Biol. Chem. Hoppe-Seyler
Vol. 369, pp. 1055–1063, September 1988

Structural Requirements of Human Preproapolipoprotein AI for Translocation and Processing Studied by Site-Directed Mutagenesis in vitro

Wilhelm STOFFEL and Erika BINCZEK

Institut für Physiologische Chemie der Universität Köln

(Received 20 June 1988)

Summary: *A full length human serum apolipoprotein AI (apo AI) cDNA clone was isolated from a human liver cDNA library. The EcoRI insertion fragment was cloned into expression vectors pDS5 and pDS12 for in vitro transcription and translation. The primary translation product is correctly translocated and the N-terminal signal sequence of the primary translation product of the wild type apo AI cleaved in the presence of dog pancreatic endoplasmic reticulum (ER) membranes releasing proapo AI.*

Ala^{-7} at the C-terminus of the signal sequence and Gln^{-1} of the prosequence were transposed by site-directed mutagenesis thus mutually exchanging the C-termini Gln^{-8}-Ala^{-7} of the presequence and Gln^{-2}-Gln^{-1} of the prosequence. The primary translation product of this mutated preproapo AI cDNA is correctly co-translationally translocated into the lumen of the ER membranes and remains uncleaved by the signal peptidase.

Deletion of the hexapeptide prosequence by site-directed mutagenesis in the preproapo AI cDNA led to a primary translation product which is cotranslationally translocated with processing to the mature apo AI polypeptide. We conclude that neither the proteolytic cleavage of the presequence nor the presence of the prosequence are structurally essential for the cotranslational translocation of apo AI. The amino-acid sequence bordering the cleavage site at the C-terminus of the presequence is without influence for the specificity of the signal peptidase.

Untersuchungen über die Strukturvoraussetzungen für die Translokation und das Processing des menschlichen Präproapolipoproteins AI mit der In-vitro-Mutagenese

Zusammenfassung: Ein die gesamte kodierende Region des menschlichen Serum-Apolipoproteins AI (Apo AI) umfassender cDNA-Klon wurde aus einer menschlichen Leber-cDNA-Bank isoliert. Das *Eco*RI-Fragment wurde in die Expressionsvektoren pDS5 und pDS12 zur In-vitro-Transkription und -Translation kloniert. Das primäre Translationsprodukt wird korrekt

---

Enzymes:
DNA ligase, Polydeoxyribonucleotide synthase (ATP), poly(deoxyribonucleotide): poly(deoxyribonucleotide) ligase (AMP-forming) (also called DNA ligase) (EC 6.5.1.1);
Alkaline phosphatase, orthophosphoric-monoester phosphohydrolase (alkaline optimum) (EC 3.1.3.1) (from calf intestinum);
Creatine kinase, ATP:creatine *N*-phosphotransferase (EC 2.7.3.2);
DNA-directed RNA polymerase, nucleoside-triphosphate:RNA nucleotidyltransferase (DNA-directed (EC 2.7.7.6) (from *E. coli*).

Abbreviations:
$NaDodSO_4$, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; kb, kilo base pairs; Tris, Tris-(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; ER, endoplasmic reticulum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; cDNA, complementary DNA; RF, replicative form; RNAsin, RNAse inhibitor (placental) (one unit of inhibitor is the amount required to inhibit by 50% the activity of 5 ng of RNAse A); SRP, signal recognition peptide; DTT, dithiothreitol.

auf die luminale Seite der endoplasmatischen Vesikel transloziert, und dabei wird die Präsequenz unter Freisetzung von Proapo AI abgespalten. Ala^{-7}, der C-Terminus der Signalsequenz und Gln^{-1}, der C-Terminus der Prosequenz, wurden durch gerichtete Mutagenese gegenseitig ausgetauscht. Damit entstanden die C-Termini Gln^{-8}-Gln^{-7} der Prä- und Gln^{-2}-Ala^{-1} der Prosequenz. Das primäre Translationsprodukt dieser mutierten Präproapo AI-cDNA wird effizient cotranslational transloziert und nicht durch die Signalpeptidase zur Proform prozessiert.

Die Deletion der Hexapeptid-Prosequenz durch gerichtete Mutagenese in der Präproapo-AI-cDNA führte zu einem primären Translationsprodukt, das unter Abspaltung der Präsequenz zum reifen Apo-AI-Polypeptid transloziert wird. Wir folgern daraus, daß weder die proteolytische Spaltung der Präsequenz noch die Anwesenheit der Prosequenz strukturell für die cotranslationale Translation des Apo AI essentiell sind. Die auf den C-Terminus der Präsequenz folgende Aminosäuresequenz ist ohne Einfluß auf die Spezifität der Signalpeptidase.

Key words: Human preproapo AI, site-directed mutagenesis of preprosequence, transcription-translation in vitro, processing and translocation.

