The Number of Identical Kringle IV Repeats in Apolipoprotein(a) Affects Its Processing and Secretion by HepG2 Cells*

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Christoph Brunner‡§, Eva-Maria Lobentanz‡, Attila Pethö-Schramm§, Angelika Ernst§, Chantal Rangi, Hans Dieplinger‡, Hans-Joachim Müller§, and Gerd Utermann‡**

From the ‡Institut für Medizinische Biologie und Humangenetik, Universität Innsbruck, Schöpfstraße 41, 6020 Innsbruck, Austria, the §Boehringer Mannheim GmbH, Department of Molecular Biology, Sandhofer Straße 116, 68305 Mannheim, Germany, and the **Institut National de la Santé et de la Recherche Médicale, U.143, Paris, France

A variable number of 5.6-kilobase kringle IV repeats in the human apolipoprotein(a) (apo(a)) gene results in a size polymorphism of the protein and correlates inversely with the plasma levels of the atherogenic lipoprotein(a) (Lp(a)). In order to analyze whether this association reflects a direct effect of kringle IV repeat number on Lp(a) plasma concentration, we have studied the expression of recombinant apo(a) (r-apo(a)) isoforms in the human hepatocarcinoma cell line HepG2. Following transient transfection of apo(a) cDNA expression plasmids that differed only in the number of kringle IV repeats, we observed a gradual decrease of Lp(a) in the medium of the cells with an increasing number of kringle IV repeats, mimicking the relationship present in humans in vivo. The analysis of apo(a) protein in the lysate and in the medium of cells that were transfected with a plasmid encoding an apo(a) isoform with 22 kringles revealed a predominant intracellular precursor with little secretion of the mature apo(a) protein. In contrast, transfection of a plasmid encoding an isoform with 11 kringles led to effective secretion of the mature peptide into the medium, indicating differential processing rates of apo(a) isoforms in the secretory pathway. The intracellular accumulation of an apo(a) precursor in the endoplasmic reticulum was demonstrated by cell fractionation and [35S]Met metabolic labeling/temperature block experiments using HepG2 cells stably transfected with recombinant apo(a). The direct and causal effect of kringle IV repeat number on the expression of recombinant apo(a) in HepG2 cells, and presumably liver cells, provides a novel mechanism for the genetic regulation of the concentration of a protein.

*Lipoprotein(a) (Lp(a)) represents a quantitative genetic trait in human plasma and consists of low density lipoprotein (LDL) and a high molecular weight glycoprotein called apolipoprotein(a) (apo(a)). (1) The apo(a) gene contains ten distinct 5.6-kilobase plasminogen-like kringle IV (K-IV) units (2), named type 1–10 (3). Nine of the K-IV units have been suggested to be unique in each apo(a) allele (4, 5), whereas one (K-IV type 2) is variable in number (5–7). Variation in the number of the kringle IV type 2 (K-IV-2) repeats in the gene results in a size polymorphism of the protein (8, 9) with apo(a) isoforms comprising from 11 to >50 K-IV domains (6, 7, 10, 11). High Lp(a) plasma levels are associated with premature atherosclerosis and its sequelae, myocardial infarction, stroke, and peripheral vascular disease (12, 13). In Caucasians, the genetic control of Lp(a) concentrations is mainly exerted by the apo(a) gene locus on chromosome 6q26–q27 (7, 14) and has been dissected into two components. The first relates to the number of K-IV type 2 repeats in apo(a) that correlates inversely with Lp(a) plasma concentrations in all human populations (7, 8, 10, 11, 14, 15). Second, it has been demonstrated by sibling-pair linkage studies that Lp(a) concentrations are almost entirely controlled by variation at the apo(a) gene locus (7, 14, 16), implying sequence variation in the apo(a) gene beyond the size variation. A pentanucleotide repeat polymorphism in the apo(a) promoter and microheterogeneity in the K-IV-2 repeats has recently indeed been associated with Lp(a) concentrations (17–19), but the influence of these sequence variations on Lp(a) concentration is not a direct effect but reflects allelic associations with unknown sequence variation within or linked to the apo(a) gene in Caucasians (19, 20).

