Intracellular processing of apolipoprotein J precursor to the mature heterodimer

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Abstract Circulating apolipoprotein J (apoJ) is a 70 kDa glycoprotein comprised of disulfide-linked α and β subunits derived from a single precursor. Post-translational modifications that occur prior to apoJ secretion were assessed, with specific focus on carbohydrate type, the timing of proteolytic cleavage, and the importance of glycosylation on the cleavage and secretion processes. ApoJ was initially resolved as a single chain, intracellular precursor of 58 kDa which contained N-linked oligosaccharide but no O-linked oligosaccharide. The precursor was converted to an intracellular 70 kDa glycoprotein, which became the major intracellular form of apoJ prior to secretion. Maturation of the 58 kDa precursor involved conversion of high-mannose carbohydrate to complex-type carbohydrate containing sialic acid, as well as intracellular cleavage to yield α and β subunits. This cleavage event occurred at a late stage of carbohydrate modification, most likely in the *trans*-Golgi or a post-Golgi compartment. The maturation and secretion of apoJ occurred rapidly, with a half-time of 30–35 min. Tunicamycin treatment of cells resulted in an unglycosylated doublet comprised of one single chain and one cleaved form of apoJ. The unglycosylated apoJ species were secreted rapidly with a half-time of 20 min. Both cleavage and secretion were independent of glycosylation. **—Burkey, B. F., H. V. deSilva, and J. A. K. Harmony.** Intracellular processing of apolipoprotein J precursor to the mature heterodimer. *J. Lipid Res.* 1991. **32:** 1039–1048.

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Human apolipoprotein J (apoJ) is a 70 kDa plasma glycoprotein in high density lipoproteins (HDL) (1). The lipoprotein association of apoJ suggests that it plays a role in lipid metabolism, although other functions of apoJ have been proposed. The rat apoJ homolog, sulfated glycoprotein-2 (SGP-2), the major secretory product of Sertoli cells (2), is present in seminiferous tubule fluid and attached to spermatozoa (3). SGP-2 levels are dramatically incraesed during prostate gland regression (4) and in hydronephrotic kidney injury (5), models of programmed cell death. SGP-2 thus appears to be important in spermatogenesis, and in the regulation of tissue damage and cellular injury. Recently, apoJ (serum protein-40,40, SP-40,40) was isolated from human plasma associated with the sC5b-9 complex (6). ApoJ is a potent inhibitor of terminal complement lysis (7), suggesting a link between lipid metabolism and the immune system with a unique role for HDL as a carrier of a protein with a potentially important immunoregulatory role.

In comparison to known apolipoproteins, the structure of apoJ is unique. ApoJ is a disulfide-linked heterodimer, consisting of two nonidentical subunits, apoJα (34–36 kDa) and apoJβ (36–39 kDa). Approximately 30% of the mass of each subunit is carbohydrate (8). The subunits are encoded by a single 1.9 kb transcript, and the translated protein undergoes cleavage between amino acid residues Arg-205 and Ser-206 to generate the subunits. The deduced amino acid sequence of apoJ reveals seven potential N-glycosylation sites, three in apoJα and four in apoJβ, and a cluster of five Cys residues in each subunit (9). All ten of the Cys residues are involved in disulfide bonds (10). The structure of apoJ is reminiscent of that of several complement proteins, especially C3 and C4, which are disulfide-linked hetero-oligomers produced by proteolytic cleavage of a precursor protein (11, 12).

The goal of this study was to determine the sequence of post-translational events that occur prior to apoJ secretion. We were specifically interested in learning whether apoJ carbohydrate is N- or O-linked, whether cleavage of apoJ occurs intra- or extracellularly, and whether glycosylation influences the cleavage process. The timing of the cleavage reaction has important implications in the deduction of apoJ function. Since the apoJ mRNA level is relatively high in human liver (9), the hepatoma cell line HepG2 was utilized as a model system to study the post-synthetic maturation of apoJ and the roles that glycosylation and proteolytic cleavage play in its production. We show that apoJ contains N-linked carbohydrate

Abbreviations: HDL, high density lipoproteins; apoJ, apolipoprotein J; SGP-2, sulfated glycoprotein-2; SP-40,40, serum protein 40,40; MEM, minimal essential medium; FBS, fetal bovine serum; PDB, plasma density buffer; SDS, sodium dodecyl sulfate.

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that is processed from a high mannose to complex-type oligosaccharide, that the proteolytic cleavage of the precursor protein into its two subunits occurs late in the intracellular trafficking sequence, and that inhibition of carbohydrate addition has little influence on the secretion of apoJ from liver cells.

METHODS

Cell culture

The human hepatoma cell line HepG2 (13) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured in 25-cm^2 tissue culture flasks (Costar, Cambridge, MA) in Eagle's Minimal Essential Medium (MEM) (Whittiker Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hazelton, Lenexa, KS) and 4 mM L-glutamine (Sigma Chemical Co., St. Louis, MO). Cultures were maintained at 37°C in a humidified incubator in 5% CO_2–95% air. Cells were passaged, using 0.25% trypsin (Whittiker Bioproducts), and reseeded at a density of 1×10^6 cells per 25-cm^2 flask. Experiments were initiated when cell monolayers reached >95% confluency.

