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Synthesis *in vitro* and Translocation of Apolipoprotein AI across Microsomal Vesicles

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Apolipoprotein AI of rat has been synthesized in a cell-free wheat germ system and cotranslationally translocated into dog pancreas microsomal vesicles. Translocation is accompanied by cleavage of a signal sequence of 18 amino acid residues and is dependent on the recently purified signal recognition protein.

The apolipoprotein AI is the principal apoprotein of serum high-density lipoprotein (HDL) and is common to all species from cyclostoma to man [1]. The 243-amino-acid-residue polypeptide of human origin [2] is characterized by its high α-helix content, which amounts to 65% when calculated on the basis of the predictive parameters of Chou and Fasman [3,4], Nagano [5] and Robson [6]. Many of the ordered segments exhibit amphipathic helices.

In aqueous solutions apolipoprotein AI behaves like a detergent. It associates with itself and binds phospholipids and cholesterol to form HDL-like particles of either disc or spherical shape, depending on the phospholipid species [7]. The lipid protein interactions are exclusively hydrophobic as demonstrated by ^{13}C-NMR spectroscopy [8,9] and photoaffinity labeling [10,11].

Apolipoprotein AI is synthesized in the liver [12] and the small intestine [13]. Being a secretory protein, it requires translocation across the lipid bilayer of the endoplasmic reticulum membrane in its biogenesis. Given its propensity for binding lipids, its mechanism of translocation could be envisioned to differ from that of other secretory proteins. However, our data here on cell-free synthesis and translocation indicate that the mechanism of translocation across the endoplasmic reticulum membrane is like that for other secretory proteins: apolipoprotein AI was found to be synthesized with a signal sequence that is cleaved upon translocation across translocation-competent microsomal vesicles; its translocation was strictly coupled to translation and was mediated by the recently discovered 'signal recognition protein'.

MATERIALS AND METHODS

[^{35}S]Methionine at 1000 Ci/mmol, [^{3}H]leucine at 110 Ci/mmol and [^{3}H]proline at 100 Ci/mmol were from New England Nuclear. Protein-A–Sepharose 4B was a product of Pharmacia Fine Chemicals. Trypsin and chymotrypsin were from Boehringer (Mannheim, FRG). Trasylol was from Bayer AG (Leverkusen, FRG).

Rat serum HDL was isolated by the sedimentation-flotation procedure in the density range 1.063–1.21 g/ml [14]. The fraction proved to be homogeneous in agarose electrophoresis. Delipidation [15] and separation of the apoprotein by DEAE-cellulose chromatography [16] with an additional Sephadex G-200 chromatography [16] yielded apolipoprotein AI in electrophoretically homogeneous form. Apolipoprotein AI antibodies were raised in rabbits. The IgG fraction was obtained by ammonium sulfate precipitation [17].

Total liver RNA was isolated by the sodium perchlorate method [18]. Protein synthesis in a cell-free wheat germ system was as described previously [19]. 100 µl of translation mix contained 24 µl of staphylococcal-nuclease-treated [20] wheat germ 23000 × *g* supernatant, 2 units of human placental RNase inhibitor [21] and 100 µCi of [^{35}S]methionine. Newly synthesized apolipoprotein AI was immunoprecipitated as described elsewhere [22]: in brief, translation products were denatured in 2% sodium dodecyl sulfate, diluted with Triton X-100 and incubated with antibody; the resulting antigen-antibody complexes were isolated by adsorption to protein-A–Sepharose 4B; desorption was by sodium dodecyl sulfate followed by reductive alkylation of the desorbed products. Analysis of the desorbed products was by polyacrylamide gradient gel electrophoresis (10–15%) in 0.1% sodium dodecyl sulfate and subsequent fluorography [23].

In some cases (specified in figure legends) the translation mix was supplemented by rough microsomal membranes, potassium-acetate-extracted microsomal membranes and signal recognition protein, all from dog pancreas, and prepared as described [24].

Partial Sequence Determination

The incubation volumes for protein synthesis in the presence of [^{35}S]methionine or one tritium-labelled amino acid were 1 ml. Newly synthesized apolipoprotein AI was immunoprecipitated and approximately equal ^{35}S and ^{3}H activities were subjected to electrophoresis in 10–15% gradient 0.1% sodium dodecyl sulfate/polyacrylamide slab gels. The slab gels were dried directly without staining and autoradiographed. The desired radioactive band was excised from the dried gel, rehydrated in 0.1% sodium dodecyl sulfate/electrophoresis buffer and electrophoretically eluted. Whale apo-myoglobin (3 mg) was added as a carrier and the proteins were precipitated by 9 vol. of acidified acetone at −20 °C. The precipitate was dried under N_2 and dissolved in 0.6 ml 60% (v/v) heptafluorobutyric acid; the resulting solution was applied to a Beckman 890C sequencer and the proteins were subjected to Edman degradation, using a dimethylallylamine program. The thiazolinones were dried under N_2 and their radioactivity measured directly in PPO scintillator, using a Beckman LS 350 scintillation counter, with a double-label counting program.

