Deletion of the propeptide of apolipoprotein A-I impairs exit of nascent apolipoprotein A-I from the endoplasmic reticulum

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Human apolipoprotein (apo) A-I is secreted as a proprotein of 249 amino acids and is processed extracellularly to the mature form (243 amino acids) by removal of a six-residue propeptide segment. We have examined the role of the apoA-I propeptide in intracellular transport and secretion using transfected baby hamster kidney cells that secreted either proapoA-I (from the wild-type cDNA, A-Iwt) or mature-form apoA-I (from A-IΔpro, a cDNA in which the propeptide sequence was deleted). Deletion of the propeptide from the apoA-I sequence did not affect the rate of apoA-I synthesis, nor did it affect the fidelity of proteolytic removal of the propeptide. However, the propeptide deletion caused mature-form apoA-I to accumulate within the cells as determined by pulse-chase experiments; the intracellular retention times for the mature-form apoA-I in which the pro-

peptide was prematurely removed was three times longer than that of proapoA-I ($t_{1/2} > 3$ h compared with ~ 50 min). There was no detectable degradation of either form of newly synthesized apoA-I. Immunofluorescence microscopy revealed that, whereas the proapoA-I was located predominantly in the Golgi apparatus, large quantities of the mature-form apoA-I were detected in the endoplasmic reticulum and very little was in the Golgi apparatus of A-IΔpro-transfected cells. These findings suggest that the propeptide sequence may be involved in the intracellular transport of apoA-I from the endoplasmic reticulum to the Golgi apparatus. We propose that the function of the propeptide sequence is to facilitate efficient transport of apoA-I through the secretory pathway.

INTRODUCTION

Human apolipoprotein (apo) A-I, a single-chain polypeptide of 243 amino acids, is the major protein component of plasma high-density lipoproteins (HDL). Aside from its well-documented function as a cofactor for the intravascular esterification of cholesterol by lecithin-cholesterol acyltransferase (reviewed in [1]), apoA-I may also mediate the interaction of HDL with plasma membranes, thereby facilitating the translocation of intracellular cholesterol to the plasma membrane for desorption on to lipoproteins [2]. Accumulating evidence indicates that the circulating level of HDL correlates inversely with the risk for developing atherosclerosis in humans [3,4] and in transgenic animals [5]. Thus, an understanding of factors that influence apoA-I production is important for developing strategies to reduce the incidence or progression of atherosclerosis.

ApoA-I is synthesized in the liver and the intestine as a preprotein of 267 amino acids [6]. PreproapoA-I is processed to the mature form by two successive proteolytic events. First, the 18-amino acid signal sequence (i.e. propeptide) is hydrolysed as the nascent apoA-I chain translocates across the membrane of the endoplasmic reticulum (ER) [7,8]. Subsequently, proapoA-I, which contains a six-residue (RHFWQQ) propeptide segment, is processed to the mature form by an endoproteolytic event in the plasma or lymph [9].

Processing of human proapoA-I to the mature form is unique in two aspects. First, unlike many proproteins in which hydrolysis of the propeptide occurs at the peptide bond C-terminal to a positively charged amino acid residue [10,11], removal of the human apoA-I propeptide sequence involves the hydrolysis of

the peptide bond following two glutamine residues [12]. Secondly, the timing of the proteolysis of the apoA-I propeptide is also unusual. Whereas many propeptides are hydrolysed before secretion, hepatocytes and enterocytes secrete apoA-I with its propeptide intact [6]. ProapoA-I is also the secretory form in several mammalian cell lines transfected with the apoA-I cDNA [13–16]. Nonetheless, the majority of proapoA-I is processed to mature-form apoA-I by an extracellular apoA-I-specific propeptidase [9], as less than 5% of the apoA-I in human plasma is proapoA-I [17].