The serum high-density lipoprotein class (HDL) with a buoyant density of 1.063 to 1.21 g/ml represents a population of spherical cholesterol and cholesterolester rich particles of around 100 Å diameter with the highest protein content of the four main lipoprotein classes. Apolipoprotein AI (apo AI), a 243 amino acid residues long and apo AII, a dimeric 77 residues long polypeptide, are the main components. An essential function of apo AI beyond its lipid binding properties is the activation of lecithin-cholesterol acyltransferase (LCAT) and possibly receptor recognition properties. The functions of apo AII are not yet defined. Like low-density lipoproteins (LDL) serum HDL particles have proceeded to a final assembly stage for cholesterol transport in cholesterol homeostasis. Unlike LDL the nascent structures from which HDL particles evolve are unknown.

The hepatocyte has been demonstrated as apo AI- and apo AII-synthesizing and -secreting cell[1,2]. In addition enterocytes are the other source of apo AI synthesis[3,4]. These two apoproteins are synthesized as preproapolipoproteins and are cotranslationally translocated to the endoplasmic reticulum and Golgi apparatus for secretion as proapolipoprotein. The mRNA of apo AI encodes a 267 amino-acid residues polypeptide which is processed to the 249 amino-acid proform loosing an octadecapeptide presequence. The mRNA of apo AII encodes a 100 residues long monomeric preproform which processes to the 82 amino-acid residues long proapo AII[2,5]. The proforms are secreted into the blood plasma and proteolytically processed by specific serum proteases to their final mature forms[6–9]. Among the ten serum apolipoproteins only apo AI and apo AII are synthesized as preproapoproteins. The function of the proapo AI hexapeptide sequence and the proapo AII pentapeptide sequence which are intracellularly stable is unknown. The C-terminal Gln^{-2}-Gln^{-1} sequence of the apo AI prosequence is unusual whereas the Arg^{-2}-Arg^{-1} C-terminus of the apo AII prosequence conforms with those of other prosequences known so far.

Site-directed mutagenesis now allows to study the influence of structures of the pre- and prosequences on the translocation, intra- and extracellular processing of the primary translation products of wild-type and of modified apolipoprotein mRNAs. Here we report on the in vitro transcription-translation and intracellular processing of a wild-type full length human apo AI cDNA and two mutagenized apo AI cDNAs modified in the preprosequence. The apo AI inserts were cloned into expression vectors (pDS5 and pDS12). Mutations at the transition sites of the pre- to the pro- and the pro- to the mature sequence (apo AI mutant 1) and the deletion of the prosequence of the apo AI (apo AI mutant 2) primary translation product have been introduced. Our studies demonstrate that

1) in mutant 1 the cleavage site of the signal peptidase is not shifted to the newly introduced specific Gln^{-2}-Ala^{-1} site of the 24 residues long preprosequence. Nevertheless the unprocessed apo AI mutant 1 is completely translocated and fully protected against proteolysis.

2) The apo AI mutant 2 with a missing prosequence but an unmutated presequence and correct length of 18 amino-acid residues is cotranslationally cleaved and releases mature apo

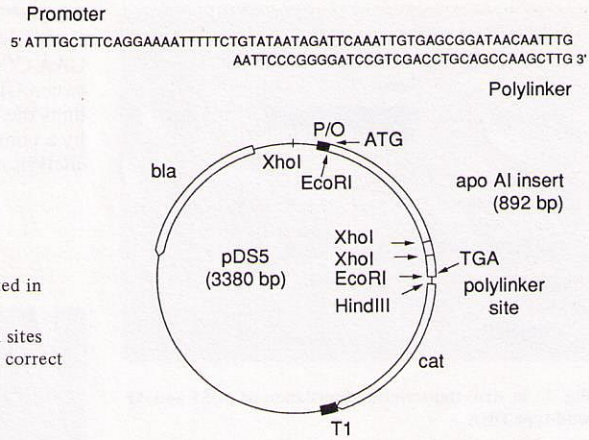

Fig. 1. Plasmid pDS5 with apo AI cDNA inserted in *Eco*RI restriction site.

Start and stop codons and two *Xho*I restriction sites which are relevant for the determination of the correct orientation of the apo AI insert are indicated.

AI with an unimpaired N-terminus. Since the prosequence and N-terminal sequence of the mature apo AI are completely different the amino acid sequence downstream the cleavage site of the normal signal peptide does not contribute to defining the processing site.

