Apolipoprotein(a): Expression and Characterization of a Recombinant Form of the Protein in Mammalian Cells†

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ABSTRACT: We have stably expressed a recombinant form of apo(a) in a human embryonic kidney cell line. The engineered protein (predicted mass of 250 kDa) contains 17 copies of the apo(a) domain, which resembles kringle 4 of plasminogen, followed by the plasminogen-like kringle 5 and protease-like domain of apo(a). The recombinant protein [r-apo(a)] was isolated from cell culture media by immunoaffinity chromatography, and its physical properties were studied. As is the case for apo(a) isolated from plasma-derived Lp(a), r-apo(a) is highly glycosylated (23% by weight), containing both N- and O-linked glycans, which results in an observed molecular mass of 500 kDa by SDS–PAGE. The high sialic acid content was reflected in a p*I* of 4.3 for the r-apo(a). Two subpopulations of r-apo(a) secreted by the permanent cell line were identified with respect to lysine–Sepharose binding; the majority of the r-apo(a) bound specifically to this matrix and was eluted with ε-aminocaproic acid (ε-ACA). When the r-apo(a) plasmid was used to transfect a human hepatoma cell line, lipoprotein particles were secreted containing the disulfide-linked complex of apoB-100 and the r-apo(a). The density of these particles was shown to be heterogeneous, with the majority of the r-Lp(a) floating in the density range of plasma-derived Lp(a).

Lipoprotein(a) [Lp(a)][1] is an independent risk factor for the development of coronary heart disease; elevated levels in plasma strongly correlate with atherosclerosis in human populations [see Scanu and Fless (1990) for a review]. Marked inherited variability has been observed in plasma Lp(a) levels, which range from <1 to >100 mg/dL in the population. Roughly 25% of the population possess Lp(a) levels greater than 20 mg/dL, which more than doubles their risk of developing heart disease (Armstrong et al., 1986; Rhoads et al., 1986; Dahlen et al., 1986; Durrington et al., 1988).

The Lp(a) particle closely resembles low-density lipoprotein (LDL) in both lipid composition and the presence of apolipoprotein B-100 (apoB-100). Lp(a) is distinguished from LDL by the presence of an additional protein component designated apolipoprotein(a) [apo(a)], which is complexed to apoB-100 by one or more disulfide linkages (Gaubatz et al., 1983; Utermann & Weber, 1983; Fless et al., 1985).

Apo(a) is a large plasma glycoprotein (28% carbohydrate by weight) (Fless et al., 1986) that is synthesized primarily by the liver (Tomlinson et al., 1989; Kraft et al., 1989). Apo(a) displays genetically determined size heterogeneity, and as such, molecular masses in individuals range from approximately 300–800 kDa (Fless et al., 1984; Gaubatz et al., 1987; Utermann et al., 1987). On the basis of partial amino acid sequence (Eaton et al., 1987), followed by complete analysis of cloned apo(a) cDNA sequence (McLean et al., 1987), extensive homology was revealed between apo(a) and plasminogen. Plasminogen is a serine protease zymogen composed of 5 kringle domains, followed by a trypsin-like protease domain. Human apo(a) consists of multiple tandem repeats of a sequence closely resembling plasminogen kringle 4, followed by sequences exhibiting approximately 90% identity to the kringle 5 and protease regions of plasminogen (McLean et al., 1987). Interestingly, the protease domain of apo(a) contains the catalytic triad and cysteine residues present in plasminogen. However, at the site where plasminogen is cleaved by activators to generate plasmin, the arginine residue in plasminogen has been replaced by serine in apo(a) (Eaton et al., 1987; McLean et al., 1987). It has been proposed that this substitution may result in failure to demonstrate plasmin-like activity with use of purified Lp(a) as a substrate (McLean et al., 1987; Eaton et al., 1987; Salonen et al., 1989).

In the current study, we describe the expression, purification, and characterization of recombinant apo(a) in cultured mammalian cells. The potential utility of this recombinant expression system as a research tool for identifying the role of apo(a) in atherosclerosis is also addressed.

Experimental Procedures

Construction of the apo(a) Expression Vector. A recombinant apo(a) [r-apo(a)] molecule containing 17 kringle 4-like domains, as well as the kringle 5-like and protease-like domains, was assembled from apo(a) cDNA clones reported by McLean et al. (1987). A 2095-bp *Eco*RI–*Hha*I fragment encoding the 5′ untranslated region, signal sequence, the first 5 kringle 4-like repeats, and 291 bp of the sixth kringle repeat of apo(a) was fused to a 4858-bp *Hha*I–*Eco*RI fragment encompassing the latter 51 bp of kringle 26, followed by kringle repeats 27–37, kringle 5-like and protease-like domains, and

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[1] Abbreviations: Lp(a), lipoprotein(a); LDL, low-density lipoprotein; apo(a), apolipoprotein(a); apoB-100, apolipoprotein B-100; r-apo(a), recombinant apolipoprotein(a); ε-ACA, ε-aminocaproic acid.

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67 bp of 3′ untranslated sequence. The apo(a) cDNA, bounded by *Eco*RI sites, was ligated into the pRK5 expression vector containing the cytomegalovirus (CMV) promoter, a hybrid CMV–immunoglobulin G intron in the 5′ untranslated region, and the SV40 early poly(A) signal and origin of replication (Suva et al., 1989). This expression construct was designated pRK5ha17.

Transfection of 293s and HepG2 Cultured Cells. Both 293s (human embryonic kidney; Graham et al., 1977) and HepG2 (human hepatocarcinoma; Knowles et al., 1982) cells were cultured in 100 mm dishes with modified Eagle's medium (MEM) supplemented with 10% fetal calf serum, and 2 mM glutamine. Cells were transfected by the method of calcium phosphate precipitation (Graham & van der Erb, 1973) by using 10 μg of the expression plasmid/100-mm dish. Plasmid DNA was purified by alkaline lysis, followed by banding in a cesium chloride density gradient. For 293s cell transfections, the precipitate was left on the cells overnight, after which time fresh media was added (see below). For transient transfections of HepG2 cells, the precipitate was removed from the cells after 5 h, at which time the cells were treated with 20% glycerol in phosphate-buffered saline (PBS) for 30 s. The glycerol was then removed, and the cells were rinsed with PBS prior to the addition of fresh MEM. Following transfection, HepG2 cells were allowed to recover overnight prior to metabolic labeling (see below).