ApoJβ antibody

ApoJβ was purified to homogeneity by reverse phase-HPLC (8), and antibodies were raised in a young New Zealand White female rabbit (1.5 kg body weight). The purity of the antigen was confirmed by NH_2-terminal sequence analysis, by SDS-PAGE analysis, and by its failure to react with apoJα specific monoclonal antibodies (8). The preimmune serum of this rabbit showed no reactivity toward human plasma proteins by immunoblot analysis. A bolus injection of apoJβ, 60 μg in complete Freund's adjuvant (Gibco, Grand Island, NY), was administered intramuscularly on day 1. After 4 and 8 weeks, the animal was boosted subcutaneously with the antigen, 20 μg in incomplete Freund's adjuvant. Antiserum was obtained 7, 10, and 13 days post-reinoculation, and the antibody was purified. Immunoglobulin was isolated by precipitation in 50% $(NH_4)_2SO_4$. The precipitate was dialyzed into 20 mM Tris-HCl, pH 7.2, 20 mM NaCl, and chromatographed on DEAE-Affi-Gel Blue (Bio-Rad, Rockville Centre, NY) (14). Protein bound to the column was eluted with a stepwise NaCl gradient (20–500 mM). Fractions containing predominantly pure immunoglobulin, as determined by SDS-PAGE, were pooled, dialyzed into 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA, and then stored at −20°C.

Electroimmunoblots

HepG2 cells cultured in 75-cm^2 flasks were incubated for the final 24 h of culture in serum-free MEM plus 4 mM L-glutamine. The conditioned medium was collected, cooled to 4°C, and concentrated 100-fold using an Amicon ultrafiltration apparatus with a YM 10 membrane (Amicon, Beverly, MA), then concentrated an additional 10-fold using a Centricon 10 (Amicon). Human plasma was diluted one part in four with 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 5 mM EDTA. Each sample was mixed with an equal volume of 2× SDS-PAGE sample buffer (40 mM Tris-HCl, pH 6.8, 4% SDS, 20% sucrose, 8 M urea, and 4 mM EDTA), and heated to 90°C for 10 min. The samples were electrophoresed (15) in a 10% acrylamide gel for 2 h at 15 mA, then transferred at 4°C to nitrocellulose (Hoefer Scientific Instruments, San Francisco, CA) by electroblotting for 2.5 h at 250 mA in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% methanol. Membranes were incubated with purified apoJβ antibody (1:5000 dilution) for 15 h in blotto (16) (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5% nonfat dry milk, 0.01% (v/v) antifoam A, and 0.001% merthiolate), washed thoroughly with blotto, incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad), and developed in blotto containing 0.8 mg/ml NiCl, 1.6 mg/ml diaminobenzamidine, and 0.2% H_2O_2.

Metabolic labeling of HepG2 proteins

HepG2 monolayers were, sequentially, washed twice with serum-free MEM, incubated in serum-free MEM for 1 h, incubated in serum-free MEM minus methionine (Flow Laboratories, McLean, VA) for 20 min, and then pulsed with [^{35}S]methionine (Amersham, Arlington Heights, IL) (>1,200 Ci/mmol) at 200 μCi/ml of MEM minus methionine/25-cm^2 flask. After 15 min, the labeling medium was removed, the cells were washed once with MEM and incubated in 1 ml of serum-free MEM containing a 10-fold excess (150 μg/ml) of unlabeled methionine (Sigma). At the end of the specified chase period, culture medium was collected, and deoxycholate (Sigma) and Triton X-100 (Sigma) were added to a final concentration of 0.5% each. Monolayers were washed once with cold phosphate-buffered saline (PBS), cells were lysed in PBS containing 0.5% each of deoxycholate and Triton X-100, and cell lysated were collected. Culture medium and cell lysate fractions were stored at −20°C.

Immunoprecipitation of apoJ and apoA-I

Samples of medium or cell lysate were centrifuged at 2000 *g* to remove cell debris. Immunoprecipitation reactions were performed in 1.5-ml microfuge tubes. Reaction mixtures contained 200–500 μl of culture medium or cell lysate, 20–40 μl of rabbit anti-apoJβ or 10 μl of rabbit anti-apoA-I (Calbiochem, San Diego, CA), 500–750 μl of plasma density buffer (PDB) (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 5 mM EDTA), 50 μl of protein A-Affi-Gel (Bio-Rad) (wet agarose: PDB ratio, 1:2 (v/v)), and

0.5% each of deoxycholate and Triton X-100. Each reaction was incubated at 4°C overnight with gentle mixing. After incubation, the protein A–Affi-Gel complex was collected by centrifugation (100 *g*), and successively washed in the following buffers: once in buffer #1 (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 140 mM NaCl, 1 mg/ml of bovine serum albumin (Sigma), 1 mg/ml of methionine (Sigma), 0.5% Triton X-100, 0.5% deoxycholate); once in buffer #2 (100 mM $NaHCO_3$, pH 7.5, 500 mM NaCl, 2 mM EDTA); and twice in buffer #3 (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.02% SDS). ApoJ or apoA-I, solubilized by boiling the agarose pellet for 5 min in 1× SDS-PAGE sample buffer, was electrophoresed in polyacrylamide gels in the presence of SDS; the gels were soaked in Enlightening (DuPont, Wilmington, DE), dried, and exposed to Kodak X-OMAT film.

To quantitate radioactive proteins resolved by SDS-PAGE, bands were cut from dried gels and enough 30% H_2O_2 was added to cover the gel pieces. After 12 h at room temperature, 5 ml of Scintiverse scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was added and samples were vortexed periodically until the gel pieces dissolved. Radioactivity was determined with a Beckman LS3801 scintillation counter.

The reported percentages reflect the cpm of immunoprecipitated apoJ or apoA-I, compared to cpm of trichloroacetic acid-precipitable protein from equal volumes of 1-h chase culture medium. The percentages were adjusted to account for the methionine content of each protein.

