Intracellular Early and Late Modifications of Human Apolipoprotein A-II. Effect of Glutamine-+1 to Leucine Substitution†

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ABSTRACT: It has been shown previously that apoA-II undergoes several intracellular modifications in HepG2 cells (Hussain & Zannis, 1990). In the present study, we have generated permanent cell lines in mouse C127 cells which express the normal apoA-II gene and a mutated form in which Gln+1 was substituted with Leu (Leu+1). This modification was designed to prevent cyclization of the N-terminal glutamine of apoA-II and thus identify the isoproteins which are precursors and products of the N-terminal cyclization reaction. The C127-expression cells were also utilized to study the cellular compartments where the apoA-II modifications occur as well as the importance of the modifications for apoA-II trafficking and secretion. We have found that apoA-II (Gln+1) synthesized by C127 and HepG2 cells had similar isoproteins. In both cell types, unmodified pro-apoA-II, designated isoprotein 3, had a similar isoelectric point as the cell-free translation product of apoA-II mRNA, suggesting that isoprotein 3 results from cleavage of the signal peptide. Isoprotein 3 represents an unmodified apoA-II isoprotein and undergoes an early modification into a more acidic isoprotein 1, which differs from isoprotein 3 by two negative charges. Brefeldin A treatment of the cells did not prevent the formation of isoprotein 1, suggesting that this modification occurs in a pre-Golgi compartment. Neuraminidase treatment of secreted apoA-II isoproteins did not affect isoprotein 1, indicating that it is not sialylated isoprotein. Isoprotein 1 undergoes further modifications which are consistent with cleavage of the propeptide, N-terminal cyclization and sialylation most likely resulting from O-glycosylation. The Gln+1 to Leu substitution prevented N-terminal cyclization as evidenced by the lack of conversion of isoprotein 1a to 0 in the intracellular and secreted mutant (Leu+1) forms of apoA-II. Furthermore, the Gln+1 to Leu substitution also impaired cleavage of the propeptide. ApoA-II undergoes extensive intracellular degradation in C127 cells and to a lesser extent in HepG2 cells. The kinetics of depletion of intracellular apoA-II were similar in HepG2 cells as well as in C127 cells expressing the wild-type and the mutant apoA-II isoproteins.

Human apoA-II is the second major protein component of HDL and is synthesized by the liver and, to a much lesser extent, by the intestine (Schonfeld et al., 1982; Hussain & Zannis, 1990). The plasma form of human apoA-II contains 77 amino acids and exists as a dimer of 2 subunits linked by an intrasulfide bond at residue 6 (Brewer et al., 1972). Despite recent advances pertinent to apoA-II, its significance for the structure and function of HDL is not fully understood. ApoA-II is an important structural component of a subpopulation of HDL particles (De Coen et al., 1988; Brasseur et al., 1992). ApoA-II is capable of displacing apoA-I from the surface of HDL (Lagocki & Scanu, 1980) and competes for the binding of HDL to the HDL binding sites on cell surfaces (Fidge & Nestel, 1985). HDL consist of two major types of lipoprotein (LP) particles containing either only apoA-I (LpAI) or both apoA-I and apoA-II (LpAI:AII) (Cheung & Albers, 1984; Kilsdonk et al., 1990). Some studies indicated that LpAI particles favor the reverse transport of cholesterol from peripheral tissues to the liver (Schultz et al., 1992) whereas LpAI:AII particles bind less efficiently to cell receptors (Kilsdonk et al., 1990) and fail to promote cholesterol efflux from mouse adipose cells (Barbaras et al., 1986, 1987) and to stimulate LCAT activity (Vanloo et al., 1992). However, other studies did not observe major differences in the efflux of cholesterol from other cell types when either LpA-I or LpA-I:AII was utilized (Johnson et al., 1991; Oikawa et al., 1993). It has been reported that the increased apoA-II levels in certain inbred strains of mice are associated with increased HDL size, suggesting that the plasma concentration of apoA-II may affect HDL structure and function(s) (Lusis, 1988). Overexpression of human apoA-II in transgenic mice, in one study, affected the size and the distribution of HDL particles (Schultz et al., 1991) as well as the ratio of apoA-I/apoA-II. In a different study, overexpression of apoA-II predisposed the mice to atherosclerosis in response to atherogenic diets (Warden et al., 1993).

Both the protein (Brewer et al., 1972) and the DNA sequences (Sharpe et al., 1984; Tsao et al., 1985) showed that apoA-II contains an 18-residue-long signal peptide with 0 overall charge and a 5-residue-long prosegment (Ala-Leu-Val-Arg-Arg). It has been reported that the prosegment is cleaved intra- and extracellularly (Hussain et al., 1990; Gordon et al., 1983, 1984) by a thiol protease displaying a cathepsin B like activity (Gordon et al., 1985). It has been shown

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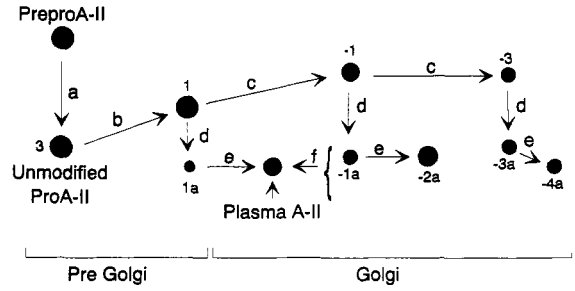

FIGURE 1: Schematic representation of the intra- and extracellular modifications of human apoA-II isoproteins. The numbers indicate the different apoA-II isoforms. a–e indicate the following: a, prepeptide cleavage; b, pre-Golgi modification of apoA-II; c, sialylation; d, cleavage of the prosegment; e, cyclization of the N-terminal Gln; f, desialylation.

previously that apoA-II synthesized by HepG2 cells undergoes a complex array of intra- and extracellular modifications (Hussain & Zannis, 1990; Remaley et al., 1993). In the present study, we have investigated further the nature of intracellular modifications and the kinetics of secretion of apoA-II in C127 cells that have been derived from a mouse mammary tumor which expresses the wild-type apoA-II as well as a mutant apoA-II isoprotein defective in the N-terminal cyclization step. Pulse and chase experiments were used with [^{35}S]methionine which labels all the isoproteins and [^{3}H]-arginine which labels only the isoproteins which contain the prosegment. The results obtained indicate that following translation apoA-II expressed in C127 cells undergoes a novel modification in the ER that generates isoprotein 1. This isoprotein is trapped in the ER in the presence of brefeldin A. Following translocation in the Golgi, apoA-II is further modified by N-terminal cyclization, propeptide cleavage, and sialylation (Figure 1). The substitution of Gln+1 with Leu+1 prevented N-terminal cyclization and inhibited propeptide cleavage, but did not affect the rate of depletion of intracellular apoA-II.