In order to establish a 293s cell line containing stable integrants, 10 μg of the pRK5ha17 expression plasmid was cotransfected with 1 μg of a plasmid containing the neomycin gene (pRSVneo; Gorman et al., 1983). The calcium phosphate precipitate was left on the cells overnight, at which time the precipitate was removed and fresh media was added. After 24 h, the selective agent G418 (800 μg/mL of media) was added to the plates. After several weeks, individual G418-resistant foci were transferred to 24-well dishes. Positive clones were identified by immunoperoxidase staining with an apo(a) monoclonal antibody (2G7), and apo(a) production was measured by ELISA (Wong et al., 1990). The isolate expressing the highest level of apo(a) was purified according to the limiting dilution method.

Purification of r-apo(a). Apo(a) secreted by the stable transformant was isolated from conditioned medium by immunoaffinity chromatography with the apo(a) monoclonal antibody 2G7 immobilized on glycerol-coated controlled-pore glass beads (CPG; Sigma) by using periodate coupling (Roy et al., 1984). The media was clarified by filtration, made 1 mM in phenylmethanesulfonyl fluoride (PMSF) and NaN_3, and then batch-adsorbed with 2G7-CPG for 4 h at 4 °C. The resin was then packed in a Bio-Rad Econo-column (5 × 10 cm) and sequentially washed with 10 column volumes of 0.05 M Tris-HCl/150 mM NaCl, pH 7.5 (buffer A), followed by 10 column volumes of the same buffer containing 1.0 M NaCl. The column was then washed with 5 volumes of buffer A prior to specific elution of apo(a) with 0.1 M glycine ethyl ester hydrochloride (pH 2.2). Eluted fractions were neutralized immediately by the addition of 1.0 M Tris-HCl, pH 7.5, to a final concentration of 50 mM and analyzed by use of SDS–PAGE.

Binding of r-apo(a) to Lysine–Sepharose. Conditioned media from the permanent cell line was clarified by filtration (0.2 μm) and concentrated 2-fold by use of a Pharmacia Ultrasette (>50-kDa retention). The retentate was made 1 mM in PMSF and batch-adsorbed onto lysine–Sepharose 4B (Pharmacia) at a ratio of 0.6 mg of protein/mL of swollen gel for 4 h at 4 °C. The resin was then packed in a Bio-Rad Econo-column (5 × 10 cm) and sequentially washed with 10 column volumes of PBS followed by 10 column volumes of PBS containing 0.5 M NaCl at a flow rate of 2 mL/min. The r-apo(a) was eluted by using 200 mM ε-aminocaproic acid (ε-ACA) containing 1.0 M NaCl, pH 7.1. Samples from all fractions were analyzed by SDS–PAGE in a 4–15% gradient gel (Bio-Rad) and visualized by silver staining.

Metabolic Labeling Studies. Cells were preincubated for 1 h in methionine/cysteine-depleted MEM media supplemented with 10% dialyzed fetal calf serum and 2 mM glutamine. [^{35}S]Cysteine (Amersham) was then added (20 μCi/mL of media). Depending on the study, supernatants or lysates were harvested either directly after the labeling period or after varying lengths of cold-chase incubations.

Preparation of Postnuclear Cell Lysates. Cell monolayers were rinsed twice with cold PBS. Cells were then scraped into cold lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.01 M EDTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF) and centrifuged in an Eppendorf centrifuge at 4 °C for 5 min in order to pellet cell nuclei. The postnuclear lysate fraction was then harvested for immunoprecipitation analysis.

Immunoprecipitations. Supernatants or lysate preparations clarified by microfuge centrifugation were incubated overnight at 4 °C with 1–5 μg of antibodies directed against apo(a) [(either monoclonal 2G7 (Wong et al., 1990) or polyclonal antibodies raised in rabbits against purified recombinant apo(a) from 293s cells) or human LDL (polyclonal; Boehringer)]. At this time, protein A–Sepharose (Pharmacia) coupled to rabbit anti-mouse IgG (Cappel) was added to monoclonal antibody incubations; protein A–Sepharose was added directly in order to precipitate polyclonal antibody complexes. Samples were incubated at 4 °C for an additional 3 h. At this time, the Sepharose was pelleted by brief centrifugation and washed three times with RIPA buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 20 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) containing 0.5 M NaCl, followed by a final wash with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Laemmli sample buffer (50 μL) (Laemmli, 1972) was added to each sample; dithiothreitol was added to a final concentration of 10 mM in order to reduce samples where required. Samples were boiled for 5 min, and solubilized proteins were separated by SDS–PAGE. Gels were then treated for 15 min with Enlightening (DuPont), dried at 60 °C under vacuum, and exposed to film.

Tunicamycin Treatment of the Permanent Cell Line. Confluent six-well dishes containing 1.5 mL of Cys/Met-depleted MEM were incubated for 3 h with 1, 2, or 5 μg of tunicamycin (Sigma; prepared as a 1 mg/mL stock in PBS). Cells were then pulsed for 2 h with [^{35}S]cysteine (see above). Supernatants were then concentrated by Centricon (Amicon) filtration to 50 μL and analyzed directly by use of SDS–PAGE and fluorography.

IEF Analysis. Samples of purified LDL and Lp(a) (ranging from 0.75 to 1 μg/sample) were subjected to agarose IEF analysis by using the PhastSystem as described by Camejo et al. (1989). The gel contained a preblended ampholine range of pH 3.5–9.5 (Pharmacia). The p*I* value for purified recombinant apo(a) (5 μg) was determined by IEF by using a 5% polyacrylamide gel matrix (0.2 mm thick) containing Pharmalyte carrier ampholytes (Pharmacia), pH range 2.5–5. The sample was visualized by using Coomassie R-250 stain.