Results

Transcription and translation in vitro of wild-type human apo AI cDNA

A full length human apo AI clone has been isolated from a human liver cDNA library by oligonucleotide screening. It comprises a 892 bp insert in the *Eco*RI site with 14 bp 5′-untranslated region, 804 bp coding region and a 74 bp untranslated 3′ region. This *Eco*RI fragment was cloned into the dephosphorylated *Eco*RI site of pDS5 vector (Fig. 1). Clones with the correct orientation of the insert were ascertained by a *Hin*dIII/*Xho*I restriction enzyme digestion which released the expected 912 bp long fragment, Fig. 2 (apo AI wild-type). DNA sequencing confirmed results published previously[10]. In vitro transcription was carried out in the presence of [^{32}P]UTP[11]. A 1 450 bp transcript was obtained which encodes the preproapo AI sequence and the cat gene of pDS5 (results not shown). The mRNA synthesized complementary to the apo AI cDNA was translated in vitro and assayed for cotranslational translocation[12,13]. The wheat germ translation system was programmed in the absence and presence of canine pancreatic microsomal membranes. The resulting proteins were assayed by immunoprecipitation with anti human apo AI anti-

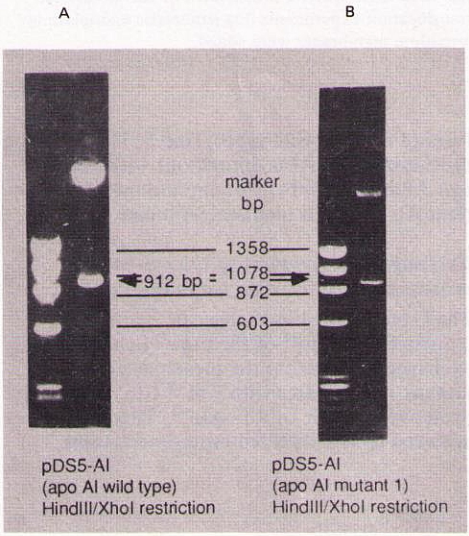

Fig. 2. Restriction analysis of wild type and mutant 1 of pDS5 apo AI clones.

Determination of the orientation of the apo AI cDNA insert by *Hin*dIII/*Xho*I restriction analysis of clones pDS5 apo AI wild-type and pDS5 apo AI mutant 1 ($Ala^{-7} \rightarrow Gln^{-1}$). Restriction fragments were separated on a 1% agarose gel (0.5 μg ethidium bromide/ml). ΦX 174/*Hae*III fragments were used as marker.

serum. A competition with apo AI in immunoprecipitation (lane 4) was carried out, too. Fig. 3 demonstrates that the microsomal membranes process the primary translation product in the transcription- translation experiment to the pro-

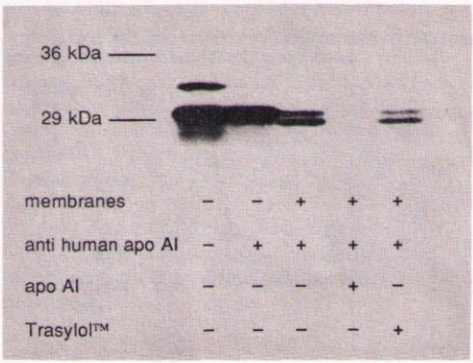

Fig. 3. In vitro transcription-translation of pDS5 apo AI wild-type DNA.

Autoradiogram of translation products separated by gradient (10–15%) $NaDodSO_4$-PAGE. [^{35}S]methionine was used as radioactive precursor. For cotranslational translocation experiments dog pancreatic endoplasmic reticulum membranes were added.

form of apo AI (wild-type), lane 3. The newly synthesized apo AI is diluted out with added apo AI in the competition immunoprecipitation (lane 4). Lane 5 is identical with lane 3.

Exchange of C-terminus of the pre- and prosequence by site-directed mutagenesis

The C-terminal amino acids Ala^{-7} of the presequence and Gln^{-1} of the prosequence were exchanged. Therefore the presequence of mutant 1 terminates with Gln^{-8}-Gln^{-7} and the prosequence with Gln^{-2}–Ala^{-1}. This was achieved by site-directed mutagenesis using the 39^{mer} oligonucleotide 5′ GGG AGC CAG CAG CGG CAT TTC TGG CAG GCT GAT GAA CCC 3′ which encodes the sequence between Gly^{-10}-Pro^{+3}. Fig. 4 schematically outlines the mutations. Direct proof was obtained by a comparison of the nucleotide sequence analysis of the wild-type and the mutant 1

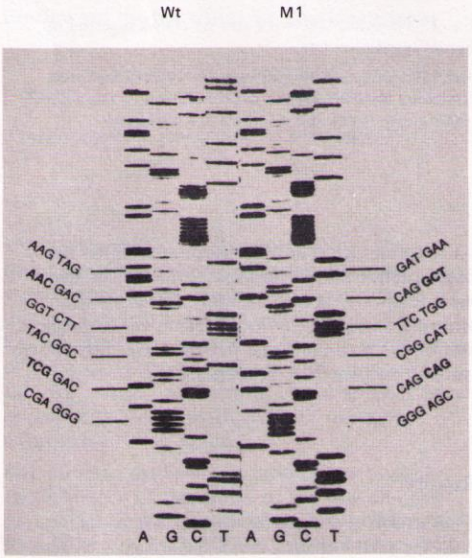

Fig. 5. Nucleotide sequence around site-directed mutation: GCT (Ala^{-7}) and CAA (Gln^{-1}) of wild-type (Wt) are mutually exchanged [CAG (Gln^{-7}) and GCT (Ala^{-1}) (M1)].