MATERIALS AND METHODS

Materials

Synthetic Oligonucleotides and DNA Fragments Generated by the Polymerase Chain Reaction (PCR). Oligonucleotides were synthesized by the solid-phase phosphite triester method using an automated oligonucleotide synthesizer (Applied Biosystems, Model 380-B), and were purified by electrophoresis on 20% polyacrylamide/7 M urea gels. DNA fragments were generated by DNA amplification using the polymerase chain reaction with the Perkin-Elmer automated thermocycler (Mullis & Faloona, 1987). The fragment generated and the primers used are shown in Table 1.

Methods

Plasmid Construction and Mutagenesis. The construction of the plasmids described below is shown schematically in Figure 2. A 3-kb *Hin*dIII/*Hin*d III fragment containing the entire apoA-II gene was obtained from a human genomic library and subcloned in plasmid pUC19 to generate pUC-AII. The pUC-AII plasmid was then digested with *Bgl*I, blunted with the Klenow fragment of DNA polymerase I, and digested with *Hin*dIII. The 1.9-kb apoA-II gene fragment was cloned into the *Sma*I and *Hin*dIII sites of Bluescript KS+/– derivative plasmid pB3. This plasmid was obtained by substitution of the original polylinker with a new polylinker sequence containing the restriction sites *Sst*II–*Xho*I–*Hin*dIII–*Sma*I–*Eco*RI–*Cla*I–*Sal*I–*Bam*HI–*Xho*I. The recombinant plasmid, designated pB3-AII, was propagated in the dam–*Escherichia coli* strain GM33 to prevent methylation of the *Bcl*I site. The pB3-AII plasmid was digested with *Xho*I, and the apoA-II gene fragment was cloned into the *Xho*I site of the BPV expression vector pBMT3X described previously (Krystal et al., 1986). This vector carries the entire human metallothionein 1A (hMT1A) as well as the mouse metallothionein 1 (mMT-1) gene with an engineered unique *Xho*I site positioned 70 bp downstream from the transcription initiation site of the mMT-1 gene. The recombinant plasmid, designated pBMT-AII, contains the promoterless human apoA-II gene fragment under the control of the mMT-1 gene promoter. The insertion and orientation of the apoA-II gene fragment in the pBMT-AII construct were determined by DNA sequencing (Sanger et al., 1977).

To mutagenize Gln+1 to Leu+1, the region between nucleotides +430 and +619 was amplified using primers 1 and 2 of Table 1. Primer 1 contains a *Bgl*II site at the 5′ end, and primer 2 contains a CAG to CTG substitution of the codon specifying Gln+1, and also contains a *Sal*II site at the 5′ end. The region between nucleotides +619 and +1146 was amplified using primers 3 and 4, which contain, respectively, an *Xho*I and a *Bcl*I site at their 5′ end. The two amplified fragments were digested with *Bgl*II and *Sal*I and with *Xho*I and *Bcl*I, respectively, and cloned into the *Bgl*II and *Bcl*I sites of the pB3-AII plasmid. This resulted in substitution of the normal for the mutant apoA-II sequences. As described above for the normal apoA-II gene fragment, the substituted apoA-II gene fragment was excised from the corresponding pB3-AII plasmid with *Xho*I and was cloned into the *Xho*I site of the pBMT3X vector, to generate the recombinant mutant pBMT-AII M (Gln+1→Leu). The sequence of the fragments and their orientation in the final constructs were determined by DNA sequencing (Sanger et al., 1977).

Cell Transfection and Selection of Stable Cell Lines. The C127 cells were maintained in log phase as a monolayer with Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) in 100-mm-diameter petri dishes. The day before the transfection, the C127 cells were split at 1×10^6 per 100-mm dish. The normal apoA-II and the mutant apoA-II (35 μg of plasmid DNA) were introduced into the mouse C127 cell line derived from a mammary tumor by phosphate–DNA coprecipitation as previously described (Lowy et al., 1978; Roghani & Zannis, 1988). After 48-h incubation at 37 °C with D-MEM containing 10% FBS, the cells were split 1:5 and plated in D-MEM supplemented with 10% FBS and 10 mM $CdCl_2$. The cells were refed every 3 days until discrete isolated colonies were generated, and the colonies were collected using cloning cylinders. Positive clones were selected by RNA analysis and apoA-II immunoprecipitation.

RNA Blotting Analysis. Approximately 80% confluent cultures of the C127 clones expressing apoA-II and HepG2 cells were scraped off flasks, and RNA was extracted using RNAzol B (Bioteck Lab). RNA was subjected to electrophoresis on 1% agarose–formaldehyde gels (Mangeney et al., 1989), transferred onto nitrocellulose filters, and subsequently hybridized with human apoA-II cDNA probe and subjected to autoradiography.

Analysis of Intracellular and Secreted ApoA-II Isoproteins. C127 cell clones expressing the normal and mutated apoA-II genes and HepG2 cells were grown to approximately 90% confluency in 25 cm^2 flasks in D-MEM containing 10% FBS, 10 μM $CdCl_2$, 1% (v/v) penicillin–streptomycin, and 2 mM

Table 1: Site-Directed Mutagenesis of the Human ApoA-II Gene by DNA Amplification Using the PCR

synthetic fragments	5′ amplification primer	3′ amplification primer
apoA-II (+430/+618) (A597→T, Gln+1→Leu)	apoA-II (+425 to +452) including a *Bgl*II site	apoA-II (+619 to +587) with A597 T substitution creating a *Sal*I site
apoA-II (+613/+1146)	apoA-II (+645 to +614) with a G616 C substitution creating an *Xho*I site	apoA-II (+1151 to +1129) including a *Bcl*I site

glutamine. The cell monolayer was rinsed twice with methionine-free D-MEM (GIBCO) supplemented with 10 μM $CdCl_2$ and 2 mM glutamine and preincubated in 2 mL of this medium for 2 h at 37 °C. The cells were then incubated in the same medium containing 0.25 mCi of [^{35}S]methionine for various times in the presence or absence of 10 μg/mL Brefeldin A. For arginine labeling, cells were preincubated in serum-free MEM supplemented with 2 mM glutamine for 2 h and in arginine-free MEM for 2 h and labeled in the same medium in the presence of 0.5 mCi of [^{14}C]arginine. The medium was collected, centrifuged to remove cell debris, and adjusted to a final concentration of 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, 1% Triton X-100, and 1% sodium dodecyl sulfate. The cell cultures were washed twice in PBS buffer and lysed in a solution containing 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, and 1% Triton X-100. The cell lysate was vortexed and centrifuged for 10 min, and the supernatant was diluted 1:1 in solution containing 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, and 1% sodium dodecyl sulfate. Protease inhibitors [benzamidine (2 mM), Pefabloc (2 mM), leupeptin (2 mM), and EDTA (5 mM)] were added to medium and cell supernatants. Immunoprecipitation was performed as described previously (Hussain & Zannis, 1990), using 20 μL of rabbit anti-human apoA-II antiserum that was prepared by conventional methods.