Density Fractionation of HepG2-Transfected Supernatants. One milliliter of labeled supernatants from transiently transfected or mock-transfected HepG2 cells was centrifuged for 2 h (15 °C) at 100000 rpm in a TL-100 centrifuge

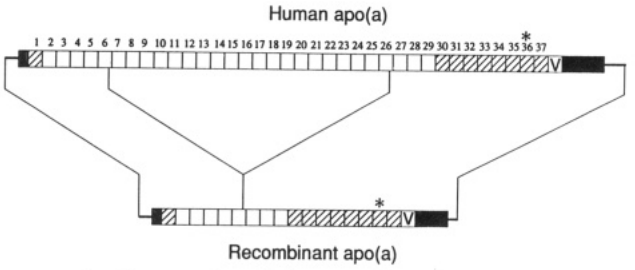

FIGURE 1: Construction of the r-apo(a) plasmid. The upper line illustrates the organization of the apo(a) cDNA reported by McLean et al. (1987). Relative to the cDNA sequence, the recombinant cDNA construction (shown on the lower line) contains the 5′ untranslated region (−) and signal sequence (shaded box), followed by 17 kringle 4-like repeats, the kringle 5-like (V) and protease-like domains (the latter represented by a solid bar), and a truncated 3′ untranslated region (−). Open boxes indicate kringle repeats of identical amino acid sequence, while hatched boxes designate kringles that contain amino acid substitutions (McLean et al., 1987). The penultimate repeat (*) contains an extra cysteine residue, postulated to form a covalent linkage with apoB-100. The construct, bounded by *Eco*RI sites, was ligated into the pRK5 expression vector containing the CMV promoter.

(Beckman). The top 150 μL (representing the VLDL fraction $d < 1.006$ g/L) was harvested. The infranatant was then adjusted to a density of 1.063 with sodium bromide and centrifuged again as above. The upper 150 μL (containing the LDL density fraction) was collected, and the infranatant was adjusted with sodium bromide to a final density of 1.2. Tubes were then centrifuged for 4 h as above. In this case, both the upper 150 μL containing the HDL fraction and the infranatants (i.e., the lipid-poor fraction) were harvested. All samples were divided in two for immunoprecipitation with either the apo(a) monoclonal antibody 2G7 or anti-LDL. Samples were then analyzed by SDS–PAGE and fluorography.

RESULTS

Isolation of 293s Stable Transformants Expressing apo(a). The plasmid pRK5ha17 was constructed in order to express a recombinant apo(a) protein containing the apo(a) signal sequence and 17 plasminogen kringle 4-like repeats, followed by the kringle 5-like and protease-like domains (see Figure 1; this construct was designed in order to include at least several copies of each kringle sequence variant and represents an isoform size that is present in the population, as discussed below). Cotransfection of 293s cells with pRK5ha17 and pRSVneo yielded over 100 colonies of stable transformants per 100-mm culture dish. Among those foci picked for analysis, approximately 50% contained apo(a) plasmid integrants on the basis of immunoperoxidase staining of the cells. With use of ELISA screening, the transformant secreting the highest level of apo(a) (100 μg from 2×10^7 cells, on the basis of a 24-h collection period) was identified. This line was subjected to a second round of cloning by limiting dilution, resulting in the isolation of the stable transformant designated as 293/apo(a).24. Preparation of RNA from this cell line, followed by Northern blot analysis using an apo(a) cDNA probe, indicated a size for the apo(a) message of approximately 7.2 kb (data not shown), in agreement with the expected size on the basis of the cDNA sequence.

Characterization of the r-apo(a) Protein Secreted from 293/apo(a).24. The r-apo(a) protein secreted into the cell medium was isolated by immunoaffinity chromatography. With use of SDS–PAGE analysis in 2.5–15% gradient gels, the mobility of the purified protein under nonreducing conditions was determined to be faster than that observed for samples reduced with 10 mM DTT (Figure 2, lanes 1 and 2,

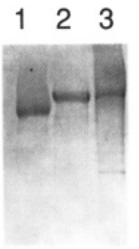

FIGURE 2: Analysis of the purified r-apo(a) isolated from the stable transformant 293/apo(a).24. The r-apo(a) was purified from the cell media by immunoaffinity chromatography. Samples of the r-apo(a) (lanes 1 and 2) and purified human LDL (lane 3) (2 μg of each) were analyzed by SDS–PAGE, with use of 2.5–15% gradient gels. DTT (10 mM) was added to the samples in lanes 2 and 3. The samples were visualized by Coomassie staining.

Table I: Summary of the Physical Properties of Purified r-apo(a)

molecular mass determination	
predicted from cDNA sequence (kDa)	249
SDS–PAGE apparent mobility (kDa)	500
estimated from gel filtration[a] (kDa)	1200–1400
extinction coefficient[b]	
$E^{0.1\%}$(theoretical)	1.94
$E^{0.1\%}$(calculated)	1.9–2.0
carbohydrate composition[c]	
fucose	18
galactosamine	36
glucosamine	67
galactose	103
mannose	30
sialic acid	97
carbohydrate by weight (%)	23
isoelectric point	4.3

[a] Gel filtration analysis was performed with use of a Superose-6 FPLC column (Pharmacia) equilibrated with 10 mM Tris-HCl plus 50 mM NaCl, pH 7.5, which was standardized by use of a mixture of Bio-Rad molecular weight markers (M_r = 1350–670 000). [b] The r-apo(a) molar extinction coefficient was determined by the absorbance at 280 nm of apo(a) in PBS, pH = 7.2. The concentration of r-apo(a) in this solution was determined by amino acid analysis on a Beckman amino acid analyzer. [c] The carbohydrate composition [moles per mole of r-apo(a)] was determined by dialyzing the purified protein against 0.1 M NH_4HCO_3 prior to a 1-h hydrolysis with 4.0 N trifluoroacetic acid (TFA) at 125 °C for monosaccharide analysis. For sialic acid determination, samples were hydrolyzed with 0.1 N TFA followed by hydrolysis at 80 °C. Samples were analyzed with use of a Dionex system and compared to monosaccharide or sialic acid standards.

respectively). On the basis of direct comparison of reduced and nonreduced samples, it appeared that the apo(a) secreted from the 293s cells was not complexed to another protein(s).