Studies of the propeptides of various secretory proteins have suggested that propeptide sequences may be important for intracellular processing of nascent proteins [18]. In some short polypeptide hormones, propeptides provide sufficient chain length to target the hormone to the ER membrane [19]. In vitamin K-dependent coagulation proteins, the propeptide sequences are involved in post-translational modification, i.e. γ -carboxylation [20]. The propeptide of bovine pancreatic trypsin inhibitor has been shown to accelerate the rate with which the appropriate conformation is achieved [21]. Thus propeptide segments are often critical components leading to the formation of a fully functional protein. However, a specific function for the propeptide of apoA-I has not yet been identified. As the apoA-I propeptide sequence is removed outside of the cell, we hypothesized that this sequence might be involved in the intracellular transport and secretion of apoA-I.

In the present work, we investigated the potential role of the propeptide in the intracellular transport and secretion of human apoA-I by deleting the propeptide sequence via genetic engineering. This alteration, which resulted in the generation

Abbreviations used: apo, apolipoprotein; HDL, high-density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; BHK, baby hamster kidney; ER, endoplasmic reticulum.

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of intracellular mature-form apoA-I, markedly reduced the efficiency of apoA-I secretion from transfected eukaryotic cells. The premature removal of the propeptide caused mature-form apoA-I to accumulate within the ER even though hydrolysis of the signal peptide was not affected. Our data suggest that the apoA-I propeptide facilitates transport of apoA-I out of the ER.

EXPERIMENTAL

Materials

Enzymes and reagents for DNA cloning and sequencing were obtained from Bethesda Research Laboratories, Pharmacia-LKB Biotechnology, Boehringer-Mannheim Corp. or United States Biochemicals. Site-specific mutagenesis was performed using Mutagene (Bio-Rad Laboratories). Oligonucleotide primers for mutagenesis and DNA sequencing were synthesized as described previously [22] and were purified by reversed-phase chromatography (Sep-Pak C18; Waters Associates). Reagents and supplies for cell culture were from Gibco-BRL. Methotrexate was purchased from Cyanamid Canada Inc. [^{35}S]Methionine (Tran ^{35}S -label; 100 $\mu\text{Ci}/\text{mmol}$) was obtained from ICN-Flow Laboratories. Agarose-immobilized Protein G (Gammabind G) was obtained from Genex Corp. (Gaithersburg, MD, U.S.A.). Horseradish peroxidase-labelled anti-(sheep IgG) and fluorescein isothiocyanate-labelled anti-(mouse IgG) were from Sigma. Texas Red-conjugated anti-(rabbit IgG) was purchased from Calbiochem. The plasmid pBL14AI containing the apoA-I cDNA [23] was a gift from B. Levy-Wilson (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, CA, U.S.A.). The expression vector pNUT [24,25] and baby hamster kidney (BHK) cells were provided by R. MacGillivray (University of British Columbia). Polyclonal antibodies against human apoA-I were purchased from Boehringer-Mannheim Corp. Monoclonal antibody 6B8 to human apoA-I was a gift from R. Milne and Y. Marcel (University of Ottawa Heart Institute, Ottawa, Ont., Canada). Rabbit antibody to bovine liver protein disulphide isomerase was a gift from M. Michalak (University of Alberta). Rabbit anti- α -mannosidase-II serum was generously provided by M. Farquhar (University of California, San Diego, CA, U.S.A.).

Mutagenesis and construction of apoA-I expression plasmids

The apoA-I propeptide deletion mutant was prepared by site-specific mutagenesis essentially as described by Kunkel et al. [26]. Briefly, the apoA-I cDNA, excised from the pBL14AI plasmid [23] by digestion with *EcoRI*, was ligated to *EcoRI*-digested M13mp18. Uracil-containing single-stranded template DNA for oligonucleotide-primed mutagenesis was generated in bacterial strain CJ236 (Mutagene). The mutagenic oligonucleotide primer (5'-ACGGGGAGCCAGGCTGATGAACCCCCCAG-3') was annealed with the DNA template and then extended with T4 DNA polymerase. Plaques were screened with ^{32}P -labelled mutagenic oligonucleotide, and mutant clones were confirmed by sequencing [27]. The mutated (A-I Δ pro) and wild-type (A-Iwt) cDNA fragments were recovered by *EcoRI* digestion, end-filled with the Klenow fragment and separately ligated to *SmaI*-digested pNUT between sequences of the mouse metallothionein promoter and the termination and polyadenylation signals of the human growth hormone gene. The correct orientation of the apoA-I cDNA insertion was established by restriction mapping. The pNUT vector contains the mutant dihydrofolate reductase gene which permits immediate selection of stable transformants by their survival in high concentrations of methotrexate [24].