Abbreviations. HDL, high-density lipoprotein, density 1.063–1.21 g/ml; VLDL, very-low-density lipoprotein, density 1.006–1.063 g/ml.

RESULTS

Translation of total rat liver RNA in the wheat germ cell-free translation system followed by immunoprecipitation of the total translation products with rabbit antiserum against apolipoprotein AI yielded (Fig. 2, lane 1) a major polypeptide (M_r 31000), larger by 3000 daltons than its mature counterpart (M_r 28000). The identity of this immunoreactive polypeptide was confirmed by competition with purified authentic apolipoprotein AI (Fig. 1, lane 2).

When translation was carried out in the presence of dog pancreas microsomal vesicles, a second major band (M_r 28000) comigrating with mature apolipoprotein AI (Fig. 1, arrow) was observed (Fig. 1, lane 4 and Fig. 2, lane 2). Like its 31000-M_r counterpart this polypeptide was immunocompeted by unlabeled authentic apolipoprotein AI (Fig. 1, lane 5).

Probing the location within or outside the microsomal vesicles of the newly synthesized immunoreactive polypeptides with proteolytic enzymes (trypsin and chymotrypsin) we found that only the 28000-M_r band, but not the 31000-M_r band (Fig. 1, lane 6) was protected. Protection depended on the integrity of the microsomal vesicle and was abolished when microsomal vesicles were lysed by the nonionic detergent Triton X-100 (Fig. 1, lane 7).

As previously demonstrated for other secretory proteins, translocation was strictly coupled to translation [25]. Completion of translation in the absence of microsomal vesicles followed by posttranslational incubation with microsomal vesicles yielded only the 31000-M_r band (Fig. 1, lane 1).

These results were in all respects analogous to those obtained previously with other secretory proteins by similar assays. They suggested that the 31000-M_r polypeptide contains a cleavable signal sequence that functions in translocation across the endoplasmic reticulum membrane and is cleaved during or shortly after translocation.

Recently, the important conjecture of the signal hypothesis [26] namely that translocation across the endoplasmic reticulum is a receptor-mediated process (whereby the receptors decode information contributed by both the signal sequence and by the ribosome) has been substantiated by the isolation [24] and characterization [27–29] of the so-called 'signal recognition protein'. This protein was purified [24] from a salt wash of microsomal vesicles [30] and was shown to bind with high affinity to ribosomes that are engaged in the synthesis of polypeptides containing a signal sequence addressed to the endoplasmic reticulum [27]. Most interestingly, in the absence of membranes, signal recognition protein was shown to cause a signal-sequence-induced arrest