Upon reduction with DTT, the purified recombinant protein (calculated polypeptide mass = 250 kDa) was found to migrate at a similar position to that of apoB-100, which has a molecular mass of approximately 550 kDa (Figure 2, lanes 2 and 3). This result suggests the large effect of glycosylation on the migration of apo(a) in polyacrylamide gels. The molecular weight of the r-apo(a) was also estimated by use of gel filtration chromatography (Table I). With use of this method, a molecular mass of 1200–1400 kDa was estimated for the recombinant protein. A molar extinction coefficient ($E^{0.1\%}$) of 1.9–2.0 was determined for purified r-apo(a), which is in good agreement with the predicted theoretical value of 1.94 (see Table I).

In order to determine the effect of N-linked glycosylation on the apparent molecular mass of r-apo(a), cells were treated with 1, 2, or 5 μg of tunicamycin, which inhibits oligosaccharide addition to the free amide group of asparagine residues (Heifetz et al., 1979) (Figure 3). This resulted in a decrease in size of the recombinant protein from 500 kDa to approximately 400 kDa, which was detected by direct SDS–PAGE analysis of the cell supernatants. The identity

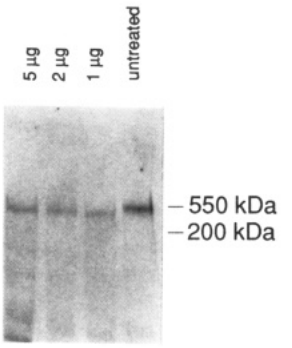

FIGURE 3: Tunicamycin treatment of the 293/apo(a).24 line. Confluent six-well dishes of the permanent cell line were incubated for 3 h with 0, 1, 2 or 5 µg of tunicamycin. Cells were then pulse-labeled for 2 h with [^{35}S]cysteine. Supernatants were harvested and concentrated; samples containing 10 mM DTT were analyzed directly by SDS–PAGE with use of a 2.5–15% gradient. The gel was treated with Enlightening, dried under vacuum, and exposed to film overnight. The positions of apoB-100 (550 kDa) and myosin (200 kDa) are shown to the right of the gel.

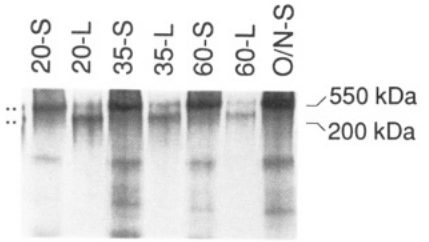

FIGURE 4: Analysis of the intracellular form of r-apo(a). Six-well dishes of the 293/apo(a).24 cell line were pulse-labeled for 1 h with [^{35}S]cysteine. Supernatants (S) or lysates (L) were analyzed after 20, 35, or 60 min or overnight (O/N) cold chase incubations by immunoprecipitation. Samples reduced with 10 mM DTT were analyzed by SDS–PAGE with use of a 2.5–15% gradient gel. The gel was then treated with Enlightening, dried under vacuum at 60 °C, and exposed to film overnight. The positions of apoB-100 (approximately 550 kDa) and myosin (200 kDa) are indicated to the right of the gel. The secreted form of r-apo(a) (500 kDa) and the intracellular species (300 kDa) are also shown (••).

of the r-apo(a) protein was confirmed by immunoprecipitation analysis (data not shown).

In order to examine the combined effect of N-linked and O-linked carbohydrate addition on apo(a) protein mobility, 293/apo(a).24 cells were pulse-labeled for 1 h with [^{35}S]-cysteine. The appearance of r-apo(a) both intracellularly and in the medium was followed by immunoprecipitation with the apo(a) monoclonal antibody 2G7. After 20 min of chase, two forms of r-apo(a) could be detected in the reduced detergent lysate (Figure 4, 20-L), the fully modified secreted form that is seen in the supernatant corresponding to this time point (Figure 4, 20-S) and a smaller, intracellular form (300 kDa) that represents the cell-associated form of r-apo(a) with incomplete carbohydrate modification. No proteins corresponding to these sizes could be detected with this antibody when nontransfected labeled 293s cells were used for immunoprecipitation analysis, demonstrating the specificity of the antibody (data not shown). With increasing chase periods, the relative amounts of the intracellular 300-kDa form decreased, corresponding to an increase in the 500-kDa species observed in the cell supernatants (see Figure 4). This indicates that the protein detected in the cell lysates represents the intracellular form corresponding to the secreted r-apo(a). By 1 h postlabeling, the majority of the labeled r-apo(a) was secreted into the medium (Figure 4, 60-S).

The carbohydrate composition of the r-apo(a) produced by the permanent cell line (approximately 23% by weight represented by fucose, galactosamine, glucosamine, galactose, mannose, and sialic acid) is summarized in Table I.