It is important to note that the inverse correlation between the number of K-IV repeats in apo(a) and Lp(a) concentration formally represents an association and that the epidemiological data do neither prove a causal relationship nor provide insight into the mechanisms underlying the association. There exist strong allelic associations between the number of K-IV repeats and sequence variation related to Lp(a) concentrations (17, 19) within the apo(a) gene, and the frequency of apo(a) null alleles is significantly associated with high K-IV repeat numbers (10). White et al. (21) have previously shown that the secretion of apo(a) by primary baboon hepatocytes relates to the size of the apo(a) isoform and that larger isoforms have a longer residence time in the endoplasmic reticulum. It is, however, unknown whether there are internal polymorphisms in the natural apo(a) alleles analyzed in this work. In the present study, we have analyzed whether the association reflects a causal relationship between the K-IV repeat number and Lp(a) concentration by transient transfection of apo(a) cDNA plasmids that differed solely in the number of K-IV repeats into the human hepatocarcinoma cell line HepG2. This allowed us to investigate the effect of isoform size on the expression of apo(a) without interference from other genetic variation existing in vivo.
**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The cloning of the cDNA expression plasmid pCMV-A18 encoding signal peptide, 17 K-IV repeats, 1 kringle V unit, and apoB-48 was obtained by recombination between pCMV-A10 and pCMV-A18 D32-35 (24) and contained the 6 apo(a) kringle IV-1, IV-2/3, IV-4, IV-9, IV-10, and V. Plasmid pCMV-A11 resulted from the insertion of a single K-IV-2 domain between K-IV-1 and K-IV-3 in pCMV-A10. The reporter gene plasmid pLUC-TFA contained a firefly luciferase cDNA (25) linked to a human transferrin promoter fragment (26) and was used for cotransfection studies. Standard cloning methods were used to insert the firefly luciferase expression cassette from pLUC-TFA into the apo(a) isoform plasmids. The resulting plasmids were named pCMV-A6-LUC, pCMV-A10-LUC, etc. and contained the luciferase and apo(a) cassettes in a head to head orientation (see Fig. 1).

**Cell Culture and Transient Transfection**—The human hepatocarcinoma cell line HepG2 was obtained from the American Type Culture Collection and maintained as described (22). HepG2 cells were transiently transfected by lipofection using the lipofectamine reagent (Life Technologies, Inc.) with monoclonal antibody 012 (Chemicon), directed against human apo(a) and K-IV-3 in pCMV-A10. The reporter gene plasmid pLUC-TFA resulted from the insertion of a single K-IV-2 domain between K-IV-1 and K-IV-3 in pCMV-A10. The reporter gene plasmid pLUC-TFA contained a firefly luciferase cDNA (25) linked to a human transferrin promoter fragment (26) and was used for cotransfection studies. Standard cloning methods were used to insert the firefly luciferase expression cassette from pLUC-TFA into the apo(a) isoform plasmids. The resulting plasmids were named pCMV-A6-LUC, pCMV-A10-LUC, etc. and contained the luciferase and apo(a) cassettes in a head to head orientation (see Fig. 1).

**Detection of Metabolically Labeled r-apo(a)/apoB Complexes from Transiently Transfected Cells**—1 × 10^6 HepG2 cells were cotransfected with equimolar amounts of apo(a) isoform plasmids and a constant amount of the luciferase reporter gene plasmid pLUC-TFA. 24 h after transient transfection, the media were replaced by MEM labeling medium minus methionine (Life Technologies, Inc.) supplemented with 10% dialyzed FCS, 2 mM glutamine, and 200 μCi/ml [35S]-s-methionine (Amersham Buchler GmbH, Braunschweig, FRG). Following another 24-h incubation, the labeling was stopped by addition of 2 mM methionine (Sigma) and 1 mM phenylmethylsulfonyl acid (Boehringer Mannheim). Media were centrifuged to remove lipoproteins and to precipitate the protein complexes. Aliquots of the supernatant were further processed as described previously (22).