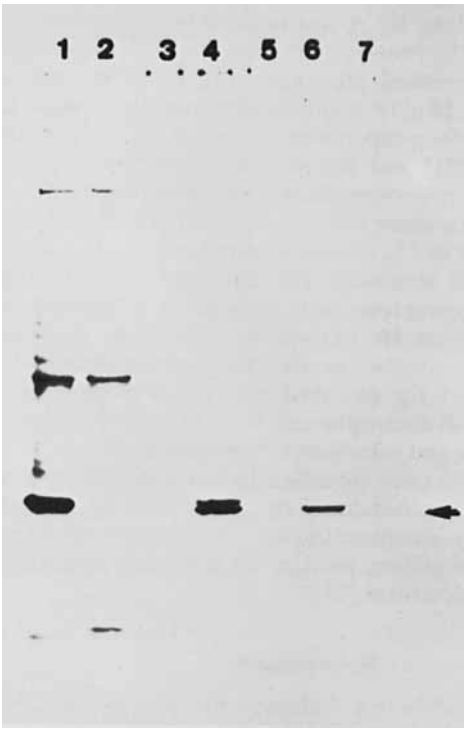

Fig. 1. *Translocation* in vitro *of newly synthesized apolipoprotein AI.* Total rat liver RNA (8 A_{260} units/ml) was translated in a wheat germ system (100 µl translation mixture) in the absence (lanes 1–3) or presence (lanes 4–7) of dog rough microsomes (final concentration 4 A_{280} units/ml). After a 90-min incubation at 26 °C cycloheximide was added to 10 µg/ml. Microsomal membranes were then added to 4 A_{280} units/ml to the tubes which did not contain membrane during translation (lanes 1–3) and the incubation of all tubes was continued for an additional 90 min. Immunoprecipitation using rabbit anti-(apolipoprotein AI) IgG (30 µg/tube) and protein-A–Sepharose (25 µl packed beads) were performed. The immunoprecipitated products were analyzed by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate and visualized by fluorography. In lanes 2 and 5 excess native apolipoprotein AI (20 µg) was added to the translation mix prior to immunoprecipitation. In lanes 3, 6 and 7 trypsin and chymotrypsin (0.3 mg/ml each) were added prior to immunoprecipitation and an additional incubation for 60 min at 0 °C was performed. In addition to the proteases, Triton X-100 (1% final concentration) was added to the sample displayed in lane 7. The arrow indicates the position of authentic apolipoprotein AI electrophoresed in our gel system

Fig. 2. *Translocation of apolipoprotein AI is dependent on signal recognition protein.* Rat liver RNA was translated in the absence (lanes 1 and 6) or the presence of either rough microsomal membranes (lane 2) or salt-extracted rough microsomal membranes (lanes 3–5). Purified signal recognition protein was added to the translation systems at 80 units/ml (lane 4) or 400 units/ml (lanes 5 and 6). The translation products were immunoprecipitated, analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and visualized by fluorography

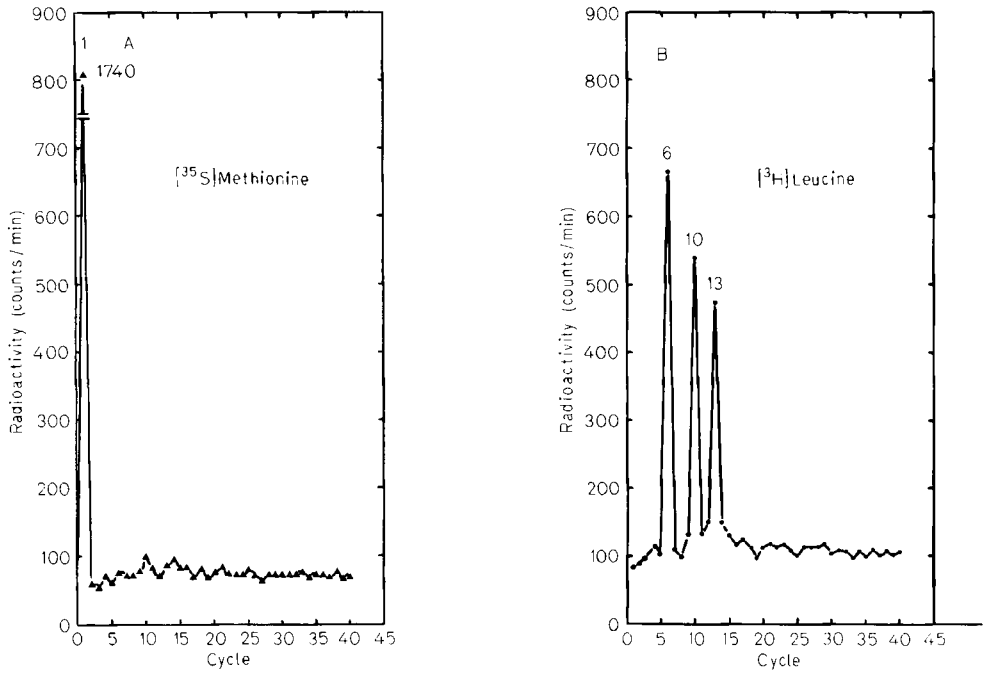

Fig. 3. *Partial sequence determination of pre-apolipoprotein AI.* 1-ml translation reactions containing 0.5 mCi of (B) [^{3}H]leucine or (A) [^{35}S]-methionine and 8 A_{260} units RNA/ml were carried out and pre-apolipoprotein AI was isolated after immunoprecipitation and preparative polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Pre-apolipoprotein AI was taken through 40 cycles of Edman degradation and the residues analyzed as described in Materials and Methods. The sequence assignments are indicated by arrows. Input radioactivity was 40000 counts/min for each isotope

in chain elongation of secretory proteins that could be released by the addition of membranes [29].