The immunoprecipitates obtained from the cell lysates and the culture media were analyzed by two-dimensional polyacrylamide gel electrophoresis and autoradiography, as described below.

Neuraminidase Treatment of ApoA-II Isoproteins. Medium from the C127 cells expressing the normal apoA-II gene that were labeled overnight with [^{35}S]methionine was adjusted to a concentration of 50 mM sodium acetate (pH 5.3) and was treated with a mixture containing 0.4 unit of neuraminidase obtained from *Clostridium perfringens* and 0.4 unit of neuraminidase obtained from *Vibrio cholera* at 37 °C for 2 h (Zannis & Breslow, 1981). The reaction mixture was dialyzed for 2 h against 85 mM NaCl/5 mM KCl and then overnight against 10 mM sodium phosphate, pH 7.2, was adjusted to a final concentration of 0.5% deoxycholate, 1% Triton X-100, and 1% sodium dodecyl sulfate, and was immunoprecipitated as described above.

Pulse–Chase Experiments: Kinetics of Intracellular Depletion of Normal and Mutant ApoA-II Isoproteins. Monolayers of C127 cell clones expressing the normal and mutant apoA-II gene grown in 25 cm^2 flasks were pulsed for the indicated time as described above, and then chased for various times with D-MEM containing 10 mM methionine and 10 μM $CdCl_2$. The cell lysate and the culture medium were immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. Autoradiograms from one-dimensional gel electrophoresis were subjected to quantitative scanning densitometry using an LKB laser densitometer. The depletion of the intracellular apoA-II was subjected to first-order kinetic analysis.

Cell-Free Translation of ApoA-II mRNA. Cell-free translation of RNA was performed with rabbit reticulocyte lysate in a total volume of 30 μL containing 5 mg of RNA and 0.05 mCi of [^{35}S]methionine for 1 h at 37 °C using a cell-free translation kit (BRL) following the reaction conditions recommended by the manufacturer. The reaction mixtures from two experiments were pooled, diluted to 1 mL, and adjusted to a final concentration of 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, 1% Triton X-100, and 1% sodium dodecyl sulfate. After immunoprecipitation, the cell-free translation products were analyzed by two-dimensional gel electrophoresis and autoradiography.

Two-Dimensional Isoelectric Focusing/Polyacrylamide Gel Electrophoresis. Equilibrium isoelectric focusing/polyacrylamide gel electrophoresis was performed according to O'Farrell (1975) with previously described modifications (Zannis et al., 1980, 1982, 1986a,b) using a minigel apparatus (Bio-Rad) under the following conditions: (A) 1500 V h with 0.75% (pH 5–8), 0.75% (pH 4–6), and 0.25% (pH 2.5–4) ampholines (LKB); (B) 400 V h with 0.62% (pH 4–6) and 1.25% (pH 3–5) Biolytes (Bio-Rad). The nonequilibrium pH gradient gels were electrophoresed in the first dimension for 1500 V h with 0.5% (pH 3.5–10), 0.25% (pH 5–8), 0.25% (pH 2.5–4), and 0.5% (pH 4–6) ampholines LKB as described by Hussain and Zannis (1990). Human apoA-I (5 μg) and apoA-II (5 μg) (Sigma) were utilized as internal standards. The isoelectric points of the apoA-I and apoA-II isoproteins have been determined previously, and thus the different isoproteins can serve as isoelectric point markers (Zannis et al., 1980; Hussain & Zannis, 1990). For the second dimension, the acrylamide strips containing the focused protein were placed on minislab gels containing 18% polyacrylamide with a 4% polyacrylamide stacking gel. Electrophoresis was performed at 30 mA for approximately 40 min, until the Bromophenol Blue dye reached the bottom of the gel. After electrophoresis, the gels were fixed in 5% (v/v) trichloroacetic acid and 10% (v/v) sulfosalicylic acid for 10 min, and stained in a solution of 0.25% (w/v) Coomassie brilliant blue, 50% (v/v) ethanol, and 10% (v/v) acetic acid for approximately 2 h. Destaining was accomplished in 50% (v/v) ethanol and 10% (v/v) acetic acid until proper background was achieved. The gels were treated with Amplify (Amersham), dried, and exposed to X-ray film.

RESULTS

Nomenclature of ApoA-II Isoproteins. To facilitate the presentation of the Results and the Discussion, we introduce the nomenclature of apoA-II isoproteins and indicate the cellular compartments in which these modifications occur. The pertinent data supporting our assignments are based on previous work (Hussain & Zannis, 1990) and the findings presented herein.

Generation of C127 Cell Lines Expressing the Normal and Mutant ApoA-II Genes. To study the physiological significance of the N-terminal cyclization of apoA-II, the CAG codon specifying Gln+1 was substituted with a CTG codon specifying Leu+1. The presence of the mutation in the recombinant expression vector was confirmed by DNA