**Immunoprecipitation**—Immunoprecipitation was performed using antibodies against apo(a) (clone 1A2) and apoB (clone 1A2). Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with the monoclonal antibodies 1A2 (28) and LM-1. Controls included a monospecific polyclonal rabbit anti-apo(a) antibody (29) and a control mixture containing 62% sucrose. To this 1M of PNS, 1.5 M of sucrose and 1 M of 25% sucrose was overlayed and filled with homogenization buffer including EDTA. Ultracentrifugation was performed in a Beckman SW 50.1 rotor at 50,000 × g for 30 min. Immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Proteins were visualized by using a monoclonal antibody against human apo(a) (Boehringer Mannheim). Fluorography was performed with commercially available solutions from DuPont (Enertense A, B²).
Expression of r-Apo(a) in HepG2 Cells—The human hepatocarcinoma cell line HepG2, which is widely used in lipoprotein research, secretes apolipoprotein B-100 (apoB) containing lipoproteins (e.g. LDL) into the medium but no apo(a) or Lp(a) (22, 34–36). Here, we have used apo(a) plasmids pCMV-A10, -A11, -A14, -A18, -A22, -A26, -A30, and -A34, which differed only in the number of K-IV repeats from null to 24 (Fig. 1), in order to study the mechanism underlying the inverse correlation of apo(a) isoform size with Lp(a) concentration. The shortest apo(a) plasmid, pCMV-A6, differed from the pCMV-A10 plasmid by an internal deletion of the apo(a) repeat domain K-IV-5 to K-IV-8 (kringle 32–35 in the nomenclature of McLean et al. (2)). In a first set of experiments, HepG2 cells were transiently transfected with plasmids pCMV-A10, -A14, -A18, and -A22 (Fig. 1). Following transfection, aliquots from the conditioned media were subjected to immunoblotting. The sizes of the secreted apo(a) isoforms corresponded to the K-IV repeat number in the transfected apo(a) plasmids (Fig. 2, lanes 1–4). To test whether all r-apo(a) isoforms are associated with lipids and form Lp(a) particles, lipoproteins were separated from the media by ultracentrifugation at density of 1.21 g/ml (at which all lipoproteins float) and subjected to immunoblotting (Fig. 2, lanes 6–9). Each r-apo(a) isoform was recovered almost quantitatively from the lipoprotein fraction as shown by ELISA analysis (not shown), indicating that it had assembled with LDL. This is consistent with our previous analysis of HepG2 derived r-apo(a) by density gradient ultracentrifugation (22). The intensity of apo(a) isoforms on the immunoblots (Fig. 2, lanes 1–4) already indicated that cells transfected with the smallest isoforms secreted apo(a) more efficiently than those transfected with the larger isoforms. However, no correction for transfection efficiency was performed in these experiments.

Dependence of r-Apo(a) Expression from K-IV Repeat Length:

RESULTS

Expression of r-Apo(a) in HepG2 Cells—The human hepatocarcinoma cell line HepG2, which is widely used in lipoprotein research, secretes apolipoprotein B-100 (apoB) containing lipoproteins (e.g. LDL) into the medium but no apo(a) or Lp(a) (22, 34–36). Here, we have used apo(a) plasmids pCMV-A10, -A14, -A18, and -A22 (Fig. 1) were transfected into HepG2 cells. 60 h after transfection, culture media (lanes 1–4) and lipoprotein fractions (<1.21 g/ml) (lanes 6–10), thereof, were analyzed by reducing SDS-PAGE and subsequent immunoblotting with the anti-apo(a) antibody IA2. An apo(a) phenotyping standard (Immuno, Heidelberg, Germany) was included as a size marker (lane 5).