In vitro translation of human apoA-I

ApoA-I mRNA transcripts were synthesized using SP6 RNA polymerase [28] from a pSPT plasmid (Pharmacia-LKB) which contained the apoA-Iwt cDNA. *In vitro* translation of the apoA-I was performed in rabbit reticulocyte lysates (Promega Biotech) containing [^{35}S]methionine with or without dog pancreatic microsomal membranes. Product apoA-I was isolated by immunoabsorption as described below.

Generation of transfected BHK cell lines expressing human apoA-I

BHK cells (in 100-mm-diam. dishes) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum in 5% CO_2 atmosphere. Stable cell lines that expressed human apoA-I were generated by co-precipitation of plasmid DNA (10 μg) with calcium phosphate [29] and selection with 500 μM methotrexate. Cell lines that secreted apoA-I were identified by immunoblot analysis of the culture medium (see below). All studies of the processing and secretion of apoA-I by transfected cells were performed after growth in the absence of methotrexate.

N-terminal amino acid sequence analysis of secreted apoA-I

Transfected BHK cells (70% confluence) were cultured in serum-free DMEM for 24 h. The conditioned medium was collected and supplemented with 100 units/ml aprotinin, 0.1 mM leupeptin and 1 mM phenylmethanesulphonyl fluoride. ApoA-I in the medium was concentrated by ultracentrifugation [104000 g (40000 rev./min); 24 h], at $d = 1.21$ g/ml [30] in the presence of egg yolk phosphatidylcholine/cholesterol vesicles (4:1 molar ratio, 250 μg of phospholipid/100 ml of medium) prepared by ethanol injection [31]. ApoA-I was recovered from the $d < 1.21$ g/ml fraction, resolved by preparative SDS/PAGE and transferred to Immobilon-P membranes (Millipore). The N-terminal amino acids were identified by automated sequential Edman degradation [32,33] using purified human plasma apoA-I [34] as reference material.

Metabolic studies of apoA-I

BHK cells in 60-mm-diam. Primaria dishes were methionine-depleted in serum-free methionine-free DMEM and labelled in the same medium containing [^{35}S]methionine (80 μCi per dish). After labelling for 0–30 min, the monolayer was recovered by cell lysis as described [35]. ^{35}S -labelled apoA-I was immuno-precipitated, and the radioactivity associated with apoA-I was quantified (see below). Total cellular proteins were precipitated on glass-fibre supports (Millipore GF/C) with 10% trichloroacetic acid, and radioactivity on the filter was determined by liquid-scintillation spectrometry. In pulse-chase experiments, cells were pulse-labelled as described above for 10 min, and chase incubations were performed in DMEM containing 10% (v/v) fetal bovine serum. At the indicated time, the medium and cell lysate samples were recovered as described [35].

The ^{35}S -labelled apoA-I in the cells and medium was purified by immunoabsorption with sheep anti-(human apoA-I) antibody and Protein G-agarose according to the manufacturer's instructions. After overnight incubation at 4 $^{\circ}\text{C}$, the immunocomplex was washed and then released from agarose beads into sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 10 mM dithiothreitol and 40% glycerol) at 90 $^{\circ}\text{C}$ for 10 min. The immunopurified samples were separated on 12% (w/v) polyacrylamide gels containing 0.1% SDS [36]. After electrophoresis the gels were stained, equilibrated with Amplify (Amersham), dried and

visualized by fluorography. Gel slices containing the apoA-I proteins were dissolved in 30% H_2O_2 at 70 °C and the apoA-I radioactivity was quantified by liquid-scintillation spectrometry in Hionic-fluor (Canberra-Packard).

