Intracellular Modification of Human Apolipoprotein AII (ApoAII) and Sites of ApoAII mRNA Synthesis: Comparison of ApoAII with ApoCII and ApoCIII Isoproteins†

M. Mahmood Hussain and Vassilis I. Zannis*

Section of Molecular Genetics, Cardiovascular Institute, Departments of Medicine and Biochemistry, Boston University Medical Center, 80 East Concord Street, R420, Boston, Massachusetts 02118

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ABSTRACT: We have studied the intracellular modifications of human apoAII by pulse–chase labeling of HepG2 cell cultures with [^{35}S]methionine or [^{3}H]arginine followed by two-dimensional analysis and autoradiography of the radiolabeled apoAII isoproteins. A short (5.0-min) pulse showed the presence of a precursor form of apoAII (p*I* = 5.75) designated proapoAII or apoAII$_3$. A 5–10-min chase resulted in a decrease in the relative concentration of the proapoAII coupled with an increase in the relative concentration of a new form (p*I* = 5.3) designated modified proapoAII or apoAII$_1$. Longer chase resulted in the appearance of the plasma apoAII form and at least five other acidic apoAII isoproteins in the cell lysate and the culture medium. Labeling with [^{3}H]arginine showed that apoAII isoproteins designated 3, 1, −1, and −3 contained the prosegment whereas isoproteins designated 1a, 0, −1a, −2a, −3a, and −4a did not. Comparison of nascent apoAII, apoCII, and apoCIII isoproteins revealed that they were distinctly different on the two-dimensional gels. Neuraminidase treatment converted the acidic apoAII isoproteins to isoproteins 1a and 0 (modified and plasma apoAII forms). The combined data are consistent with the following intra- and/or extracellular modifications of apoAII: (a) modification of the apoAII which results in the net loss of two positive charges; (b) glycosylation of the modified proapoAII with carbohydrate chains containing sialic acid; (c) proteolytic removal of the prosegment and cyclization of the N-terminal glutamine. Analysis of apoAII mRNA distribution in 13 fetal human tissues as well as in cell lines of human origin showed abundance of apoAII mRNA in liver and HepG2 cells and only traces in the intestine.

Apolipoprotein AII is the second major protein of high-density lipoprotein (HDL) (Kostner & Alaupovic, 1971; Scanu et al., 1969, 1971) which is reported to be synthesized by the liver (Zannis et al., 1982) and the intestine (Rachmilewitz et al., 1978). In plasma, apoAII resides in a subfraction of HDL that has an apoAI to apoAII molar ratio of approximately 2 (Cheung & Albers, 1984). Similar to other apolipoproteins, binding of apoAII to phospholipids is associated with an increase in its helical content (Assmann & Brewer, 1974; Mao et al., 1977, 1981). The lipid binding domain of apoAII has been localized at the carboxy-terminal region and probably involves amino acid residues 50–55 (Mao et al., 1981). The putative functions of apoA-II as a ligand for the HDL receptor (Hwang & Menon, 1985), for the activation of hepatic triglyceride lipase (Jahn et al., 1983; Shinomiya et al., 1982) and lecithin:cholesterol acyltransferase (Fielding et al., 1972; Scanu et al., 1982; Soutar et al., 1975), require further clarification. The plasma form of human apoAII consists of two identical polypeptide chains of 77 amino acids each linked by a disulfide bond at residue 6 (Brewer et al., 1972). Protein (Gordon et al., 1983, 1984) and DNA sequence analyses of the apoAII gene (Knott et al., 1984; Lackner et al., 1984; Sharpe et al., 1984; Tsao et al., 1985) have shown that the primary translation product of apoAII mRNA consists of 100 amino acids including an 18-residue-long signal peptide (Gordon et al., 1983, 1984; Knott et al., 1984; Lackner et al., 1984; Sharpe et al., 1984; Tsao et al., 1985). The newly secreted apoAII consists of 82 residues and contains a 5-residue-long N-terminal prosegment with the sequence AlaLeuValArgArg (Gordon et al., 1983). This prosegment is cleaved extracellularly by a thiol protease to produce the mature plasma apoAII form (Gordon et al., 1984). Minor apoAII isoproteins (Gordon et al., 1984; Lackner et al., 1985; Schmitz et al., 1983), presumably the products of posttranslational modification (Lackner et al., 1985), have been reported recently. The chemical nature of these isoproteins as well as the function and the physiological significance of apoAII remains unknown. The present study was undertaken in order to establish the sites of synthesis of human apoAII and the relationship between the newly synthesized apoAII form to its plasma counterparts.

Materials and Methods

Materials. Bovine serum albumin, ovalbumin, lysozyme, trypsin inhibitor, neuraminidase (*Clostridium perfringens*), deoxycholate, Triton X-100, 2-mercaptoethanol, *N,N,N′,N′*-tetramethylenediamine, hydrogen peroxide (30% w/w), and Tris were purchased from Sigma. Ampholines pH 2.5–4, 5–8, and 3.5–10 were purchased from LKB Instruments, Inc., Rockville, MD. Nonidet P-40 was purchased from Particle Data Laboratories, Ltd. Acrylamide was obtained from Serva Fine Chemicals. Glycine, methanol, and glacial acetic acid were purchased from Fisher Scientific Co. Sodium dodecyl sulfate, bis(acrylamide), ammonium persulfate, Coomassie brilliant blue, bromophenol blue, Biolytes pH 4–6, and agarose were obtained from Bio-Rad. Urea ultrapure grade was a product of Schwarz/Mann. [^{35}S]Methionine (1150 Ci/mmol), [^{3}H]arginine (15–30 Ci/mmol), [α-^{32}P]dATP, [α-^{32}P]dCTP, and [α-^{32}P]dTTP were obtained from New England Nuclear. Nitrocellulose filters and DE-52 were obtained from Schleicher & Schuell, Inc., and Whatman, respectively. X-ray film

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Cronex 4 was purchased from DuPont Instruments, Wilmington, DE. Methionine-free Eagle's minimum essential medium (MEM) and L-glutamine were purchased from Gibco. A staphylococcal protein A preparation (IgGSorb) was obtained from the Enzyme Centre, Boston, MA. Goat anti-human apoAII, apoCII, and apoCIII were gifts from Dr. Peter Herbert, Miriam Hospital, Providence, RI. Anti-human apoE was obtained from Atlantic Antibody Inc.

