Molecular Basis for "Null" Lipoprotein(a) Phenotypes and the Influence of Apolipoprotein(a) Size on Plasma Lipoprotein(a) Level in the Baboon*

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Ann L. White*, James E. HixsonO, David L. Rainwater§, and Robert E. Lanford††

From the †† Department of Virology and Immunology and the ‡ Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, Texas 78228-0147

High plasma levels of lipoprotein(a) (Lp(a)) and its unique apolipoprotein, apo(a), are an independent risk factor for cardiovascular disease. Plasma Lp(a) levels vary over a 1000-fold range and are determined by the apo(a) locus, which has at least 34 alleles expressing apo(a) isoforms with molecular weights from <300,000 to >800,000. In addition, "null" apo(a) alleles produce no detectable plasma apo(a). We used primary cultures of baboon hepatocytes to investigate the molecular basis for null apo(a) phenotypes. Immunoprecipitation of apo(a) after radiolabeling of hepatocytes revealed that some null alleles gave rise to intracellular protein products that were not secreted. Pulse-chase analysis and endoglycosidase digestion demonstrated that these proteins were retained in the endoplasmic reticulum. We also examined the molecular basis for the documented inverse correlation between apo(a) size and plasma Lp(a) concentration. Steady-state labeling and pulse-chase analysis of hepatocytes from animals expressing two isoforms of apo(a) revealed that the endoplasmic reticulum residence time of secreted apo(a) isoforms was determined by their size. This accounted for the inverse relationship between isoform size and level of secretion. We conclude that the efficiency of post-translational processing of apo(a) is a major determinant of plasma Lp(a) concentration.

Lipoprotein (a) (Lp(a)) was first described by Berg in 1963 (1) and consists of a modified form of low density lipoprotein in which apolipoprotein (apo) B100 is disulfide-linked to an additional, high molecular weight glycoprotein, apo(a) (2). High plasma levels of Lp(a) represent an independent risk factor for the development of atherosclerotic cardiovascular disease (3). However, the function of this lipoprotein, its role in coronary disease, and factors that regulate its production and removal from the circulation remain poorly understood.

Apo(a) is synthesized by the liver (4–6) and is present as a number of isoforms that vary in size from <300 to >800 kDa (7, 8). Apo(a) is highly homologous to plasminogen and contains a single copy of the plasminogen kringle 4 and protease domains preceded by multiple copies of plasminogen kringle 4-like repeats (9). The size of the apo(a) protein is determined by the number of kringle 4 domains encoded (10–12), which varies from approximately 12 to 51 (13). So far, 34 apo(a) isoforms have been resolved by gel electrophoresis of human plasma (14). Apo(a) thus represents the most polymorphic plasma protein known to date. Apo(a) is also highly glycosylated, with 28% of its weight contributed by carbohydrate (15).

Plasma Lp(a) concentrations show tremendous variation (<1 to >100 mg/dl) among individuals, are highly heritable, and are remarkably constant throughout an individual’s lifetime (16). The apo(a) locus contributes to >50% of the variation in plasma Lp(a) levels (17). Apo(a) isoform size is inversely correlated with plasma Lp(a) concentration (7, 8, 12) and accounts for 19–70% of the variation depending on the ethnic group (18). However, exceptions to the correlation between apo(a) size and plasma Lp(a) level exist such that a particular isoform size may be associated with widely different Lp(a) concentrations (8, 12, 19). This suggests that sequence variation at the apo(a) locus, other than the number of encoded kringle 4 repeats, has a significant effect on plasma Lp(a) concentration.

Metabolic studies demonstrate that plasma Lp(a) levels are determined by the rate of Lp(a) production rather than its rate of catabolism (21). In cynomolgous monkeys, hepatic apo(a) mRNA concentration and apo(a) glycoprotein size together account for up to 58% of the variation in plasma Lp(a) levels. The two effects are independent, however, as no association between apo(a) mRNA levels and protein size was found (22). Thus, both transcriptional and post-transcriptional mechanisms play an important role in determining levels of Lp(a) synthesis.

The absence of detectable Lp(a) in the plasma of some individuals led to the proposal that "null" apo(a) alleles, which do not give rise to detectable apo(a) proteins, are present in the population (7). Studies using more sensitive Lp(a) assays suggest that very few true Lp(a) negative plasmas exist (8, 23). However, null alleles are still required to explain the high frequency of single apo(a) isoform phenotypes in the population (8, 24) compared with the very low frequency of homozygous genotypes (12). Studies in baboons demonstrate that two classes of null apo(a) alleles exist, "transcript negative null" alleles, which do not produce a detectable hepatic apo(a) mRNA transcript or plasma protein product and "transcript positive null" alleles, which do not give rise to a detectable plasma protein despite the presence of substantial levels of hepatic apo(a) mRNA (10, 25). Null alleles, therefore, represent extreme examples of both transcriptional and post-transcriptional regulation of plasma Lp(a) levels.