Incubation of apoJ with glycosidases

Samples (300 μl) of culture medium or cell lysate were incubated for 16 h at 37°C with exoglycosidases. Amounts of the enzymes used were as follows: 1,000 mU of neuraminidase (Gibco Laboratories), 20 μU of endoglycosidase H (Boehringer Mannheim, Indianapolis, IN), 0.5 μU of N-glycanase (Genzyme, Boston, MA), or 2 mU of O-glycanase (Genzyme). Since O-glycanase cleavage requires the removal of sialic acid prior to the cleavage of O-linked sugars (17), samples were treated with neuraminidase for 10 h prior to O-glycanase addition. ApoJ was immunoprecipitated as described.

Treatment of HepG2 cells with tunicamycin or monensin

Tunicamycin (Calbiochem, San Francisco, CA) was dissolved in 25 mM NaOH to yield a stock solution of 2.5 mg/ml. Confluent monolayers of HepG2 cells were washed twice and incubated for 1 h in serum-free MEM. Cells were then incubated with 15 μg/ml tunicamycin for 70 min prior to metabolic labeling. Tunicamycin was maintained in cultures throughout the metabolic labeling. Monensin (Calbiochem) was dissolved in 100% ethanol to yield a stock solution of 1 mg/ml. Confluent monolayers were treated as above except cells were pretreated with 3 μg/ml of monensin for 30 min prior to metabolic labeling. ApoJ was immunoprecipitated as described.

RESULTS

HepG2 cells produce apoJ

To determine whether HepG2 cells produce apoJ similar to that found in plasma, secreted HepG2 apoJ was compared to human plasma apoJ by immunoblot analysis (**Fig. 1**). Proteins in human plasma and in concentrated HepG2 culture medium were resolved in SDS-PAGE under nonreducing and reducing conditions. Human plasma apoJ consisted of 70 kDa and 35 kDa proteins under nonreducing and reducing conditions, respectively, confirming the report of deSilva et al. (1). HepG2 apoJ was similar to plasma apoJ under both conditions, except that the immunoreactive protein bands were broader and had slightly higher average molecular masses.

Metabolically labeled HepG2 apoJ was isolated from cell lysate and culture medium fractions by immunoprecipitation, using apoJβ-specific antibody (**Fig. 2**). When resolved by 10% SDS-PAGE under nonreducing conditions, cell lysate contained two proteins of 58 and 70 kDa (top panel). In contrast, culture medium contained a single apoJ protein of 70 kDa (bottom panel). The immunoprecipitation was specific for apoJ since purified apoJ from human plasma, but not albumin, competed with both the labeled 58 and 70 kDa HepG2 apoJ in the im-

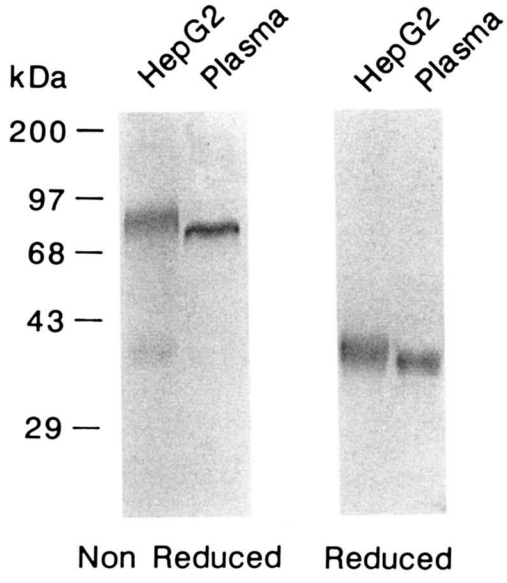

Fig. 1. HepG2 and plasma apoJ are similar. ApoJ in concentrated HepG2 culture medium (100 μg protein) and in human plasma (80 μg protein) were compared by immunoblotting after electrophoresis in the absence or presence of 1% β-mercaptoethanol. Proteins transferred to nitrocellulose were immunoblotted with rabbit anti-apoJβ, and visualized with HRP-conjugated goat anti-rabbit antibody.

munoprecipitation reaction. This result further established the similarity between human plasma and HepG2 apoJ. Preimmune serum did not remove a 58 or 70 kDa protein from cell lysate or culture medium. The amount of apoJ secreted by HepG2 cells in 1 h was 0.5% of the total secreted TCA-precipitable protein. ApoA-I, which is 5.4% of the total secreted protein, is therefore about 10 times more concentrated in HepG2 medium.

ApoJ is rapidly secreted

To assess the relationship of the intracellular 58 kDa and 70 kDa forms of apoJ and the relationship of each to the 70 kDa secreted apoJ, a pulse-chase kinetic study was performed (**Fig. 3**). HepG2 cells were pulsed for 15 min with [^{35}S]methionine, then chased with medium containing a 10-fold excess of unlabeled methionine. ApoJ, immunoprecipitated from cell lysate (top panel) and culture medium (bottom panel), was analyzed by SDS-PAGE. In cell lysate at zero chase time, apoJ was predominantly 58 kDa. With increasing chase the amount of radiolabeled 58 kDa apoJ decreased as that of the 70 kDa apoJ increased; by 45 min the 70 kDa protein was the predominant intracellular form of apoJ. The disappearance of the 58 kDa form of apoJ with concomitant appearance and accumulation of the 70 kDa form suggests a precursor-product relationship between the two. Neither the