Labeling of HepG2 Cells and Organ Cultures and Immunoprecipitation of Radiolabeled Apolipoproteins. HepG2 cells were grown in 35- or 60-mm-diameter tissue culture dishes containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. On day 4, approximately 90% confluent cultures were washed twice with serum-free MEM and incubated in the same medium for 18 h. The cultures were then washed twice with methionine-free MEM supplemented with 2 mM glutamine and incubated in the same medium for 1 h. The cells were then washed with methionine-free MEM supplemented with 2 mM glutamine and incubated in the same medium for the desired time in the presence of 0.25 or 0.50 mCi of [^{35}S]-methionine per 35- or 60-mm dish, respectively (pulse). The [^{35}S]methionine-containing medium was removed at the appropriate times. The cultures were washed twice with MEM (chase) and incubated in 1 mL of the same medium for various times. For arginine labeling, 35-mm-diameter 90% confluent cultures were preincubated in serum-free MEM supplemented with 2 mM glutamine for 18 h and in Arg-free MEM for 2 h and labeled in the same medium in the presence of 0.5 mCi of [^{3}H]arginine. At the end of the pulse or chase period, the medium was collected, centrifuged in a microcentrifuge to remove cell debris, and adjusted to a final concentration of 10 mM sodium phosphate pH 7.2, 85 mM NaCl, 5 mM KCl, 0.25% deoxycholate, 0.5% Triton X-100, and 0.5% sodium dodecyl sulfate. The cell cultures were washed twice with phosphate-buffered saline 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 5 min in a microcentrifuge, and the supernatant was diluted 1:1 with a solution of 10 mM sodium phosphate, pH 7.0, 85 mM NaCl, 5 mM KCl, and 1% sodium dodecyl sulfate. An aliquot of 50 μL of 10% IgGSorb in 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, 1% sodium dodecyl sulfate, and 1% Triton X-100 suspension was added to 1 mL of cell lysate or culture medium prepared as described above, and the mixture was incubated at 4 °C for 1 h. The mixture was centrifuged for 5 min in a microcentrifuge. To the supernatant was added 20 μL of goat anti-human apoAII (or anti-apoCII or anti-apoCIII), and the mixture was incubated overnight at 4 °C. Next, a 50-μL aliquot of 10% IgGSorb suspension in 10 mM sodium phosphate at pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, 1% sodium dodecyl sulfate, and 1% Triton X-100 was added and incubated for an additional hour at 4 °C. The mixture was centrifuged for 30 s in a microcentrifuge. The pellet was washed once by resuspension and 30-s centrifugation in a buffer containing 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.5% deoxycholate, 1.0% Triton X-100, 1.0% sodium dodecyl sulfate, 10 mM L-methionine, and 1 mg/mL bovine serum albumin and then 3 times in the same buffer without albumin. The immunoprecipitates obtained from the cell lysates and the culture media were dissolved in O'Farrell's lysis buffer (O'Farrell et al., 1977) [9.5 M urea, 2% w/v Nonidet P-40, 2.1% ampholines (1.2% pH 5–8, 0.5% pH 2.5–4, and 0.4% pH 4–6, and 5% (v/v) β-mercaptoethanol] and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography as described (O'Farrell, 1975; Zannis et al., 1982). Organ cultures of fetal human and adult monkey intestine were performed as described previously (Zannis et al., 1980, 1982).

Immunoprecipitation of Apolipoproteins from Human Serum. The amount of antiserum required for optimum precipitation of human apoAII, apoCII, and apoCIII from an aliquot of 50 μL of human serum was determined as follows. Samples of 5, 10, 20, 40, 80, and 160 μL of antiserum were added to the human serum, and all samples were adjusted to the same final concentration with normal saline. After 4 days at 4 °C, the immunoprecipitate was collected by centrifugation for 5 min in a microcentrifuge and was washed 3 times with normal saline. The immunoprecipitate was then dissolved in O'Farrell's lysis buffer (O'Farrell et al., 1977) and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis (Zannis et al., 1982, 1986).

Neuraminidase Treatment of Nascent Apolipoproteins. Medium from the HepG2 cultures that were labeled for 4 h with [^{35}S]methionine was adjusted to a concentration of 0.1 M sodium acetate, pH 5.5, and was treated with 2 units of neuraminidase (*Clostridium perfringens*) at 37 °C for 2 h (Zannis & Breslow, 1981). The reaction mixture was dialyzed against 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, and 5 mM KCl and was adjusted to a final concentration of 0.25% deoxycholate, 0.5% Triton X-100, and 0.5% sodium dodecyl sulfate and used for immunoprecipitation of apolipoproteins as described above.

Charge Relationship of ApoAII Isoproteins. The charge differences among the various forms of apoAII were estimated in two different ways. The first way was to compare the focusing positions of apoAII isoproteins with those of human apoAI which have known isoelectric points (Zannis et al., 1980). The second method involved carbamylation of the newly synthesized apoAII isoproteins by boiling for 5 min in a solution of 9 M urea. This process generates new isoproteins which differ from the original protein by 1, 2, 3, etc. negative charges (Zannis & Breslow, 1980).

Isolation, Cell-Free Translation, and Blotting Analysis of RNA. Isolation and analysis of RNA from human and monkey tissues and cell lines of human origin have been described previously (Goldberg, 1980; Zannis et al., 1982). Cell-free translation of RNA was performed in a total volume of 30 μL containing 5 μg of RNA and 0.05 mCi of [^{35}S]-methionine for 1 h at 37 °C using a cell-free translation kit (Bethesda Research Labs). The reaction conditions were those recommended by the manufacturer except that Mg^{2+} was omitted. The reaction mixtures from two experiments were pooled, diluted to 1 mL, adjusted to a final concentration of 10 mM sodium phosphate, pH 7.2, 85 mM NaCl, 5 mM KCl, 0.25% deoxycholate, 0.5% Triton X-100, and 0.5% sodium dodecyl sulfate, and immunoprecipitated using IgGSorb as described above (Zannis et al., 1986). Blotting analysis of RNA was performed as described (Zannis et al., 1985). The nitrocellulose filter was sequentially hybridized with apoAII, apoE, and apoCIII cDNA probes (Zannis et al., 1985). Prior to each hybridization, the radioactive band was erased by boiling the filter for 5 min with 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The apoAII cDNA probe used for hybridization was a generous gift of Dr. Jan L. Breslow, Rockefeller University. The probe which contained the 3′ region and extended to the 5′ *Pst*I site of the apoAII cDNA clone (Knott et al., 1984; Lackner et al., 1984; Sharpe et al., 1984) was

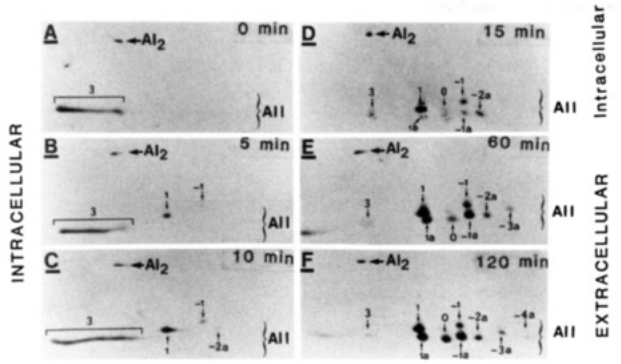

FIGURE 1: Identification of intracellular and secreted forms of apoAII by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography. HepG2 cells were labeled with [^{35}S]methionine for 5 min and chased for the indicated times. ApoAII was immunoprecipitated from the cell lysate and the culture medium with goat anti-human apoAII and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography as described under Materials and Methods. The figure shows the autoradiogram obtained from this analysis. Panels A–D show the intracellular apoAII forms obtained at the indicated chase times. Panels E and F show the secreted apoAII forms after 1 and 2 h of chase. The positions of the nascent apoAII and apoAI isoproteins are indicated. Note the conversion of proapoAII to modified proapoAII after 5-min chase and the generation of other modified forms. In this and subsequent figures, the cathode is on the left, and the anode is on the right.

