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Transient Expression of Wild Type and Mutant Human Apolipoprotein AI in COS Cells

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Summary: A human apolipoprotein AI (apo AI) minigene and two mutants were cloned into the vector pUHD10-1 for expression studies in COS cells under the control of the strong CMV (cytomegalovirus) enhancer and the own apo AI promoter. In the mutated apo AI minigene (mutant M1) the positions of the triplets of Gln^{-2}-Gln^{-1} at the C-terminus of the prosequence were exchanged against Gln^{-8}-Ala^{-7}, the recognition site of the signal peptidase of the wild type human apo AI. The prosequence has been deleted in mutant M2 and the presequence linked directly to the N-terminus of the mature apo AI form.

We report here on expression studies in COS cells, a cell line, which does not express apo AI. They were transfected by electroporation with pUHD10-1 constructs, which contain a) the wild type apo AI minigene and b) the two mutant apo AI minigenes with mutations described above.

The following results were obtained: a) the wild type and mutant apo AI constructs were efficiently transcribed and translated in COS cells, b) the expression of the wild type preproapo AI minigene in COS cells led to the secretion of proapo AI (29 kDa), that of the mutant (M2) gene, devoid of the prosequence of mature apo AI (28.4 kDa), whereas the product of mutant gene M1 (31 kDa) with the recognition site of the signal peptides transposed to the C-terminus of the prosequence remained uncleaved within the COS cells.

These in vivo results confirm and extend our previous in vitro expression-translation findings on the processing and secretion using wild type and the same mutations in the preprosequence of human apo AI cDNA constructs. The prosequence of apo AI is neither required for intracellular processing nor secretion.

COS cells secreting apolipoprotein AI after transfection offer a system to study the expression and secretion of apolipoproteins and the assembly of lipoprotein particles.

Transiente Expression von Wildtyp- und mutiertem menschlichem Apolipoprotein AI in COS-Zellen

Zusammenfassung: Wir beschreiben die Konstruktion eines Minigens des menschlichen Apolipoproteins AI und zweier seiner Mutanten im Vektor pUHD10-1. Die Expression des Gens steht unter der Kontrolle des starken CMV-Promoter-Enhancers. In dem mutierten Apo AI-Minigen (Mutante M1) sind

Enzymes:
CIP, Alkaline phosphatase (from calf intestinum), orthophosphoric-monoester phosphohydrolase (alkaline optimum) (EC 3.1.3.1); LCAT, Phosphatidylcholine–sterol acyltransferase, phosphatidylcholine:sterol *O*-acyltransferase (EC 2.3.1.43) (in this paper named lecithin-cholesterol acyltransferase).

Abbreviations:
Apo AI, apolipoprotein AI of human serum high density lipoprotein; CE, cholesterol ester; CMV, cytomegalovirus; COS cells, SV40 transformed African green-monkey kidney (ATCC CRL 1650); DMEM, Dulbecco's minimal essential medium; DMSO, dimethylsulfoxide; EDTA, ethylendiaminetetraacetic acid; ER, endoplasmic reticulum; FCS, fetal calf serum; HDL, serum high density lipoprotein; MCS, multiple cloning site; NP-40, ethylphenylpolyethyleneglycol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.4; PCR, polymerase chain reaction; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; SV40, simian virus 40.

die Positionen der beiden Tripletts Gln^{-2}-Gln^{-1} am C-Terminus der Prosequenz gegen Gln^{-8}-Ala^{-7} am C-Terminus der Erkennungssequenz für die Signalpeptidase ausgetauscht. In der anderen Mutante (M2) ist die Prosequenz deletiert.

Die Expressionsstudien in COS-Zellen, einer Zellinie, die kein Apo AI exprimiert, führten zu folgenden Ergebnissen: a) nach Transfektion mit pUHD10-1-apo-AI-Konstrukten, die den Wildtyp des Mini-Apo-AI-Gens und die beiden mutierten Apo-AI-Minigene enthielten, fand eine effiziente Transkription und Translation der drei Minigene in den COS-Zellen statt, b) die mit dem Wildtyp durch Elektroporation transfizierten COS-Zellen sezernierten Proapo AI (29 kDa), die mit der Δpro-Mutante (M2) reifes Apo AI (28.4 kDa), wohingegen das Translationsprodukt (31 kDa) in den mit der Mutanten M1 transfizierten Zellen unprozessiert internalisiert verblieb. Die Translationsprodukte wurden durch Immunpräzipitation isoliert und charakterisiert.

