Hoppe-Seyler's Z. Physiol. Chem.
Bd. 364, S. 227–237, März 1983

Cell-Free Translation of Human Liver Apolipoprotein AI and AII mRNA Processing of Primary Translation Products

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(Received 18 November 1982)

Dedicated to Professor A. Butenandt on the occasion of his 80th birthday

Summary: Human liver apolipoprotein AI and A II poly(A+) mRNA has been translated in the cell-free rabbit reticulocyte lysate system.

The structures of the two primary translation products of these two main protein components of human serum high-density lipoprotein (HDL) have been characterized. The products of the synthesis in vitro are preproapolipoproteins. The signal sequence (pre-sequence) of the primary translation product of human apo AI mRNA consists of 18 amino acids, that of apo AII of 17 amino acids. The cotranslational translocation into dog microsomal vesicles is associated with the cleavage of these sequences by the signal peptidase releasing the proapolipoproteins AI and AII, both extended by an N-terminal hexapeptide. Preproapolipoprotein AII is synthesized in its monomeric form consisting of 100 amino acids. Pro-apo AII is present in the vesicles of the endoplasmic reticulum also as monomer.

Sequencing of the radiolabelled signal sequences of both pre-forms revealed their strongly hydrophobic nature. Despite the high affinity of HDL-apolipoproteins for complex lipids their secretion requires these hydrophobic signal sequences for translocation. Internal recognition sequences in the native apoproteins are not responsible for the transmembrane transport.

Zellfreie Translation von menschlicher Leber-Apolipoprotein-AI- und -AII-mRNA. Processing des primären Translationsprodukts

Zusammenfassung: Apolipoprotein-AI- und -AII-(Apo-AI- und -AII-) -Poly(A+)-mRNA wurde aus menschlicher Leber isoliert und im zellfreien Kaninchen-Retikulozyten-Lysat translatiert. Die primären Translations-Produkte der Hauptproteinkomponenten der menschlichen Serum-High-Density-Lipoproteine (HDL) wurden charakterisiert und der Translokationsmechanismus analysiert.

Die In-vitro-Synthese-Produkte sind Präproapoproteine. Die Signalsequenz (Sequenz der Prä-Form) des primären Translationsproduktes der menschlichen Apo-AI-mRNA besteht aus 18, die des Apo AII aus 17 Aminosäuren. Diese Sequenzen werden während der kotranslationalen Translokation in Hunde-Mikrosomen-Vesikel durch die Signal-Peptidase abgespalten, wodurch die Proapolipoproteine AI und AII, beide mit

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Abbreviations:
apo AI, II = apolipoprotein AI and AII of human serum high-density lipoprotein; ER = endoplasmic reticulum; Pth = phenylthiohydantoin; HDL = high-density lipoprotein; poly(A+) mRNA = poly(A)-rich mRNA; oligo(dT)$_{12-18}$-cellulose = 12–18-nucleotides long oligo(dT), bound to cellulose; $\overline{\text{Glu}}$ = pyroglutamic acid.

0018-4888/83/0364-0227$02.00

einem Hexapeptid am N-Terminus versehen, freigesetzt werden.

Präapolipoprotein AII wird in seiner monomeren Form, die aus 100 Aminosäuren besteht, synthetisiert. Auch das Proapolipoprotein AII liegt in den Vesikeln des endoplasmatischen Retikulums in monomerer Form vor.

Sequenz-Analyse der radioaktiv markierten Signale der beiden Prä-Formen bewies ihre stark hydrophoben Eigenschaften. Trotz der hohen Bindungsaffinität der HDL-Apolipoproteine für komplexe Lipide erfordert ihre Translokation diese hydrophoben Signalsequenzen. Interne Erkennungssequenzen der nativen Apoproteine sind nicht für den transmembranalen Transport verantwortlich.

Key words: Human liver mRNA, cell-free translation and immunoprecipitation of translation products, radioactive sequencing of signal peptide and prosequence of apo AI and AII.

The principal apolipoproteins of human serum high-density lipoprotein (HDL) are apolipoprotein AI and AII. Both apoproteins exhibit a propensity for binding phospholipids and cholesterol, and therefore possess a key function in the lipid transport system of the blood circulation.

