction of the pro-protein and the RER membranes might provide the necessary signals limiting proteolysis. However, until the complete sequences of all of the pre proteins are known, speculation concerning the type of protease and the recognition signal for the protease is difficult.

As yet, the number, type, and peptide bond specificities of the enzymes which process pre-proteins are entirely unknown. Moreover, the mechanism of removal of the amino acids of the pre piece is inapparent. Processing could occur by (i) one or more endoproteolytic events, (ii) repeated exoproteolytic cleavages, or (iii) a combination of the two. Purification and characterization of the proteolytic enzymes will clarify the events involved in pre-protein processing and may provide insight into the importance of these events in the regulation of synthesis and secretion of pre-proteins.

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REFERENCES


The entire sequence of the pre-piece of pre-hPL has not yet been established. However, the -COOH-terminal sequence (7 residues) of the pre piece of pre-hPL does not appear to contain serine, glycine, alanine, or phenylalanine residues (S. Birken, R. Canfield, and I. Bonne, unpublished data).
In Vitro Conversion of Pre-proalbumin to Proalbumin

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