Tripletts are written bold faced.

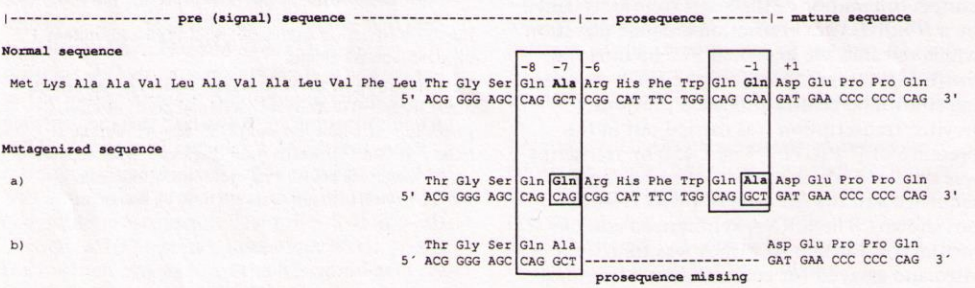

Fig. 4. Schematic drawing of site-directed mutagenesis at the C-termini of the pre- and prosequence of human serum apolipoprotein A-I and the respective cDNA.

a) Amino acid and nucleotide sequence of apo AI mutant 1 with the exchanged C-termini of pre- and prosequence of the wild-type (upper two lines) are encased.

b) The deletion of the prosequence is indicated by the dashed line.

around the sites of the mutation, Fig. 5. The mutated *Eco*RI fragment was isolated from the RF form of M13 phage and inserted into the *Eco*RI site of pDS5 in the correct orientation which was confirmed by *Hin*dIII/*Xho*I restriction analogous to the wild-type experiment.

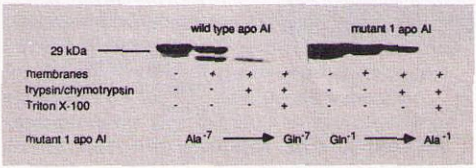

Fig. 6. Comparative in vitro transcription-translation of pDS5 apo AI wild-type and mutant 1.

Autoradiogram of translation products separated by gradient (10–15%) NaDodSO$_4$-PAGE. Rabbit pancreatic ER membranes were added for translocation and processing in the assay.

The comparative in vitro transcription-translation of the wild-type apo AI cDNA and this mutated apo AI cDNA (mutant 1) were carried out. Fig. 6 demonstrates that the translation product does not change its molecular mass when the translation was carried out in the presence of microsomal membranes. This product is resistant to trypsin/chymotrypsin digestion and therefore must have been translocated to the luminal side of the ER membrane. The mutagenized preproapo AI is not cleaved by the signal peptidase when cotranslationally translocated due to the dislocation of the recognition sequence Gln^{-8}-Ala^{-7} to a more distant position. Addition of the permeabilizing detergent Triton X-100 renders the translocated mutated preproapo AI susceptible to complete proteolysis. The translocated mutant preproapo AI was analysed by Edman degradation after electroelution. [^{35}S]Methionine appeared in the first cycle which indicated that the N-terminus of the presequence is also protected after translocation.

Deletion of the prosequence from preproapo AI-specific cDNA

The influence of the prosequence of apo AI, a hexapeptide bordering with Gln^{-2}-Gln^{-1} on the N-terminal Asp^{1}-Glu^{2}-Pro^{3} of the mature form was studied by site-directed mutagenesis. The prosequence coding the octadecameric sequence is shown in Fig. 4. The 30^{mer} oligonucleotide 5′ ACG GGG AGC CAG GCT GAT GAA CCC CCC CAG 3′ which hybridized with 15 nucleotides at the 5′ end coding the five C-terminal residues of the apo AI presequence and 15 nucleotides coding Asp Glu Pro Pro Gln of the first five N-terminal residues of the mature form was used for the oligonucleotide-directed construction of the deletion mutation via gapped duplex DNA to generate the Δpro AI clone. Dot blot analysis of DNA from M13mp9 apo AI clones probed with the octadecameric oligonucleotide (5′ CGG CAT TTC TGG CAG CAA 3′) corresponding to the deleted prosequence revealed one out of 24 clones with the deletion. The mutated insert was excised by *Eco*RI restriction out of the RF form of the M13mp9 clone and cloned into the *Eco*RI site of pDS12. Clones with the correct orientation of the apo AI cDNA were selected by restriction analysis. *Bam*HI/*Xho*I restriction released the expected 787-bp fragment from mutant 2 with the deleted prosequence, (results not shown). The nucleotide sequence derived from the insert ligated into the *Eco*RI site of M13mp9 is shown in Fig. 7. It clearly demonstrates the deletion of the 18 nucleotides long prosequence. pDS12 apo AI cDNA was transcribed and translated in vitro.