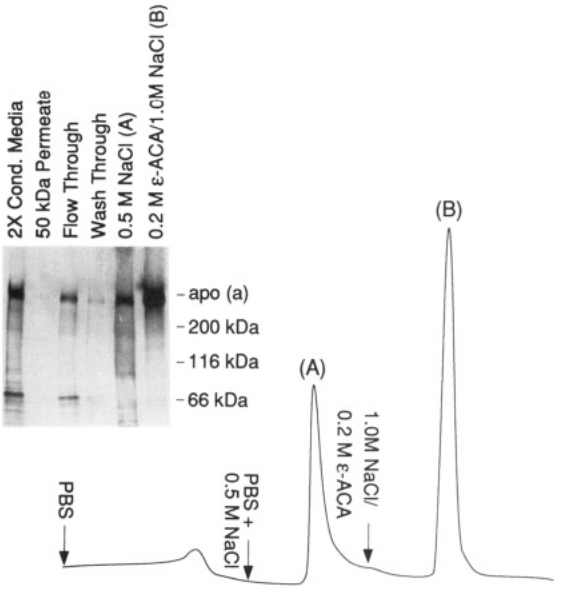

FIGURE 5: Binding or r-apo(a) to lysine–Sepharose. Conditioned media from the permanent cell line 293/apo(a).24 was harvested and concentrated 2-fold (2× Cond. Media). The retentate was then batch-adsorbed to lysine–Sepharose. The Sepharose was recovered and washed with 10 column volumes of PBS prior to column packing. The column was then washed sequentially with PBS, PBS containing 0.5 M NaCl, and 0.2 M ε-ACA in 1 M NaCl; the A_{280} trace for the washing and elution steps is shown. Absorbance peak A corresponds to material eluted with 0.5 M NaCl; specific elution with ε-ACA is represented by peak B. Twenty microliters of each fraction was analyzed by SDS–PAGE with use of a 4–15% gradient gel and visualized by silver staining; these data are shown in the inset. Molecular mass markers are shown to the right of the gel.

Binding of the r-apo(a) Protein to Lysine–Sepharose. The binding and elution profile of r-apo(a) to lysine–Sepharose is shown in Figure 5. The majority of the r-apo(a) bound the resin and remained associated following extensive washing with PBS. A variable percentage (5–30% as estimated by SDS–PAGE) of the r-apo(a) that initially bound the matrix could be eluted with 0.5 M NaCl. (When rechromatographed, this fraction remained unbound in 0.5 M NaCl.) The majority of the bound r-apo(a) was eluted with 200 mM ε-ACA in 1.0 M NaCl. Both subpopulations of r-apo(a) had the same apparent mobility as determined by SDS–PAGE (Figure 5), and both showed cross-reactivity with the monoclonal antibody 2G7 (data not shown).

Isoelectric Focusing of r-apo(a). With use of an IEF agarose matrix, the isoelectric points of purified human LDL and human Lp(a) were determined to be approximately 5.5 and 5.2, respectively (Figure 6, lanes 2 and 3). The p*I* value for r-apo(a) was estimated to be 4.3 (Figure 6, lane 1) by using a polyacrylamide gel matrix containing ampholytes of pH range 2.5–5.5.

Secretion of r-Lp(a) Particles from Transiently Transfected HepG2 Cells. HepG2 cells were transfected by using the pRK5ha17 expression plasmid as described above. At 17 h posttransfection, cells were pulse-labeled for 4 h, followed by an overnight cold chase with serum-depleted MEM. At this time, cell supernatants were harvested for immunoprecipitation, followed by SDS–PAGE with 2.5–15% gradient gels. In addition to free apo(a), a high molecular weight complex was observed in the cell supernatants, which was immunoprecipitable with either apoB-100 (raised againt LDL) or apo(a)-specific (2G7) antibodies (Figure 7, lanes 6 and 8, respectively). This suggested that the higher molecular weight

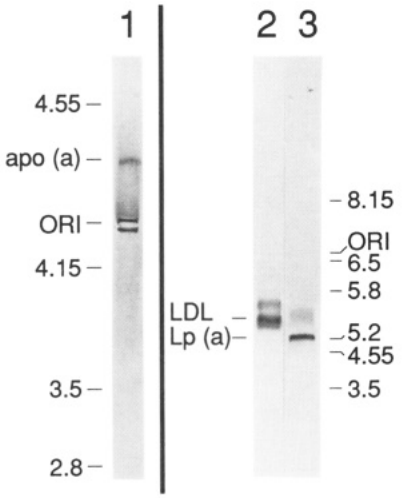

FIGURE 6: Isoelectric focusing of r-apo(a), LDL, and Lp(A). Isoelectric focusing of purified r-apo(a) (1 μg) is shown in lane 1. The sample well (ORI) and the positions of the ampholyte markers (Pharmacia Low p*I* Calibration Kit; pH 2.5–6.5) are indicated to the left of lane 1. (Artifactual staining bordered the origin in this polyacrylamide gel system, independent of the position of the origin.) Isoelectric focusing of LDL and Lp(a) (purified by density-gradient centrifugation) are shown in lanes 2 and 3, respectively, utilizing the agarose IEF PhastGel system (Camejo et al., 1989). Sample loading positions (ORI) and the positions of ampholyte markers (Pharmacia preblended ampholytes; pH range 3.5–9.5) are indicated to the right of the gel. The slight contamination of the Lp(a) sample with LDL is visible in lane 3.

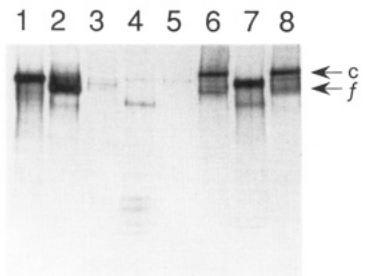

FIGURE 7: Analysis of r-Lp(a) particle formation in HepG2 cells. HepG2 cells were transfected ith the pRK5ha17 construct and labeled for 2 h with [^{35}S]cysteine. Following an overnight cold chase incubation, supernatants were harvested and immunoprecipitated with either the apo(a) monoclonal antibody 2G7 or an apoB-100-specific polyclonal antibody (raised against human LDL). Immunoprecipitates were analyzed by SDS–PAGE with use of a 2.5–15% gradient gel. For visualization, the gel was treated with Enlightening, dried under vacuum, and exposed to film overnight. Lanes 3–5 contain mock-transfected controls, immunoprecipitated with either apoB-100 (lane 3) or 2G7 (lanes 4 and 5) antibodies. The sample in lane 4 was reduced with 10 mM DTT. Lanes 6–8 contain r-apo(a)-transfected cell supernatants, immunoprecipitated with either apoB-100 (lane 6) or 2G7 antibodies (lanes 7 and 8). The sample in lane 7 was reduced with 10 mM DTT. Labeled r-apo(a) immunoprecipitated from the 293/apo(a).24 permanent line is shown in lanes 1 and 2; the sample in lane 1 contains 10 mM DTT. The positions of uncomplexed r-apo(a) and the r-Lp(a) complex are indicated by labeled arrows (f and c, respectively). A band of unknown origin that is smaller in size than that of free r-apo(a) is detectable in lanes 4 and 7.