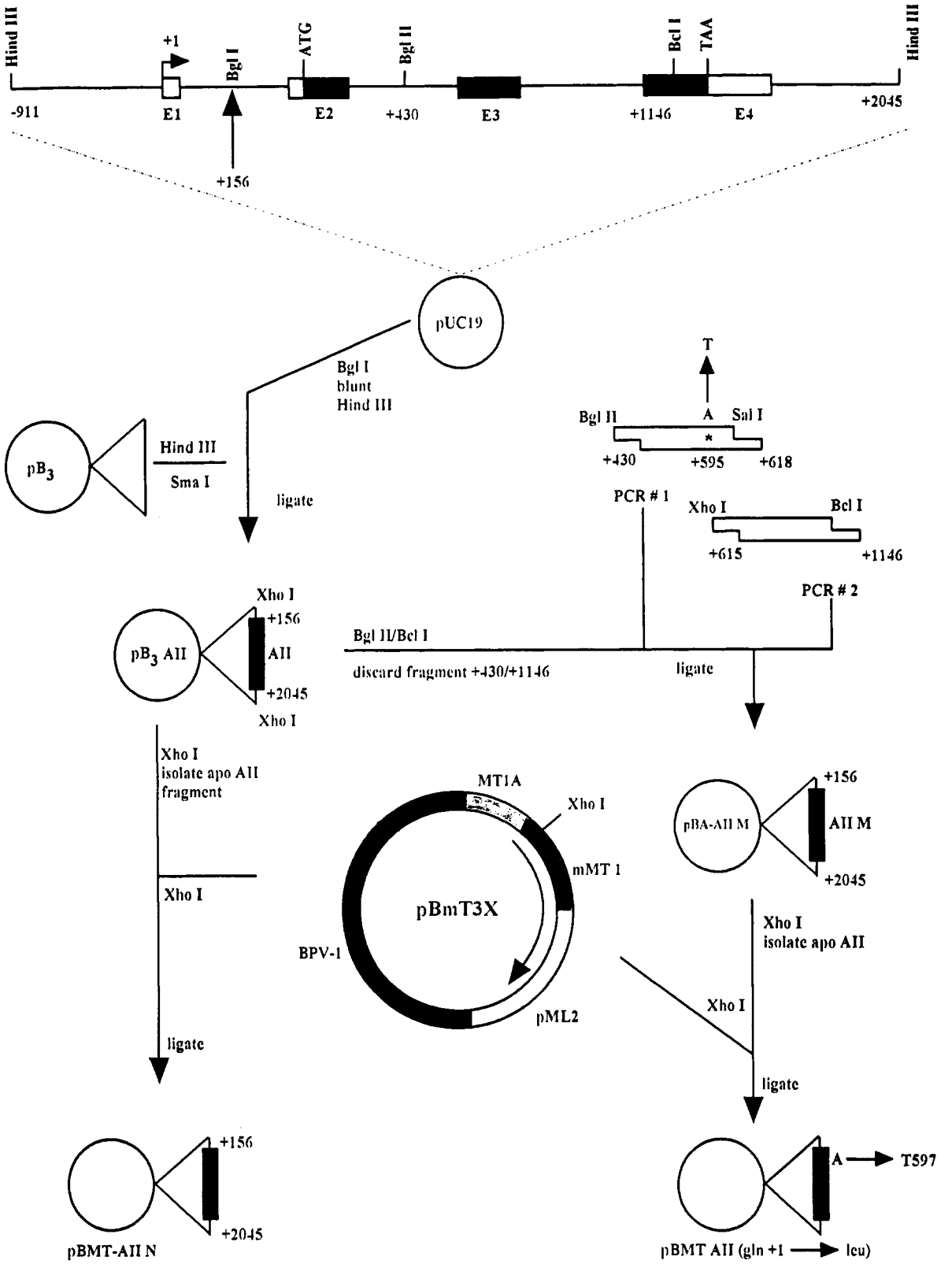

FIGURE 2: Schematic representation of the strategy used to generate expression constructs containing the normal and a mutated apoA-II gene carrying a Gln+1 to Leu+1 substitution. The open and filled rectangles represent nontranslated and translated mRNA sequences, respectively. The position of the transcription initiation site is indicated by +1 and corresponds to nucleotide 912 of the published genomic sequence (Tsao et al., 1985). The initiation and termination codons are also indicated.

sequencing (data not shown). To express the normal and mutated apoA-II genes, a 1.9-kb fragment containing the entire coding sequence of the human apoA-II gene was placed in the pBMT3X vector (Figure 2), under the control of the mMT-1 promoter, which is inducible by heavy metals, to generate the recombinant expression vectors pBMT-AII-N and pBMT-AII-M, respectively. Transfection of mouse C127 cells permitted the selection of several cell clones resistant to 10 μM $CdCl_2$. These cell clones were found to produce apoA-II mRNA and protein. A representative RNA blotting analysis is shown in Figure 3. The size of the major apoA-II mRNA produced by C127 clones is slightly larger than that of HepG2

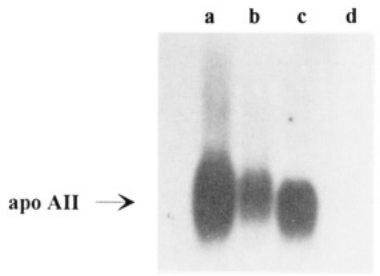

FIGURE 3: Blotting analysis of RNA obtained from cells expressing the apoA-II gene. Lanes a–d contain 20 μg of total RNA isolated from C127 cell clones expressing the normal apoA-II gene (a), as well as C127 cell clones expressing a mutated apoA-II gene carrying the Gln+1 to Leu substitution (b), HepG2 cells (c), and nontransfected C127 cells (d).

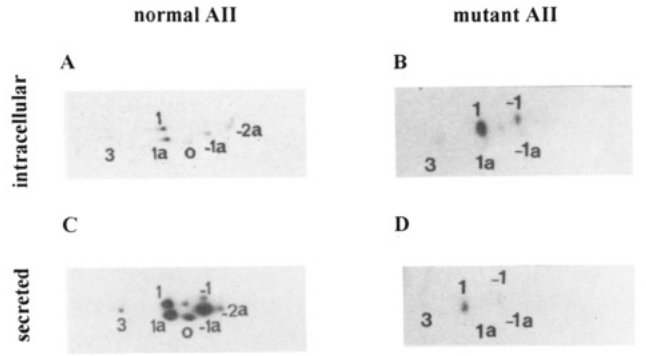

FIGURE 4: Identification of intracellular and secreted apoA-II forms produced by C127 cell clones expressing the normal and mutated apoA-II genes. Cells were labeled with [^{35}S]methionine for 4 h. ApoA-II was immunoprecipitated from the cell lysate and the culture medium with rabbit anti-human apoA-II antiserum and analyzed by two-dimensional polyacrylamide gel electrophoresis and autoradiography as described under Materials and Methods. The figure shows the autoradiogram obtained from this analysis. Panels A and B show the intracellular forms and panels C and D the secreted apoA-II forms produced by C127 clones expressing the normal and mutated forms, respectively. Note that apoA-II produced by C127 clones displays the same isoprotein pattern as HepG2 cells (Hussain & Zannis, 1990) and that the Gln+1 to Leu+1 substitution results in impairment in the cyclization of N-terminal glutamine and in cleavage of the propeptide. The numbers 3, 1, 1a, 0, etc. refer to the different apoA-II isoproteins as indicated in Figure 1.

cells, as already observed in C127 clones expressing the human apoA-I gene in pBMT3X (Roghani & Zannis, 1988). This size is consistent with utilization of the transcription initiation site of the mMT-1 promoter and with utilization of the polyadenylation signal of the mouse mMT-1 gene (Glanville et al., 1981).