Although perfusion experiments of rat liver[1] and analyses of chyle from rat thoracic duct[2] suggest hepatocytes and intestinal mucosa cells as sites of the biosynthesis of apolipoprotein AI and AII, we have recently given direct evidence for rat liver as a main site of apolipoprotein AI synthesis by the cell-free translation of rat liver mRNA[3]. Similar experiments, using rat intestinal RNA[4], demonstrated the cell-free synthesis of apo AI.

We found that rat serum high-density apo AI is synthesized with a signal sequence which is processed during the cotranslational translocation across competent microsomal membranes. The translocation was mediated by the signal recognition particle[5,6]. Therefore, our studies proved that the primary translation product of rat apo AI mRNA required a signal peptide sequence for transmembrane transport. Internal recognition sequences were not responsible for the translocation through the rough endoplasmic reticulum membranes.

In addition, the signal peptide sequence of the primary translation product of rat intestinal apolipoprotein AI mRNA is followed by a prosegment consisting of a hexapeptide.

In this paper we describe the isolation of poly(A+) mRNA from human liver, the translation of apolipoprotein AI and AII mRNA in a cell-free system and the characterization of the two primary translation products, the molecular masses of which exceed those of the native apolipoproteins AI and AII by about 3 kDa.

The precursor of human liver apolipoprotein AI contains an extension of 24 amino acids and that of apolipoprotein AII of 23 amino acids. The apo AII precursor is formed in its monomeric form (mol. mass about 12 kDa).

Upon translocation across translocation-competent rough endoplasmic reticulum (ER) membranes a signal sequence of 18 amino acid residues of the precursor apo AI and 17 amino acids of the apo AII precursor are cleaved. The two translocated apolipoproteins are present in the vesicles of the endoplasmic reticulum as pro-apo AI and pro-apo AII, each with an additional N-terminal hexapeptide. Pro-apo AII is still present in its monomeric form after translocation. Radioactive amino acids were used for labelling the precursor apoproteins and their signal and prosegment sequences were partially determined by automated Edman degradation.

Materials and Methods

[^{35}S]Methionine at 600 Ci/mmol, [^{35}S]cysteine at 600 Ci/mmol, [^{3}H]leucine at 190 Ci/mmol, [^{3}H]proline at 130 Ci/mmol, [^{3}H]phenylalanine at 130 Ci/mmol,

[^{3}H]glutamine at 30 Ci/mmol, [^{3}H]aspartic acid at 60 Ci/mmol and [^{3}H]lysine at 100 Ci/mmol were purchased from Amersham-Buchler Braunschweig. [^{3}H]Alanine at 85 Ci/mmol was purchased from New England Nuclear, Dreieich.

Heat-killed, formalin-treated *Staphylococcus aureus* (Cowan strain) were prepared for immunoadsorption according to the method of Kessler[7]. Trypsin and chymotrypsin were from Boehringer Mannheim GmbH, Trasylol from Bayer AG, Leverkusen, oligo(dT)$_{12-18}$-cellulose was purchased from Bethesda Research Laboratories, Neu-Isenburg.

Human serum HDL was isolated by repeated sedimentation/flotation in the density range between 1.063–1.21 g/ml in homogeneous form[8], as demonstrated by agarose electrophoresis. After delipidation of HDL with chloroform/methanol[9] apoproteins AI and AII were separated and purified to homogeneity by DEAE-cellulose chromatography[8] followed by Sephadex G-150 chromatography[10]. Antibodies were raised against human HDL, apo AI and carboxymethylated monomeric apo AII (apo AII/2) in rabbits according to an established immunization scheme[11]. The latter antibody cross-reacted with dimeric apo AII.

The IgG fraction was isolated by ammonium sulfate precipitation[12]. Dog pancreatic rough microsomal membranes were prepared as described[13]. Human placental ribonuclease inhibitor was isolated in pure form according to the two-step purification procedure described by Blackburn et al.[14]. Total human liver RNA was isolated either by the guanidine thiocyanate method[15] or the phenol method[16] and poly(A+) RNA, obtained from these preparations by oligo-(dT)$_{12-18}$-cellulose affinity chromatography[17]. Poly-(A+) RNA samples were separated at 4 °C in vertical agarose slab gel (1.5%) electrophoresis in Tris/borate (pH 8.2), urea at 4 °C, staining with ethidium bromide (1 μg/ml) and examined under UV light[18].