Demonstration of Metabolically Labeled r-Apo(a)-ApoB Complexes—Next we tried to quantify metabolically labeled r-apo(a) of different length in the HepG2 medium. However, employing several conditions for metabolic labeling including >12-h labeling periods, we realized that r-apo(a) was only insufficiently labeled (not shown). In contrast, apoB was efficiently labeled. Therefore, we quantified r-apo(a) as r-apo(a)-apoB complexes by non-reducing SDS-PAGE. HepG2 cells were transfected with equimolar amounts of the four apo(a) isoform plasmids pCMV-A10, -A14, -A18, and -A22 (Fig. 1) and cotransfected with the luciferase reporter gene plasmid pLUC-TFA. 24 h after transfection, the cells were labeled with [35S]methionine. Following a 24-h labeling period, media were collected, and apoB containing lipoproteins was precipitated with anti-apoB antibody. This resulted in the coprecipitation of all apo(a) from the media as measured by ELISA (not shown). The immunoprecipitates were subjected to SDS-PAGE under non-reducing conditions, and gels were autoradiographed prior to semiquantitative determination of r-apo(a)-apoB complexes by densitometry. All HepG2 cells, whether transfected or not, produced free apoB in similar amounts (Fig. 3a, lanes 1–5). Cells transfected with the A11, A14, and A18 plasmids in addition produced r-apo(a)-apoB complexes that varied in size depending on the expressed apo(a) isoform. The intensity of these complexes decreased with the length of the apo(a) isoform present in the complex (Fig. 3a, lanes 1–4). Following transfection with the
largest (A22) plasmid, the cells produced only barely detectable amounts of r-apo(a)-apoB complexes (Fig. 3A, lane 4). Densitometric determination of the r-apo(a)-apoB band intensities and normalization for cytosolic luciferase activities revealed a clear inverse relationship between r-apo(a) isoform size and r-apo(a)-apoB complexes in the media of transfected cells (Fig. 3B). The relative amounts of r-apo(a)-apoB complexes after transfection of apo(a) isoform plasmids was determined by densitometric scanning. The intensity of the 11 kringle apo(a)-apoB complex was taken as 100%. Luciferase normalization was performed to account for varying transfection efficiencies.

**Demonstration of Secreted Apo(a) in HepG2 Media by Immunoblotting**—Theoretically, the different sizes of the apo(a) isoform plasmids pCMV-A11, A14, A18, and A22 were transfected into HepG2 cells followed by metabolic labeling of newly synthesized proteins with [35S]methionine. Anti-apoB antibody was used to immunoprecipitate apoB-containing lipoproteins. After fractionation of the immunoprecipitates by non-reducing SDS-PAGE, the dried gel was subjected to autoradiography. A, the exposure shows strong apoB bands of similar intensity (B) in transfected (lanes 1–4) and in untransfected (lane 5) cells. Bands corresponding to r-apo(a)-apoB complexes were only visible in the transfected cells, with a gradual decrease in the band intensity from the shortest A11 isoform (lane 1) to the longest A22 isoform (lane 4). B, the relative amounts of r-apo(a)-apoB complexes after secretion of apo(a) isoforms plasmids was determined by densitometric scanning. The intensity of the 11 kringle apo(a)-apoB complex was taken as 100%. Luciferase normalization was performed to account for varying transfection efficiencies.

**Fig. 3. Quantification of metabolically labeled r-apo(a)-apoB complexes in the medium of transfected HepG2 cells.** The apo(a) isoform plasmids pCMV-A11, A14, A18, and A22 were transfected into HepG2 cells followed by metabolic labeling of newly synthesized proteins with [35S]methionine. Anti-apoB antibody was used to immunoprecipitate apoB-containing lipoproteins. After fractionation of the immunoprecipitates by non-reducing SDS-PAGE, the dried gel was subjected to autoradiography. A, the exposure shows strong apoB bands of similar intensity (B) in transfected (lanes 1–4) and in untransfected (lane 5) cells. Bands corresponding to r-apo(a)-apoB complexes were only visible in the transfected cells, with a gradual decrease in the band intensity from the shortest A11 isoform (lane 1) to the longest A22 isoform (lane 4). B, the relative amounts of r-apo(a)-apoB complexes after secretion of apo(a) isoforms plasmids was determined by densitometric scanning. The intensity of the 11 kringle apo(a)-apoB complex was taken as 100%. Luciferase normalization was performed to account for varying transfection efficiencies.