form represents a complex containing both endogenously expressed LDL and r-apo(a). The complex disappeared completely upon sample reduction (Figure 7, lane 7), suggesting that the protein association involved one or more disulfide linkages. However, in lane 6, a band is detected with apoB-100 antibody that comigrates with nonreduced apo(a) precipitated with the apo(a) antibody (indicated with an arrow labeled f in Figure 7). We interpret this as consistent with some apo(a) that becomes noncovalently associated with apoB-100 during immunoprecipitation but that dissociates without reduction

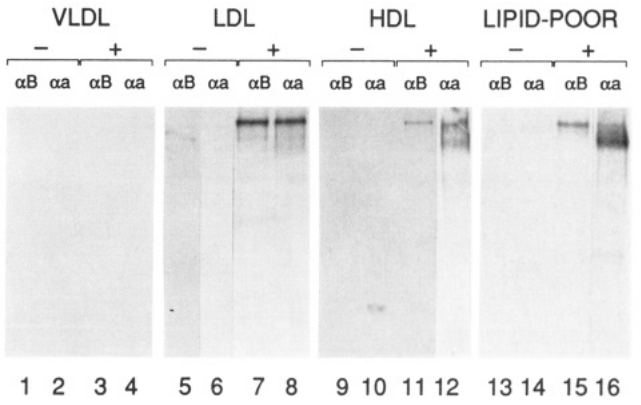

FIGURE 8: Density-gradient fractionation of the r-Lp(a) particles. One milliliter of [^{35}S]cysteine-labeled supernatants from either mock-transfected HepG2 cells (−) or cells transfected with the pRK5ha17 construct (+) was subjected to ultracentrifugation at increasing densities in order to isolate different lipoprotein classes. Isolates from each lipoprotein group were subjected to immunoprecipitation analysis, with either the monoclonal antibody 2G7 (αa) or the apoB-100-specific polyclonal antibody (αB). Immunoprecipitates were analyzed by SDS–PAGE with use of 2.5–15% gradient gels, followed by autoradiography. All samples were nonreduced.

in SDS–PAGE (see Discussion). The antibodies used in this experiment were tested by using mock-transfected HepG2 cell supernatants for immunoprecipitations with either the apoB-100 or apo(a)-specific antibodies (Figure 7, lanes 3–5). No cross-reactivity was observed to proteins of the same molecular mass as those synthesized as a result of r-apo(a) transfection. As expected, endogenous apoB-100 was detectable in mock-transfected HepG2 cells, with the apoB-100-specific antibody (Figure 7, lane 3). This band is only faintly visible because the labeling of the apoB-100 with [^{35}S]cysteine was less efficient than that observed for r-apo(a), since apoB-100 contains 25 cysteine residues per molecule, compared with 126 cysteines per molecule of apo(a). For direct comparison using a non-hepatocyte-derived host cell line, 293s/apo(a).24 cells were labeled with [^{35}S]cysteine for 4 h, at which time cell supernatants were immunoprecipitated with the apo(a) 2G7 monoclonal antibody. Analysis of reduced versus nonreduced samples (Figure 7, lanes 1 and 2, respectively) indicated that apo(a) does not form homodimers under these conditions.

Characterization of the r-Lp(a) Particle with Density Gradient Centrifugation. The density of the r-Lp(a) particles secreted from HepG2 cells was determined by ultracentrifugation of transfected and mock-transfected cell supernatants at increasing densities, followed by immunoprecipitation of the various fractions (Figure 8). No labeled proteins immunoprecipitable with antibodies against either apo(a) or LDL were detectable in the VLDL density range ($d < 1.006$) (lanes 1–4). The ultracentrifugation studies showed that the majority of both the endogenous LDL and r-Lp(a) floated at a density of 1.063 (Figure 8, lanes 5–8). Both LDL and r-Lp(a) were also detectable in lesser amounts in the Lp(a) plus HDL density range ($1.063 < d < 1.2$; lanes 9–12). In the fraction containing proteins with densities > 1.2 (designated as the lipid-poor fraction), the majority of the uncomplexed apo(a) was detected, in addition to apo(a) bound to apoB-100 (lanes 13–16). Free apoB-100 was virtually undetectable in this fraction (lane 13).

Discussion

We have established a stably transformed embryonic kidney cell line that expresses recombinant apo(a). The secreted recombinant protein contains 17 kringle 4-like domains, as well

as the kringle 5-like and protease-like domains. Although the molecular mass of the r-apo(a) polypeptide was calculated to be 250 kDa on the basis of the cDNA sequence, the apparent mass of the secreted protein corresponded to approximately 500 kDa as determined by SDS–PAGE under reducing conditions. This discrepancy is primarily due to the high degree of both N- and O-linked glycosylation. It has been reported that, in addition to increasing the molecular mass of proteins, a high carbohydrate content may also result in misleading migration in SDS–PAGE (Produslo, 1981). With gel filtration chromatography, the molecular mass of the r-apo(a) protein was estimated to be 1200–1400 kDa. One possible explanation for this inflated value is that the r-apo(a) either self-associates or forms complexes with other proteins, resulting in inaccurate estimations of molecular mass with gel filtration. This is unlikely, however, since the purified protein used for this study was analyzed by SDS–PAGE and visualized as a single band by silver staining (data not shown), suggesting that r-apo(a) was not associated with other proteins. Additionally, the purified r-apo(a) does not appear to aggregate in solution, since preliminary sedimentation velocity experiments indicate that r-apo(a) behaves as a monomer in PBS at pH 7.2 (S. Shire and T. Zioncheck, unpublished results). On this basis, the estimated molecular mass of apo(a) from gel filtration may reflect a nonglobular configuration for the protein in solution, resulting in nonideal behavior. Inhibition of N-linked sugar addition with use of tunicamycin resulted in a decrease in molecular mass of the r-apo(a) protein from 500 kDa to approximately 400 kDa. Since the size of the intracellular form (Figure 4) was estimated to be 300 kDa, this suggests the contribution of O-linked glycans, in addition to N-linked sugars, in order to reconcile the difference between the calculated and apparent molecular mass of r-apo(a).