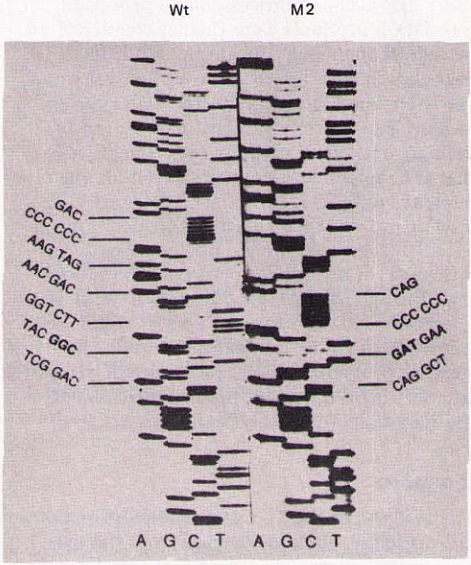

Fig. 7. Nucleotide sequence of wild-type apo AI (Wt) and apo AI mutant 2 cDNA around the deleted prosequence of the wild-type.

The prosequence starts with CGG (Arg^{-6}), the mature sequence with GAT (Asp^{+1}).

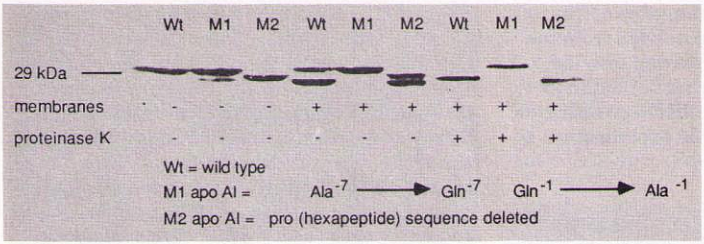

Fig. 8. Comparative in vitro transcription-translation of pDS5 apo AI wild type (Wt), mutant 1 (M1) and pDS12 apo AI mutant 2 (M2).

Translation products unprocessed and processed in the presence of dog pancreatic ER membranes were separated by gradient (10–15%) NaDodSO$_4$-PAGE and autoradiographed.

The primary translation products of wild-type (Wt), mutant 1 (M1) and mutant 2 (M2) with the deleted prosequence compared in gradient polyacrylamide gel electrophoresis (10–15%) clearly indicates the difference in molecular size of the primary translation product of preapo AI with the deleted sequence and preproapo AI (Wt), Fig. 8.

Translation in the presence of dog pancreatic ER membranes led to a product with a molecular size approximately 2 kDa smaller than the proapo AI but identical with mature apo AI (28.5 kDa). The unprocessed and processed translation products were further characterized by radiosequencing. The automated Edman degradation was carried out over 30 cycles and the N-terminal sequences of the wild type and mutant translation products unambiguously defined. The result of these epxeriments was that the signal peptidase has cleaved off the presequence with its C-terminal Gln^{-2}-Ala^{-1}. The missing prosequence and the sequence C-terminal of the cleavage site are obviously without influence on the correct cleavage. The processing of the translation product apo AI of mutant 2 led to the mature form of apo AI which is translocated to the luminal side of the ER vesicles, protected against proteinase K attack and only after detergent permeabilization of the membranes proteolytically cleaved.

Discussion

The mRNAs of the 10 serum apolipoproteins known so far whether secreted from the liver or intestine all encode N-terminal signal peptides for cotranslational translocation and secretion. Different from the others apolipoprotein AI contains a hexapeptide and AII a pentapeptide as prosequence interposed between the signal (pre-) and the mature sequence. These proforms are intracellularly stable and processed to the mature form only extracellularly after secretion. The machinery involved in the unidirectional translocation and processing of secretory proteins is being widely understood. Pivotal evidence came from the faithful reproduction in vitro of the processes involved in the translation/translocation[12,13], the receptor-mediated translocation of the nascent polypeptide chain recognized by the signal recognition peptide (SRP)[14,15] and the SRP receptor in the membrane of the endoplasmic reticulum[16,17]. Apolipoprotein AI was one of the early examples in which the now well established function of pancreatic SRP for the segregation of secretory proteins has been established[11].

In contrast to this well understood recognition the translocation mechanism of the polypeptides through the membrane has not yet been elucidated. However, the recent isolation and characterization of the signal peptidase as a hexameric complex of integral microsomal membrane proteins[18] together with the notion of permanently complex-associated ribophorins[19] and transient protein-protein interactions with the integral SRP receptor might form a proteinaceous pore for the translocation of the growing polypeptide chain.

In this context apo AI with its signal peptide and prosequence as cleavable N-terminal extensions and the possibility to change these sequences at critical sites by site-directed mutagenesis offers an attractive system to describe parameters related to the structure and cleavage of signal peptides and cooperatively the translocation.