Diese Resultate von In-vivo-Expressionsexperimenten unterstreichen und erweitern unsere früheren In-vitro-Expressions-Translations-Befunde zur Prozessierung und Sekretion der Wild-Typ und der Präpro-Mutanten der menschlichen Apo-AI-cDNA-Konstrukte. Die Prosequenz des Apo AI ist weder für das intrazelluläre Processing noch für die Sekretion erforderlich.

COS-Zellen, die nach Transfektion Apolipoprotein AI sezernieren, stellen ein geeignetes System dar, die Expression und Sekretion von Apolipoproteinen und die Aggregation von Lipoproteinpartikeln zu studieren.

Key terms: Apolipoprotein AI, COS cell transfection, minigene construction, secretion, site-directed mutagenesis.

Human serum HDL has drawn considerable attention because of its function in reverse cholesterol transport and therefore in the homoeostasis of cholesterol metabolism. The main protein constituent of HDL is apolipoprotein AI (apo AI). Apo AI has been recognized as the activator of lecithin cholesterol acyltransferase (LCAT)[1]. This enzyme catalyses the transfer of the *sn*-2-acyl group of phosphatidylcholine to cholesterol yielding lysolecithin and cholesterol ester (CE). The activation of the enzyme occurs at the surface of the HDL particle. The CE is thought to be deposited in the hydrophobic core of the HDL particle for transport to the liver. Apo AI is synthesized in the hepatocyte[2] and enterocyte[3,4] as preproprotein with 267 amino-acid residues. Its signal sequence (18 residues) is processed by a signal peptidase of the ER during the cotranslational translocation. Proapo AI is stable intracysternally. It is secreted and finally processed[5] by a serum proteinase at the unusual Gln-Gln dipeptide to the 243 amino-acid residues protein (28 kDa). Nothing is known about the function of the N-terminal hexapeptide extension of the proform of apo AI, whether it is required for the intracellular transport, as secretory signal or for the assembly of the HDL primary particle. In vitro transcription-translation experiments with wild-type apo AI cDNA and two mutants M1 and M2 have been carried out[6]. Fig. 1 outlines the amino-acid sequence of the preprosequence of apo AI after site-directed mutagenesis.

We describe in this report the construction of an apo AI wild-type minigene and two mutants. In mutant M1 the Gln-Ala C-terminus of the presequence has been exchanged with the Gln-Gln sequence at the C-terminus, the cleavage site of the prosequence. In mutant M2 the prosequence has been deleted by site-directed mutagenesis. These minigenes were ligated into the eukaryotic expression vector pUHD10-1 under the influence of the strong CMV (human cytomegalovirus) enhancer/promoter. Transfection by electroporation with these three DNAs led to the transient expression in COS cells: transfected COS cells efficiently synthesized the wild type, mutant 1 and mutant 2 preapo AI polypeptides respectively. Wild type preproapo AI was processed and secreted as proapo AI, mutant 1 apo AI remained unprocessed and internalized in the COS cells whereas mutant 2 was secreted as mature apo AI.

The COS cell system described here mimics essentially the cotranslational translocation of apo AI in hepatocytes and intestinal cells. The results reported here verify those derived from the in vitro transcription/translation system: the prosequence is neither demanded for cotranslational translocation nor for accurate processing of the signal sequence and the secretion in vivo. They underline the restriction for the signal peptidase with respect to the position, the correct distance from the N-terminus and/or possibly the conformation around the recognition site (Gln-Ala). Furthermore this system allowed to study in a non apo AI secreting cell line apolipoprotein expression, secretion and the assembly of lipoprotein particles.