The data shown in Fig. 2 indicate that signal recognition protein was required for the translocation of nascent pre-apolipoprotein AI and that signal recognition protein, in the absence of membranes, inhibited synthesis of apolipoprotein AI, presumably via arrest of chain elongation [29]. Thus, in the presence of salt-extracted membranes and in the absence of signal recognition protein there was synthesis primarily of the 31000-M_r form and little synthesis of the 28000-M_r form (Fig. 2, lane 3); in the presence of salt-extracted rough endoplasmic membrane and of increasing amounts of signal recognition protein (Fig. 2, lanes 4 and 5) there was increasing synthesis of the 28000-M_r band at the expense of the 31000-M_r band; and in the presence of signal recognition protein, but in absence of salt-extracted rough endoplasmic membrane synthesis of a completed 31000-M_r band could not be detected (Fig. 2, lane 6).

To prove that the 31000-M_r band contained an NH_2-terminal signal sequence that is cleaved upon translocation into the microsomal vesicles we undertook partial NH_2-terminal sequence analysis of both the 31000-M_r and the 28000-M_r band. Consecutive automated Edman degradation (40 cycles) of the 31000-M_r band labeled with [^{35}S]methionine and [^{3}H]leucine yielded (Fig. 3) Met at position 1, and Leu at positions 6, 10 and 13. Consecutive automated Edman degradations (15 cycles) of the 28000-M_r band labeled with [^{35}S]methionine and [^{3}H]proline yielded (Fig. 4) Pro at position 9. After completion of this work we learned that Strauss and co-workers [31] had undertaken similar studies. From their almost complete sequence data [31] and our partial data here (Fig. 5), it is apparent that Pro-9 of the signal peptidase-processed form aligns with Pro-27 of the 31000-M_r precursor

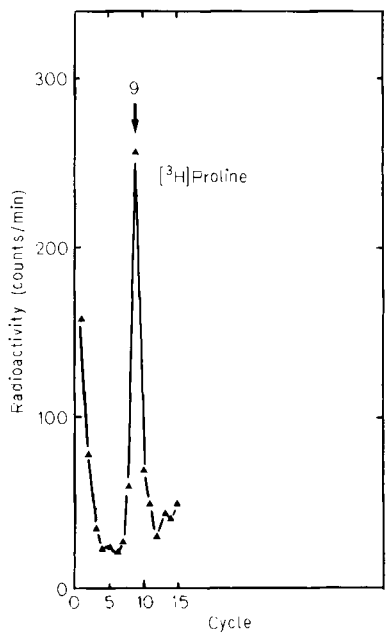

Fig. 4. *Partial sequence determination of apolipoprotein AI translocated* in vitro. A 1-ml translation reaction containing 1 mCi [^{3}H]proline, 8 A_{260} units RNA, 4 A_{280} units of rough endoplasmic membrane and 100 units signal recognition protein was carried out. Apolipoprotein AI translated *in vitro* was isolated and subjected to 15 cycles of Edman degradation as described (see Materials and Methods). The sequence assignment is indicated by an arrow. Input radioactivity was 15000 counts/min. A similar sequence determination employed [^{3}H]leucine and [^{35}S]methionine revealed no Leu or Met in the first 20 residues of processed apolipoprotein AI

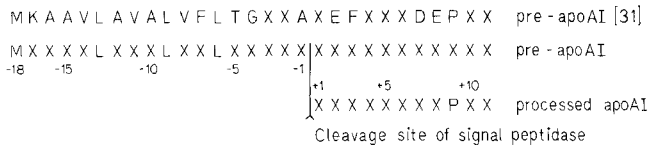

Fig. 5. *Alignment of partial sequence data for apolipoprotein AI translocated* in vitro *and pre-apolipoprotein AI*

suggesting a signal sequence of 18 residues, in agreement with data by Strauss and co-workers (A. Strauss, personal communication).

DISCUSSION

Our data here show that the mechanism of translocation of the apolipoprotein AI across the endoplasmic reticulum membrane is indistinguishable from that of other secretory proteins.

Thus, translocation of apolipoprotein AI is initiated by an NH_2-terminal signal sequence, 18 residues long, that is cleaved upon translocation across the microsomal membrane. Strauss and co-workers [31] and Chan and co-workers [32] have independently obtained similar results. Further, Chan et al. [33] had previously demonstrated the presence of a signal sequence (and determined its primary structure) for another apolipoprotein, apolipoprotein AII of VLDL.

Translocation of apolipoprotein AI was found to be strictly coupled to translocation and was dependent on the recently purified [24] and characterized [27–29] 'signal recognition protein'. This protein (consisting of six polypeptides) embodies the features of a signal sequence and a ribosome receptor, the existence of which were predicted in the signal hypothesis [26]. The dependence on signal recognition protein of apolipoprotein AI translocation across the microsomal membrane provides not only an additional example (previously only bovine pituitary prolactin was used as a model secretory protein) for receptor-mediated translocation of secretory proteins in general, but specifically extends this concept to include also lipophilic secretory apolipoprotein. Alternative hypotheses that have proposed that protein translocation across membranes is a spontaneous process that does not require receptor mediation (for most recent examples of such hypotheses see [34, 35]) can thus be ruled out also for the case of a lipophilic secretory protein.