Analysis of the Intracellular and Secreted ApoA-II Isoproteins Produced by C127 Cells. Figure 4A–D compares the isoprotein composition of intracellular and secreted apoA-II produced by C127 clones expressing the normal (panels A and C) and the mutated (panels B and D) human apoA-II gene. Following 4-h labeling with [^{35}S]methionine, this analysis showed that the normal apoA-I synthesized by C127 cells contains several isoproteins designated 3, 1, 1a, 0, –1, –1a, and –2a (Figure 1). In contrast, the mutant apoA-II Leu+1 consists predominantly of isoproteins 1 and –1 and totally lacks the plasma form of apoA-II (isoprotein 0). The isoprotein pattern of intracellular and secreted apoA-II produced by C127 clones expressing the normal apoA-II gene was similar to that of HepG2 cells (Hussain & Zannis, 1990), although the ratio of isoproteins 1a/1 and –1a/–1 was lower in C127 cells than in HepG2 cells. This suggests that the apoA-II propeptide may be cleaved somewhat slower in C127 cells than in HepG2 cells. In addition, unmodified pro-apoA-II (isoprotein 3) was detectable in the medium of C127 cells, suggesting that a small part of pro-apoA-II could, in this cell type, escape the intracellular processing and be secreted.

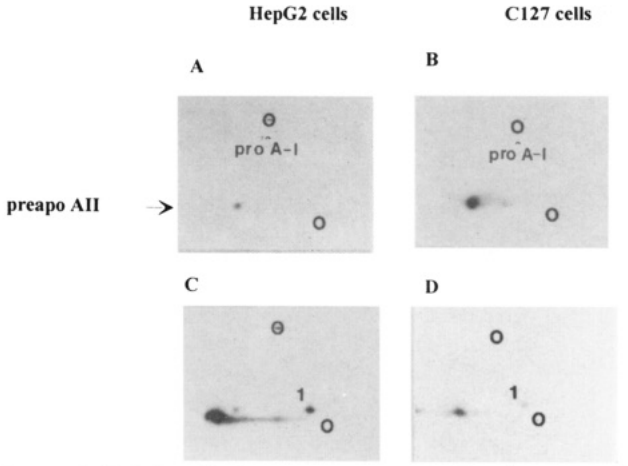

FIGURE 5: Relationships between prepro-apoA-II and pro-apoA-II in C127 and HepG2 cells. Panels A and B show the [^{35}S]methionine-labeled cell-free translation product of apoA-II mRNA obtained from HepG2 and C127 cells, respectively. Panels C and D show the intracellular apoA-II isoproteins following a 10-min labeling of HepG2 and C127 cells, respectively, with [^{35}S]methionine. The position of pro-apoA-I is indicated on the top of the figure. The open circle at the bottom of the figure shows the position of the plasma apoA-II (isoprotein 0). The numbers 1 and 0 etc. refer to the different apoA-II isoproteins as indicated in Figure 1.

The absence of isoprotein 0 in cells expressing the mutant apoA-II Leu+1 is consistent with the previous hypothesis (Hussain & Zannis, 1990) that this isoprotein results from N-terminal cyclization of the glutamine residue of isoprotein 1a which is generated from isoprotein 1 by cleavage of the propeptide. In addition, the ratio of isoproteins 1a/1 and isoproteins –1a/–1 was drastically decreased in C127 clones expressing the mutated as compared to the normal apoA-II. The finding suggests that the Gln+1 to Leu substitution also impairs the cleavage of the propeptide.

Relationships between Early and Late Modified Isoproteins of ApoA-II in C127 and HepG2 Cells. HepG2 cells and C127 cell clones expressing the normal and the mutated apoA-II gene were labeled with [^{35}S]methionine for 10 min, and the intracellular and secreted forms were immunoprecipitated with a rabbit anti-human apoA-II antiserum and analyzed by two-dimensional isoelectrofocusing/polyacrylamide gel electrophoresis followed by autoradiography. Figure 5A–D compares the cell-free translation product of apoA-II mRNA prepared from HepG2 and C127 cells (panels A and B) to intracellular apoA-II isoproteins present after a 10-min pulse (panels C and D). In both cell types, prepro-apoA-II and unmodified pro-apoA-II (isoprotein 3) migrated similarly, as compared to mature forms of apoA-I and apoA-II used as markers. This suggests that isoprotein 3 results from cleavage of the signal peptide. During the 10-min labeling period, isoprotein 3 was partially converted to isoprotein 1 in both cell types.

To determine the intracellular localization of the early modification of pro-apoA-II (isoprotein 3) into isoprotein 1, C127 cells were labeled for 1 h with [^{35}S]methionine in the absence or in the presence of 10 μg/mL Brefeldin A (Figure 6A–D), which is known to block the transport of secretory proteins from the ER to the Golgi apparatus (Davis et al., 1989). Only pro-apoA-II isoproteins 3 and 1 were detected in C127 cells incubated in the presence of Brefeldin A for 1 h (Figure 6C) and 2 h (results not shown), whereas all other isoproteins were also present in the absence of the inhibitor

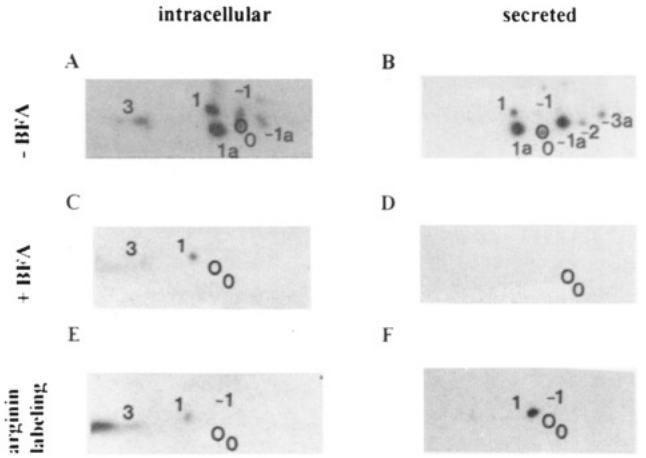

FIGURE 6: Relationships between early and late modified isoproteins of apoA-II in C127 cells. Comparison of different apoA-II forms was performed by two-dimensional electrophoresis of the proteins labeled with [^{35}S]methionine in the presence or absence of 10 μg/mL Brefeldin A, or with [^{14}C]arginine. Panels A and C show the intracellular forms and panels B and D the secreted apoA-II forms following 60-min labeling with [^{35}S]methionine, in the absence or presence of 10 μg/mL Brefeldin A, respectively. Panels E and F show the intracellular and secreted apoA-II forms following 2-h labeling with [^{14}C]arginine, respectively. Arginine labeling reveals only the apoA-II isoproteins 3, 1, –1, and –3, which contain the propeptide, whereas methionine labeling shows in addition to the above isoproteins forms with lower molecular weight (1a, –1a, and –3a) which apparently originate from the 1, –1, and –3 isoproteins by cleavage of the prosegment. Note that Brefeldin A trapped apoA-II isoproteins in the ER and prevented their secretion as well as the formation of the late modified isoproteins. The open circle at the bottom of the figure indicates the position of the plasma apoA-II form. The numbers 3, 1, 1a, 0, etc. refer to the different apoA-II isoproteins as indicated in Figure 1.