The baboon shows very similar characteristics to humans in terms of plasma Lp(a) levels and apo(a) isoform sizes (19, 26). Eleven apo(a) isoform size classes have been identified in the baboon and are designated A (the largest) through L (the smallest) (19). We have established primary baboon hepatocyte cultures for the analysis of Lp(a) biogenesis (4, 27, 28). The aim of...
the current study was to use this system to identify post-transcriptional mechanisms involved in determining levels of Lp(a) production. In particular, we investigated the molecular bases for (a) the absence of detectable plasma apo(a) proteins associated with transcript positive null apo(a) alleles and (b) the inverse relationship between apo(a) size and plasma Lp(a) concentration. The results demonstrated that transcript positive null apo(a) phenotypes were caused by the production of defective apo(a) proteins, which were retained inside the ER. In addition, variable ER retention of secreted apo(a) isoforms accounted for the inverse relationship between apo(a) size and plasma Lp(a) level. Thus, the efficiency of post-translational processing of apo(a) is a major determinant of plasma Lp(a) concentration.

**EXPERIMENTAL PROCEDURES**

*Materials*—(35S)Cysteine and Expre[35S]S label were from DuPont NEN. Sheep anti-human apoB polyclonal antibody was from Boehringer Mannheim, and goat anti-human Lp(a) was from Biodesign, Kennebunkport, ME. The rabbit anti-human apo(a) polyclonal antibody has been described previously (19). This antibody was prepared against purified Lp(a), and anti-apo(a) was isolated by removing antibodies that bound apoB. Methionine and cysteine-free Williams medium E was purchased from Life Technologies, Inc. N-Glycanase (peptide-N-glycanase) and Neuraminidase (acylneuraminyl hydrolase) were from Genzyme Corp. (Cambridge, MA), and endoglycosidase H (endo-β-N-acetyl-glucosaminidase H) was from ICN Immunobiologicals (Costa Mesa, CA). All other chemicals were of analytical grade.

**Hepatocytic Isolation and Culture**—Hepatocytes were isolated and cultured in a serum-free medium formulation (formula III) as described previously (27), except for the omission of thyrotropin releasing factor. All experiments were performed on confluent 60-mm dishes of cells, which had been in culture for 7–10 days.

**Radiolabeling and Immunoprecipitation**—For steady-state labeling experiments, hepatocytes were incubated for 20 h in methionine and cysteine-free serum-free medium supplemented with 50 μM each of unlabeled l-methionine and l-cystine plus 125 μCi/ml each of l-[35S]cysteine and Expre[35S]S label. For pulse-chase studies, cells were preincubated for 2 h in methionine- and cysteine-free serum-free medium, labeled for 10 min with 2 ml of the same medium supplemented with 125 μCi/ml each of l-[35S]cysteine and Expre[35S]S label, then chased for 1, 3, or 10 min in serum-free medium. Radioabeled samples were harvested and analyzed by immunoprecipitation and 3–10% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after reduction with 2-mercaptoethanol, exactly as described previously (28). For indicated samples, the percentage of apo(a) in the mature form was determined using an LKB laser densitometer.

**Endoglycosidase Digests**—Apo(a) was immunoprecipitated from cell lysates, as described above, and samples were eluted from protein A-agarose in 112 μl of 0.5% SDS and 8 μl of 10% 2-mercaptoethanol for 5 min at 100 °C. Aliquots of the samples (20 μl) were incubated at 37 °C for 16 h after addition of one of the following: 1) 10 μl of 7.5% Nonidet P-40, 30 μl of 0.4% Tris, pH 8.6, and 5 units of N-glycanase; 2) 40 μl of 50 mM sodium acetate, pH 5.7, and 3 μIU of endoglycosidase H; 3) 10 μl of 7.5% Nonidet P-40, 30 μl of 30 mM sodium acetate, pH 6.5, and 10 μl of neuraminidase; 4) 40 μl of phosphate-buffered saline (control).

**Immunoblotting**—Aliquots of sera (1 μl) and culture media were analyzed for apo(a) isoforms by immunoblotting after reduction with 2-mercaptoethanol as described previously (28), except that where indicated goat anti-human Lp(a) and rabbit anti-goat IgG were the primary and secondary antibodies, respectively. Detection was with [125I]-protein A.