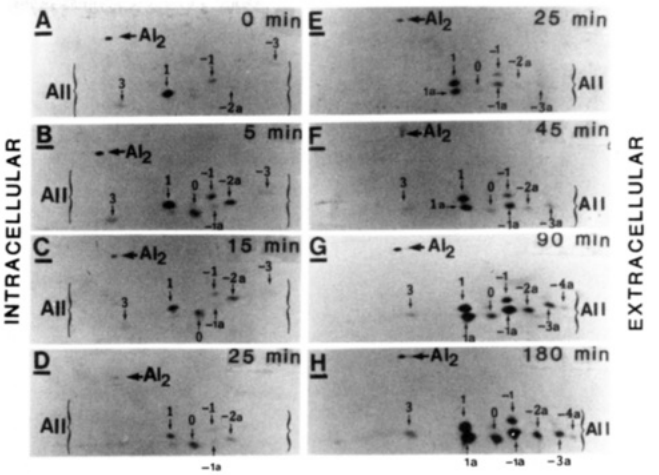

FIGURE 2: Relationship between different apoAII isoproteins. HepG2 cells were labeled with [^{35}S]methionine for 12 min and chased for the indicated times. ApoAII was immunoprecipitated and analyzed as described under Materials and Methods and in Figure 1. The figure shows the autoradiogram obtained from this analysis. This experiment shows the difference between the intra- and extracellular apoAII isoproteins. Note the abundance of isoproteins 1, 0, −1, −2a, and −3 in the cell lysate and of isoproteins 1a, −1a, and −3a in the culture media.

labeled with ^{32}P by nick translation (Rigby et al., 1977). The apoE and apoCIII probes have been described previously (Zannis et al., 1985).

Two-Dimensional Isoelectric Focusing/Polyacrylamide Gel Electrophoresis. Equilibrium isoelectric focusing/polyacrylamide gel electrophoresis was performed according to the method of O'Farrell (1975) with previously described modifications (Zannis et al., 1980, 1982, 1986). The ampholines used were 1.5% (pH 5–8), 0.2% (pH 2.5–4), and 0.5% (pH 4–6 Biolytes). Nonequilibrium pH gradient gel electrophoresis was performed according to O'Farrell et al. (1977) with the following modifications. The ampholines used were 0.5% (pH 3.5–10), 0.25% (pH 5–8), 0.25% (pH 2.5–4), and Biolyte (Bio-Rad) 0.5% (pH 4–6). The samples were electrophoresed for 2150 V h. For the second dimension, the focused cylindrical gels were thawed quickly and placed on slab gels. The slab gels (19.5 × 20.5 cm with a thickness of 1.5 mm) consisted of a polyacrylamide gradient (10–18%) [13.7% acrylamide and 0.37% bis(acrylamide)] and the stacking gel [4.4% acrylamide and 0.12% bis(acrylamide)]. Electrophoresis was performed at 100 V until the bromophenol blue dye reached the bottom of the gel. After electrophoresis, the gels were fixed in 50% (v/v) methanol and 10% (v/v) acetic acid for 1 h and stained in a solution of 0.25% Coomassie brilliant blue, 50% methanol, and 10% acetic acid for 30 min to an hour. Destaining was accomplished by sequential exposure of the gel for a period of 1 to fixing solution followed by water until proper stain background was achieved. The gels were treated with Enhance (New England Nuclear), dried, and exposed to Cronex-4 film.

RESULTS

Intra- and Extracellular Forms of ApoAII. The intra- and extracellular modifications of apoAII were studied by short-pulse and pulse–chase labeling of HepG2 cells. The [^{35}S]-methionine- or [^{3}H]arginine-labeled intracellular and secreted, apoAII isoproteins produced by HepG2 cells were immunoprecipitated and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography. A 5-min pulse revealed a single intracellular form of apoAII which focused as a broad band designated proapoAII or apoAII$_3$ (Figure 1A). A 5-min chase resulted in the appearance of an additional higher molecular weight, acidic apoAII form designated modified proapoAII or apoAII$_1$ (Figure 1B). A 10-min chase resulted in a decreased apoAII$_3$ concentration with a concomitant increase in apoAII$_1$ concentration as well as the appearance of another high molecular weight, acidic isoprotein designated apoAII$_{-1}$ (Figure 1C). After a 15-min chase, the apoAII$_3$ decreased with a concomitant increase of apoAII$_1$ and of several more acidic proteins designated as 1a, 0, −1, −1a, and −2a (Figure 1D). These data suggest that apoAII is synthesized as apoAII$_3$ and is subsequently modified to apoAII$_1$, apoAII$_{-1}$, and several other acidic isoproteins differing in molecular weight and isoelectric point from the original apoAII$_3$ forms. The secreted apoAII consisted of several isoproteins designated 1, 1a, 0, −1, −1a, −2a, −3a, and −4a (Figure 1E,F). To characterize further the formation of acidic and lower molecular weight isoproteins, 1a, 0, −1a, −2a, −3a, and −4a, we pulsed the HepG2 cells for 12 min and chased for longer time intervals. A 12-min pulse showed the presence of apoAII isoproteins 3, 1, −1, −2a, and −3 (Figure 2A). Five-minute chase increased the relative concentration of apoAII isoproteins 0, −1, and −2a (Figure 2B). A longer chase resulted in a decrease in the relative concentration of all apoAII isoproteins (Figure 2C,D), suggesting their secretion into the medium. Comparison of intracellular and secreted apoAII isoproteins (compare Figure 1D with Figure 1F and Figure 2B with Figure 2H) shows that the relative concentrations of isoproteins 1a, 0, −1a, −3a, and −4a are substantially increased in the culture media. Furthermore, comparison of the secreted apoAII isoproteins isolated at various time points shows that the relative concentrations of isoproteins 1 and −1 decrease with concomitant increases of isoproteins 1a, 0, −1a, −3a, and −4a. These data suggest that the lower molecular weight apoAII isoproteins 1a, 0, −1a, −2a, −3a, and −4a are generated at least partially by extracellular modification of the corresponding higher molecular weight forms (1, −1, and −3).