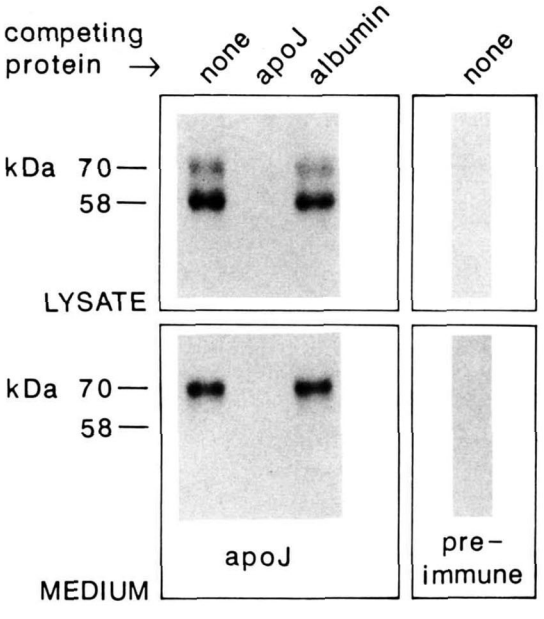

Fig. 2. Immunoprecipitation of apoJ; specificity of anti-apoJβ. ApoJ was immunoprecipitated from 20-min chase cell lysate (300 μl) (top panel), and 45-min chase culture medium (300 μl) (bottom panel). HepG2 cells were pulse-labeled for 15 min with [^{35}S]methionine (200 μCi/ml). Immunoprecipitation was performed in the absence of competing protein or in the presence of 40 μg of purified apoJ or 30 μg of human albumin (Sigma). Rabbit preimmune antiserum (20 μl) was used as a control.

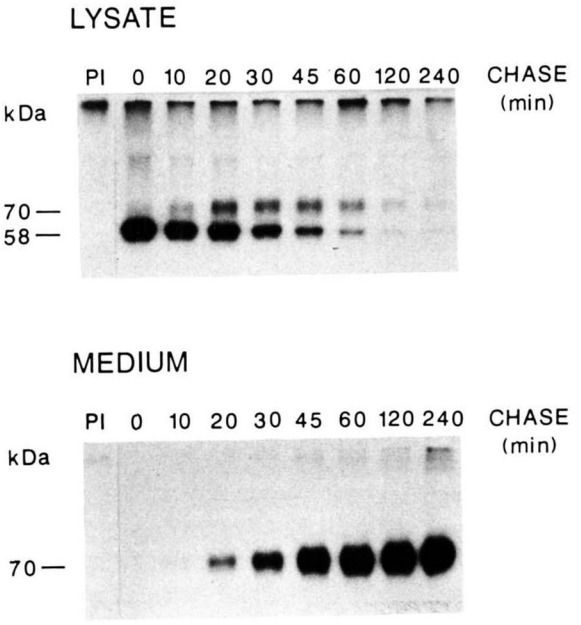

Fig. 3. Secretion of apoJ by HepG2 cells. ApoJ from cell cultures of increasing chase time was analyzed by SDS-PAGE. Chase time in min is indicated above each lane. ApoJ was immunoprecipitated from 200 μl of cell lysate or culture medium. The immunoprecipitates was resolved by SDS-PAGE under nonreducing conditions, followed by fluorography. The PI lane represents the control immunoprecipitation from 30-min chase cell lysate or 30-min chase culture medium, using 20 μl of preimmune serum.

radiolabeled 58 nor 70 kDa forms of apoJ were detected in cell lysates after 60 min of chase. In the culture medium 70 kDa apoJ was present within 30 min, and its amount increased through 120 min. ApoJ was secreted with a half-time of 30–35 min (**Fig. 4,** top). This secretion rate was comparable to that of apoA-I (Fig. 4, bottom), which is also rapidly secreted from HepG2 cells (18).

ApoJ is a glycoprotein with N-linked carbohydrate

To test the hypothesis that the maturation of the 58 kDa precursor involves addition and modification of carbohydrates, labeled proteins from cell lysate and culture medium were subjected to glycosidase digestion prior to apoJ immunoprecipitation (**Fig. 5**). Endoglycosidase H, an enzyme specific for the removal of high mannose-type oligosaccharides (19), reduced the size of the intracellular 58 kDa form of apoJ to approximately 45 kDa. In contrast, the sizes of both the intracellular and secreted 70 kDa forms of apoJ were not affected by endoglycosidase H. Neuraminidase, which cleaves terminal sialic acid residues (20), did not alter the size of the intracellular 58 kDa form of apoJ, whereas the intracellular and secreted 70 kDa forms of apoJ were each reduced by 3–5 kDa. N-Glycanase effectively digested carbohydrate from every form of apoJ, reducing the size down to 40 to 45 kDa, whereas O-glycanase had no effect on apoJ migration. These results indicate that apoJ contains only N-linked