A rabbit reticulocyte lysate, obtained from *N*-acetylphenylhydrazine-treated rabbits, was nuclease-treated and conditioned following exactly the procedure outlined by Pelham and Jackson[19].

Analytical translation mixtures contained 7 μl lysate and 100 μCi [^{35}S]methionine in a total volume of 15 μl, preparative incubation of 100 μl lysate and 1 mCi [^{35}S]methionine in 1 ml total volume. Lysates were fortified with 19 amino acids except the ^{35}S-(methionine, cysteine) or ^{3}H-labelled amino acid (1 mCi) for radiolabelled sequence determination. Poly(A+) RNA concentration ranged between 10 to 15 μg/ml. Human placental ribonuclease inhibitor (spec. act. 10000 units/mg) was added at a concentration of 100 units/ml (for definition of unit see ref.[14]).

The translation products were denatured in 2% sodium dodecyl sulfate. Triton X-100 was added in a five-fold excess (w/w) over sodium dodecyl sulfate and either the IgG fraction or the complete antiserum against the respective apolipoprotein antigen added and the formation of the antibody-antigen complex completed by overtop rotation in the cold room. The complex was adsorbed to a 100 μl of heat-inactivated formalin-fixed *Staphylococcus aureus* (Cowan strain) suspension (10%) and washed extensively (10 times) with 10 vol. of a wash solution (0.5% Nonidet P-40, 5mM EDTA, 50mM Tris/HCl, pH 7.5; NaCl in decreasing concentration from 0.5M to 0).

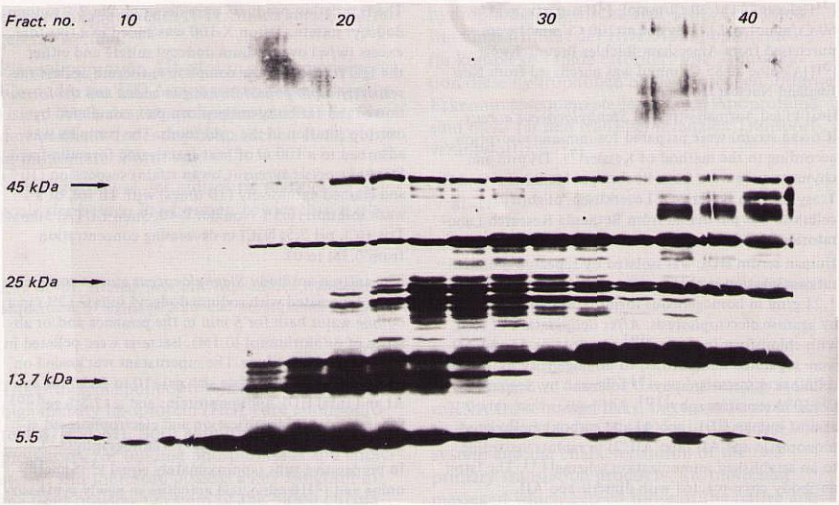
The antigen-antibody *Staphylococcus aureus* complexes were dissociated with sodium dodecyl sulfate (2%) in a boiling water bath for 5 min in the presence and/or absence of dithiothreitol (0.1M). Bacteria were pelleted in an Eppendorf Minifuge. The supernatant was loaded on a gradient polyacrylamide slab gel (10 to 15%) for apo AI and total HDL apolipoproteins and a 17.5% gel[20] for the apo AII identification and electrophoresed at 20 mA overnight for subsequent fluorography[21].

In preparative runs (approximately equal [^{35}S]methionine and [^{3}H]amino acid activities in newly synthesized apolipoproteins were subjected to gradient or 17.5% polyacrylamide slab gel electrophoresis, 0.1% sodium dodecyl sulfate. The slab gels were either dried directly or fixed and stained with Coomassie blue and dried for autoradiography. The radioactive band was excised, rehydrated in the same electrophoresis buffer and eluted electrophoretically. 3 mg whale myoglobin was added as carrier, the solution dialysed overnight against 1mM EDTA at 4 °C, lyophilised and the sodium dodecyl sulfate extracted with 10 volumes acetone/1M acetic acid 9:1 (v/v) at – 20 °C. The dried precipitate was dissolved in 60% formic acid and subjected to automated Edman degradation, using a 0.2M Quadrol program in the Beckman sequenator 890 C. Aliquot portions of the thiazolinones were converted to the phenylthiohydantoine derivatives for the determination of the repetitive yield by quantitating the recovery of the myoglobin residues by high-performance liquid chromatographic analysis. Yields ranged between 95% and 98%.