Two-dimensional gel-electrophoretic analysis

Immunoprecipitated apoA-I samples were mixed with plasma apoA-I (as a marker for the mature protein) before electrophoresis. Two-dimensional separations were achieved as described [37] using pH 4–6 Ampholytes (Pharmacia-LKB) in the first dimension and SDS/12% polyacrylamide in the second.

Immunoblot analysis of recombinant apoA-I

Medium or cellular protein samples were resolved by electrophoresis in 12% polyacrylamide gels and transferred to nitrocellulose filters. The filters were blocked and incubated first with a sheep anti-(human apoA-I) antibody, and then with horseradish peroxidase-conjugated anti-(sheep IgG). Immunocomplexes on the nitrocellulose filters were visualized by chemiluminescence (ECL; Amersham).

Immunofluorescence microscopy

Transfected BHK cells were incubated overnight on poly-L-lysine-coated coverslips in DMEM containing 10% (v/v) fetal bovine serum. After washes with PBS, cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 [38]. Cellular distribution of apoA-I was examined using monoclonal anti-(human apoA-I) antibody 6B8 followed by fluorescein-labelled goat anti-(mouse IgG). Rabbit antibodies to protein disulphide isomerase and α -mannosidase-II were used to localize the ER- and Golgi-resident proteins respectively. Texas Red-coupled antibodies to rabbit IgG were used to visualize these marker proteins. Images were obtained with a Zeiss Axiovert fluorescence microscope equipped with filters for differential visualization of fluorescein and Texas Red.

Analytical techniques

Protein was quantified as described [39] using BSA as a standard.

RESULTS

Generation of stably transfected BHK cells expressing human apoA-I

Stable BHK cell lines were generated by transfection with the wild-type apoA-I cDNA construct (A-Iwt) or a construct (A-I Δ pro) in which the sequence encoding the propeptide segment (RHFWQQ) was deleted (Figure 1a). The mutant construct retained the apoA-I signal peptide (i.e. prepeptide) in-frame with respect to the mature apoA-I coding sequence. Therefore primary translation products from both apoA-I constructs (Figure 1b) would be targeted to the secretory pathway. BHK cells were chosen not only because this cell does not express endogenous apoA-I but also because the propeptide of human apoA-I was not hydrolysed efficiently within these cells. Thus this cell line allowed us to test directly whether or not the propeptide is required for accurate and efficient cellular transport and secretion of apoA-I.

Human apoA-I is synthesized and secreted from transfected BHK cells

Of the multiple methotrexate-resistant colonies that were transfected with either the A-Iwt or A-I Δ pro construct, one cell line from each transfection was selected for detailed analysis. Each cell line secreted a similar amount of apoA-I protein as determined by immunoblot analysis. The cells transfected with A-Iwt or A-I Δ pro cDNA did not differ in growth rate or morphology.

The intracellular apoA-I produced by each transfected cell line was analysed to determine the extent of N-terminal proteolytic processing. A single species of approx. 29 kDa was detected in the cell lysate of each transfected cell line (Figure 2a, lanes 2 and 3). The apparent molecular mass of the apoA-I produced by the BHK cell (a, b in Figure 2a) was identical with proapoA-I produced by *in vitro* translation of the apoA-Iwt mRNA in the presence of dog pancreatic microsomal membranes (a in Figure 2b, lane 3). ApoA-I in the BHK cells was smaller than the 31 kDa *in vitro* translation product in the absence of microsomal membranes (preproapoA-I, x in Figure 2b, lane 2). These data provide evidence that the signal sequence (i.e. prepeptide) is proteolytically removed in both transfected cell lines. As removal of the signal sequence occurs on the luminal side of the ER [40], these results suggest that deletion of propeptide did not impair translocation of nascent apoA-I across the ER membrane. ProapoA-I and mature-form apoA-I could not be resolved by PAGE (a and b in Figure 2a).