To study the cleavage of apoAII propeptide, HepG2 cells were pulsed for the indicated times with [^{3}H]arginine. The intra- and extracellular isoproteins were immunoprecipitated

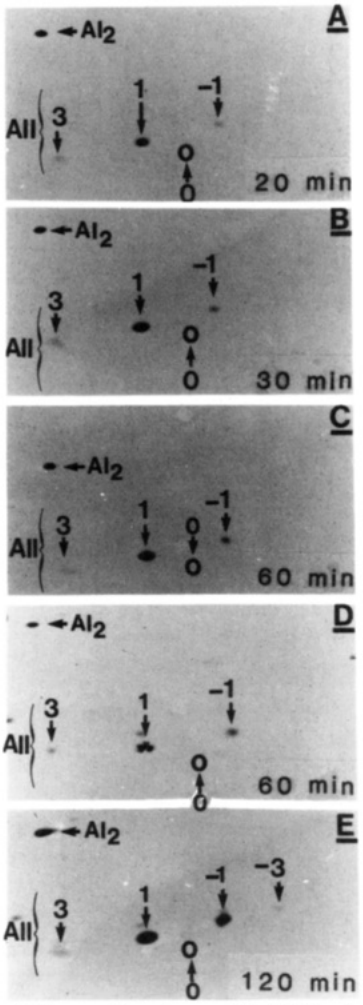

FIGURE 3: ApoAII isoproteins observed by [^{3}H]arginine labeling of HepG2 cells. Panels A–C show intracellular apoAII and panels D–H extracellular apoAII. HepG2 cells were labeled with 0.5 mCi of [^{3}H]arginine for the indicated times. ApoAII isoproteins were immunoprecipitated and analyzed as described under Materials and Methods and in Figure 1. The figure shows the autoradiogram obtained from this analysis. Note the presence of apoAII isoproteins 3, 1, −1, and −3 and the absence of 0, −1a, −2a, −3a, and −4a isoproteins. This finding suggests that the low molecular weight isoproteins do not contain arginine.

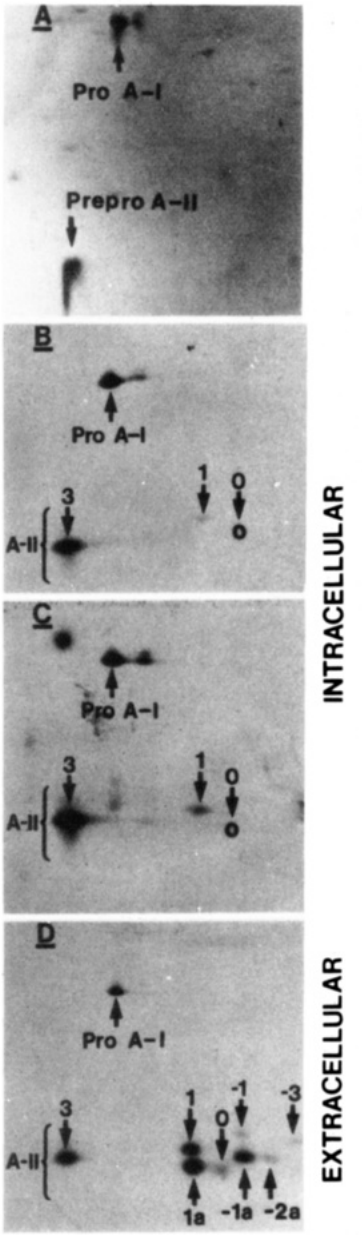

FIGURE 4: Comparison of preproapoAII with unmodified and modified proapoAII isoproteins by nonequilibrium pH gradient gel electrophoresis and autoradiography. The reaction mixture containing the cell-free translation products of fetal human liver mRNA was immunoprecipitated with goat anti-human apoAII and was mixed with [^{35}S]methionine-labeled proapoAII prepared as described in Figure 1. The mixture was analyzed by nonequilibrium pH gradient gel electrophoresis and autoradiography. The figure shows the autoradiogram obtained from this analysis. Panel A shows the cell-free translation product of apoAII mRNA. Panels B and C show the intracellular apoAII forms following 5-min pulse and 0- and 5-min chase. Panel D shows the secreted apoAII forms after 60-min chase. The positions of apoAII and apoAI isoproteins are indicated. Note that under the nonequilibrium conditions used, preproapoAII and the proapoAII migrate similarly. However, both proteins migrate faster than proapoAI.

and analyzed by two-dimensional isoelectric focusing/polyacrylamide gel electrophoresis and autoradiography. A 20-, 30-, or 60-min pulse revealed the intracellular presence of apoAII isoproteins 3, 1, and −1 (Figure 3A–C). Analysis of extracellular apoAII isoproteins showed the presence of 3, 1, −1, and −3 isoproteins (Figure 3D,E). Comparison of [^{3}H]-arginine-labeled and [^{35}S]methionine-labeled isoproteins showed that low molecular weight isoproteins (1a, 0, −1a, −2a, −3a, and −4a) were not labeled with [^{3}H]arginine (compare Figure 3D,E with Figure 1E,F and Figure 2E–H). This finding suggests that these low molecular weight isoproteins were generated from their higher molecular weight counterparts, 1, −1, and −3, by cleavage of the propeptide followed by cyclization of the N-terminal glutamine.

The present data do not allow us to establish the exact precursor–product relationship of the higher molecular weight isoproteins with their lower molecular weight counterparts.

Comparison of ProapoAII with the Cell-Free Translation Product of ApoAII mRNA. The cell-free translation product of apoAII mRNA (preproapoAII) was compared to the apoAII$_3$ isoprotein by nonequilibrium pH gradient gel electrophoresis. This was necessary because the cell-free translation product of apoAII mRNA focused as a diffused band similar to apoAII$_3$ in Figure 1A (data not shown). Nonequilibrium pH gradient gel electrophoresis showed that preproapoAII and proapoAII, isolated after a 5-min pulse, are single isoproteins and migrate similarly in this system (Figure 4A,B). ProapoAII was modified partially to apoAII$_1$ after 5-min chase (Figure 4C). Analysis of extracellular apoAII isoproteins showed the presence of all the apoAII isoproteins observed in Figures 1 and 2 (Figure 4D).

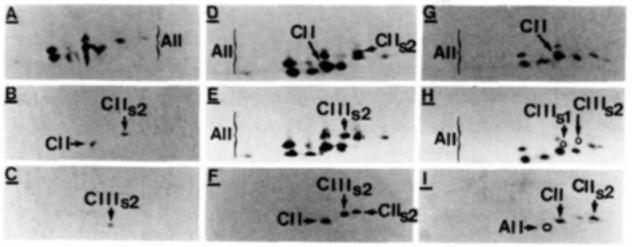

FIGURE 5: Comparison of nascent and plasma apoAII, apoCII, and apoCIII isoprotein forms. HepG2 cells were labeled with [^{35}S]-methionine for 4 h. ApoAII, apoCII, or apoCIII was individually immunoprecipitated and analyzed as described under Materials and Methods and in Figure 1. The figure shows the autoradiogram obtained from this analysis. Panels A–C represent the individual apoAII, apoCII, and apoCIII isoproteins. Panels D–F represent the mixtures of ^{35}S-labeled nascent apoAII and apoCII, apoAII and apoCIII, and apoCII and apoCIII. Panels G and H represent mixtures of ^{35}S-labeled nascent apoAII with plasma apoCII and apoCIII, respectively. Panel I represents a mixture of ^{35}S-labeled nascent apoCII with plasma apoAII. The positions of plasma apolipoproteins are represented as open circles and were established by superimposing the autoradiogram on the corresponding Coomassie Blue stained gel. Note that the isoproteins of human apoCII and apoCIII are distinctly different from those of apoAII.