We first addressed the specificity of the signal peptidase for preproapo AI since apo AI and AII are the only serum apolipoproteins with a prepro amino-terminal extension. By site-directed

mutagenesis we exchanged Ala^{-7}, the C-terminal residue of the signal sequence, against Gln^{-1}, the C-terminus of the prosequence and thereby extended the signal peptide (Gln^{-2} Ala^{-1}) to 24 residues with the C-terminus of the signal peptide six residues downstream but in front of the mature apo AI sequence. On the other hand the Gln^{-2}-Gln^{-1} sequence, which in wild-type preproapo AI forms the C-terminus of the prosequence, terminates the 18-residues long N-terminal presequence. Following empirical rules for the recognition of the endoprotease (signal peptidase) no specific amino-acid sequence determines the cleavage site but typical amino-acid residues with small side chains at position -1 and -3[20,21]. In our apo AI mutant 1 Ala^{-1} would follow this rule but Trp^{-3} is rather space-filling. Due to the small consensus in presequences a marginal essential specific conformation of the amino-acid sequence around the cleavage site has been postulated[22–24]. We studied the influence of the mutations by the predictive rules of Chou and Fasman[25–27]. The first 13 residues of the 18 amino-acid residues long presequence of wild-type apo AI and likewise of the apo AI mutant 1 are strong helix formers. Another predicted α-helix spans from Ala^{-7} to Gln^{-1} through the prosequence. These two α-helices are linked by a β-turn sequence thus forming a bent, hair pin-like structure which may expose the cleavage site of the wild-type with the small side chains of Ala^{-7}, Gln^{-8} and Ser^{-9} in the β-turn structure for proteolytic attack whereas in apo AI mutant 1 apparently the side chain of Gln^{-7} prohibits access to the peptide bond.

When the α-helical prosequence is deleted the presequence is fused to the mature sequence. The hydrophobic α-helical presequence remains identical with that of the wild-type and continues into the β-turn and a very short α-helix with the cleavage site followed by the random structure of the N-terminal mature sequence.

The cleavage of the signal sequence occurs at the luminal side of the ER membrane[28]. The presequence and its hydrophobic domain have dimensions which would satisfy the proposed transmembranal orientation with the N-terminus on the cytosolic side and the cleavage site for the signal peptidase presented on the luminal side[29]. Fig. 6 proves that the mutation in apo AI mutant 1 with the substitution of Ala^{-7} by Gln^{-1} prohibits the cleavage but not the complete translocation to the luminal space which renders the mutated preproapo AI then fully protected against the proteolytic attack.

Edman degradation of [^{35}S]Met-labelled translocation product refers to the integrity of the mutated 24-residue preprosequence of the translocated translation product. Whether the N-terminal sequence is still integrated in the membrane or also threaded through and detached from the ER membrane cannot be concluded from these experiments.

The site-directed mutagenesis leading to the apo AI mutant 1 and to mutant 2 with the deletion of the prosequence of preproapo AI cDNA yielded a primary translation product of apo AI which was cotranslationally translocated and lost by accurate cleavage by the signal peptidase in a yield comparable with that of the wild-type. The results derived from apo AI mutant 1 and mutant 2 allows the following conclusions: a) the prosequence is of no relevance for the processing of the signal sequence and therefore neither contributes to a putative linear topogenic sequence nor to a topogenic site in a folded tertiary structure around the cleavage site and b) the sequence following the signal peptide neither the pre- nor the N-terminal mature sequence – which differ considerably in the case of apo AI – influence the processing.

The function of the prosequence remains unclear. A possible adaptor function in preproapo AII has been deduced from cotranslational translocation and processing experiments with apo AII mutant in which the prosequence was deleted[30]. Signal peptidase recognized Ala^{+2} of the mature sequence instead of Gly^{-1} of the signal sequence as cleavage site. It is more likely that the prosequence serves another recognition process e.g. intracellularly for the dichotomy in the posttranslational pathway to a secretory protein or in the targeting process required for the assembly with lipids to supramolecular structures of the still undefined secretory particle. Also extracellular recognition processes could be facilitated by the propeptide structure (see Note added in proof, p. 1062).

These mutants can now be used to establish transfected cell lines with permanent expression of apo AI and its mutants which could give insight into the function of the pre- and propeptide sequences for the intracellular transport, the secretion and the assembly process of lipoprotein particles.

Materials and Methods

Restriction endonucleases, T_4 DNA ligase and calf intestinal phosphatase were purchased from Boehringer-Mannheim GmbH (D-6800 Mannheim), *E. coli* RNA polymerase and 7mGpppA from Pharmacia (D-7800 Freiburg), [^{35}S]methionine, ^{32}P-labelled ribo- and desoxyribonucleotides and wheat germ extract from Amersham (D-3300 Braunschweig).