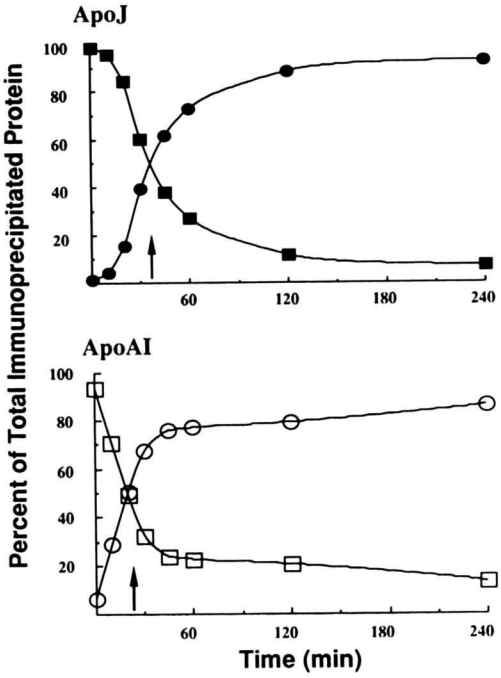

Fig. 4. Rates of secretion of apoJ and apoA-I. Secretion of apoJ (top panel) and apoA-I (bottom panel) during a pulse-chase experiment is shown. Immunoprecipitated apoJ or apoA-I, resolved by SDS-PAGE, was excised, solubilized, and the cpm of [^{35}S]methionine determined. Squares represent cell lysate fractions and circles represent culture medium fractions. The arrows designate the times at which 50% of the immunoprecipitable protein was both cell-associated and secreted.

oligosaccharides, which are converted from a high-mannose type to a complex-type carbohydrate containing sialic acids.

Proteolytic cleavage of apoJ precursor occurs just prior to secretion

To determine when proteolytic cleavage of the apoJ precursor into its subunits occurs in the secretory pathway, apoJ immunoprecipitated from cell lysates of increasing chase times was analyzed by SDS-PAGE under nonreducing and reducing conditions (**Fig. 6**). Under nonreducing conditions, apoJ showed the expected 58 kDa to 70 kDa transition as it traversed the cell to be secreted. Upon reduction, the 58 kDa form was not altered, while most but not all of the 70 kDa form was reduced to the 35 kDa subunits. This result suggests that proteolytic cleavage of apoJ occurs late in the trafficking pathway, at a point after which most of the carbohydrate has ben converted from high-mannose to complex-type. Almost all of the radiolabeled apoJ that was secreted into culture medium was converted to the 35 kDa subunits by reduction. A minor component of secreted apoJ (less than 5%) remained at 70 kDa. This may represent apoJ that was secreted without undergoing proteolytic cleavage or that was cleaved but failed to be reduced under the experimental conditions. In the presence of reductant, a minor 70 kDa form of apoJ is also detected in human plasma by electroimmunoblot (1).

The timing of proteolytic cleavage was determined more precisely by incubating HepG2 cells with monensin, a carboxylic ionophore that disrupts movement from medial to *trans*-Golgi cisternae (21). Cells were grown in the absence or presence of monensin, and apoJ was immunoprecipitated from lysate and medium fractions of increasing chase times and resolved by SDS-PAGE under nonreducing and reducing conditions (**Fig. 7**). Nonreduced apoJ (top panel) accumulated intracellularly as a heterogeneous mass ranging from 58 to 68 kDa. Secretion of apoJ in the presence of monensin occurred after a lag of 60 min. A similar lag in secretion time from monensin-treated HepG2 cells has been reported for apoE (22). The molecular mass of apoJ secreted by monensin-treated cells was about 2–3 kDa smaller than that secreted by control cells. To assess proteolytic cleavage, molecular masses were evaluated in the presence of reductant (bottom panel). The molecular mass range of lysate fractions was not altered by reduction, and only a minor portion of the secreted apoJ was reduced.

Tunicamycin inhibits N-linked glycosylation without preventing cleavage or secretion

The importance of glycosylation in the post-translational cleavage and secretion of apoJ was assessed with tunicamycin, an antibiotic that inhibits the addition of N-acetylglucosamine phosphate to dolichol phosphate and thus prevents N-linked oligosaccharide addition (23).

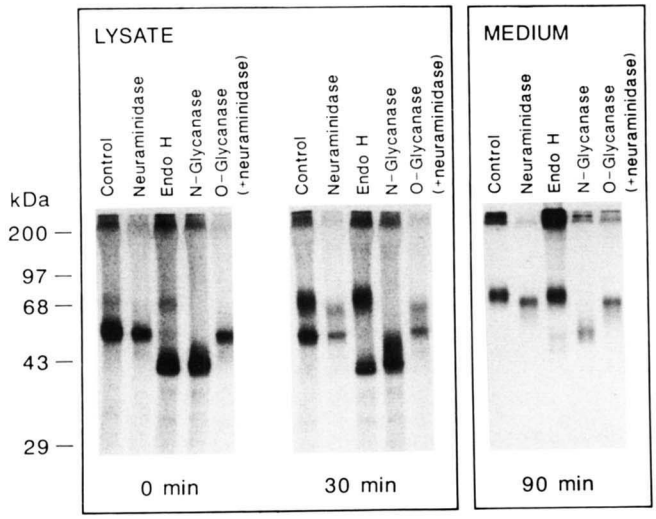

Fig. 5. Analysis of apoJ carbohydrate by exoglycosidase digestion. ApoJ was analyzed by SDS-PAGE after incubation with exoglycosidases. HepG2 cells were pulse-chased as described in Methods. Cell lysates and culture media were treated with exoglycosidases for 16 h at 37°C. ApoJ was immunoprecipitated from 0- and 30-min chase cell lysates (left) and from 90-min chase culture media (right) and analyzed by SDS-PAGE under nonreducing conditions, followed by fluorography.