Comparison of ApoAII Isoproteins with ApoCII and ApoCIII Isoproteins. Control experiments were performed to eliminate the possibility that some of the putatively identified apoAII isoproteins were due to contamination by apoCIII or apoCII isoproteins. In these experiments, apoAII, apoCII, and apoCIII were isolated by immunoprecipitation with monospecific antisera from the culture medium of HepG2 cells labeled with [^{35}S]methionine and analyzed by two-dimensional isoelectric focusing/polyacrylamide gradient gel electrophoresis and autoradiography. Analysis of apoAII showed the presence of several isoproteins (Figure 5A) described earlier in Figures 1 and 2. Secreted apoCII consisted of two isoproteins designated apoCII and apoCII$_{s2}$ (Figure 5B), and secreted apoCIII consisted of a single isoprotein designated apoCIII$_{s2}$ (Figure 5C). Mixing of apoAII and apoCII showed that apoCII has a slightly higher molecular weight and a more basic isoelectric point than apoAII isoprotein −1a. ApoCII$_{s2}$ has the same p*I* but higher molecular weight than apoAII isoprotein −3a (Figure 5D). Mixing of apoAII and apoCIII isoproteins revealed that apoCIII$_{s2}$ focused between isoproteins −1 and −3a (Figure 5E). Mixing of apoCII and apoCIII shows that apoCIII$_{s2}$ focuses between the two apoCII isoproteins (Figure 5F). To further characterize the relationship between nascent and plasma isoproteins, apoAII, apoCII, and apoCIII were isolated from culture media of HepG2 cells grown in the presence of [^{35}S]methionine and from normal human serum. The apoAII and apoCII isolated from serum consisted of single isoproteins apoCII and AII (Figure 5G,I). In contrast, apoCIII consisted of two isoproteins designated apoCIII$_{s1}$ and apoCIII$_{s2}$ (Figure 5H). The positions of plasma apoCII, apoAII, and apoCIII are indicated in panels G, I, and H, respectively, of Figure 5 by open circles. Mixing of labeled apoAII isoproteins with serum apoCII shows that serum apoCII has a slightly higher molecular weight and a more basic isoelectric point than apoAII isoprotein −1a (Figure 5G). Comparison of the labeled apoAII isoproteins with serum apoCIII isoproteins shows that serum apoCIII isoproteins CIII$_{s1}$ and CIII$_{s2}$ have slightly higher molecular weight and a more acidic isoelectric point than apoAII isoproteins −1a and −2a, respectively (Figure 5H). Mixing of serum apoAII with labeled apoCII isoproteins showed that serum apoAII has a lower molecular weight and a more basic p*I* than apoCII (Figure 5I).

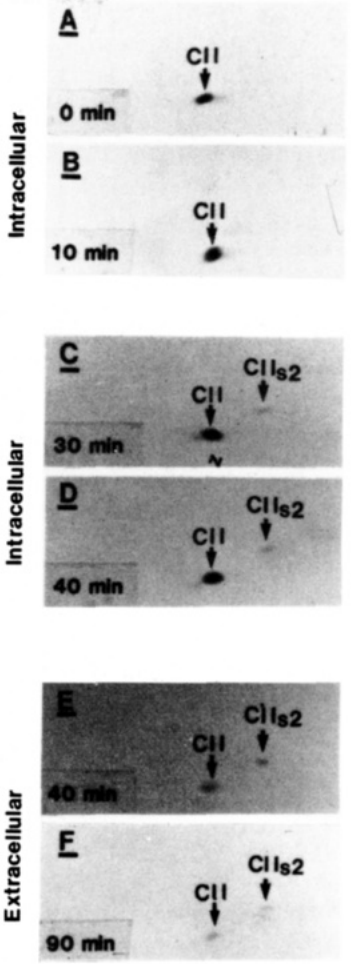

FIGURE 6: Pulse–chase labeling of human apoCII. HepG2 cells were labeled with [^{35}S]methionine for 5 min and chased for the indicated times. ApoCII was immunoprecipitated from the cell lysate and the culture medium with anti-human apoAII and analyzed by two-dimensional isoelectric focusing/polyacrylamide gradient gel electrophoresis and autoradiography as described under Materials and Methods. The figure shows the autoradiogram obtained from this analysis. Panels A–D show the intracellular apoCII forms, and panels E and F show the secreted apoCII forms. The positions of the isoproteins are indicated. Note that apoCII is synthesized as a single protein which is modified intracellularly and that both apoCII forms are secreted into the medium.

Intracellular Modification and Secretion of Human ApoCII. To further confirm the intracellular origin of apoCII isoproteins, we performed pulse–chase experiments using HepG2 cells. The [^{35}S]methionine-labeled intracellular and secreted apoCII isoproteins were immunoprecipitated with monospecific antiserum and analyzed by two-dimensional isoelectric focusing/polyacrylamide gradient gel electrophoresis and autoradiography. A 5-min pulse revealed a single intracellular form of apoCII (Figure 6A). A 30–40-min chase resulted in the appearance of another isoprotein designated apoCII$_{s2}$ (Figure 6C,D). The secreted apoCII consisted of two isoproteins apoCII and apoCII$_{s2}$ (Figure 6E,F). These results suggest that apoCII is partially modified intracellularly. Both unmodified and modified apoCII isoproteins are secreted into the medium. Similar phase–chase experiments showed that apoCIII is synthesized and modified intracellularly to apoCIII$_{s2}$ (data not shown). Only modified apoCIII was secreted into the medium (Figure 5C).

Isoelectric Points and Charge Relationship of ApoAII, ApoCII, and ApoCIII Isoproteins. The comparison of nascent and plasma apoAI, apoAII, apoCII, and apoCIII isoproteins described in Figure 5 allowed estimation of the relative iso-

Table I: Apparent Isoelectric Points of Different Isoproteins of ApoAII, ApoCII, and ApoCIII

isoprotein	isoelectric point	charge difference from corresponding plasma forms
$apoAI_2$ (proapoAI)	6.00	1
$apoAI_4$ (plasma apoAI)	5.64	0
preproapoAII	5.75	3
$apoAII_3$ (proapoAII)	5.75	3
$apoAII_1$ (modified proapoAII)	5.3	1
$apoAII_0$ (plasma apoAII)	4.95	0
$apoAII_{-1}$ (disialo-modified proapoAII)	4.80	−1
$apoAII_{-2}$	4.65	−2
$apoAII_{-3}$ (tetrasialo-modified proapoAII)	4.50	−3
$apoAII_{-4a}$	4.35	−4
apoCII (plasma apoCII)	4.82	0
$apoCII_{s2}$ (disialo-apoCII)	4.50	−2
apoCIII (asialo-apoCIII)	4.95	0
$apoCIII_{s1}$ (monosialo-apoCIII)	4.80	−1
$apoCIII_{s2}$ (disialo-apoCIII)	4.65	−2

electric points and charge differences of the various apoAII, apoCII, and apoCIII isoproteins, and the results are shown in Table I. The charge difference between apoAII isoproteins estimated by this method, and by carbamylation of $apoAII_1$ (data not shown), indicated that apoAII isoproteins 3, 1, −1, −2, −3, and −4a differ from plasma apoAII ($apoAII_0$) by +3, +1, −1, −2, −3, and −4 charges, respectively (Table I). ApoCII and $apoCII_{s2}$ differed from plasma apoCII by 0 and −2 charges. ApoCIII, $apoCIII_{s1}$, and $apoCIII_{s2}$ differed from plasma apoCIII by 0, −1, and −2 charges, respectively.