**Northern Blotting**—Liver samples were obtained and stored at −80 °C. Total RNA was extracted from homogenized liver samples and analyzed by Northern blotting, as described previously (10), using a human apo(a) (1p159Da41.2) probe provided by Richard Lown (9).

**RESULTS**

*Transcript Negative Null and Transcript Positive Null Apo(a) Phenotypes*—An example of a transcript negative null and a transcript positive null allele is illustrated in Fig. 1 in a comparison of plasma apo(a) isoforms and hepatic apo(a) transcripts from three baboons heterozygous at the apo(a) locus. A, plasma apo(a) isoforms were analyzed by electrophoresis of sera in 3–10% gradient SDS-polyacrylamide gels under reducing conditions followed by immunoblotting with a rabbit anti-apo(a) antibody, as described under "Experimental Procedures." We have previously demonstrated the specificity of this antibody in immunoblotting, enzyme-linked immunosorbent assay, and immunoprecipitation assays (19, 28, 40). The lower limit of detection of apo(a) corresponded to a plasma Lp(a) concentration of 0.4 mg/dl. The positions of apo(a) isoforms and of apoB, are indicated; a single B isoform was detected for animal 1, an A and an I isoform were detected for animal 2, and a single K isoform was detected for animal 3. A small amount of apoB was also detected in each lane due to residual anti-apoB reactivity in the antibody preparation. B, total hepatic RNA from each animal was analyzed by Northern blotting using a radiolabeled apo(a) cDNA probe, as described under "Experimental Procedures." The probe used cross-reacts with domains in plasminogen mRNA (Fig. 1) with homology to apo(a) transcripts. From the data shown and from knowledge of the pedigree of each animal (see "Results"), it can be inferred that animal 1 was heterozygous for a B and a transcript negative null apo(a) allele, animal 2 was heterozygous for an A and an I allele, and animal 3 was heterozygous for a K and a transcript positive null apo(a) allele.
FIG. 2. Transcript positive null apo(a) phenotypes result from the inability of apo(a) to exit the ER. Experiments were performed on hepatocytes isolated from a baboon known to possess a K and a transcript positive null apo(a) allele (Fig. 1, animal 3). Panel A, cells were labeled to steady state with \[^{35}S\]methionine and \[^{35}S\]cysteine. ApoB (B) and apo(a) (\(a\)) were immunoprecipitated from the cell lysate and culture medium and were analyzed by 3–10% SDS-PAGE and fluorography, as described under “Experimental Procedures.” We have previously demonstrated the specificity of the apo(a) antibody for immunoprecipitation by immunocompetition and use of control antibodies (28). Panel B, cells were labeled as described in panel A, and apo(a) was immunoprecipitated from the cell lysate and treated with phosphate-buffered saline (1), Endo H (2), or N-glycanase (3) as described under “Experimental Procedures.” Samples were analyzed by 3–10% SDS-PAGE and fluorography. The positions of plasminogen (Plg.), which cross-reacts with the anti-apo(a) antibody, of apoB, the mature and precursor forms of the K isoform of apo(a) (apo(a) \(K\) and \(pr\) apo(a) \(K\)), respectively, and of an additional intracellular form of apo(a) (apo(a) \(X\)) are indicated.

A survey of 25 null Lp(a) phenotype baboons by Northern blotting of hepatic RNA revealed a frequency of 0.26 for transcript positive null alleles. Both apo(a) transcript size and level varied between animals (25).

Transcript Positive Null Phenotypes Result from the Inability of Apo(a) to Exit the ER—The molecular basis for transcript positive null apo(a) phenotypes was investigated in primary cultures of baboon hepatocytes. We have previously demonstrated that apo(a) is synthesized as a lower molecular weight precursor, which, after a prolonged residence time in the ER, is processed to a mature form and secreted (28). Thus for each secreted apo(a) isoform, two proteins, representing the precursor and mature forms of apo(a), are present in cell lysates, whereas only the mature form is present in the culture medium (28).