The apoA-I proteins synthesized by the two cell lines were further characterized by two-dimensional gel-electrophoretic analysis. Again, a single apoA-I species was identified in each cell line (Figure 2c). The apoA-I species from cells transfected with A-Iwt cDNA exhibited a more basic isoelectric point (a in Figure 2c) than mature-form apoA-I from plasma (dotted circle in Figure 2c). This observation is consistent with the charge difference arising from basic amino acid residues in the propeptide (RHFWQQ). The apoA-I produced by cells transfected with A-I Δ pro (b in Figure 2c) was identical with the mature-form apoA-I (dotted circle in Figure 2c) in both charge and size. Compared with the two-dimensional gel-electrophoretic pattern of the *in vitro* translocation products from the wild-type apoA-I mRNA, where both preproapoA-I (x in Figure 2d) and proapoA-I (a in Figure 2d) were shown, it was clear that BHK cells (Figure 2c) did not contain preproapoA-I (x in Figure 2d). Thus these data indicate that cells transfected with the wild-type cDNA produce predominantly proapoA-I, and cells transfected with the mutant cDNA produce mainly mature-form apoA-I.

The secreted forms of apoA-I were also identified. Immunoblot analysis of the medium from cells transfected with A-Iwt or A-I Δ pro (Figure 3) showed that the apparent molecular mass of the cellular and secreted forms of apoA-I did not differ. N-terminal amino acid sequence analysis of the secreted forms of apoA-I revealed that cells transfected with A-Iwt secreted predominantly proapoA-I. Approx. 80% of the secreted apoA-I was proapoA-I and a minor fraction (20%) of the N-terminal sequence was the mature form. Sequence analysis demonstrated that cells transfected with A-I Δ pro secreted exclusively the mature-form apoA-I.

Rate of synthesis and secretion of human apoA-I

Figure 3 shows that the level of apoA-I accumulated in the medium of A-I Δ pro-transfected cells after 24 h incubation was similar to that of A-Iwt-transfected cells, and, conversely, the steady-state level of mature form apoA-I in the A-I Δ pro-transfected cells was much higher than that of proapoA-I in A-

(a) Deletion mutagenesis

(b) Translation products

Figure 1 Human apoA-I expression plasmids

(a) The deletion mutant (A-Δpro) and wild-type (A-lwt) cDNA sequences are shown. The propeptide-coding sequence (indicated by the boxed nucleotides in A-lwt) was deleted using the 30-mer oligonucleotide indicated by the double line below A-Δpro. Dashes indicate continuous regions of the cDNA sequence for which nucleotides are not shown. Single-letter amino acid designation for each codon is shown below the nucleotide sequence. (b) Schematic representation of the predicted primary translation products of the A-lwt and A-Δpro cDNAs. Hatched and solid bars represent the signal peptide (pre) and propeptide (pro) respectively. Open bar designates the mature apoA-I polypeptide. The propeptide amino acid sequence is indicated by the single-amino acid designation above the linear schematic.

(a) BHK cells

(b) In vitro translation

(c) BHK cells

(d) In vitro translation

Figure 2 Expression of human apoA-I in stably transfected BHK cells

(a) Cells transfected with A-lwt or A-Δpro were labelled with [³⁵S]methionine, and newly synthesized apoA-I was isolated from cell lysates by immunoprecipitation. The immunocomplex was separated by SDS/PAGE and visualized by fluorography. Control, non-transfected BHK cells. (b) Fluorogram of apoA-I translated *in vitro*. Transcription and translation of human apoA-I was performed as described in the Experimental section. [³⁵S]-labelled apoA-I was immunoprecipitated, resolved by SDS/PAGE and visualized by fluorography. (c) Two-dimensional electrophoretic analysis of [³⁵S]-labelled apoA-I isolated from transfected BHK cells as described in (a). (d) Two-dimensional gel-electrophoretic analysis of *in vitro* translated apoA-I isolated as described in (b). The dotted circle indicates the position of the mature form of apoA-I purified from plasma; x, preproapoA-I; a, proapoA-I; b, mature-form apoA-I.