Effect of Neuraminidase Treatment on ApoAII, ApoCII, and ApoCIII Isoproteins. In order to determine whether the acidic isoproteins of apoAII, apoCII, and apoCIII result from glycosylation, we treated the culture medium of HepG2 cells containing the [^{35}S]methionine-labeled apolipoproteins with *Clostridium perfringens* neuraminidase. ApoAII, apoCII, apoCIII, and apoE were immunoprecipitated from neuraminidase-treated and untreated medium and analyzed by two-dimensional isoelectric focusing/polyacrylamide gradient gel electrophoresis and autoradiography. This treatment converted the acidic apoAII isoproteins to $apoAII_{1a}$ and $apoAII_0$ isoproteins, but not to $apoAII_3$ (Figure 7A,B). Neuraminidase treatment converted $apoCII_{s2}$, $apoCIII_{s2}$, and $apoE_s$ to apoCII, apoCIII, and apoE, respectively (Figure 7C–F). These findings suggest that the acidic forms of apoAII, apoCII, and apoCIII result from posttranslational modification with carbohydrate chains containing sialic acid residues.

Proposed Pathway of Intra- and Extracellular Modification of Human ApoAII, ApoCII, and ApoCIII. Figure 8A–C shows a schematic representation of the intra- and extracellular modifications of human apoAII, apoCII, and apoCIII. The proposed pathway of modification of apoAII is consistent with the data of Figures 1–7 and represents one possible mechanism by which apoAII isoproteins can be generated.

Figure 8B,C, shows that apoCII and apoCIII, respectively, are synthesized as single isoproteins, are modified intracellularly with carbohydrate chains containing two sialic acid residues, and are subsequently desialylated in plasma.

Determination of the Sites of ApoAII mRNA Synthesis. The sites of apoAII mRNA synthesis were determined by blotting analysis of RNA isolated from fetal human and adult monkey tissues as well as from cell lines of human origin. This analysis showed that apoAII mRNA is present in human and monkey liver and HepG2 cells. Traces of apoAII mRNA were also detected in human intestine (Figure 9). Dot blot analysis of the intestinal and hepatic apoAII mRNA indicated that the

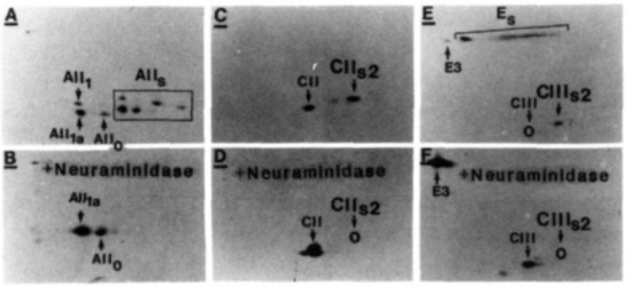

FIGURE 7: Neuraminidase treatment of secreted apoAII, apoCII, apoCIII, and apoE isoproteins. Culture medium obtained from one 60-mm culture of HepG2 cells grown for 4 h in [^{35}S]methionine-containing medium was adjusted to 0.1 M sodium acetate, pH 5.5. Half of the sample was treated with *Clostridium perfringens* neuraminidase as described under Materials and Methods, and half was used as a control. ApoAII, apoCII, and apoCIII were precipitated individually from 300 μL of the medium as described under Materials and Methods from the neuraminidase-treated and the control sample, and the precipitates were analyzed by two-dimensional isoelectric focusing/polyacrylamide gradient gel electrophoresis and autoradiography. Panels A, C, and E show autoradiograms corresponding to the untreated and panels B, D, and F corresponding to neuraminidase-treated samples, respectively. Note that the neuraminidase treatment converts $apoE_{3s}$ to apoE3, $apoCII_{s2}$ to apoCII, $apoCIII_{s2}$ to apoCIII, and apoAII isoprotein to $apoAII_{1a}$ and $apoAII_0$ and not to $apoAII_3$ isoproteins.

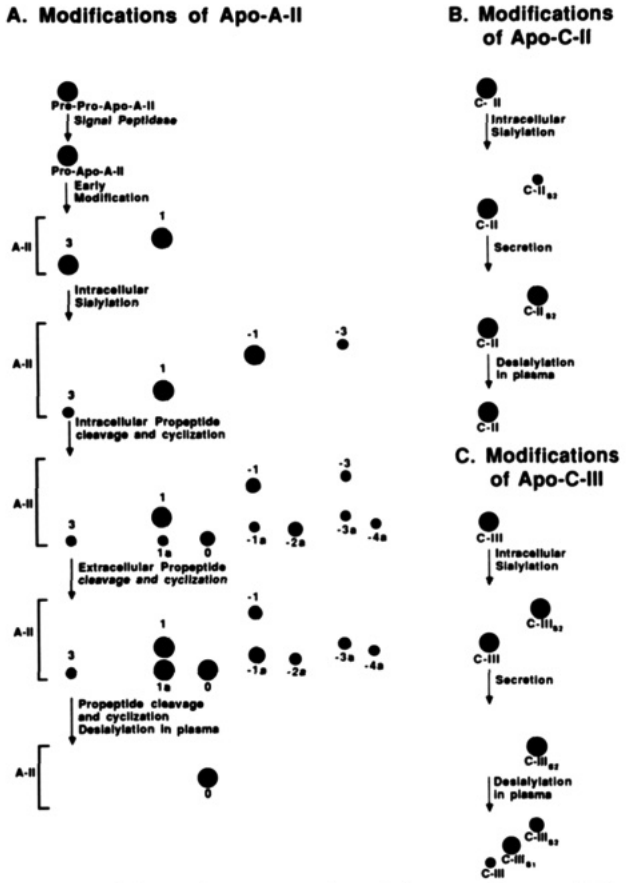

FIGURE 8: Schematic representation of intra- and extracellular modifications of human apoAII, apoCII, and apoCIII. Panel A shows a schematic representation of the intracellular and extracellular modifications of human apoAII. Panels B and C show the intra- and extracellular modification of human apoCII and apoCIII, respectively.

steady-state apoAII mRNA levels of the fetal intestine are less than 0.5% of that found in liver (expressed per milligram of total RNA). The steady-state apoAII mRNA levels of HepG2 cells are approximately 40% of that found in the human liver. ApoAII mRNA was undetectable in a variety of other tissues and cells including adrenal, brain, spleen, ovary,