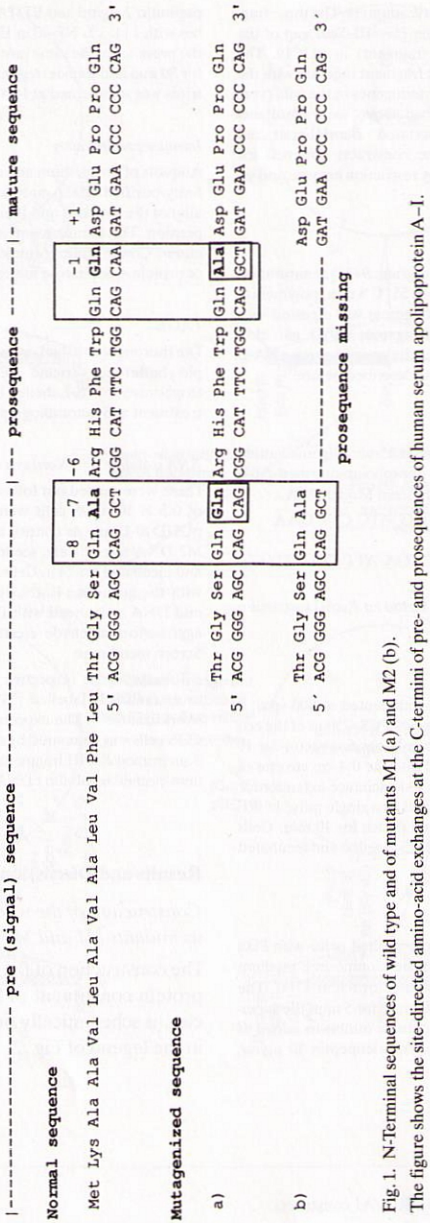


Fig. 1. N-Terminal sequences of wild type and of mutants M1 (a) and M2 (b).
The figure shows the site-directed amino-acid exchanges at the C-termini of pre- and prosequences of human serum apolipoprotein A–I.

Materials and Methods

Construction of wild type and apo AI mutants M1 and M2

The plasmid pUHD10-1 was kindly provided by *Dr. Bujard*, ZMBH Heidelberg. This vector contains the strong CMVenhancer and the SV40 polyadenylation site. We used the *Bam*HI site of the multiple cloning site for the insertion of the following fragments: a 355 bp fragment comprising the 5′-nontranslated regulatory region of the human apo AI gene described before[7] synthesized by PCR using the 5′ antisense oligonucleotide primer I and the 3′ sense primer oligo II, described below, and the *Hin*dIII-*Sau*I fragments (371 bp) of apo AI cDNA of wild type, M1 and M2 (353 bp) encoding exons 2 and 3 which were isolated from their respective pSV2 cat constructs. The apo AI full length cDNAs were inserted

into the *Hin*dIII site by 5′ and 3′ synthetic adapters. The three fragments were isolated and ligated into the *Hin*dIII-*Sau*I gap of the genomic apo AI clone (2.2 kb *Pst*I fragment) in pUC19. The *Bam*HI-*Eco*RI (355 bp) restricted PCR fragment together with the *Eco*RI-*Bam*HI inserts of the respective minigenes of the wild type, M1 and M2 mutants in pUC 19, described above, were simultaneously ligated into the dephosphorylated *Bam*HI-restricted pUHD10-1 vector and three minigene constructs obtained for transfection. They were characterized by restriction enzyme and sequence analysis.

PCR[8]

30 cycles were run with the following program: 94 °C denaturation 2 min, cooling for annealing temperature 55 °C 2 min, polymerase chain reaction 72 °C 2 min. The PCR fragment was digested with *Eco*RI and *Bam*HI and purified by agarose (1%) gel electrophoresis and collection on a DEAE cellulose membrane NA45 (Schleicher & Schuell, W-5160 Düren) as described before[9].

Oligonucleotide primers for PCR

The following 30mer oligonucleotide I and 35mer oligonucleotide II were synthesized with the solid phase phosphoamidite method[10] using an Applied Biosystem DNA-synthesizer, Model 380A.