Iwt-transfected cells. The elevated intracellular mass of mature-form apoA-I was not attributable to increased synthetic rate, because measurement of [³⁵S]methionine incorporation into

apoA-I (proapoA-I or mature-form apoA-I) (Figure 4a) and total cellular protein (Figure 4b) revealed no differences between the two cell lines.

Figure 3 Identification of secreted apoA-I in transfected BHK cell cultures

Cells were maintained in serum-free medium for 24 h, and medium and cell samples were recovered for analysis. Cell and medium samples were fractionated by SDS/PAGE, transferred to nitrocellulose, and apoA-I was displayed by immunoblot analysis. A-lwt, cells transfected with wild-type cDNA; A-lΔpro, cells transfected with A-lΔpro cDNA. a and b indicate the mobility of proapoA-I and mature-form apoA-I respectively.

Figure 4 Synthesis of apoA-I and total protein in transfected BHK cells

BHK cell monolayers were grown overnight in 60-mm-diam. dishes in DMEM containing 10% (v/v) fetal bovine serum. The medium was removed and replaced with serum-free methionine-free DMEM containing 80 μ Ci of [35 S]methionine. After the indicated incubation period, the cells were recovered by lysis and apoA-I was isolated by immunoprecipitation and PAGE as described in the Experimental section. (a) Radioactivity in the apoA-I band was quantified by liquid-scintillation counting; (b) radioactivity in total protein was determined after precipitation with 10% trichloroacetic acid. Data points are means $\pm$ S.D. of triplicate dishes for a representative experiment. ●, Cells transfected with A-lwt cDNA; ○, cells transfected with A-lΔpro cDNA.

We performed pulse-chase experiments to examine (i) the efficiency of apoA-I secretion, (ii) the retention half-time ($t_{1/2}$) of apoA-I and (iii) intracellular degradation of newly synthesized apoA-I proteins. Figure 5 shows that secretion of proapoA-I (A-lwt) was approx. 2.7-fold higher than secretion of mature-form apoA-I (A-lΔpro). At the end of a 2 h chase, more than 80% of the newly synthesized proapoA-I was secreted, whereas only 30% of the mature-form apoA-I was recovered in the medium. Conversely, the intracellular $t_{1/2}$ of proapoA-I was approx. 2.7-fold shorter than that of mature-form apoA-I (Figure 5): 53 min for proapoA-I and 180 min (assuming linear secretion) for mature-form apoA-I. The $t_{1/2}$ for proapoA-I in BHK cells is comparable with the value of approx. 1 h observed in rat [35] and human [41] hepatoma cell lines. Recovery of the radiolabelled

Figure 5 Pulse-chase analysis of recombinant apoA-I in transfected BHK cells

Cells (in 60-mm-diam. dishes) were pulse-labelled for 10 min with [35 S]methionine (300 μ Ci per dish) in serum-free methionine-free DMEM. The labelling medium was then replaced with DMEM containing 10% (v/v) fetal bovine serum for the chase incubation. After the indicated time, the medium and cells were recovered and apoA-I was isolated by immunoprecipitation and SDS/PAGE with fluorography (c and d). Radioactivity associated with apoA-I was quantified (a and b) by liquid-scintillation counting. A-lwt, cells transfected with A-lwt cDNA; A-lΔpro, cells transfected with A-lΔpro cDNA. A representative experiment which was repeated on three occasions is shown.