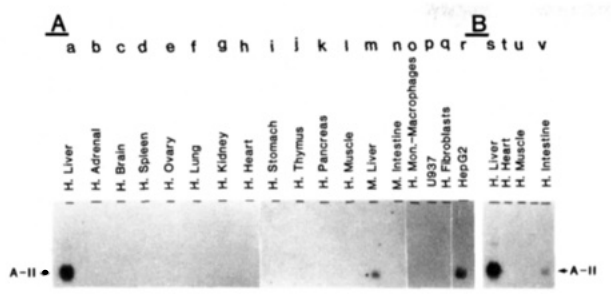

FIGURE 9: Blotting analysis of RNA isolated from various human tissues, monkey liver and intestine, and various cell lines of human origin. H indicates fetal human, and M indicates monkey mRNA samples. Collection of the fetal human tissues was performed by approved protocols in 1981 as described (Zannis et al., 1982). (Panel A) Lanes a–l contain 20 μg of total RNA obtained from the following tissues: (a) liver; (b) adrenal gland; (c) brain; (d) spleen; (e) ovaries; (f) lung; (g) kidney; (h) heart; (i) stomach; (j) thymus; (k) pancreas; (l) muscle. Lanes m and n contain 20 μg of total RNA obtained from cynomolgus monkey liver and intestine, respectively. Lanes o and p contain 5 and 10 μg of total RNA obtained from 8-day cultures of peripheral blood human monocyte–macrophages and U937 human macrophage like cell line. Lanes q and r contain 20 μg of total RNA obtained from SV40-transformed human fibroblasts and HepG2 cells, respectively. (Panel B) Lanes s, t, u, and v contain 20 μg of RNA obtained from fetal human liver, heart, muscle, and intestine, respectively. The autoradiogram of panels A and B were exposed for 2 and 15 days, respectively.

lung, kidney, heart, stomach, thymus, pancreas, muscle human monocyte macrophage culture (MM), macrophage line U937 cells, and cultured human skin fibroblasts. Control experiments showed that the apoE and/or apoCIII mRNAs of the tissues lacking apoAII mRNA were intact. Organ cultures of human and monkey intestine and liver showed apoAII synthesis by the hepatic cultures but failed to detect synthesis by intestinal cultures. However, apoAII could be detected in the culture medium of the human carcinoma cell line CaCo2 (data not shown).

Discussion

Description of Intracellular and Extracellular Modifications of Human ApoAII. Apolipoprotein A-II is a protein of known primary structure (Brewer et al., 1972) but unknown function. It is secreted as a proform containing 82 amino acids and is converted to the mature form by proteolytic cleavage of a 5 amino acid long prosegment (Gordon et al., 1984).

In the present study, we observed that proapoAII and preproapoAII migrate similarly in nonequilibrium pH gradient gel electrophoresis system. This is consistent with the published sequence of the apoAII signal peptide, which contains one positive and one negative charge (Knott et al., 1984; Lackner et al., 1984; Sharpe et al., 1984; Tsao et al., 1985). Thus, removal of signal peptide does not change the charge of proapoAII as compared to preproapoAII. Pulse–chase studies also demonstrate several subsequent intracellular modifications of apoAII. The earliest of these to appear is the conversion of proapoAII (apoAII$_3$, pI = 5.75) to a modified form of proapoAII (designated apoAII$_1$, pI = 5.3). We have estimated by charge calibration experiments that the relative loss of charge going from the unmodified proapoAII (pI = 5.75) to the modified proapoAII (pI = 5.3) was approximately two positive charges. Such loss of positive charges could be achieved by a variety of posttranslational modifications including sialylation, acylation, or alkylation of the amino group of Lys and ADP-ribosylation of Arg (Uy & Wold, 1977; Wold, 1981). The quantities of apoAII produced by HepG2 cells do not allow chemical characterization of this early apoAII modification. However, this may become feasible in the future by generation through transfection of new cell lines producing large amounts of this protein.

The proapoAII form subsequently undergoes several additional modifications. The pulse–chase experiments of Figures 1 and 2 and the neuraminidase treatment experiments of Figure 7 suggest that glycosylation of apoAII$_1$ provides apoAII$_{-1}$ and apoAII$_{-3}$ isoproteins which contain two and four sialic acid residues, respectively. The [^{3}H]Arg labeling experiment of Figure 3 suggests that forms 1a, −1a, and −3a result from removal of the propeptide of the higher molecular weight apoAII isoproteins. The present data do not allow us to establish the exact precursor–product relationship of the proapoAII forms, 1, −1, and −3, with the lower molecular weight forms, 1a, −1a, and −3a. Finally, we propose that the low molecular weight isoproteins, 1a, −1a, and −3a, undergo cyclization of the N-terminal glutamine. This modification has been documented previously in the plasma apoAII form (isoprotein O; Brewer et al., 1972). Cyclization is expected to decrease the positive charge of the apoAII isoproteins by 1 without changing the molecular weight, resulting in the conversion of 1a, −1a, −3a isoproteins to 0, −2a, and −4a, respectively. The finding that significant quantities of isoproteins 0 and −2a accumulate intracellularly suggests either that the major portion of isoprotein 2 arises from intracellular sialylation of isoprotein 0 or that intracellular cyclization occurs rapidly. Rapid intracellular cyclization is consistent with recent findings suggesting that the cyclization is an enzymatic process (Busby et al., 1987). Furthermore, the nonsialylated (isoprotein 0) and the sialylated (isoprotein −2a) forms are both observed after 12–17-min labeling, suggesting that cyclization and sialylation occur in the same subcellular compartment, possibly the Golgi apparatus.

The desialylation of −1a, −2a, −3a, and −4a results in the generation of isoproteins 1a and 0 (Figure 7A,B) but not of isoprotein 3. This suggests that the conversion of apoAII$_3$ to apoAII$_1$ may not involve addition of sialic acid residues. The plasma form of apoAII (isoprotein 0) could then be produced by a series of intracellular processes involving propeptide cleavage and cyclization of the N-terminal glutamine. The plasma form of apoAII (isoprotein 0) is then secreted into the extracellular space, however. The various nascent modified apoAII isoproteins are also secreted into the extracellular space, suggesting that the plasma form could also be produced by the same series of extracellular processes involving propeptide cleavage and cyclization as well as by desialylation. Thus, following propeptide cleavage, the cyclization of the lower molecular weight apoAII isoproteins, 1a, −1a, and −3a, would generate isoproteins 0, −2a, and −4a. Plasma apoAII could then be formed by desialylation of isoproteins −2a and −4a. Isoproteins 1a, −1a, and −3a accumulate in appreciable quantities extracellularly, suggesting that the extracellular cyclization is a slower process. This is consistent with recent findings showing that the nonenzymatic cyclization of Gln in model peptides is 160-fold slower than the enzymatic process (Busby et al., 1987).