Screening of λgt11 human liver cDNA

Approximately 5 x 10^5 λgt11 plaques were screened with a ^{32}P-labelled 30^{mer} oligonucleotide coding the N-terminal 10 amino-acid residues of mature apo AI. The positive clone carrying a 892-bp long *Eco*RI insert was sequenced completely. It encoded the complete apo AI sequence including the preprosequence. The 5′ non-coding region contained 14 bp and the 3′ non-coding region 74 bp. The *Eco*RI fragment from the λDNA digest was separated by agarose gel electrophoresis, isolated and purified by collecting the restriction fragments on a DEAE cellulose membrane NA 45 (Schleicher & Schüll, D-5160 Düren) as described before[31,32]. The *Eco*RI cDNA insert of the selected phage clone was subcloned into M13mp9, pUC9, pDS5 and pDS12 respectively. Plasmids and phages were purified as described by Maniatis et al.[33].

In vitro transcription

In vitro transcriptions of the pDS5 and pDS12 based vectors were carried out as described before[11]: 1–3 μg plasmid DNA was incubated at 37 °C for 30 min in a 100-μl reaction mixture. The mixture was 20mM Hepes/KOH, pH 7.9, 200mM potassium acetate, 10mM magnesium acetate, 5mM DTT, 0.2mM spermidine, 0.1mM EDTA, 2 U RNAsin, 0.5mM in ATP, GTP and CTP, 100μM UTP, 0.05μM [α-^{32}P]UTP (3000 Ci/mmol), 0.6 U *E. coli* RNA polymerase. The reaction was terminated by the addition of EDTA to the final concentration of 40mM. The RNA sample was prepared for electrophoresis by phenol extraction and ethanol precipitation.

DNA-sequence analysis

Sequence analysis was carried out according to Sanger et al.[34].

RNA and DNA electrophoresis

100 μg/ml DNA fragments were separated after digestion by agarose gel electrophoresis at 2–5 V/cm with Tris borate (0.05M) as buffer system. The agarose gels (1%) contained ethidium bromide (0.5 μg/ml), RNA was electrophoretically separated on denaturing formaldehyde agarose gels[35].

In vitro transcription-translation

The coupled transcription-translation was carried out as described by Stüber et al.[11].

Translation reactions (25 μl total volume) were programmed with in vitro transcribed apo AI-specific mRNAs in the wheat germ extract system [30% (v/v)] and the following final concentrations: 20mM Hepes/KOH, pH 7.4, 110mM potassium acetate, 3mM magnesium acetate, 1mM dithiothreitol, 1mM ATP, 10mM creatine phosphate, 100 μg/ml creatine kinase, 5μM *S*-adenosyl methionine, 20μM unlabelled 19 amino-acid mixture and 1 mCi/ml [^{35}S]methionine. Translation was carried out at 25 °C for 1 h. Dog pancreatic membranes were included at a concentration of 0.05 A_{280} units per assay where indicated.

Immunoprecipitation and analysis of translation products

The translation mixtures were boiled in $NaDodSO_4$ buffer for 3 min and incubated in the detergent mix with antiserum and non-immune serum as described before[1]. The immune complex was adsorbed to formaldehyd-treated *Staphylococcus aureus* and the mixture centrifuged after 2–4 h at 10000 rpm for 2 min. The pellet was thoroughly washed with the buffer detergent mix and then dissociated with $NaDodSO_4$ sample buffer by boiling for 5 min. Proteins were separated by gradient (10–15% $NaDodSO_4$ polyacrylamide gel electrophoresis for autoradiographic analysis[36].

Automated Edman degradation of radiolabelled wild-type, mutant 1 and mutant 2 apo AI was carried out in a Beckman 890C spinning cup sequencer using the Quadrol program as described before[1]. Two wash cycles were initially prerun before the first cleavage reaction was started.

Oligonucleotide-directed mutagenesis

Mutations at the predetermined positions of the wild-type leading to the pDS5 apo AI mutant 1 and the deletion of the prosequence in pDS12 apo AI mutant 2 were generated with the gapped duplex DNA approach described by Kramer et al.[37]. The mutagenic oligonucleotides for mutant 1 was [5′ GGG AGC CAG CAG CGG CAT TTC TGG CAG GCT GAT GAA CCC 3′] and for the apo AI deletion mutant 2 [5′ ACG GGG AGC CAG GCT GAT GAA CCC CCC CAG 3′]. One apo AI mutant 1 clone was obtained out of 12 and one apo AI mutant 2 out of 24. The two mutants were completely sequenced to confirm the programmed mutations.

Note added in proof: In an independent study Folz and Gordon[38] reported on the effect of the deletion of the prosequence of human preproapoAI and made the same observation that the signal-sequence peptidase cleaved the presequence of their pre(Δpro)apo AI construct with high fidelity.

We gratefully acknowledge the support of this work by the *Deutsche Forschungsgemeinschaft*, SFB 74, and the *Fonds der Chemischen Industrie*. We thank *Prof. H.J. Fritz* for providing the *E. coli* strains BMH 71-18, 71-18 mut S, MK30-3, M13mp9 ds DNA, M13mp9 rev ds DNA and *Prof. Bujard*, ZMBH Heidelberg, for providing the plasmids pDS5 and pDS12; *Dr. H. Giersiefen* for protein and *J. Hülsmann* for DNA sequence analyses.