Oligo I: 5′ CCA GGG ACA GAG CGG ATC CTT GAA CTC TTA 3′

Oligo II: 5′ CG CAG CTT TCA TCC TGA ATT CCC GTG GGG GAC CTG 3′

The primers contained a *Bam*HI (oligo I) and an *Eco*RI restriction site (oligo II), which are underlined.

Electroporation

COS cells (2×10^7) were trypsinized, sedimented at 800 rpm, 5 min, and resuspended in 1 m*l* DMEM + 5% FCS. 250 μl of the cell suspension was incubated with 30 μg non-linearized vector for 10 min in an ice bath and then transferred into the 0.4-cm cuvette of the Biorad Gene Pulser equipped with a capacitance extender for electroporation. The sample was subjected to a single pulse of 960 μF and 160 V. The cuvette was incubated on ice for 10 min. Cells were then suspended in 10 m*l* DMEM/FCS, seeded and incubated for 16 h at 37 °C.

Labelling experiments

Cells transfected by electroporation were washed twice with PBS and incubated with 4 m*l* of serum and methionine-free medium supplemented with 200 μCi [^{35}S]methionine for 6 h at 37 °C. The medium was collected, centrifuged at 800 rpm for 5 min, the supernatant adjusted to 0.5% NP-40 and proteinase inhibitors added to a final concentration of aprotinin 30 μg/m*l*, leupeptin 10 μg/m*l*, pepstatin 2 μg/m*l* and EDTA of 2mM. Cells were lysed in petri dishes with 1 m*l* 1% NP-40 in 150mM NaCl, 50mM Tris/HCl, pH 8.0 in the presence of the same proteinase inhibitor concentrations at 4 °C for 30 min and transferred into Eppendorf tubes. The insoluble detritus was sedimented at 14000 rpm for 10 min at 2 °C.

Immunoprecipitation

Aliquots of the medium and cell lysate were incubated with 5 μg affinity-purified sheep anti human apo AI antibody. To another aliquot the antibody and 10 μg apo AI were added for immunocompetition. The immunocomplex was adsorbed to a *Staphylococcus aureus* Cowan strain (protein A)[11] suspension in 2mM methionine or protein A Sepharose as described before[12].

PAGE

The thoroughly washed immuno complex was dissociated with sample buffer for Tricine SDS-polyacrylamide gel (10%) electrophoresis[13]. ^{35}S-Labelled bands were visualized by PPO/DMSO treatment and autoradiography using a Kodak XAR-5 film.

RNA isolation and Northern blot hybridization analysis

These were carried out following established procedures: Aliquots of 0.5×10^7 COS cells were transfected in 250 μl medium with pUHD10-1 only as control and pUHD10-1 with apo AI, M1 and M2 DNA respectively, seeded in petri-dishes with 10 m*l* medium and incubated for 24 h. Cells were washed with PBS, RNA isolated with the guanidine-isothiocyanate method[14] and persistent plasmid DNA hydrolysed with DNAse I. RNA was separated by 1% agarose/formaldehyde electrophoresis[15] and blotted to a Gene Screen membrane.

α-Tubulin in the respective RNA preparation was visualized by using randomly labelled [^{32}P] α-tubulin cDNA for Northern blot hybridization[16]. The expression of the three apo AI constructs in COS cells was compared by quantitative laser densitometry. A random primed *Eco*RI fragment (892 bp) of the apo AI cDNA and random-primed α-tubulin cDNA were used for the hybridization.