It has previously been reported that apo(a) contains 28% carbohydrate by weight (Fless et al., 1986), which is in good agreement with the value of ~23% determined for r-apo(a). Each of the kringle 4-like repeats contains a potential N-linked glycosylation site (McLean et al., 1987). Additionally, peptide sequence analysis has suggested that many of the repeated apo(a) kringles also contain six potential O-linked carbohydrate attachment sites per kringle (Kratzin et al., 1987). The oligosaccharide composition of apo(a) also suggests the presence of a number of O-linked glycans in the apo(a) protein (Fless et al., 1986), which is consistent with our findings. The carbohydrate composition of the r-apo(a) is overall very similar to that reported for apo(a) by Fless et al. (1986). One difference of note, however, is that unlike the analysis of Fless et al. (1986), which shows the absence of fucose in apo(a), we observe a small amount of this sugar moiety [18 mol/mol of apo(a)] in the secreted r-apo(a) (see Table I). This apparent difference in glycosylation may result from the synthesis of r-apo(a) in kidney cells as opposed to plasma-derived apo(a), which is synthesized by the liver. It has previously been reported that apo(a) contains a high degree of sialic acid (Fless et al., 1986; Gaubatz et al., 1987), present as a terminal oligosaccharide component of either N- or O-linked glycans. The r-apo(a) contains 97 moles of sialic acid per molecule of protein, which renders a net negative charge to apo(a). This is supported by the p*I* value of 4.3 for r-apo(a), as determined by isoelectric focusing. Purified Lp(a) was shown to have a p*I* of approximately 5.2, which is intermediate between that determined for r-apo(a) (4.3) and purified human LDL (5.5); the latter value is in good agreement with that previously reported by Camejo et al. (1989). Since most carbohydrate attachment sites are located in the kringle domains of apo(a), we expect that some of these values would vary with isoform size.

The mobility of r-apo(a) by SDS–PAGE has allowed us to estimate the variation in the numbers of repeated kringle domains that exist in apo(a) proteins in the population. Apo(a) size heterogeneity is due to allelic variation in the number of repeated domains in the apo(a) gene (Koschinsky et al., 1990; Hixson et al., 1989; Gavish et al., 1990; Lindahl et al., 1990). The originally cloned and sequenced apo(a) cDNA was estimated to contain 37 kringle repeats (McLean et al., 1987), but plasma was unavailable from this individual for protein size determination. The recombinant apo(a) containing 17 kringle repeats migrates at approximately the position of apoB-100. Since individual isoforms are known to range in migration from well above to below that of apoB-100 (300–800 kDa), we estimate that apo(a) variants with as few as ~15 and as many as ~37 kringle repeats can be found in individuals. With reference to kringle organization, we cannot conclusively demonstrate that the r-apo(a) corresponds to a naturally occurring protein isoform. However, the examined properties of the recombinant molecule closely resemble plasma-derived Lp(a) (see below), thereby pointing to the utility of this expression system in the analyses of structure–function relationships of Lp(a).

A difference in mobility was observed between reduced and nonreduced forms of r-apo(a), with the nonreduced form migrating faster in SDS gels. A similar pattern has been observed for t-PA, which contains two kringle domains (Vehar et al., 1984). This difference in mobility likely occurs as a result of the unfolding of the kringle units under reducing conditions. Although apo(a) can be isolated from plasma-derived Lp(a) by reduction, it is likely that this disrupts the integrity of the apo(a) secondary structure. As such, apo(a) purified by reduction does not exhibit the same degree of binding affinity to either endothelial cells (Hajjar et al., 1989) or apoB-100-containing lipoproteins (Trieu et al., 1990) that is characteristic of native Lp(a). This, coupled with the technical difficulties of working with Lp(a) due to its tendency to aggregate when highly purified, undescores the importance of the r-apo(a) expression system as a research tool. With this approach, r-apo(a) can be purified from cell culture supernatants without reduction and mimics Lp(a) with respect to its ability to bind to endothelial cells (J. Tomlinson, unpublished results), apoB-containing lipoproteins (V. N. Trieu, T. F. Zioncheck, R. M. Lawn, and W. J. McConathy, unpublished results), and degraded fibrin (Eaton et al., 1990).

It is well established that like plasminogen Lp(a) can bind to lysine–Sepharose (Eaton et al., 1987). As has been reported for plasma-derived Lp(a) (Armstrong et al., 1990), we have shown that r-apo(a) secreted from kidney cells is heterogeneous in terms of its ability to bind to lysine–Sepharose. Although the vast majority of the r-apo(a) secreted from 293s cells specifically binds lysine–Sepharose, a variable fraction of the protein elutes from the column with 0.5 M NaCl, in the absence of ϵ-ACA. The observed heterogeneity in lysine–Sepharose binding may reflect subtle differences in the degree and/or type of carbohydrate modification that were not detectable by isoelectric focusing. This would be consistent with reports that different glycosylation patterns in both plasminogen (Hays et al., 1975) and t-PA (Vehar et al., 1984) result in altered affinities of these proteins for lysine–Sepharose.