Hepatocytes were isolated from animal 3 in Fig. 1, which expressed a K and a transcript positive null apo(a) allele. To determine whether the transcript positive null allele gave rise to an apo(a) protein product, cells were labeled to steady state with \[^{35}S\]methionine and \[^{35}S\]cysteine, and apoB and apo(a) were immunoprecipitated from the cell lysate and culture medium and analyzed by SDS-PAGE (Fig. 2A). A single form of apoB was recovered from the cell lysate and the culture medium. Three forms of apo(a) were recovered from the cell lysate. Only one apo(a) protein, which co-migrated with the middle intracellular form, was recovered from the cell lysate (Fig. 2A). This is consistent with our earlier studies, which demonstrated that the association between apo(a) and apoB to form Lp(a) occurs after secretion (28). Due to limited degradation of apoB in the culture medium, apo(a) co-immunoprecipitating with apoB could not be seen. A third protein (labeled with an asterisk), which migrated below apo(a) on SDS-PAGE, was also recovered from the culture medium with the anti-apo(a) antibody. This is a nonspecific product of the immunoprecipitation reaction, which binds to protein A-agarose, and has been observed by other investigators using liver cell lines (29–31). Since the secreted form of apo(a) co-migrated with the middle intracellular apo(a) protein, this experiment suggested that the two smaller forms of apo(a) inside the cell represented the precursor and mature forms of the secreted K isoform. The largest intracellular apo(a) protein appeared to represent a product of the transcript positive null allele.

To confirm the relationship between the three intracellular forms of apo(a), endoglycosidase digests were performed. N-Glycanase, which removes all N-linked sugars from proteins (32), increased the mobility of all three intracellular forms of apo(a) on SDS-PAGE, confirming the presence of N-linked carbohydrate and demonstrating that each protein had at least been translocated into the ER (Fig. 2B, lane 3 versus lane 1). Endo H increased the mobility of the largest and smallest intracellular forms of apo(a) but not that of the middle form (Fig. 2B, lane 2 versus lane 1). Endo H only removes high mannose \(^{N}\)-linked sugar (33). Resistance to Endo H is acquired in the medial Golgi apparatus (34). The largest and the smallest apo(a) proteins were, therefore, ER- or early Golgi-associated proteins thus representing immature forms of apo(a). The intermediate-sized protein had at least traversed the medial Golgi apparatus consistent with it being a mature apo(a) polypeptide (28). These data confirm that the two smallest intracellular forms of apo(a) represented the precursor and mature forms of the secreted K isoform (28). The largest intracellular form of apo(a) represented a product of the transcript positive null apo(a) allele, which remained in an Endo H sensitive form, was not processed to a mature form, and was not secreted. The size of this protein was consistent with the size of the mRNA product from the transcript positive null allele in this animal (Fig. 1). A null apo(a) phenotype may, therefore, result from the synthesis of a defective apo(a) protein that is unable to exit the ER.

Null Apo(a) Proteins of Different Sizes Are Present in the Population—Hepatocytes were isolated from another animal, known to possess at least one null apo(a) allele, since the mother expressed no detectable plasma Lp(a) (data not shown). The phenotype of the father was unknown as his plasma was not available for analysis. Analysis of the hepatocytes revealed that null alleles that give rise to ER-retained apo(a) proteins of different sizes exist within the population. Immunoprecipitation of intracellular and secreted apo(a) after steady-state labeling with \[^{35}S\]methionine and \[^{35}S\]cysteine revealed two apo(a) proteins inside the cell; no apo(a) was recovered from the medium (Fig. 3A). This was not due to a general defect in protein secretion since plasminogen (which cross-reacts with
nullapo(a) proteins of different sizes are present in the population. Experiments were performed on hepatocytes isolated from a baboon known by pedigree analysis to possess at least one null apo(a) allele (see text). Panel A, cells were labeled to steady state with [35S]methionine and [35S]cysteine, and apo(a) (a) and apoB (B) were analyzed by immunoprecipitation, SDS-PAGE, and fluorography, as described under "Experimental Procedures." Panel B, cells were pulsed for 10 min with 125 μCi/ml each of [35S]methionine and [35S]cysteine and were chased for 10, 30, or 60 min or 5 h, as described under "Experimental Procedures." Apo(a) was immunoprecipitated from the cell lysates and culture media and was analyzed by 3–10% SDS-PAGE and fluorography. Panel C, cells were labeled as described in panel A. Apo(a) was immunoprecipitated from the cell lysate and was treated with phosphate-buffered saline (lane 1), endoH (lane 2), or neuraminidase (lane 3), as described under "Experimental Procedures." Samples were analyzed by SDS-PAGE and fluorography. The positions of apo(a), apoB, and plasminogen (Plg.), which cross-reacts with the anti-apo(a) antibody, are indicated. *, a protein nonspecifically recovered in the immunoprecipitates.