Results and Discussion

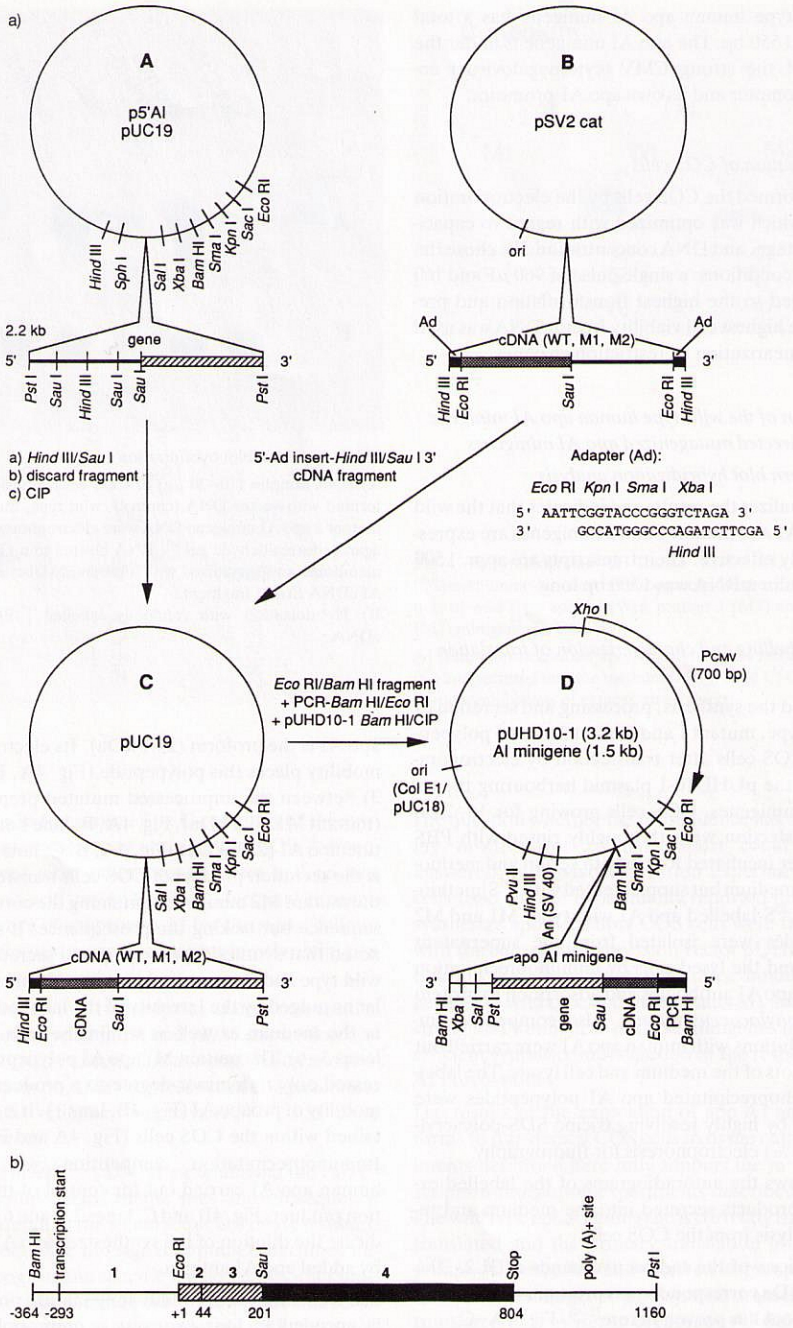
Construction of the wild-type apo AI minigene and its mutants M1 and M2

The construction of the minigenes of apo AI, the main protein constituent of high density apoprotein particles, is schematically outlined in Fig. 2 and described in the legend of Fig. 2.

Fig. 2. a) Schematic drawing of pUHD10-1/AI constructs. ▷

A) p5′AI: The genomic 2.2 kb *Pst*I fragment encoding the human apo AI gene was cloned into the *Pst*I site of the MCS of pUC 19. The vector was restricted with *Hin*dIII and *Sau*I, the excised fragment discarded, the linearized vector dephosphorylated. B) Donor vectors of the cDNA *Hin*dIII-*Sau*I fragments of the wild type and mutants 1 and 2. The mutations have been introduced as described before[6]. The *Eco*RI cDNAs were ligated via 5′ and 3′ adapters into the *Hin*dIII restriction site of pSV2 cat. C) The *Hin*dIII-*Sau*I fragments of wild type, M1 and M2 were ligated into the *Hin*dIII-*Sau*I gap of vector p5′AI (A). D) pUHD10-1, *Bam*HI-restricted and dephosphorylated. The 5′ *Bam*HI-3′ *Eco*RI-restricted PCR fragment (355 bp) and the *Eco*RI-*Bam*HI fragments of construct C were simultaneously ligated into the pUHD10-1-*Bam*HI. This vector harbours the complete human apo AI minigenes of the wild type and mutants 1 and 2.