Literature

1 Stoffel, W., Blobel, G. & Walter, P. (1981) *Eur. J. Biochem.* **120**, 519–522.
2 Stoffel, W., Krüger, E. & Deutzmann, R. (1983a) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 227–237.
3 Gordon, J.I., Smith, D.P., Andy, R., Alpers, D.H., Schonfeld, G. & Strauß, A.W. (1982) *J. Biol. Chem.* **257**, 971–978.

4 Gordon, J.I., Sims, H.F., Lentz, S.R., Edelstein, C., Scanu, A.M. & Strauß, A.W. (1983) *J. Biol. Chem.* **258**, 4037–4044.
5 Gordon, J.I., Budelier, K.A., Sims, H.F., Edelstein, C., Scanu, A.M. & Strauß, A.W. (1983) *J. Biol. Chem.* **258**, 14054–14059.
6 Stoffel, W., Bode, C. & Knyrim, K. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 439–445.
7 Stoffel, W., Knyrim, K. & Bode, C. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 1631–1640.
8 Gordon, J.I., Sims, H.F., Edelstein, C., Scanu, A.M. & Strauß, A.W. (1984) *J. Biol. Chem.* **259**, 15556–15563.
9 Gordon, J.I., Sims, H.F., Edelstein, C., Scanu, A.M. & Strauß, A.W. (1985) *J. Biol. Chem.* **260**, 14824–14831.
10 Breslow, J.L., Ross, D., Mc Pherson, J.M. & Williams, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6861–6865.
11 Stüber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. & Bujard, H. (1984) *EMBO J.* **3**, 3143–3148.
12 Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
13 Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 852–862.
14 Walter, P. & Blobel, G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7112–7116.
15 Walter, P. & Blobel, G. (1981) *J. Cell Biol.* **91**, 551–556.
16 Meyer, D.I. & Dobberstein, B. (1980) *J. Cell Biol.* **87**, 498–502.
17 Gilmore, R., Walter, P. & Blobel, G. (1982) *J. Cell Biol.* **95**, 470–477.
18 Evans, E.A., Gilmore, R. & Blobel, G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 581–585.
19 Kreibich, G., Ulrich, B.C. & Sabatini, D.D. (1978) *J. Cell Biol.* **77**, 464–487.
20 Austen, B.M. & Ridd, D.H. (1981) *Biochem. Soc. Symp.* **46**, 235–258.
21 van Hejne, G. (1984) *J. Mol. Biol.* **173**, 243–251.
22 Perlman, D. & Halvorson, H.O. (1983) *J. Mol. Biol.* **167**, 391–409.
23 Makower, A., Dettmer, R., Rapoport, T.A., Knospe, S., Behlke, J., Prehn, S., Franke, P., Etzold, G. & Rosenthal, S. (1982) *Eur. J. Biochem.* **122**, 339–345.
24 Rapoport, T.A. (1986) *Crit. Rev. Biochem.* **20**, 73–137.
25 Chou, P.Y. & Fasman, G.D. (1974) *Biochemistry* **13**, 211–221.
26 Chou, P.Y. & Fasman, G.D. (1974) *Biochemistry* **13**, 222–245.
27 Chou, P.Y. & Fasman, G.D. (1978) *Adv. Enzymol.* **47**, 43–148.
28 Jackson, R.C. & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5598–5602.
29 Lipp, J. & Dobberstein, B. (1986) *Cell* **46**, 1103–1112.
30 Folz, R.J. & Gordon, J.I. (1986) *J. Biol. Chem.* **261**, 14752–14759.
31 Dretzen, G., Bellard, M., Sassone-Corsi, P. & Chambon, P. (1981) *Anal. Biochem.* **112**, 295–298.
32 Young, R.A., Bloom, B.R., Grosskinsky, C.M., Ivany, J., Thomas, D. & Davis, R.W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2583–2587.
33 Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) *Molecular Cloning – A Laboratory Manual*, Cold Spring Harbor, New York, Cold Spring Harbor Press.
34 Sanger, F., Nicklen, S. & Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
35 Lehrach, H., Diamond, D., Wozney, J.M. & Boedtker, H. (1977) *Biochemistry* **16**, 4743–4751.
36 Erickson, A.H. & Blobel, G. (1983) *Methods Enzymol.* **96**, 38–50.
37 Kramer, W., Drutsa, V., Jansen, H.W., Kramer, B., Pflugfelder, H. & Fritz, H.J. (1984) *Nucleic Acids Res.* **12**, 9441–9456.
38 Folz, R.J. & Gordon, J.I. (1987) *J. Biol. Chem.* **262**, 17221–17230.

W. Stoffel and E. Binczek, Institut für Physiologische Chemie der Universität zu Köln, Joseph-Stelzmann-Str. 52, D-5000 Köln 41.