(Figure 6A). The lack of conversion of isoprotein 3 to 1 in the present study indicates that any recycling between ER and Golgi under the experimental conditions used was not appreciable to cause sufficient conversion of isoprotein 3 to 1. Furthermore, treatment of C127 cells with Brefeldin A prevented the secretion of all apoA-II isoprotein in the medium following 1-h (Figure 6A,D) and 2-h treatment (data not shown). These findings suggest that the modification of pro-apoA-II into isoprotein 1 occurs in a pre-Golgi compartment whereas other modifications, i.e., sialylation, propeptide cleavage, and N-terminal cyclization, occur later along the secretory pathway.

The cleavage of the propeptide was studied by [^{14}C]arginine labeling, considering that human apoA-II contains Arg residues only in amino acids –1 and –2 of the prosegment (Brewer et al., 1972; Gordon et al., 1985). After a 2-h labeling of C127 cells, only isoproteins 3, 1, and –1 were faintly labeled whereas isoproteins 1a, –1a, 0, and –2a were undetectable in intracellular (Figure 6E) and in secreted (Figure 6F) apoA-II, as previously shown in HepG2 cells (Hussain & Zannis, 1990). Comparison of the apoA-II isoprotein pattern observed after [^{35}S]methionine and [^{14}C]arginine labeling of intracellular (Figure 6A vs Figure 6E) and secreted (Figure 6B vs Figure 6F) apoA-II isoproteins confirms that isoproteins 3, 1, and –1 contain the five-residue propeptide and that the other isoproteins result from the cleavage of the propeptide.

Figure 7 shows the effect of neuraminidase treatment of apoA-II isoproteins secreted by C127 cells utilizing a mixture of *Clostridium perfringens* enzyme and of *Vibrio cholerae* enzyme. This treatment resulted in the conversion of the sialylated apoA-II isoproteins into isoproteins 1a and 0.

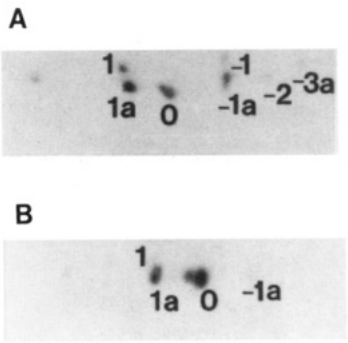

FIGURE 7: Neuraminidase treatment of apoA-II isoproteins. Culture medium obtained by labeling of C127 cells expressing the normal apoA-II gene with [^{35}S]methionine for 16 h was treated with a mixture of *Clostridium perfringens* and *Vibrio cholerae* neuraminidase as described under Methods. Panels A and B show untreated and treated apoA-II isoproteins, respectively. Note that neuraminidase treatment converts apoA-II forms containing O-linked modifications into isoproteins 1a and 0. A protein with an isoelectric point similar to that of 1 and 1a exists in plasma and may represent either an apoA-II form or a 1α isoprotein which escaped enzymatic cyclization in the Golgi. The numbers 3, 1, 1a, 0, etc. refer to the different apoA-II isoproteins as indicated in Figure 1.

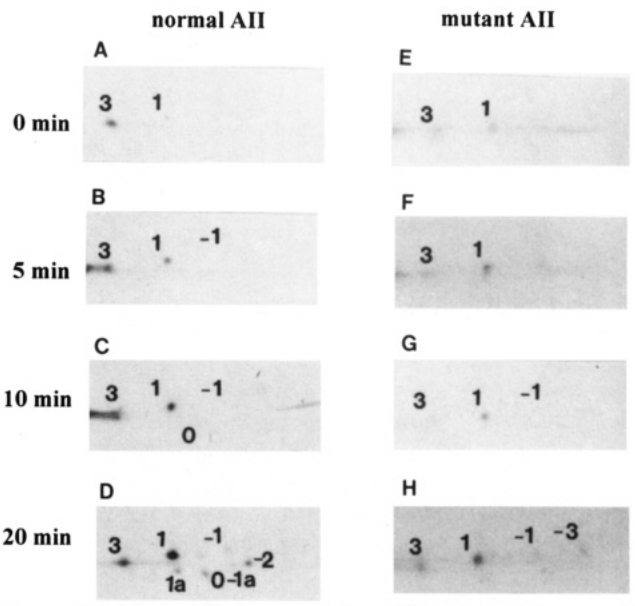

FIGURE 8: Kinetics of early and late modifications of the normal (Gln+1) and mutated (Leu+1) apoA-II isoproteins. C127 cells expressing the normal apoA-II gene (panels A–D) and the mutated apoA-II gene (panels E–H) were pulsed for 10 min and chased for the indicated times. The intracellular apoA-II isoprotein was immunoprecipitated and analyzed by two-dimensional gel electrophoresis and autoradiography. Note that the early modifications appear with similar kinetics in both C127 cell clones, whereas isoproteins resulting from propeptide cleavage are not detectable when Gln+1 is substituted with Leu.

Isoprotein 0 is the major mature desialylated isoprotein of apoA-II in plasma. In contrast, neuraminidase treatment did not affect isoproteins 1 and 1a, thus confirming earlier findings in HepG2 cells (Hussain & Zannis, 1990) which showed that the early modification of pro-apoA-II into isoprotein 1 does not involve sialylation of apoA-II isoproteins.