Figure 6 Secretion of endogenous proteins by transfected BHK cells

Cells (in 60-mm-diam. culture dishes) were incubated with DMEM containing 10% (v/v) fetal bovine serum and 250 μ Ci of [35 S]methionine (50 μ M). At the indicated time, medium samples were fractionated by electrophoresis on a 3–15% polyacrylamide gradient gel containing 0.1% SDS and the 35 S-labelled proteins were visualized by fluorography. Migration of molecular-mass markers (kDa) is indicated on the right. Proteins which were secreted in a time-dependent manner are indicated by the closed arrowheads. The apoA-I band is indicated by the arrow. Open arrowheads indicate unidentified 35 S-labelled species present in the medium with similar intensity throughout the labelling period.*

apoA-I from either cell line was complete (97–109%) during the entire 2 h chase, indicating that no intracellular degradation of newly synthesized apoA-I proteins had occurred. These results

Figure 7 Immunofluorescence microscopy of transfected BHK cells

Transfected BHK cells were grown overnight on poly-L-lysine-coated coverslips in DMEM containing 10% (v/v) fetal bovine serum. The cells were washed twice with PBS, fixed with paraformaldehyde and permeabilized with Triton X-100. ApoA-I was localized by sequential incubations with mouse anti-(human apoA-I) (6B8) and fluorescein-conjugated goat antibody to mouse IgG (**a**, **c**, **e** and **g**). The same cell preparations were then incubated with rabbit antibodies to either protein disulphide isomerase (**b** and **f**) or α -mannosidase-II (**d** and **h**). The latter markers were visualized with Texas Red-conjugated goat antibodies to rabbit IgG.

suggested that the elevated intracellular mass of mature-form apoA-I was attributable to its markedly reduced secretion efficiency.

We then examined whether or not the reduced rate of mature-form apoA-I secretion was caused by a general secretion defect in the transfected cell line. Secretion of recombinant apoA-I was compared with the bulk secretion of endogenous BHK proteins. As shown in Figure 6, apoA-I (solid arrow) was the most prominent protein secreted by both cell lines. Several BHK proteins (indicated by closed arrowheads) were secreted into the medium in a time-dependent manner; neither the pattern of these proteins nor their rates of secretion appeared to be different between the two cell lines. This result suggests that the reduced rate of mature-form apoA-I secretion is specific for apoA-I. Additional species of 15 and 40 kDa (open arrowheads) were observed in the medium and showed no change in labelling as a function of time; they appear to bind to and retain the ^{35}S -containing compounds in the labelling medium.

Intracellular localization of apoA-I in transfected BHK cells

We employed an immunohistochemical approach to determine the intracellular distribution of the recombinant apoA-I proteins. Figure 7 shows that cells transfected with A-Iwt (Figures 7a and 7c) had a lower steady-state level of apoA-I than did cells

transfected with A- Δ pro (Figures 7e and 7g). More importantly, propeptide deletion had a profound effect on the intracellular distribution of apoA-I protein. Whereas proapoA-I was found mainly in the juxtannuclear region of the cell (Figures 7a and 7c) and co-localized with the *cis*-Golgi apparatus marker [42] α -mannosidase-II (Figures 7c and 7d), mature-form apoA-I was found throughout the cells extending from the nucleus to the cell margins (Figures 7e and 7g) and co-localized with the ER marker protein disulphide isomerase (Figures 7e and 7f). Control studies showed that proapoA-I was not concentrated in the ER (Figures 7a and 7b), and mature-form apoA-I was not evident within Golgi structures containing α -mannosidase-II (Figures 7g and 7h). Subcellular-fractionation analysis of the transfected cells confirmed that both apoA-I proteins were concentrated in the microsomal fraction (not shown). However, topology of apoA-I associated with the microsomes has not been determined.

DISCUSSION

The present studies have provided evidence suggesting a role for the propeptide in the transport and secretion of human apoA-I. By comparing the rate of proapoA-I secretion (from cells transfected with the wild-type cDNA) with mature-form apoA-I secretion (from cells transfected with a modified cDNA lacking the propeptide coding region) we found that deletion of the

propeptide impairs the efficiency of apoA-I secretion. Premature removal of the propeptide appears to affect the intracellular transport of nascent apoA-I specifically, as neither the rate of apoA-I synthesis nor the fidelity of signal peptide proteolysis was affected. Immunofluorescence studies indicate that the impairment may occur at an early step in the secretory pathway by slowing the exit of nascent apoA-I from the ER. Our data strongly suggest that the apoA-I propeptide segment may play an important role in regulating the rate of transport of apoA-I between the ER and the Golgi apparatus. Impaired secretion or prolonged retention of proteins by propeptide deletion has been observed for albumin [43] and type-I collagen [44]. The current work provides additional evidence that propeptide sequences are involved in intracellular protein transport.