Thiazolinones were dried under nitrogen and their radioactivity measured directly in PPO-scintillator in a Beckman LS scintillation counter with counting efficiencies of 80% for ^{35}S and 40% for ^{3}H.

Pth derivatives of amino acids were separated and quantitated by high-performance liquid chromatography on mixed-bed Nucleosil 5 CN and Nucleosil 7 phenyl columns (4 × 200 mm) with a sodium acetate-acetonitrile gradient.*

* Stoffel, W. & Deutzmann, R., unpublished.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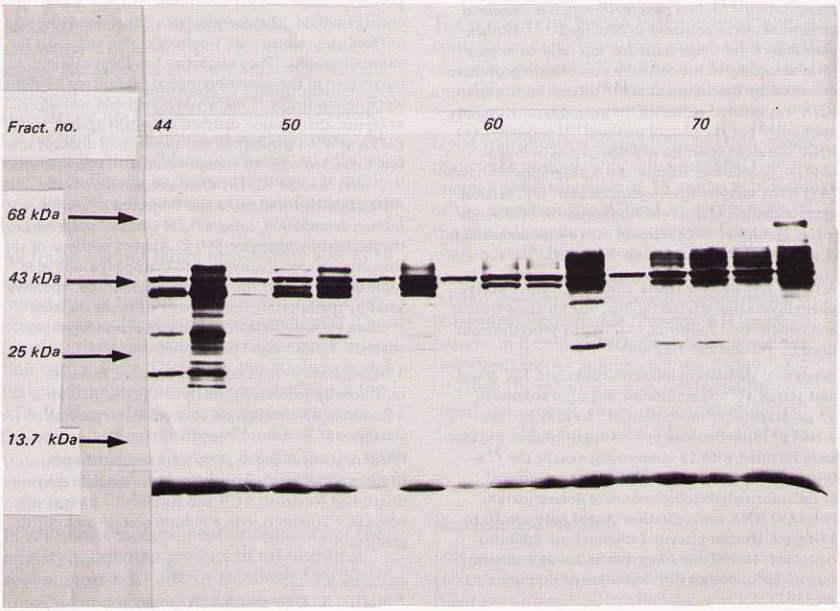
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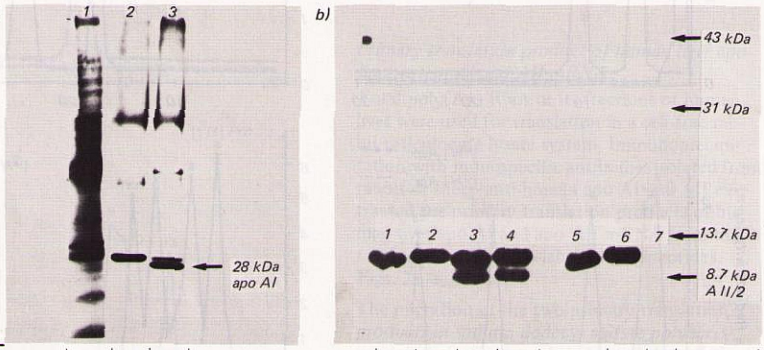
Fig. 1. Cell-free translation of human liver RNA previously fractionated by polyacrylamide gel electrophoresis.

10 A_{260} units poly(A+) RNA from human liver were separated by polyacrylamide gel electrophoresis [cylindrical gel: 4 cm height, 0.8 cm diameter; separating gel: 3.0% polyacrylamide, 1.0 cm height, 0.8 cm diameter, stacking gel: 2.5% polyacrylamide (19:1 acrylamide/bis(acrylamido)methane)]. Buffer: Tris/borate EDTA (0.2M, 5mM EDTA), pH 8.3 (TBE-buffer); voltage: 100 V. RNA bands were eluted continuously with TBE/buffer (8–10 ml/h in fractions of 0.8 ml. RNA was precipitated with 2 ml 96% ethanol.

0.01 A_{260} units poly(A+) RNA of fractions indicated above the lanes were used for the programming of the analytical reticulocyte lysate system (10 µl). [^{35}S]Methionine-labelled products were separated and visualized by autoradiography as described under Materials and Methods. Figures above lanes are numbers of fractions. Figures at the left side are molecular mass standards.