Kinetics of Intracellular Modifications of the Normal and Mutant Forms of ApoA-II. A 10-min pulse of C127 cells expressing the normal apoA-II gene with [^{35}S]methionine (Figure 8A) revealed the presence of isoprotein 3, which corresponds to the unmodified pro-apoA-II, and an additional spot corresponding to modified pro-apoA-II (isoprotein 1). A 5–10-min chase resulted in an increase in the intensity of

isoprotein 1 (Figure 8B,C). After a 20-min chase, the intensity of isoprotein 3 decreased whereas the concentrations of isoproteins 1a, –1a, 0, and –2a increased (Figure 8D). The kinetics of appearance of the different isoproteins of apoA-II combined with the results of treatment with Brefeldin A indicated that isoprotein 3 is the precursor of isoprotein 1 which in turn is the precursor of all other isoproteins. Furthermore, the conversion of isoprotein 1 to the other forms occurs in the Golgi system. Consistent with previous observations (Hussain & Zannis, 1990), the kinetics of appearance of the early and late modified isoproteins of the mutant Leu+1 apoA-II are similar to those of the wild-type protein except that isoprotein 0 was not formed and isoproteins which result from cleavage of the propeptide such as 1a appeared in trace amounts (Figure 8E–H). The findings indicate that the substitution of Glu+1 by Leu+1 impaired cleavage of the propeptide and prevented N-terminal cyclization.

Kinetics of Depletion of Intracellular ApoA-II in HepG2 and C127 Cells. C127 cells were pulsed for 30 min with [^{35}S]methionine and chased for 5–120 min. ApoA-II was immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. This analysis showed that the concentration of intracellular apoA-II decreased very rapidly and reached very low levels within 60 min (Figure 9A,B). The kinetic analysis showed that the depletion of intracellular apoA-II follows first-order kinetics with an average residence half-time of approximately 15 min in all three cell lines (Figure 9C). Quantitation of the intracellular and secreted apoA-II at different time points indicated that the majority of the newly synthesized apoA-II is degraded intracellularly in C127 cells whereas approximately 40% of the newly synthesized apoA-II in HepG2 cells is secreted (Figure 9A,B). The degradation is greater in the cell lines expressing the mutant as compared to the wild-type apoA-II form.

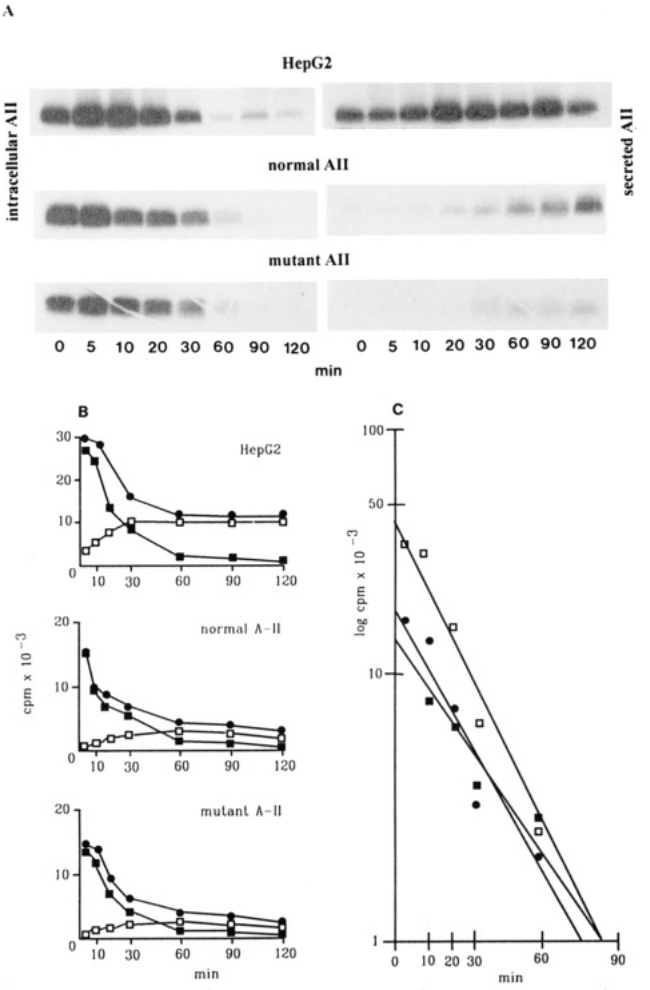

FIGURE 9: Kinetics of depletion of the intracellular apoA-II and of the appearance of secreted apoA-II in HepG2 cells and in C127 cells expressing the normal (Gln+1) and mutated (Leu+1) apoA-II. Cells were pulsed for 15 min and chased for the indicated time. The cells, lysates, and culture media were collected separately, immunoprecipitated, and analyzed by one-dimensional SDS–PAGE and autoradiography. Panel A shows the intracellular and secreted forms of apoA-II from HepG2 cells and C127 cells expressing the normal and the mutated apoA-II, respectively. The autoradiograms were analyzed by quantitative scanning densitometry, and data were expressed as cpm/dish = scanning arbitrary units × radioactivity counts associated to apoA-II at the end of the pulse. Panel B shows quantitative analysis of the depletion of intracellular apoA-II and the accumulation of secreted apoA-II. The amount of radioactivity recovered in the cell and in the medium is shown by closed and open squares, respectively. Total radioactivity is shown by the closed circles. Panel C shows first-order kinetics of the loss of intracellular apoA-II from HepG2 cells (open squares) or from C127 cells expressing the Gln+1 (closed squares) or the Leu+1 apoA-II (closed circles). Note that the majority of the newly synthesized apoA-II is degraded intracellularly in C127 cells whereas 60% of the apoA-II is secreted by HepG2 cells.

DISCUSSION

Background. Apolipoprotein A-II is synthesized by the liver and, to a much lesser extent, by the intestine (Schonfeld et al., 1982; Hussain & Zannis, 1990). Though apoA-II is found in the plasma as a desialylated isoprotein (Gordon et al., 1984), it has been shown to undergo a very complex set of intra- and extracellular posttranslational modifications in HepG2 cells (Hussain & Zannis, 1990). These various precursors of apoA-II isoproteins are eventually converted into the mature plasma apoA-II isoprotein designated isoprotein 0 by desialylation (Hussain & Zannis, 1990).

To further dissect the posttranslational modifications of apoA-II, we generated permanent C127 cell lines overexpressing the wild-type form and a mutant form of the human apoA-II gene. The protein synthesized by the apoA-II producing C127 clones displayed the same pattern of isoproteins as those observed in HepG2 cells. This observation suggests that the posttranslational modifications of apoA-II are not tissue-specific and that the enzymes responsible for these modifications are expressed in different cell types. The lower ratios of isoproteins 1a/1 and –1a/–1 observed in C127 cells as compared to HepG2 cells suggest that the cathepsin B-like protease responsible for the cleavage of the propeptide may be less effective in C127 cells.