We have expressed r-apo(a) in HepG2 cells and demonstrated the ability of the recombinant protein to associate covalently with endogenous apoB-100. In 293s cells, which do not synthesize apoB-100, we did not observe protein

multimers of apo(a). This might be due to charge repulsion between negatively charged apo(a) molecules. In native Lp(a) particles, apo(a) is thought to be bound to apoB-100 by one or more disulfide linkages (Gaubatz et al., 1983; Fless et al., 1984). However, the site of this proposed linkage remains unclear at present. It has been hypothesized that a seventh cysteine residue unique to the penultimate kringle 4-like repeat (also present in the recombinant construct) may be involved in this covalent linkage (McLean et al., 1987). Interestingly, this cysteine residue is also present in analogous position in the rhesus apo(a) protein sequence (Tomlinson et al., 1989). Recently, the existence of significant noncovalent hydrophobic interactions between apo(a) and apoB-100 have been demonstrated in vitro (Trieu & McConathy, 1990). This is consistent with Figure 7, lane 6, in which uncomplexed apo(a) is observed in the absence of DTT following immunoprecipitation with the apoB-100-specific antibody. This likely results from the noncovalent association of apo(a) with apoB-100 during immunoprecipitation, which is then dissociated during SDS–PAGE in the absence of a reducting agent. Thus, Lp(a) particle assembly may reflect both covalent and strong noncovalent interactions between the apo(a) and apoB-100 protein components. In preliminary studies, we have been unable to detect assembled r-Lp(a) particles intracellularly, with use of the HepG2 expression system described in this paper (M. Koschinsky, unpublished results). Whether covalent and/or noncovalent associations involved in the assembly of Lp(a) particles occur intracellularly is currently being addressed.

The density of the r-Lp(a) particles was shown to be heterogeneous, with the majority of the r-Lp(a) complex coincident in density with that of endogenous LDL particles ($1.006 < d < 1.063$ fraction). However, some of the r-Lp(a) particle in addition to a small amount of LDL was observed in the high-density range ($1.063 < d < 1.2$ fraction). This observation is in agreement with previous reports that cite a range of density for native Lp(a) particles of 1.05–1.12. The observed absence of r-Lp(a) in the VLDL density fraction ($d < 1.006$) is not informative since HepG2 cells produce little or no VLDL-like particles (Thrift et al., 1986).

In the fraction designated as lipid poor ($d > 1.2$), the majority of the uncomplexed apo(a) was seen, in addition to some apo(a)–apoB complex. The presence of lipid-poor apo(a)–apoB has also been observed in the $d > 1.2$ fraction of plasma (Gries et al., 1987). However, since there is no direct evidence that Lp(a) can be dissociated during ultracentrifugation, Fless et al. (1990) have suggested that the lipid-poor apo(a)–apoB complex may be synthesized de novo by the liver.

The development of an expression system for generating r-apo(a) is critical for the identification of key functional domains of the protein. In addition, the ability to generate recombinant Lp(a) particles in vitro will allow us to address the steps involved in the process of particle assembly.

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Migration of Small Molecules through the Structure of Hemoglobin: Evidence for Gating in a Protein Electron-Transfer Reaction†

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ABSTRACT: It has previously been shown that the rates and activation energies for migration molecules of different sizes through myoglobin are very similar. The results were interpreted in terms of conformational changes in the protein structure that facilitate the passage of the different molecules to a similar extent. Here we ask whether the quaternary structural changes that accompany the binding of ligands (O_2 or CO) to hemoglobin might influence the migration rate from the solution into the protein's binding site. As a model for the R state of hemoglobin, we used the protein in which the Fe protoporphyrin (FePP) in the α subunit was substituted by Zn protoporphyrin (ZnPP) and the oxidized heme was ligated by CN^-. The T state of hemoglobin was represented by the protein in which all four FePP groups were substituted by ZnPP. The quenching rate of the excited ZnPP triplet state within the hemoglobin by oxygen, methyl viologen, and anthraquinonesulfonate served as a measure of the migration rate through the protein into the binding site. It was found that the activation energies for all three quenchers were very similar and closely resembled those in myoglobin, suggesting that the migration rates are determined by the subunit structure only and that the quaternary configurational changes do not influence the quenching rates. The implications of the results for electron transfer in proteins are briefly discussed.

The reaction between a protein and small molecules, for example, a substrate, depends not only on the specific reaction rate constant(s) but also on the diffusional migration of the small molecule through the protein matrix toward the reaction site. What is usually measured is the overall process. Therefore, in order to understand fully the reaction within a biological macromolecule, it is necessary to individually determine the two components of the above complete reaction.

The binding of ligands to myoglobin and to hemoglobin has been studied extensively by following the photodissociation of the bound ligand and its subsequent rebinding at the heme site (Frauenfelder & Debrunner, 1982; Ansari et al., 1986; Henry et al., 1984; Murray et al., 1988; Marden et al., 1986; Friedman, 1985). In myoglobin, it was found that the ligand must overcome one or more potential barriers on its way toward the binding site (Austin et al., 1978; Marden, 1986) before the actual binding step occurs with its own specific activation energy. In hemoglobin, the question has been raised whether the quaternary structural changes upon ligand binding affect the reentry rate of the latter into the heme pocket. It was suggested by a group at NIH (Henry et al., 1984; Murray et al., 1988) that the diffusion rate into the protein is not responsible for the differences in the ligand association rates of the hemoglobin R and T states.

We shall concern ourselves here with the migrational movement of the potential ligand O_2 and other, somewhat larger, molecules through the structure of hemoglobin.

Various experiments, such as hydrogen exchange (Lumry & Rosenberg, 1976), fluorescence quenching (Lakowicz & Weber, 1973), and X-ray diffraction (Frauenfelder et al., 1979; Artymiuk et al., 1979) and calculations (Karplus & McCammon, 1981) indicate that proteins are dynamic entities that undergo structural fluctuations. It is these movements within the protein that are thought to enable molecules like oxygen to migrate through the otherwise compact protein structure. Following the above seminal work by Weber and Lakowicz, the fluorescence and also the triplet-state quenching of tryptophan residues in proteins by different quenchers have been extensively studied (Calhoun et al., 1988; Ghiron, 1988; Papp & Vanderkooi, 1989). While it is generally agreed that oxygen efficiently penetrates most proteins and thus quenches easily the excited tryptophans, the mechanism for larger quenchers is less well understood. The phosphorescence-quenching rate constants for a number of proteins by various

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