Lane	1	2	3		1	2	3	4	5	6	7	
	Total											
Cotranslational membranes			+				+	+				
Posttranslational membranes									+	+		
Dithiothreitol					–	+	–	+	–	+		
Trypsin											+	

Fig. 2. Autoradiography of [^{35}S]methionine-labelled translation products of human poly(A+) mRNA in reticulocyte lysate system.

a) Cell-free translated human apo AI mRNA (analyzed in 10–15% polyacrylamide gel).

Lane 1, total human liver poly(A+) RNA;

Lane 2, immunoprecipitation products of size-fractionated poly(A+) RNA (Fraction 28 of Fig. 1) with rabbit anti-human apo AI;

Lane 3, cotranslational cleavage of product of Lane 2 by dog pancreas microsomal membranes.

b) Cell-free translated human apo AII mRNA (analyzed in 17.5% polyacrylamide gel).

Lane 1, immunoprecipitated primary translation product, untreated (–DTT, dithiothreitol);

Lane 2, *reductively carboxymethylated (+DTT);*

Lane 3, cotranslational cleavage product of Lane 1 (–DTT);

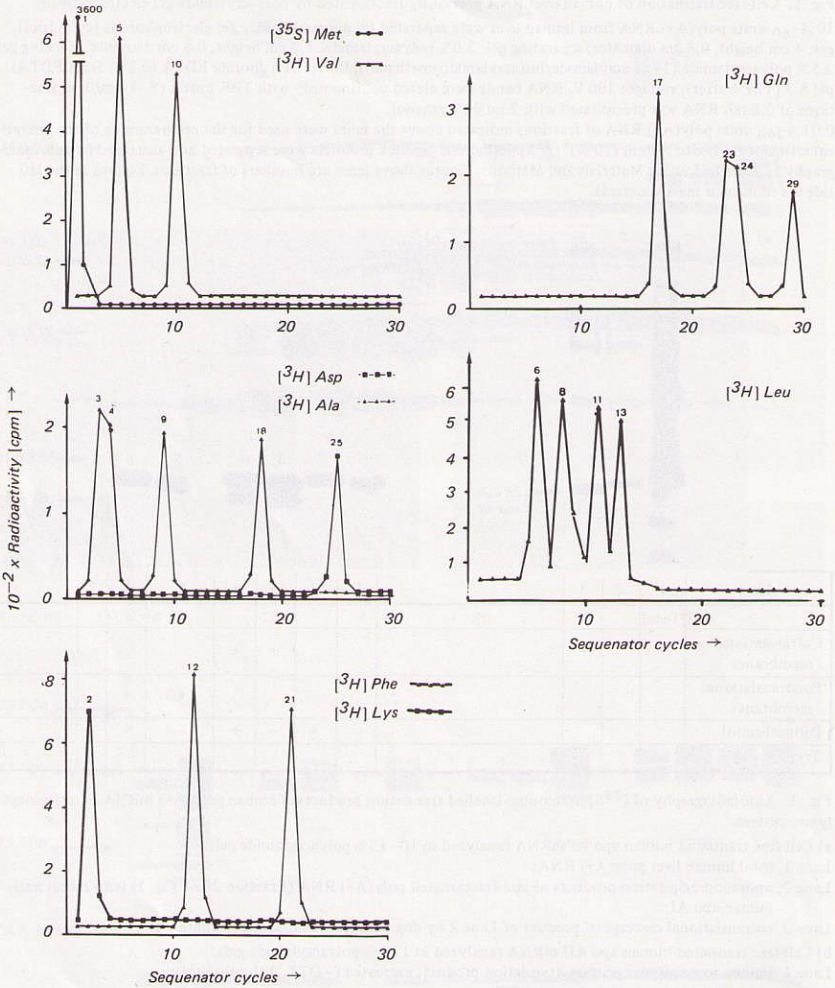
Lane 4, cotranslational cleavage product of Lane 1 (+DTT);

Lane 5, posttranslational addition of membranes (–DTT);

Lane 6, same as 5 (+DTT);

Lane 7, trypsin treatment of primary translation product of Lane 1.