**Fig. 4. Immunoblot analysis of r-apo(a) isoforms.** Media of HepG2 cells transfected with eight different plasmids encoding apo(a) isoforms, which contain from 6 to 34 kringle domains, were analyzed by immunoblotting employing either the K-IV-specific antibody 1A2 (panel A) or a kringle V-specific antibody LM-1 (panel B). The latter antibody detects an epitope in the kringle V domain of apo(a). This domain occurs as a single copy in apo(a) (2), which allows apo(a) quantification independent from the isoform size. HepG2 cells transfected with the smaller isoform produced significantly more apo(a) in the medium than cells transfected with large isoforms (Fig. 4, A and B). The inverse relation between isoform size and signal intensity on the immunoblot was clearly demonstrated by both antibodies but was more impressive after kringle V detection with the LM-1 antibody (Fig. 4B). The latter antibody detects an epitope in the kringle V domain of apo(a). This domain occurs as a single copy in apo(a) (2), which allows apo(a) quantification independent from the isoform size. HepG2 cells transfected with the smaller isoform produced significantly more apo(a) in the medium than cells transfected with large isoforms (Fig. 4, A and B). The inverse relation between isoform size and signal intensity on the immunoblot was clearly demonstrated by both antibodies but was more impressive after kringle V detection with the LM-1 antibody (Fig. 4B).
Antibody 1A2 revealed a gradual decrease in the concentration of r-apo(a) with increasing length of the apo(a) isoform.

The results of the above experiments raise the question of what the underlying mechanism of isoform-dependent expression of r-apo(a) might be. In order to evaluate differential mRNA stability as one candidate mechanism to explain the isoform-dependent expression of r-apo(a) in the media and steady-state levels of r-apo(a) mRNA in lysates from transfected HepG2 cells, Apo(a)-LUC plasmids encoding the isoforms A11, A14, A18, and A22 were transfected into HepG2 cells. 60 h after transfection, total RNA from the transfected cells was subjected to a competitive RT-PCR analysis (under "Materials and Methods") in order to quantitate apo(a) and luciferase mRNAs (panel A). This experiment was performed three times. Mean normalized apo(a) mRNA levels in the three experiments were: A11, 100%; A14, 85%; A18, 97%; and A22, 64%. Characterization of cell culture media from the same experiment as in panel A by reducing panel B or non-reducing (panel C) SDS-PAGE and subsequent immunoblotting with the monoclonal antibody IA2 revealed a gradual decrease in the concentration of r-apo(a) with increasing length of the apo(a) isoform.

or A22. This was corroborated by statistical analysis (Pearson's product moment correlation, \( r = 0.137 \), Fig. 5A). In contrast, we observed a clear inverse correlation between r-apo(a) isoform size and r-apo(a)/r-Lp(a) concentrations in the media of transfected cells (Fig. 5, B and C). Taken together, the mRNA and the protein data indicate that the isoform-dependent expression of r-apo(a) cannot be explained by differences in the steady-state concentrations of apo(a) mRNAs.

Subcellular Fractionation of HepG2 Cells—As demonstrated above, apo(a) is not metabolically labeled under the conditions of our transient transfection experiments. In order to localize and to establish the precursor-product relation of intracellular apo(a) isoforms (see below), HepG2 cell lines that stably expressed apo(a) isoforms with 14 and 22 K-IV repeats, respectively, were generated. Cells were homogenized and PNS were prepared by centrifugation at 1000 \( \times g \). No apo(a) immunoreactivity was observed in the PNS pellet (data not shown).