The mature-form apoA-I derived from A-IΔpro cDNA is not a mutant protein *per se* as it is produced *in vivo*. Why would removal of apoA-I propeptide affect its exit from the ER? One possible explanation is that the propeptide is required for the nascent chain to achieve an appropriate conformation; correct folding is essential for exit from the ER [45]. Propeptides of 100–300 amino acid length have been shown to act as templates on which the folding of the mature protein can occur [46,47]. Folz and Gordon [8] showed *in vitro* that, in the absence of the propeptide, apoA-I appeared to attain a correctly folded conformation more slowly. Although it is unlikely that the six-residue propeptide of apoA-I is a folding template, it may facilitate accurate folding by other mechanisms. The 13-residue propeptide of bovine pancreatic trypsin inhibitor accelerates the rate of correct folding by favouring correct disulphide bond formation [21]. The apoA-I propeptide may also mediate correct folding within the ER even though apoA-I contains no cysteine residues. Correct conformation could be achieved by interaction with accessory proteins or by post-translational modifications in the ER lumen. The apoA-I propeptide may provide structural information required for these processes to initiate or accelerate folding. Studies of naturally occurring mutants of apoA-I have indicated that substitution of a proline residue C-terminal to the propeptide-cleavage site affects the efficiency of extracellular processing to mature apoA-I but does not significantly affect apoA-I secretion [48]. Therefore it appears that the presence of propeptide segment on the nascent apoA-I, rather than its processing, determines secretion efficiency.

Intracellular transport of other apolipoproteins may also involve N-terminal regions, although this possibility has not yet been evaluated. Sequences that are identical to the cleavage site of the apoA-I propeptide (QQDE) occur near the N-terminus of other apolipoproteins, but not all of these pseudo-propeptide sequences are proteolytically removed. Human apoC-II contains the sequence QQDE at the N-terminus, but minimal amounts of processed apoC-II are found in human plasma [49]. The sequence QQDE is not completely conserved in the apoA-I sequence of non-human species [50,51]. In rabbit (QRDE) and chicken (QHDE), the glutamine residue at the –1 position (numbered from the mature N-terminus) is replaced by a positively charged residue. This difference may reflect a different proteolytic mechanism in the chicken, as it has been reported that the chicken apoA-I propeptide segment is hydrolysed intracellularly [51]. Conversely, proteolysis of the human and rabbit propeptides appears to occur by a similar mechanism, as rabbit plasma contains hydrolytic activity which can process both proproteins [52]. Thus, despite possible differences in the proteolytic mechanisms, propeptides of the other apolipoproteins may also regulate intracellular transport as described here for apoA-I.

Previous studies with stably transfected mammalian cells have suggested that the propeptide is not required for apoA-I secretion

from mammalian cells. In C127 cells [15] and AtT-20 cells [16] secretion of apoA-I was independent of the propeptide segment. We cannot, at present, conclude that cell line differences are responsible for these contrasting results. It has been shown, however, that the function of the collagen propeptide is cell-line-specific; its deletion markedly alters secretion efficiency in Chinese hamster lung cells but does not affect secretion in mouse fibroblast cells [44]. It is possible that the transport function of the apoA-I propeptide, like the collagen propeptide, is cell-specific. Thus the physiological role of the propeptide should be further defined by studies using hepatocyte or enterocyte model systems.

In conclusion, the present work provides the first evidence that the propeptide of human apoA-I may function to facilitate efficient secretion. Our results demonstrate that premature removal of the propeptide causes apoA-I to accumulate in the ER, suggesting that the propeptide is required for its exit from the ER. Further investigation will be required to establish the molecular basis of the functional role of apoA-I propeptide in the eukaryotic secretory pathway.

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