a) Prepro-apo AI



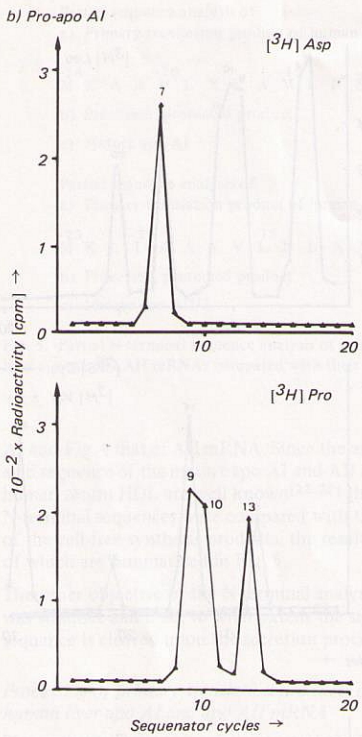


Fig. 3. N-Terminal sequence analyses by automated Edman degradation of primary and cotranslationally cleaved products of human apo AI mRNA, synthesized in parallel incubations in the presence of single labelled amino acids and purified by immunoprecipitation using monospecific rabbit anti human apo AI antibodies and *Staphylococcus aureus* Cowan's strain.

Labelled products were recovered from polyacrylamide gel slices by electroelution. Whale myoglobin (3 mg) was added as a carrier for sequential Edman degradation and determination of repetitive yields. a) Analysis of preproapolipoprotein AI; b) of proapolipoprotein AI.

Results

Total human liver RNA was isolated by either the guanidinium thiocyanate method[15] or the phenol method[16] and the poly(A+) mRNA isolated by oligo(dT)-cellulose affinity chromatography[17]. A further size fractionation was achieved by polyacrylamide gel electrophoresis. The total translation products separated by gradient polyacrylamide gel electrophoresis and visualized by autoradiography are shown in Figure 1.

Primary translation product of human liver apolipoprotein AI and AII mRNA

Total poly(A+) RNA or its fractions of human liver were used for translation in a cell-free rabbit reticulocyte lysate system. Immunoprecipitation with monospecific antibodies isolated from rabbit or sheep anti-human apo AI and AII extracted the primary translation products of human liver apo AI and apo AII mRNA from the total mixture of radio-labelled polypeptides. Figs. 2a and b.

The migration of the two primary translation products in sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated their differences in molecular masses from the mature plasma HDL-apoproteins in the following way: the cell-free product of apo AI mRNA was about 3000 Da larger than the mature apo AI (28 kDa), that of apo AII mRNA with about M_r = 12000 differed also by 3000 Da from the mature monomeric apo AII. Analogous to our previous finding[3] we concluded that the two in vitro translation products are synthesized with an N-terminal signal sequence. One objective of the partial N-terminal sequence analysis was to investigate whether human liver apolipoproteins AI and AII are synthesized with a transient signal sequence at the N-terminus. The two apolipoproteins were synthesized in the presence of [^{35}S]methionine and a number of ^{3}H-labelled amino acids (Ala, Val, Leu, Phe, Gln, Pro, Asp, Lys) and [^{35}S]Cys and the primary translation products subjected to up to 30 cycles of automated Edman degradation after isolation of the purified labelled products. Fig. 3 depicts the results of the Edman degradation of the cell-free translation product of human liver apolipoprotein