Extent of Intra- and Extracellular Processing of Human ApoAII: Isoproteins. The observation of intracellular lower molecular weight forms 1a, 0, −1a, and −2a (Figure 1D,E and Figure 2E–H) suggests that the proapoAII to apoAII conversion occurs in part intracellularly. This notion is supported further by the observation that the apoAII secreted after 12-min pulse and 25-min chase (Figure 1E) consists of approximately 50% of the lower molecular weight isoproteins 1a, 0, −1a, and −3a which lack the prosegment. The same conclusion was originally reached by Gordon et al. (1983) but was revised later to suggest almost exclusive extracellular proa-

poAII to apoAII conversion by a proteolytic activity secreted by HepG2 cells (Gordon et al., 1984). As discussed earlier, a significant portion of plasma apoAII results from extracellular propeptide cleavage and desialylation of the acidic apoAII isoproteins which were not accounted for in the previous studies (Gordon et al., 1983, 1984). The pulse–chase experiments with HepG2 cells indicated time-dependent depletion of the secreted higher molecular weight forms 1, −1, and −3 coupled with the concomitant accumulation of the corresponding lower molecular weight forms 1a, −1a, and −3a. These data are consistent with the hypothesis that the pro-apoAII to apoAII conversion occurs partially extracellularly.

Intracellular and Extracellular Modifications of Human ApoCII and ApoCII: Comparison of ApoAII, ApoCII, and ApoCIII Isoproteins. Analysis of the isoprotein patterns of human apoCII and apoCIII following treatment with neuraminidase indicated that both proteins are glycosylated with carbohydrate chains containing two sialic acid residues. Similar modifications have been described previously for apoE (Zannis et al., 1986). O-Linked carbohydrate chains containing GalNac-linked either Gal, GalNac, or GlucNac and sialic acid have been described in a variety of human glycoproteins (Cummings et al., 1983; Hounsell et al., 1985). ApoCII is secreted both as nonsialylated ($apoCII_0$) and as disialylated ($apoCII_{s2}$) isoprotein whereas apoCIII is secreted exclusively as the disialylated ($apoCIII_{2s}$) isoprotein. None of the apoCII or apoCIII isoproteins overlap on the two-dimensional gels with the apoA-II isoproteins.

Three apoCIII isoproteins ($apoCIII_0$, $apoCIII_{s1}$, and $apoCIII_{s2}$) have been described in human plasma. The observation that the nascent apoCIII consists of the disialylated ($apoCIII_{s2}$) form implies that the other two isoproteins ($apoCIII_0$ and $apoCIII_{s1}$) result from extracellular desialylation.

Four apoCII isoproteins have been described in human plasma by immunoblotting techniques (Fojo et al., 1986). Some of these forms may be the products of extracellular proteolysis of the nascent $apoCII_0$ and $apoCII_{s2}$ isoproteins.

The different degrees of sialylation observed in the various apoproteins (apoAII, apoE, apoCII, and apoCIII) may reflect different affinities of these proteins for the O-glycosylating enzymes of the Golgi complex. The observation that unmodified apoCII and apoAII isoproteins are secreted into the medium implies that modification of both proteins may not be a prerequisite for secretion.

Comparison of the kinetics of modification of apoAII with apoCII, apoCIII, and apoE showed that the $apoAII_3$ to $apoAII_1$ modification preceded the sialylation of apoCII, apoCIII, and apoE (compare Figure 1 with Figure 6), since sialylation of nascent proteins is known to occur in the trans Golgi complex (Hubbard & Ivatt, 1981; Lennarz, 1983; Kornfeld & Kornfeld, 1985). The first modification of apoAII may take place in a compartment earlier than the trans Golgi complex, possibly in the rough endoplasmic reticulum or the cis Golgi.

Physiological Significance of Apolipoprotein Modifications. It has been shown that the apoAII prosegment is cleaved extracellularly by a thiol protease (Gordon et al., 1984) which is immunologically related to cathepsin B (Gordon et al., 1985). Since the carboxy-terminal amino acid of the prosegment is Arg, one would expect that the removal of the prosegment would be mediated by a trypsin-like protease (Morihara, 1974). It is then possible that modifications of residues in the vicinity of the scissile bond may result in the cleavage of the prosegment by a thiol rather than by a trypsin-like serine protease. The role of the apoAII prosegment is not known. It is possible that the prosegment may serve as a signal for apoprotein targeting along the secretory pathway as well as for intra- (Folz & Gordon, 1986) or extracellular (Gordon et al., 1984) processing. The prosegment may also serve as a signal leading to the formation and maturation of nascent lipoprotein particles.

Sites of Synthesis of Human ApoAII. It has been proposed that the putative sialylation of apoAII is a tissue-specific modification occurring in intestinal but not in hepatic (HepG2) cells (Lackner et al., 1985). In the present study, we have analyzed the distribution of apoAII mRNA in fetal human tissues, in cell lines of human origin, and in adult cynomolgus monkey liver and intestine. Similar to previous findings, apoAII mRNA was present in fetal liver (Sharpe et al., 1984). However, RNA isolated from fetal intestine contained less than 0.5% of the apoAII mRNA levels found in liver, whereas apoAII mRNA was undetectable in adult cynomolgus monkey intestine. Organ cultures of fetal human and adult cynomolgus monkey intestine also failed to show apoAII synthesis (data not shown). These findings differ from previous reports suggesting that apoAII mRNA represents 0.2% of the translatable mRNA obtained from adult human jejunal epithelial cells (Gordon et al., 1983). However, we cannot rule out the possibility of active apoAII mRNA synthesis by intestinal epithelial or other intestinal cells under certain physiological conditions. ApoAII mRNA was undetectable in a variety of other tissues and cells including adrenal, brain, spleen, ovary, lung, kidney, heart, stomach, thymus, pancreas, muscle, human monocyte macrophage cultures (MM), macrophage line U937 cells, and cultured human skin fibroblasts. Considering the difference in mass and size between the liver and the intestine and the relative abundance of apoAII mRNA in the two tissues, we conclude that the contribution of the intestine to the overall plasma apoAII pool is very small.

Conclusions. On the basis of the present data and previous work by us (Zannis et al., 1981, 1982) and others (Brewer et al., 1972; Gordon et al., 1983, 1984), we propose that in humans apoAII is synthesized predominantly in liver. Following synthesis, apoAII undergoes a complex pattern of intracellular modifications. An early modification results in the loss of two positive charges. Subsequently, apoAII is glycosylated with O-linked carbohydrate chains containing two or four sialic acid residues. The various forms of apoAII are converted to the plasma form by intra- and extracellular proteolysis of the prosegment and N-terminal cyclization as well as by extracellular desialylation, proteolysis of the prosegment, and N-terminal cyclization. ApoCII and apoCIII are also modified with carbohydrate chains containing two sialic acid residues. ApoCII is desialylated totally in plasma whereas apoCIII is desialylated partially to give rise to the mono- and asialo-apoCIII forms. The physiological significance of the modifications of apoAII, apoCII, and apoCIII remains to be determined.

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