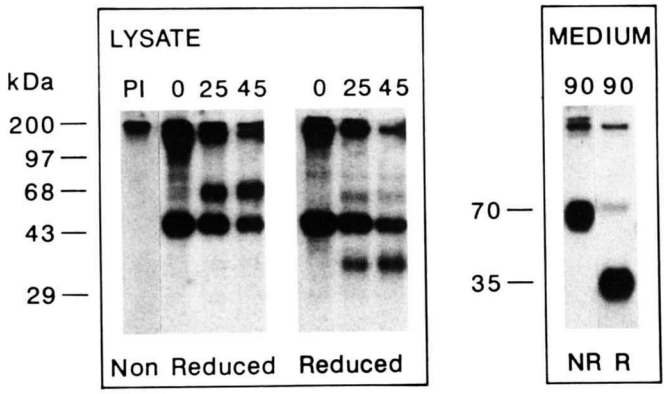

Fig. 6. Intracellular proteolytic cleavage of apoJ. Immunoprecipitates of apoJ were analyzed by SDS-PAGE under nonreducing or reducing conditions (1% β-mercaptoethanol). HepG2 cells were pulse-chase labeled and apoJ was immunoprecipitated from cell lysates and medium as described in Methods. Chase times (min) are indicated above each lane. Preimmune serum (PI) was used as a control.

HepG2 cells cultured in the presence of tunicamycin synthesized apoJ as an unglycosylated doublet (**Fig. 8**). Immediately after the [^{35}S]methionine pulse, the size of apoJ in cell lysate was predominantly 41 kDa. By 15 min, apoJ was a doublet of 41 and 43 kDa. The amount of this doublet decreased over the 60-min chase, and it was not detected in the cell after 60 min. Unglycosylated apoJ was secreted into the medium within 15 min and accumulated with increasing chase time. The rate of trafficking of unglycosylated apoJ in tunicamycin-treated HepG2 cells was 50% faster than in untreated cells, having a half-time of 20–25 min compared to 30–35 min for apoJ secreted from untreated cells (**Fig. 9**). The decrease in immunoprecipitable apoJ from tunicamycin-treated cells consistently paralleled a general decrease in overall protein secretion from tunicamycin-treated cells relative to control cells. Thus tunicamycin is not a specific inhibitor of apoJ secretion.

To determine whether tunicamycin, and hence the absence of glycosylation, alters apoJ subunit production, secreted apoJ was immunoprecipitated and evaluated by SDS-PAGE (**Fig. 10**). Unglycosylated apoJ secreted into HepG2 culture medium consisted of a 41 and 43 kDa doublet under nonreducing conditions. Upon reduction the 43 kDa form was resolved into proteins of 28 and 24 kDa, whereas the apparent molecular mass of the 41 kDa form remained unchanged. The size of the 28 and 24 kDa proteins corresponds to the calculated molecular masses of the β and α subunits, respectively.

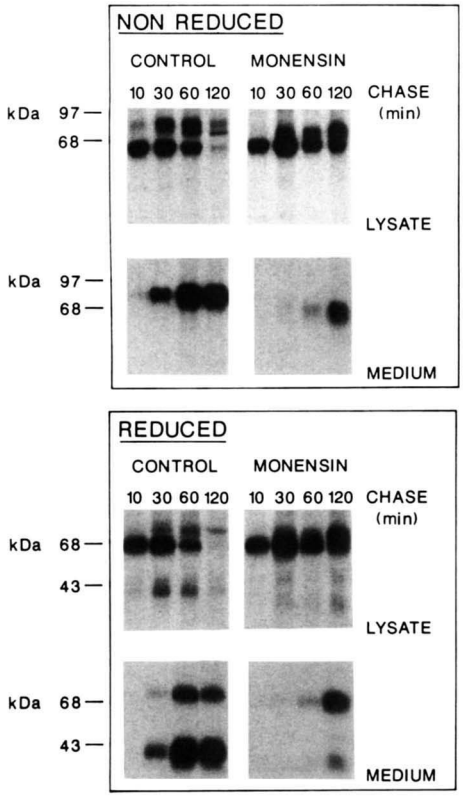

Fig. 7. The effect of monensin on the maturation and secretion of apoJ. ApoJ immunoprecipitated from HepG2 cells, cultured in the absence or presence of 3 μg/ml of monensin, was analyzed by SDS-PAGE. Cells were pretreated 30 min with monensin, then pulse-chased as described in Methods; monensin was present throughout the pulse-chase. ApoJ was resolved by SDS-PAGE under nonreducing conditions (top panel) or in the presence of 1% β-mercaptoethanol (bottom panel), followed by fluorography.

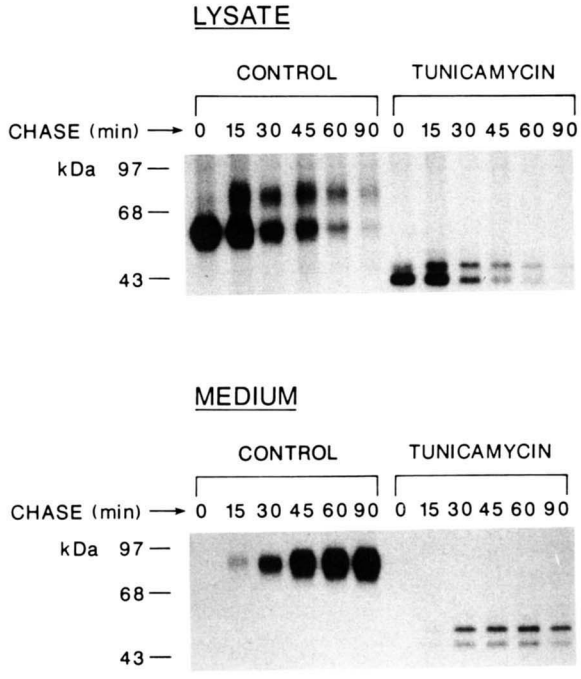

Fig. 8. The effect of tunicamycin on the maturation and secretion of apoJ. ApoJ immunoprecipitated from HepG2 cells, cultured in the absence or presence of 15 μg/ml of tunicamycin, was analyzed by SDS-PAGE. Cells were pretreated 90 min with tunicamycin, then pulse-chased as described in Methods; tunicamycin was present throughout the pulse-chase. ApoJ was resolved under nonreducing conditions, followed by fluorography.