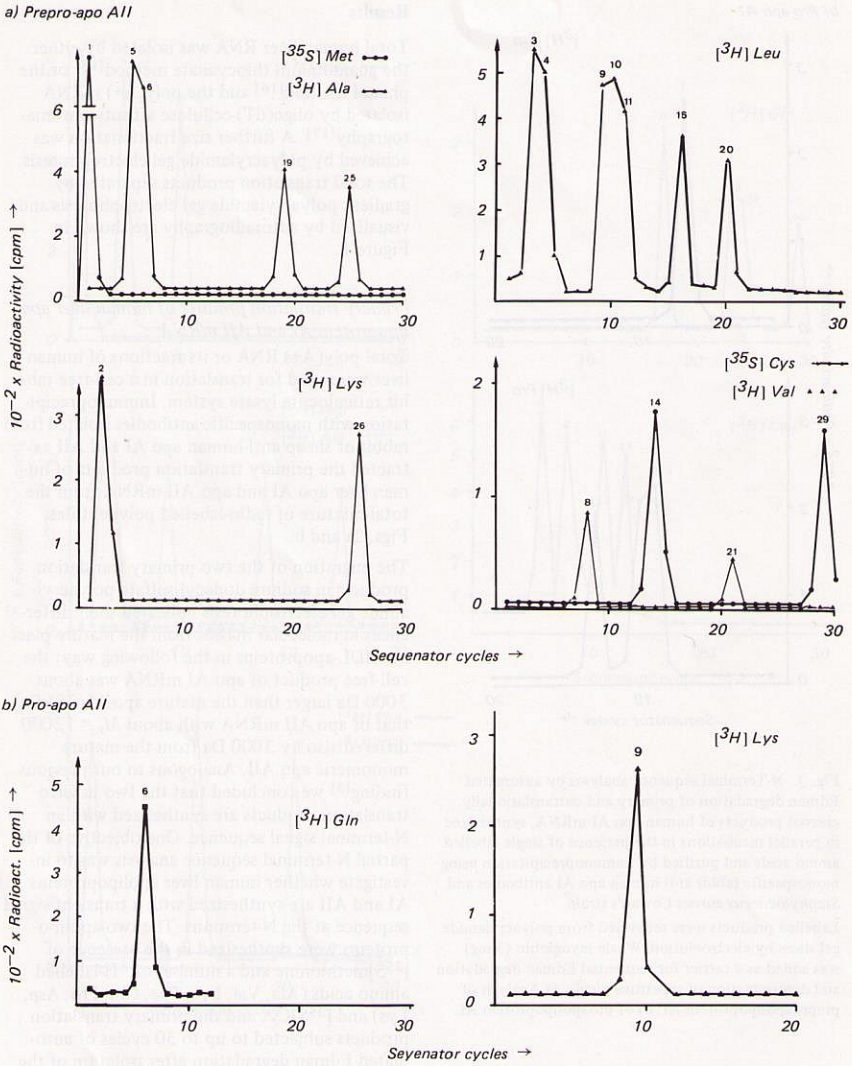


Fig. 4. N-Terminal sequence analyses by automated Edman degradation of primary and cotranslationally cleaved products of human apo AII mRNA.

a) Analysis of preproapolipoprotein AII; b) of proapolipoprotein AII.

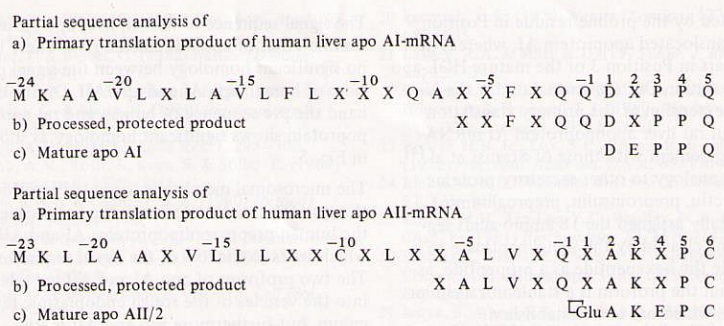


Fig. 5. Partial N-terminal sequence analysis of primary and processed and protected translation products of human liver apo AI and AII mRNAs compared with their mature (serum) forms.

AI and Fig. 4 that of AII mRNA. Since the amino acid sequence of the mature apo AI and AII of human serum HDL are well known[22,23] their N-terminal sequences were compared with those of the cell-free synthesis products, the results of which are summarized in Fig. 5.

The other objective of the N-terminal analysis was whether and if so, to what extent the signal sequence is cleaved upon the secretion process.

Processing of primary translation products of human liver apo AI and apo AII mRNA

We synthesized the two apolipoproteins in the presence of added rough microsomal membranes from dog pancreas[24] (cotranslational cleavage) or added the microsomal membranes after the synthesis to the primary rat apolipoprotein AI. It is evident that similar to our previous finding[3] the signal sequence is cleaved cotranslationally in the case of apo AI and AII (Fig. 2a, Lane 3, Fig. 2b, Lanes 3 and 4). It should be noted that the cotranslational translocation product of apo AII mRNA within the microsomal vesicles is still the monomeric form of apo AII (molecular mass about 9 kDa).