Conversion of Isoprotein 3 to Isoprotein 1 Occurs in Pre-Golgi and Involves Addition of Two Negatively Charged Groups to the ApoA-II Prosegment. To further determine the nature of posttranslational modifications of apoA-II, we treated C127 cell cultures with Brefeldin A, a drug known to block the translocation of proteins from the ER to the Golgi compartment (Orci et al., 1991). This treatment prevented the secretion of apoA-II, and produced only intracellular isoproteins 3 and 1. Previous studies also showed that treatment of cell cultures with Brefeldin A inhibited the O-glycosylation of the LDL receptor without affecting its N-glycosylation (Shite et al., 1990). This finding suggests that the conversion of isoprotein 3 to isoprotein 1 is an early event which occurs in a pre-Golgi compartment and is consistent with the effects of neuraminidase on the different apoA-II isoproteins. This treatment did not have any effect on isoproteins 3, 1, and 1a, whereas it converted other acidic forms to isoproteins 1a and 0. The conversion of isoprotein

3 to 1 resulted in an increase in the molecular mass of isoprotein 1 as well as in an increase in its positive charge by 2 units. Arginine labeling of apoA-II isoproteins indicated that subsequent cleavage of the propeptide converts isoproteins 1, −1, and −3 to isoprotein forms 1a, −1a, and −3a which have smaller molecular weights but identical isoelectric points to the original isoprotein forms 1, −1, and −3. This indicates that the propeptide is not charged despite the fact that it contains two Arg residues. Thus, the 3 to 1 modification involves residues of the apoA-II prosegment and results in the addition of two negative charges to it. The possibility that this modification occurs in the Arg residues is currently under investigation by *in vitro* mutagenesis and expression studies.

Isoprotein 0 Is the Result of Cyclization of the N-Terminal Glutamine of Isoprotein 1a. Substitution of Glu+1 by Leu Impairs the Cleavage of the Propeptide. The generation of C127 permanent cell clones expressing a mutated form of apoA-II carrying a Gln+1 to Leu+1 substitution allowed us to demonstrate that isoprotein 0 is the result of cyclization of the N-terminal glutamine of mature apoA-II. In the present study, isoprotein 0 was detectable in C127 cells expressing the normal apoA-II after 20 min (10-min pulse + 10-min chase), and isoproteins 0 and −2a accumulated intracellularly after 20 min of chase. In contrast, isoproteins 0 and 2a were not detectable after a 20-min chase as well as after a 4-h labeling of C127 cells expressing the mutant (Leu+1) apoA-II. Furthermore, isoproteins −1 and −3, which result from sialylation of isoprotein 1, did appear within 20 min (10-min pulse, 10-min chase).

Interestingly, the Gln+1 to Leu substitution not only, as expected, prevented the N-terminal cyclization of Gln but also impaired the cleavage of the propeptide to such an extent that isoproteins 1a and −1a were barely detectable in cells and medium after a 4-h labeling. ApoA-II propeptide contains two N-terminal Arg residues and is similar to propeptides which are cleaved by the cathepsin B-like proteases (Gordon et al., 1984). Endoproteolytic cleavage by cathepsin B is reported to be favored by the presence of bulky hydrophobic residues as opposed to Arg residues at positions P1 and P2 which fit into the S1 and S2 subsites, respectively, of the active site (McKay et al., 1983). Thus, it is possible that modification of the Arg residues may render pro-apoA-II a substrate for the propeptide processing enzyme. The substitution of Gln+1 by Leu+1 not only precludes N-terminal cyclization but also diminishes the catalytic rate of the processing protease.

Pulse–chase experiments showed that the impairment of propeptide cleavage, as a result of the Gln+1 to Leu substitution, did not affect the intracellular trafficking or the secretion rate of apoA-II produced by C127 clones. Our results indicate that the apoA-II prosegment sequence is not involved in apoA-II routing to the secretory pathway. It was suggested that the presence of the prosegment lessens the association of apoA-II to HDL (Gordon et al., 1984). The production of large quantities of mutated Leu+1 apoA-II which contain mostly the pro-apoA-II form will allow us to assess this possibility.

ApoA-II Is Subject to Rapid Intracellular Degradation. The pulse–chase experiments also demonstrated that apoA-II was subjected to a rapid intracellular degradation. Kinetic analysis showed that the rate of depletion of intracellular apoA-II was similar in HepG2 and the two apoA-II-expressing C127 cell lines ($t_{1/2}$ = 15 min). Most of the loss in the C127 cell lines was due to intracellular degradation which was more pronounced in the cell line expressing the mutant apoA-II form. In HepG2 cells, the intracellular degradation was less than in C127 cells, but it accounted for 60% of the newly synthesized apoA-II. In contrast, most of the newly synthesized apoA-I and apoE is secreted in the culture medium (Zannis et al., 1986; Roghani & Zannis, 1988). Extensive intracellular degradation of apoB in the ER has been reported to be the major mode of regulation of apoB synthesis in rat hepatocytes as well as in HepG2 cells (Borchardt & Davis, 1987; Davis et al., 1989; Sato et al., 1990a,b). In the ER, a large fraction of apoB is associated with the membrane whereas apoA-I, as well as albumin and α_1-antitrypsin, is mostly recovered in the lumen (Borstrom et al., 1986; Wong & Pino, 1987; Bamberger & Lane, 1988). This suggests that the membrane-associated apoB may be susceptible to intracellular degradation. In this regard, it has been reported that apoA-II binds more tightly than apoA-I or apoE to phospholipids in HDL (De Coen et al., 1988; Brasseur et al., 1992). It is possible that apoA-II may also associate more tightly to the ER membranes than apoA-I or apoE and thus be more susceptible to proteolytic cleavage. Association of apoA-II with membrane bilayers has been demonstrated (Folz & Gordon, 1986).

In conclusion, we have demonstrated that the early modification of apoA-II which converts isoprotein 3 into isoprotein 1 occurs in pre-Golgi compartment and does not involve sialylation. The generation of C127 cells clones expressing a mutated apoA-II carrying a Gln+1 to Leu+1 substitution allowed us to conclude with certainty that isoprotein 0 is the result of cyclization of the N-terminal Gln+1. The substitution of Glu+1 by Leu+1 impaired cleavage of the propeptide, indicating that the residue at the P1 position influences the activity of the cathepsin B-like protease which cleaves the prosegment. Finally, apoA-II is subject to intracellular degradation to a lesser extent in HepG2 cells which are capable of secreting lipoprotein particles as opposed to C127 cells which do not synthesize lipoproteins.

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