Synthesis in vitro and Translocation of Apolipoprotein A1 across Microsomal Vesicles

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Apolipoprotein A1 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 A1 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 a-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 A1 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 13C-NMR spectroscopy [8,9] and photoaffinity labeling [10,11].

Apolipoprotein A1 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 A1 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

[35S]Methionine at 1000 Ci/mmol, [3H]leucine at 110 Ci/mmol and [3H]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 A1 in electrophoretically homogeneous form. Apolipoprotein A1 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 [35S]methionine. Newly synthesized apolipoprotein A1 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 volume for protein synthesis in the presence of [35S]methionine or one tritium-labelled amino acid were 1 ml. Newly synthesized apolipoprotein A1 was immunoprecipitated and approximately equal 35S and 3H 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. The apo-apomyoglobin (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 N2 and dissolved in 0.6 ml 60% (ν/ν) heptfluorobutyric 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 N2 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 antiseraum against apolipoprotein A1 yielded (Fig. 2, lane 1) a major polypeptide (M, 31000), larger by 3000 daltons than its mature counterpart (M, 28000). The identity of this immunoreactive polypeptide was confirmed by competition with purified authentic apolipoprotein A1 (Fig. 1, lane 2).

When translation was carried out in the presence of dog pancreas microsomal vesicles, a second major band (M, 28000) comigrating with mature apolipoprotein A1 (Fig. 1, arrow) was observed (Fig. 1, lane 4 and Fig. 2, lane 2). Like its 31000-M, counterpart this polypeptide was immuno-competed by unlabeled authentic apolipoprotein A1 (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, band, but not the 31000-M, 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, 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, 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
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 A1 and that signal recognition protein, in the absence of membranes, inhibited synthesis of apolipoprotein A1, 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, form and little synthesis of the 28000-M, 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, band at the expense of the 31000-M, band; and in the presence of signal recognition protein, but in absence of salt-extracted rough endoplasmic membrane synthesis of a completed 31000-M, band could not be detected (Fig. 2, lane 6).

To prove that the 31000-M, band contained an NH2-terminal signal sequence that is cleaved upon translocation into the microsomal vesicles we undertook partial NH2-terminal sequence analysis of both the 31000-M, and the 28000-M, band. Consecutive automated Edman degradation (40 cycles) of the 31000-M, band labeled with [35S]methionine and [3H]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, band labeled with [35S]methionine and [3H]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, precursor

Fig. 3. Partial sequence determination of pre-apolipoprotein A1. 1-ml translation reactions containing 0.5 mCi of (B) [3H]leucine or (A) [35S]methionine and 8 A260 units RNA/ml were carried out and pre-apolipoprotein A1 was isolated after immunoprecipitation and preparative polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Pre-apolipoprotein A1 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.

Fig. 4. Partial sequence determination of apolipoprotein A1 translated in vitro. A 1-ml translation reaction containing 1 mCi [3H]proline, 8 A260 units RNA, 4 A260 units of rough endoplasmic membrane and 100 units signal recognition protein was carried out. Apolipoprotein A1 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 [3H]leucine and [35S]methionine revealed no Leu or Met in the first 20 residues of processed apolipoprotein A1.
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 A1 across the endoplasmic reticulum membrane is indistinguishable from that of other secretory proteins.

Thus, translocation of apolipoprotein A1 is initiated by an NH2-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] have previously demonstrated the presence of a signal sequence (and determined its primary structure) for another apolipoprotein, apolipoprotein AII of VLDL.

Translocation of apolipoprotein A1 was found to be strictly coupled to translation and was dependent on the recently purified [24] and characterized [27-29] 'signal recognition protein'. This protein (consisting of six glycoproteins) 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 A1 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 (stil to be identified) constitutes the endoplasmic reti culum'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 proteinaeous 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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