The precise mechanism of protein passage across the endoplasmic reticulum membrane is unknown. It is clear that signal recognition protein in conjunction with integral membrane protein(s) of the endoplasmic reticulum (still to be identified) constitutes the endoplasmic reticulum's translocation machinery [29]. The propensity of apolipoprotein to bind lipids may be expressed only following passage across the endoplasmic reticulum. During passage, the nascent chain may be prevented from interacting with lipids because passage occurs in a proteinaceous environment [26] and/or because the nascent protein traverses the membrane in an unfolded state not yet competent to bind lipids.

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REFERENCES

1. Chapman, M. J. (1980) *J. Lipid Res. 21*, 789–853.
2. Brewer, H. B., Fairwell, R., La Rue, A., Ronan, R., Hauser, R. & Bonzert, T. J. (1980) *Biochem. Biophys. Res. Commun. 80*, 623–630.
3. Chou, P. Y. & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–278.
4. Chou, P. Y. & Fasman, G. D. (1974) *Biochemistry, 13*, 211–211, 222–245.
5. Nagano, K. (1973) *J. Mol. Biol. 75*, 401–420.
6. Robson, B. (1976) *J. Mol. Biol. 107*, 327–356.
7. Stoffel, W., Därr, W. & Salm, K. P. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 1–11.
8. Stoffel, W., Zierenberg, O., Tunggal, B. & Schreiber, E. (1974) *Proc. Natl Acad. Sci. USA, 71*, 3696–3700.
9. Stoffel, W., Salm, K. P. & Tunggal, B. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 523–528.
10. Stoffel, W., Därr, W. & Salm, K. P. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 453–462.
11. Stoffel, W., Metz, P. & Heller, R. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1319–1325.
12. Felder, T. E., Fainarze, M., Hamilton, R. L. & Havel, R. J. (1977) *J. Lipid Res. 18*, 465–473.
13. Green, P. H. R., Tall, A. R. & Glickman, R. M. (1978) *J. Clin. Invest. 61*, 528–534.
14. Shore, V. G. & Shore, B. (1969) *Biochemistry, 8*, 4510–4516.
15. Scanu, A. M. (1966) *J. Lipid Res. 7*, 295–306.
16. Scanu, A. M., Toth, J., Edelstein, C., Koga, S. & Stiller, E. (1969) *Biochemistry, 8*, 3309–3316.
17. Heide, K. & Schwick, H. G. (1978) in *Immunochemistry* (Weir, D. M., ed.) vol. 1, chapter 7.1–7.11, Blackwell Scientific, Oxford.
18. Lizardi, P. M. & Engelberg, A. (1979) *Anal. Biochem. 98*, 116–122.
19. Goldman, B. M. & Blobel, G. (1981) *J. Cell Biol. 90*, 236–242.
20. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.
21. Blackburn, P., Wilson, G. & Moore, S. (1977) *J. Biol. Chem. 252*, 5904–5910.
22. Goldman, B. M. & Blobel, G. (1978) *Proc. Natl Acad. Sci. USA, 75*, 5066–5070.
23. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
24. Walter, P. & Blobel, G. (1980) *Proc. Natl Acad. Sci. USA, 77*, 7112–7116.
25. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol. 67*, 852–862.
26. Blobel, G. (1980) *Proc. Natl Acad. Sci. USA, 77*, 1496–1500.
27. Walter, P., Ibrahimi, I. & Blobel, G. (1981) *J. Cell Biol.*, in the press.
28. Walter, P. & Blobel, G. (1981) *J. Cell Biol.* in the press.
29. Walter, P., & Blobel, G. (1981) *J. Cell Biol.* in the press.
30. Warren, G. & Dobberstein, B. (1978) *Nature (Lond.) 273*, 569–571.
31. Gordon, F. I., Alpers, D. H., Schonfeld, G., Andy, R., Smith, D. P. & Strauss, A. (1981) *Fed. Proc. 40*, 1635.
32. Lin-su, M.-H., Lin-Lee, Y.-C., Bradley, W. A. & Chan, L. (1981) *Biochemistry, 20*, 2470–2475.
33. Chan, L., Bradley, W. A. & Means, A. (1980) *J. Biol. Chem. 255*, 10060–10063.
34. Wickner, W. (1979) *Annu. Rev. Biochem. 48*, 23–45.
35. Engelman, D. M. & Steitz, T. A. (1981) *Cell, 23*, 411–422.

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