In order to establish the sequence of the N-termini of the translocated apo AI and apo AII incubations in vitro on a preparative scale with different labelled amino acids were performed in the presence of dog microsomal membranes and the membrane-protected translocated apoproteins isolated for automated Edman degradation, Fig. 3 and 4. The partial sequence data integrated into Fig. 5 indicate that the N-terminal extension of the primary translation product of human liver apo AI mRNA is a 24 amino acid sequence, that of apo AII mRNA a 23 amino acid sequence. Cotranslational translocation occurs with a cleavage of 18 amino acids by the signal peptidase in the case of the preform of apo AI and of 17 amino acids in the case of the apo AII monomer. Therefore, processed apo AI and AII are present within the microsomal vesicles in their proforms with an N-terminal extension by a hexapeptide. The proapolipoproteins are fully protected against trypsin/chymotrypsin cleavage.

Discussion

In our previous communication[3] we characterized for the first time the primary translation product of rat liver apolipoprotein AI mRNA. Apolipoprotein AI is translocated by means of a hydrophobic 18 residues long N-terminal signal sequence, that is cleaved upon permeation across the microsomal membrane. Similar results were obtained independently by Chan et al.[25] and Strauss et al.[4]. Beyond this signal sequence a hexapeptide extends the N-terminus of the mature apo AI present in the microsomal vesicles

as indicated by the proline residue in Position 9 of this translocated apoprotein AI, whereas proline appears in Position 3 of the mature HDL-apo AI in the serum. Our sequence data for the N-terminal extension of the primary translation product of rat liver apolipoprotein AI mRNA were in agreement with those of Strauss et al.[4], who – in analogy to other secretory proteins (preprolactin, preproinsulin, preproalbumin) – operationally assigned the 18 amino acids segment being cleaved by the signal peptidase to a prepeptide the hexapeptide as a propeptide, assuming that the proform is a stable intracellular precursor, which has to be established.

In our previous study[3] the integrating function of the signal recognition particle (SRP) into the microsomal membranes for secretory proteins was also demonstrated. As initial steps of our studies on the organisation and regulation of the human apolipoproteins on the genomic level human liver poly(A+) RNA, total and size-fractionated by preparative polyacrylamide gel electrophoresis was translated in the cell-free reticulocyte system analogous to our previous study. The primary translation products of the two main apolipoproteins of human serum high-density lipoproteins AI (mol. mass 28.3 kDa) and AII (mol. mass 17 kDa) were isolated by immunoprecipitation with monospecific antibodies against these antigens. The respective mRNAs belong to the abundant species in total liver mRNA (about 1–3% and 1% respectively).

The two primary translation products differred from their mature apolipoproteins AI and AII by N-terminal extension of 24 and 23 amino acids respectively. The prepieces of prepro-apolipoprotein AI and AII are 18 and 17 amino acids sequences, respectively. Therefore, the propieces at the N-terminus of apo AI and AII are hexapeptides. The common feature of the N-terminal signal peptides sequenced so far is their strong hydrophobicity required for the initiation of the transfer process of secretory proteins or transmembrane proteins, regardless of whether the transmembrane translocation is mediated by postulated tunnel-forming membrane proteins or the direct translocation by a loop mechanism of the signal sequence[26–30]. This principle is again verified for the two signal sequences although only partially determined.

The signal sequence of human liver apo AI is alanine-, that of apo AII leucine-rich. There is no significant homology between the signal peptides of human apo AI and apo AII. On the other hand the pre-sequence of human and rat apolipoprotein shows significant homology as shown in Fig. 5.

The microsomal membrane-mediated translocation process is associated with the processing of the human preproapolipoproteins AI and AII which leads to the loss of the signal sequences. The two proforms of apo AI and AII are released into the vesicles of the rough endoplasmic reticulum, but furthermore pro-apo AII is still present in its monomeric form. Besides the cleavage of the hexapeptide pro-sequences of apo AI and AII also the dimerisation to mature apo AII must occur as a further posttranslational process. The isolation of an apo VLDLII from avian very low density lipoproteins has been described previously[31] and recently synthesized in vitro[32] and its signal sequence determined. This translation product of cockeral hepatic mRNA differs from the two primary translation products of the human apolipoproteins of the A-family mRNA and of rat apo AI mRNA[4] in its N-terminal extension. This signal sequence has been postulated to be 23 amino acids long, no prosegment has been described.

Our studies describe for the first time the initial steps of the biosynthesis and secretion process of human serum HDL-apolipoproteins AI and AII. The final posttranslational steps, cleavage of the different hexapeptide pro-sequence and the formation of the disulfide bond between two apo AII monomers and the subsequent binding of cellular lipids remain to be elucidated in future studies.

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