b) Minigene of human apo AI regulatory 5′ region (*Bam*HI-*Eco*RI) obtained by PCR of genomic clone λAI (exon 1), 5′-coding region of cDNA clones (*Eco*RI-*Sau*I, exons 2 and 3) and genomic sequences (*Sau*I-*Pst*I, exon 4).


a)
A
p5'AI
pUC19
Hind III
Sph I
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Eco RI
2.2 kb
gene
5'
3'
Pst I
Sau I
Hind III
Sau I
Sau I
Pst I
B
pSV2 cat
ori
Ad
cDNA (WT, M1, M2)
Ad
5'
3'
Hind III
Eco RI
Sau I
Eco RI
Hind III
a) Hind III/Sau I
b) discard fragment
c) CIP
5'-Ad insert-Hind III/Sau I 3'
cDNA fragment
Adapter (Ad):
Eco RI Kpn I Sma I Xba I
5' AATTCGGTACCCGGGTCTAGA 3'
3' GCCATGGGCCCAGATCTTCGA 5'
Hind III
C
pUC19
Eco RI/Bam HI fragment
+ PCR-Bam HI/Eco RI
+ pUHD10-1 Bam HI/CIP
Xho I
Pcmv
(700 bp)
D
pUHD10-1 (3.2 kb)
AI minigene (1.5 kb)
ori
(Col E1/
pUC18)
Sal I
Xba I
Bam HI
Sma I
Kpn I
Sac I
Eco RI
Pvu II
Hind III
An (SV 40)
Bam HI
Sma I
Kpn I
Sac I
Eco RI
cDNA (WT, M1, M2)
apo AI minigene
5'
3'
3'
5'
Hind III
Eco RI
cDNA
Sau I
Pst I
Bam HI
Xba I
Sal I
Pst I
gene
Sau I
cDNA
Eco RI
PCR
Bam HI
b)
Bam HI
transcription start
1
Eco RI
2
3
Sau I
4
Stop
poly (A)+ site
Pst I
-364 -293
+1 44
201
804
1160

The wild-type human apo AI minigene has a total length of 1550 bp. The apo AI minigene is under the control of the strong CMV (cytomegalovirus) enhancer-promoter and its own apo AI promotor.

Transformation of COS cells

We transformed the COS cells by the electroporation method which was optimized with regard to capacitance, voltage, and DNA concentration. We chose the following conditions: a single pulse of 960 μF and 160 V which led to the highest transformation and preserved the highest cell viability. Vector DNA was used without linearization by restriction enzymes.

Expression of the wild-type human apo AI minigene and site-directed mutagenized apo AI minigenes

a) Northern blot hybridization analysis

Fig. 3 visualizes the results and indicates that the wild type apo AI and mutant 1 and 2 minigenes are expressed equally effective. Their transcripts are appr. 1500 bp, α-tubulin mRNA was 1300 bp long.

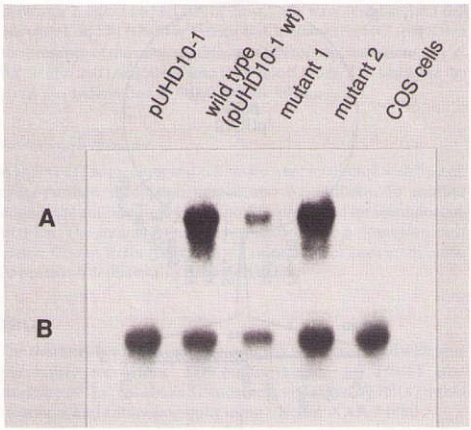


Fig. 3. Northern blot hybridization analysis.

A) RNA samples (10–20 μg) of COS cells, transiently transformed with vector DNA (control), wild type, mutant 1 and mutant 2 apo AI minigene DNA were electrophoresed in a 1% agarose-formaldehyde gel[14], RNA blotted to a Gene Screen membrane and hybridized with a randomly labelled [^{32}P] apo AI cDNA *Eco*RI fragment.