FIG. 5. Representative experiment showing r-apo(a) isoforms in the media and steady-state levels of r-apo(a) mRNA in lysates from transfected HepG2 cells. Apo(a)-LUC plasmids encoding the isoforms A11, A14, A18, and A22 were transfected into HepG2 cells. 60 h after transfection, total RNA from the transfected cells was subjected to a competitive RT-PCR analysis (under "Materials and Methods") in order to quantitate apo(a) and luciferase mRNAs (panel A). This experiment was performed three times. Mean normalized apo(a) mRNA levels in the three experiments were: A11, 100%; A14, 85%; A18, 97%; and A22, 64%. Characterization of cell culture media from the same experiment as in panel A by reducing panel B or non-reducing (panel C) SDS-PAGE and subsequent immunoblotting with the monoclonal antibody IA2 revealed a gradual decrease in the concentration of r-apo(a) with increasing length of the apo(a) isoform.

Antibody 1A2 revealed a gradual decrease in the concentration of r-apo(a) with increasing length of the apo(a) isoform.

For subcellular fractionation, the postnuclear supernatant (PNS) was prepared from homogenized HepG2 cells by low speed centrifugation and fractionated by ultracentrifugation in a floating sucrose gradient as described under "Materials and Methods." Subcellular organelles were identified by their marker enzymes, NADPH-cytochrome c-reductase (ER, open squares) and UDP-galactosyltransferase (Golgi, filled circles). Densities are given by open circles. Insert, medium (M), PNS and fractions 1–11 from the sucrose gradient were subjected to 4% SDS-PAGE under reducing conditions, followed by immunoblotting with monoclonal antibody IA2 to apo(a). Arrows point at the precursor (pr-apo(a)) and mature forms of apo(a).
14 K-IV repeats under temperature conditions that are known to inhibit the secretory pathway at the ER to Golgi transport (15 °C) and at the TGN (20 °C) level (37, 38). Cells were first metabolically labeled with [35S]methionine, chased for 6 h at 15 °C, 20 °C, and 37 °C, lysed, and subjected to reducing SDS-PAGE together with samples from their respective culture supernatants (Fig. 7). At 15 °C, all the labeled apo(a) was exclusively found in the form of the low molecular weight precursor; whereas at 20 °C, approximately half of the labeled apo(a) was processed into the large form, but none was secreted. At 37 °C, most of the apo(a) was found in the medium, and only some was retained intracellularly predominantly in the form of the precursor (Fig. 7). These results confirm the precursor product relation and the predominant localizations of the precursor and mature forms of intracellular apo(a) in the ER and Golgi-TGN fractions, respectively.

**Posttranscriptional Processing of Apo(a) Is Different for r-Apo(a) Isoforms of Different Length**—The next experiments were designed to detect possible differences in the intracellular processing and secretion of apo(a) isoforms from HepG2 cells. We transiently transfected the apo(a) plasmids pCMV-A11-LUC and pCMV-A22-LUC either alone or in combination. 85 h after the transfection cell lysates and media were characterized by immunoblotting. As for the stable transfections the immunoblot analysis of HepG2 lysates after transfection with the pCMV-A11-LUC plasmid revealed two intracellular apo(a) species with different apparent molecular masses a minor band representing mature r-apo(a) glycoprotein and the predominant lower molecular mass apo(a) precursor (Fig. 8). Transfection of the larger pCMV-A22-LUC plasmid yielded the apo(a) precursor as the predominant intracellular apo(a) species with little mature protein detectable in the cell. The media of transfected cells contained exclusively mature apo(a) glycoprotein with at least a tenfold higher amount of apo(a) for the smaller (A11) as compared to the larger (A22) isoform. Cotransfection of the pCMV-A11-LUC and pCMV-A22-LUC plasmids confirmed the results from the single transfections (Fig. 8). There was significantly more of the large precursor than of the small one found intracellularly, whereas in the medium there was significantly more of the small than of the large mature apo(a).