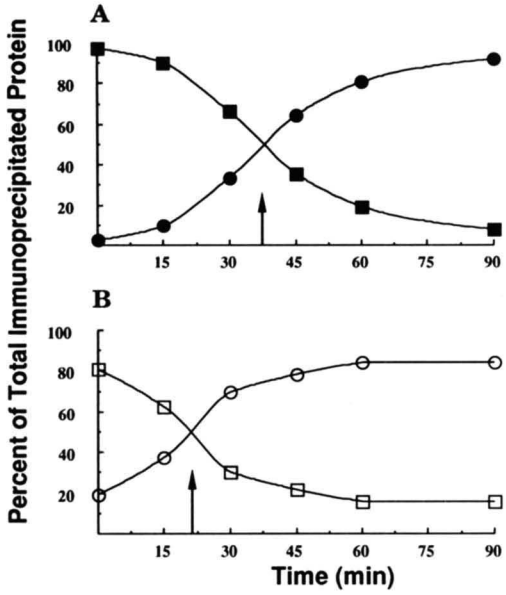

Fig. 9. The rate of secretion of unglycosylated apoJ. Rates of secretion of apoJ from control (A) and tunicamycin-treated (B) HepG2 cells were determined from pulse-chase experiments. Immunoprecipitated apoJ, resolved by SDS-PAGE, was excised, solubilized, and the dpm of [^{35}S]methionine was determined. Squares represent cell lysate fractions and circles represent culture medium fractions. The arrows designate the half-times of secretion, the time at which 50% of the immunoprecipitable apoJ is both cell-associated and secreted.

DISCUSSION

It is well documented that the HepG2 cell line is a useful model for the study of differentiated liver function and the production of liver-specific plasma proteins (13, 24). On the basis of similarity in size and electrophoretic mobility between secreted HepG2 apoJ and human plasma apoJ, we conclude that HepG2 cells are also a reasonable model for studying the processing of apoJ. Human HepG2 cells synthesize, process, and secrete apoJ. Almost all of the translated apoJ is secreted, and secretion is rapid with a half-time comparable to that of apoA-I. ApoJ normally undergoes post-translational cleavage to its subunits just prior to secretion. Its high content of N-linked carbohydrate is not necessary for proteolytic cleavage or secretion.

The molecular mass of the first detectable apoJ is 58 kDa. This form is processed to a 70 kDa form prior to secretion. Secreted apoJ is 70 kDa, more than 95% of which can be reduced to its 35 kDa subunits. ApoJ is rapidly secreted from HepG2 cells with a half-time of 30 to 35 min. Furthermore, secretion is complete, with greater than 90% of apoJ secreted within 90 min. The 10% of labeled apoJ that remains cell-associated after a 90-min chase may be due to residual [^{35}S]methionine, resulting in a "leaky" chase. Cell-association may also be due to reuptake of secreted apoJ. ApoJ falls into a class of rapidly secreted hepatic proteins such as albumin, indicating that apoJ is constitutively secreted from HepG2 cells and is unlikely to be retained or compartmentalized. Other hepatic proteins, such as alpha-1-antichymotrypsin and transferrin, have longer half-times of secretion, where exit of the protein from the endoplasmic reticulum is the rate-limiting step (25).

Carbohydrate accounts for 30% of the molecular mass of plasma apoJ. HepG2 apoJ is susceptible to hydrolysis catalyzed by N-glycanase but not O-glycanase, indicating that the carbohydrate of HepG2 apoJ is Asn-linked. This result is consistent with the existence of seven potential N-linked glycosylation sites in the primary structure of apoJ (9). The N-linked oligosaccharides are enzymatically processed, producing both the precursor (58 dKa) and mature (70 kDa) forms of apoJ. The 58 kDa intracellular form contains high-mannose oligosaccharide, as determined by its susceptibility to endoglycosidase H. The 70 kDa mature form contains complex-type carbohydrate as determined by endoglycosidase H resistance and neuraminidase sensitivity. This mature form of apoJ is then rapidly secreted. Differences in the sialic acid content may be responsible for the difference in size of secreted HepG2 apoJ and plasma apoJ.

Three lines of evidence demonstrate that proteolytic cleavage of apoJ occurs intracellularly, and not consequent to secretion. First, the apparent molecular mass of the intracellular 58 kDa form of apoJ is not influenced by reductant, whereas the 70 kDa form is, in part reduced to 35 kDa species. This result demonstrates that the 58 kDa

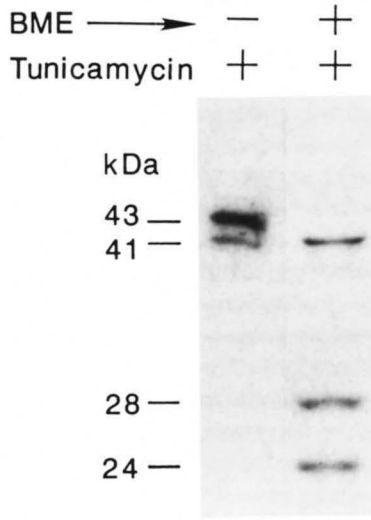

Fig. 10. SDS-PAGE analysis of the apoJ doublet produced in the presence of tunicamycin. ApoJ, immunoprecipitated from culture medium of 60-min chase tunicamycin-treated HepG2 cells (see Fig. 8), was analyzed in the absence or presence of 1% β-mercaptoethanol. The molecular mass in kDa is indicated to the left.