B) Hybridization with randomly labelled [^{32}P] α-tubulin cDNA.

b) ^{35}S Labelling and characterization of translation products

We studied the synthesis, processing and secretion of the wild type, mutant 1 and mutant 2 apo AI polypeptides in COS cells after transfection by electroporation with the pUHD10-1 plasmid harbouring the respective minigenes. COS cells growing for 16–24 h after transfection were thoroughly rinsed with PBS and further incubated for 6 h with serum and methionine-free medium but supplemented with [^{35}S]methionine. The ^{35}S-labelled apo AI wild type, M1 and M2 polypeptides were isolated from the supernatant medium and the lysed cells by immunoprecipitation with anti apo AI antibodies and adsorption to protein A of *Staphylococcus aureus*. Also competitive immunoinhibitions with human apo AI were carried out with aliquots of the medium and cell lysate. The labelled immunoprecipitated apo AI polypeptides were separated by highly resolving tricine SDS-polyacrylamide (10%) electrophoresis for fluorography.

Fig. 4 shows the autoradiograms of the labelled expression products secreted into the medium and released by lysis from the COS cells.

The positions of the radioactive bands at 31.2, 29.1 and 28.4 kDa correspond to prepro-, pro- and mature human apo AI as proven before[2–4], Fig. 4A–C.

Transiently transfected COS cells effectively transcribed the apo AI minigenes and translated, processed and secreted the respective apo AI isoforms into the medium. The secretion product of the wild type apo AI is the proform (29.1 kDa). Its electrophoretic mobility places this polypeptide (Fig. 4A, B, C, lane 3) between the unprocessed mutated preproapo AI (mutant M1, 31.2 kDa), Fig. 4A, B, lane 1 and the mature apo AI (28.4 kDa), Fig. 4A, B, C, lane 5), which is the secretion product of COS cells transfected with the mutant M2 minigene containing the correct signal sequence but lacking the prosequence. It should be noted that synthesis, processing and secretion of the wild type and the mutant 2 polypeptide are very similar as judged by the intensity of the labelled products in the medium as well as within the cells (Fig. 4A, lanes 3–6). The mutant M1 apo AI polypeptide is processed only to a minor degree to a product with the mobility of proapo AI (Fig. 4B, lane 1). It is mostly retained within the COS cells (Fig. 4A and B, lane 1). Immunoprecipitation competition with mature human apo AI carried out for control of the translation products Fig. 4B and C, lanes 2, 4 and 6 clearly indicate the dilution of the synthesized apo AI proteins by added apo AI antigen.

The 243 amino-acid residue long mature polypeptide is encoded in four exons as a prepropolypeptide. Among the serum apolipoproteins only apo AI and apo AII are synthesized as preproforms. Unlike apo AII, which looses the signal and pro sequence intracellularly, apo AI is secreted as proapo AI and proces-