**FIG. 8.** Immunoblot analysis of lysates and media from transiently transfected HepG2 cells. HepG2 cells were transfected with equimolar amounts of the apo(a) isoform plasmids pCMV-A11 (panel A), pCMV-A22 (panel B), or a combination of both plasmids (panel C). 85 h after transfection, cell lysates and media were fractionated by reducing SDS-PAGE. Immunoblotting with the anti-apo(a) antibody 1A2 revealed four bands representing the mature apo(a) isoforms (a11 and a22) and the corresponding precursor molecules (p11 and p22).

Baboon hepatocytes (21). This precursor is processed into the mature form and secreted into the media more effectively for small than for large isoforms explaining the differences in extracellular mature isoform accumulation between cells transfected with apo(a) isoform plasmids. For the large intracellular apo(a) precursor with 22 kringles there was also evidence for intracellular degradation as seen from anti-apo(a) immunoreactive bands with increased electrophoretic mobility (Fig. 8). Corresponding bands remained undetectable within cells that contained comparable amounts of small apo(a) precursor with 11 kringle domains.

**DISCUSSION**

Here we demonstrate that the number of transcribed and translated K-IV repeats in apo(a) has a direct effect on the secretion of r-apo(a) from HepG2 cells. The gradual decrease in the secretion of r-apo(a) from short isoforms to long isoforms mimics the inverse correlation of apo(a) isoform size (i.e., K-IV repeat number) with Lp(a) plasma concentrations seen in vivo in humans (10, 11, 15). Since the inverse relation of the length of apo(a) constructs with the concentration of Lp(a) in the media from transfected HepG2 cells might be an artifact due to different transfection efficiencies of plasmids of different size, we have performed experiments using constructs that contained apo(a) and a luciferase reporter gene together on the same plasmid. These experiments clearly demonstrated that the observed results are not artifactual.

The mechanism underlying the association of Lp(a) concentration with repeat length in humans has not yet been elucidated. White et al. (21) have demonstrated that the processing and secretion of apo(a) isoforms by primary baboon hepatocytes correlates with the length of the isoform. In vivo turnover studies in humans show that apo(a) secretion rate into plasma is slower for large than for small isoforms (39) and that differences in plasma Lp(a) concentrations are entirely explained by differences in apo(a) synthesis rather than by differences in the catabolism of the particle (39, 40). In particular, the experiments with baboon hepatocytes have been considered as conclusive evidence that the effect of K-IV repeat number on Lp(a) concentration is direct and causal. The apo(a) alleles analyzed in these studies were, however, of undefined sequence and defined by isoform size only. They may well have differed in sequence. Therefore, these experiments do not prove an effect of kringle IV-2 repeat number on apo(a) secretion. Theoretically, there exist at least three alternative explanations for the association of apo(a) size with Lp(a) concentration. (i) The copy number of identical K-IV repeats in the gene might indeed have a direct effect on the production of apo(a) by liver cells. So far,