form is the intact precursor protein, while part of the 70 kDa form has undergone proteolytic cleavage into α and β subunits. Comparison of the broad intracellular 70 kDa band before and after reduction (Fig. 6) reveals that the lower half of the original 70 kDa band is unchanged whereas the upper half migrates to the 35 kDa position, suggesting that proteolytic cleavage occurs during late stages of carbohydrate maturation. Second, monensin causes intracellular accumulation of a smaller, heterogeneous 58–68 kDa species of apoJ, indicative of incomplete carbohydrate maturation. Since the apparent molecular mass of the smaller form of apoJ is not altered by reductant, we conclude that proteolytic cleavage of apoJ occurs during the terminal maturation of oligosaccharides. The most likely time is that coincident with the addition of sialic acid residues in the *trans*-Golgi or a post-Golgi compartment (26, 27). Finally, uncleaved apoJ secreted from monensin-treated cells remains uncleaved in culture medium, even after a 3-h incubation, suggesting that cleavage cannot occur extracellularly. Thus, the conversion of apoJ precursor to mature heterodimer occurs at a late stage of protein trafficking, making apoJ cleavage similar in timing to that of C3, C4, and proalbumin. These proteins are proteolytically cleaved from single chain precursors into mature forms in the Golgi compartment (28, 29). In contrast, the proteolytic conversion of prohaptoglobin is thought to occur in the endoplasmic reticulum (30).

The high carbohydrate content of apoJ led us to speculate that it plays a role in proper trafficking and secretion of apoJ. Precedent for such a situation exists. Alterations in the carbohydrate of C4 affect its proteolytic processing and secretion (31), its hemolytic activity (32), and the acquisition of disulfide-stabilized conformation (33). Unglycosylated apoJ appears as a doublet of 41 and 43 kDa in HepG2 cells grown in the presence of tunicamycin. This doublet is trafficked and secreted more rapidly than glycosylated apoJ. The intracellular unglycosylated 41 kDa protein appears first in tunicamycin-treated cells, suggesting that it is a precursor of the 43 kDa species. Under reducing conditions, only the 43 kDa form of apoJ is resolved into 28 and 24 kDa subunits; the 41 kDa protein is unaffected by reductant. Thus the 41 kDa precursor appears to be proteolytically cleaved, producing the 43 kDa species. The 2 kDa shift of mobility of the cleaved form may be due to the disulfide-linked dimer assuming a larger, more open conformation. The portion of the unglycosylated apoJ that is secreted in an uncleaved form may represent a portion that is aberrently trafficked through a cellular compartment lacking the appropriate protease or that is lacking the structural conformation necessary for proteolysis. The fact that over 50% of apoJ can be proteolytically cleaved and all of it can be secreted in the absence of carbohydrate addition indicates that oligosaccharide is not required for proper trafficking of apoJ. The carbohydrate may, however, be important in apoJ function and/or catabolism.

The carbohydrate structure and content of apoJ are different from that of most apolipoproteins that are either unglycosylated or contain primarily O-linked oligosaccharides. ApoE has several acidic isoforms, due in part to varying numbers of sialic acid residues added to an O-linked carbohydrate core (22). ApoC-III contains a short O-linked oligosaccharide attached to Thr-74 (34), and apoD contains O-linked carbohydrate that accounts for approximately 18% of its molecular mass (35). The only major apolipoprotein that contains N-linked oligosaccharides (4–10%) is apoB, where variations in carbohydrate structure and content may generate subclasses of LDL particles that have unique metabolic fates (36).

Since apoJ can associate with terminal complement components (6, 7, 37), it is significant that apoJ structure and post-translational processing are less like that of known HDL-associated proteins and more like that of complement proteins. Most HDL-associated proteins circulate in plasma as monomers, with the exception of apoA-II homodimers and rare apoA-II-apoE heterodimers (38). The subunits of these dimers are translated from separate RNAs. The major HDL components apoA-I and apoA-II can both undergo post-translational proteolysis but, rather than cleavage in the middle of the polypeptide chain, cleavage results in the removal of short propeptides. An apoA-I hexapeptide prosegment is removed after secretion and prior to integration of apoA-I into HDL (39, 40), and an apoA-II pentapeptide prosegment is, in part (45%), cleaved during secretion from HepG2 cells (41). In contrast, apoJ circulates as a disulfide-linked heterodimer, similar to C3 heterodimers, and C4 and C8 heterotrimers. The subunits of apoJ, like the subunits of C3 and C4, arise from proteolytic cleavage of a precursor protein translated from a single mRNA. C3 undergoes intracellular proteolytic processing just prior to secretion to yield a dimer (12). C4 is proteolytically cleaved intracellularly to yield a trimer (11). ApoJ also shares amino acid homology in its cysteine-rich region with the NH_2-terminal ends of complement components C7, C8, and C9 (37), regions that may be important for proper protein-protein interaction in terminal complement complex formation.

The delineation of the apoJ secretory pathway, using hepatoma cells, permits further investigation of the apoJ-containing lipoprotein particles derived from liver cells. Such studies may illuminate potential functional roles of plasma apoJ in lipid metabolism and immunoregulation, and may also allow for comparison of structural differences of apoJ produced from tissues of different origin. Such differences would indicate alternate functions of apoJ derived from different tissues.

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