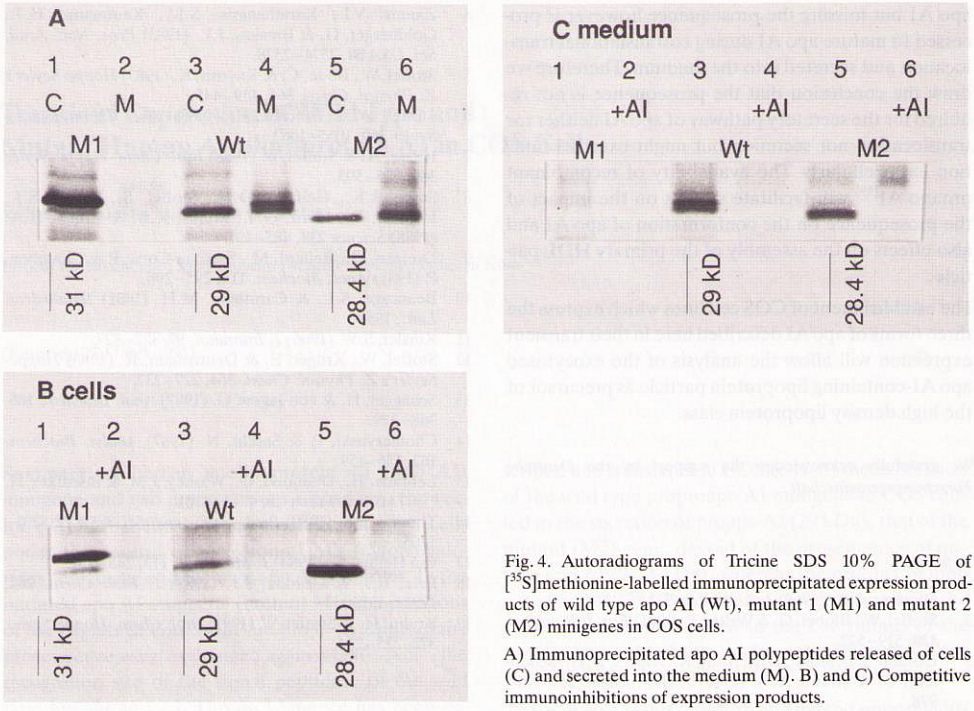


Fig. 4. Autoradiograms of Tricine SDS 10% PAGE of [^{35}S]methionine-labelled immunoprecipitated expression products of wild type apo AI (Wt), mutant 1 (M1) and mutant 2 (M2) minigenes in COS cells.

A) Immunoprecipitated apo AI polypeptides released of cells (C) and secreted into the medium (M). B) and C) Competitive immunoinhibitions of expression products.

sed further in the serum. The C-terminus of the prosequence of apo AI is an unusual Gln^{-2}-Gln^{-1} dipeptide whereas most other prosequences end on two basic amino-acid residues. The C-terminal recognition sequence of preproapo AI for the signal follows the general rules of signal sequences mostly ending on small side-chain residues[17].

In previous in vitro transcription-translation studies with the procaryotic pSP6 and pDS5 expression systems the correct transcription and translation and processing to proapo AI in the presence of dog pancreatic endoplasmic reticulum membranes[2-4,6] has been demonstrated.

We mutagenized the cDNA by exchanging the two C-terminal amino acids of the pre- and prosequence thereby extending the signal sequence by six residues. As a consequence the signal peptidase becomes unable to recognize the specific sequence. Another mutant apo AI construct had the presequence linked directly to the mature sequence, devoid of the prosequence. The preapo AI was accurately processed and translocated into the microsomal vesicles as mature apo AI. Similar results were reported by Folz and Gordon[18].

The question whether the prosequence has any targeting function for cellular export could only be answered by in vivo expression experiments. COS cells used for the experiments reported here do not synthesize apo AI. These COS cells were transfected with the eucaryotic expression vector pUHD10-1 harbouring the above-mentioned three human apo AI genes, the wild type and the mutants M1 and M2. The strong CMV enhancer of these minigenes induced an efficient synthesis and secretion of the respective apo AI polypeptides.

The results of the expression of apo AI and mutant forms in transfected COS cells in tissue culture experiments described here fully support the in vitro transcription-translation experiments described before[6]. The wild type apo AI minigene is correctly transcribed, translated and the primary translation product preproapo AI processed and secreted as proapo AI. The mutant M1 minigene, however, is transcribed and translated to a missense preproapo AI which can no longer be translocated and processed or secreted by the ER system of COS cells.

The apo AI minigene of mutant M2 with the presequence directedly fused to the mature sequence of

apo AI but missing the prosequence however is processed to mature apo AI during cotranslational translocation and secreted into the medium. Therefore we draw the conclusion that the prosequence is not required for the secretory pathway of apo AI neither for translocation nor secretion but might exert its function extracellularly. The availability of recombinant proapo AI[19] will facilitate studies on the impact of the prosequence on the conformation of apo AI and also effects on the assembly of the primary HDL particle.

The establishment of COS cell lines which express the three forms of apo AI described here in their transient expression will allow the analysis of the exocytosed apo AI-containing lipoprotein particle as precursor of the high density lipoprotein class.

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