**FIG. 7.** Analysis of [35S]Met-labeled apo(a) in lysates and media from HepG2 cells incubated at different temperatures. Stably transfected HepG2 cells expressing an apo(a) isoform with 14 kringle IV were metabolically labeled for 16 h with 500 μCi/ml [35S]Met followed by a 6-h chase at 15 °C, 20 °C, and 37 °C. Apo(a) was immunoprecipitated from media and lysates with a polyclonal antibody against apo(a). The immunoprecipitates were subjected to a 4% SDS-PAGE under reducing conditions followed by fluorography. Non-transfected HepG2 cells served as control (C). Apo(a) precursor (pr-apo(a)) and mature forms and apoB are indicated by arrows.
such a situation has not been described and would represent a novel mechanism for the genetic regulation of concentrations of a gene product. (ii) There might be allelic association (linkage disequilibrium) between the K-IV repeat number and sequence variation in apo(a) that affects Lp(a) levels. Allelic associations of the K-IV repeat polymorphism with sequence variation in kringle IV-10 (41) and with microheterogeneity in K-IV-2 (17) have indeed been demonstrated. The experiments reported for baboon hepatocytes (21), and the in vivo turnover data from humans (39, 40) may well be explained by such a phenomenon. (iii) Apo(a) genes with a high copy number of repeats may have accumulated more deleterious mutations that negatively affect the concentration of the protein. A higher likelihood for large isoforms to harbor such mutations would also result in an inverse correlation between isoform size and expression level and might completely explain the epidemiological data. Though null alleles are present within each apo(a) size category (10, 11), they are significantly associated with the higher K-IV repeats in all populations studied by us (10). Such a situation might also exist in nonhuman primates. Therefore, it has been unclear whether there is a direct effect of K-IV repeat length on Lp(a) concentration or not and, if so, what the mechanism might be. The data presented herein clearly demonstrate that differences in the number of K-IV repeats are sufficient to cause differences in Lp(a) concentrations in the media from transfected HepG2 cells. All the transfected plasmids contained the same foreign promoter, the same heterologous intron upstream of the apo(a) coding sequence, and the same heterologous (SV40) polyadenylation site downstream of the apo(a) coding sequence (22). Therefore, differential initiation of transcription or differential apo(a) mRNA processing cannot be responsible for the observed length dependence of apo(a) secretion. With the exception of the A6 plasmid, all other apo(a) plasmids differed solely in the number of K-IV-2 repeats (Fig. 1). It is conceivable that the relation observed in our in vitro cell culture system also exists in human liver cells in vivo. In the HepG2 in vitro model, we observed about a 10-fold difference in the expression levels of r-apo(a) with 10/11 versus 22 K-IV repeats, which is in the same range as seen in epidemiological studies in humans in vivo (1). This suggests a basic cellular mechanism that is responsible for the in vivo variation in Lp(a) plasma levels. Our study also provides some insights into this mechanism. For each expressed apo(a) allele, two main species of intracellular apo(a) have been demonstrated in primary baboon hepatocytes (21), a smaller precursor molecule and a mature apo(a) with the size of extracellular apo(a). Similar observations have been made for r-apo(a) in transfected cells (34) and in the liver from apo(a) transgenic mice (42). In the present work, we have cotransfected apo(a) isoforms plasmids with 11 and 22 K-IV repeats in order to compare the amounts of intracellular apo(a) precursor and of intra- and extracellular mature r-apo(a) between large and small isoforms. Intracellularly, we found a much higher concentration of large versus small r-apo(a) precursors (Fig. 6). In contrast, the amount of intra- and extracellular mature r-apo(a) was much higher for the small isoform. Together with the metabolic labeling experiments (Fig. 3), these data suggest that large pools of r-apo(a) precursors exist in the cell. Cell fractionation and temperature block experiments in HepG2 cells, stably transfected with apo(a) confirmed the presence of a large pool of apo(a) precursor in the ER and a precursor-product relationship between the two intracellular forms. Taken together, this suggests that the ER-residence times differs markedly by isoforms. This is consistent with the data from baboon hepatocytes (21) and most likely reflects differences in retention times required for proper folding and glycosylation of apo(a) isoforms.

We also want to point out that our in vitro model is limited in its capability to detect differences in transcription and mRNA processing between isoforms and that our data do not exclude the possibility that there are further differences in the cellular processing of r-apo(a) in the secretory pathway or elsewhere. We have used cDNA rather than genomic DNA in our experiments. Hence in vivo, such effects may add to those observed here. The presence of transfected repeat units in genes is a widespread phenomenon in eucaryotes. There are only a few genes with identical large transfected repeats. Examples include the size polymorphism of the human keratin 10 chain (43), the length variation in the PUM genes (44) and in the human proline-rich protein (45). Apart from the apo(a) gene, there is no other known gene with such an enormous variation in the number of large identical repeats. Finally, there exists, to our knowledge, no other protein where the number of transfected units in the gene determines the concentration of the protein secreted from the cell. What might be the biological meaning of such a phenomenon if there is one at all? In combination with the high number of alleles and heterozygosity at the apo(a) locus, one possibility might be the capacity for a rapid evolutionary adaptation of Lp(a) concentrations and/or repeat length to different environments.

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