To determine whether both intracellular polypeptides represented immature forms of apo(a) or a precursor and mature form of a single apo(a) allelic variant, pulse-chase studies were performed. Cells were labeled with [35S]methionine and [35S]cysteine for 10 min and then chased for times between 10 min and 5 h in an excess of unlabeled amino acids. Apo(a) was immunoprecipitated from the cell lysates and culture media. By 10 min of chase, both forms of apo(a) could already be detected inside the cell. The rapid appearance of both forms of apo(a) supported the contention that they were independently synthesized, immature polypeptides since maturation of apo(a) requires at least 30 min (see below and Ref. 28). At later chase time points, the amount of apo(a) detectable in the cell lysates decreased, until at 5 h of chase, virtually no apo(a) remained inside the cells (Fig. 3B). At no time was any of the smaller form of apo(a) recovered from the medium. However, on a long (16 day) exposure of the autoradiograph, it appeared that a very small amount of the upper intracellular form of apo(a) shifted to a mature form by the 5-h time point, and a trace amount was recovered from the medium (data not shown). All of the smaller form of apo(a) and >90% of the large form thus appears to be retained inside the cell in an immature form and eventually degraded. Again, the presence of plasminogen in the culture medium ruled out a general defect in secretion (Fig. 3B).

To confirm that the two intracellular forms of apo(a) represented independently synthesized, immature proteins, endoglycosidase digests were performed. In this case, Endo H was used to determine the state of the N-linked sugar on each of the proteins, and neuraminidase digestion was used to determine the presence of terminal sialic acid residues. Sialic acid is added to N- and O-linked sugar in the trans-Golgi apparatus (35), and, thus, a shift in mobility on SDS-PAGE of either protein following neuraminidase treatment is an indication of maturation (28). Endo H produced an increase in the mobility of both proteins, whereas neuraminidase had no effect (Fig. 3C). Both apo(a) proteins thus appear to represent immature, ER-associated, or early Golgi-associated proteins.

The smaller intracellular form of apo(a) seen in these cells appears to represent the product of a null allele. This protein was retained in the ER and eventually degraded inside the cell. Since only correctly folded proteins are permitted to exit the ER (36), this may reflect the presence of a mutation that prevents proper folding. This null protein is considerably smaller than the null apo(a) protein analyzed in Fig. 2. Analysis of hepatocytes from another animal known to have a Wnull phenotype and, thus, a shift in mobility on SDS-PAGE of either protein following neuraminidase treatment is an indication of maturation (28). Endo H produced an increase in the mobility of both proteins, whereas neuraminidase had no effect (Fig. 3C). Both apo(a) proteins thus appear to represent immature, ER-associated, or early Golgi-associated proteins.

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Aand an Lapo(a) allele (animal 2). A, immunoblots of culture media man Lp(a) followed by rabbit anti-goat IgG. Detection was with protein A. B, cells were labeled to steady state, and apo(a) was immunoprecipitated from cell lysates and sera isoforms, respectively, are indicated.

**Fig. 4. Effect of apo(a) size on plasma Lp(a) level is post-translational.** Sera and hepatocytes were obtained from an animal expressing an A and an I apo(a) allele (animal 1) and an animal expressing an A and an L apo(a) allele (animal 2). A, immunoblot of culture media (m) and sera (s). Aliquots of sera (1 ml) and culture media were electrophoresed on a 3–10% gradient SDS-polyacrylamide gel after reduction with 2-mercaptoethanol and immunoblotted for apo(a) using goat anti-human Lp(a) followed by rabbit anti-goat IgG. Detection was with 125I-protein A. B, cells were labeled to steady state, and apo(a) was immunoprecipitated from cell lysates (c) and culture media (m) as in Fig. 2A. Samples were analyzed by 3–10% SDS-PAGE and fluorography. The positions of molecular weight markers (apoB100, 550 kDa; myosin, 200 kDa) and of the mature (apo(a) A, apo(a) I, apo(a) L) and the immature (pr apo(a) A, pr apo(a) I, pr apo(a) L) forms of the A, I, and L apo(a) isoforms, respectively, are indicated.

detectable in pulse-chase analysis (see below). This protein may, therefore, represent the product of a second transcript positive null apo(a) allele or of an allele that gives rise to very low plasma levels of Lp(a) due to the presence of a sequence polymorphism independent of size, which affects the ability of the protein to undergo post-translational processing. Unfortunately, no plasma from this animal was available in which to measure circulating levels of this apo(a) isoform.

**Isoform Size Influences Apo(a) Transport from the ER**—The results described above demonstrate that the ability of apo(a) to move out of the ER may have a profound effect on the level of Lp(a) in plasma. Null proteins may represent one extreme of a continuum in the extent of post-translational regulation, since we have previously demonstrated that even secreted apo(a) proteins have a prolonged residence time in the ER (28). The size polymorphism of apo(a) is due to variation in the number of plasminogen kringle 4-like domains, each of which must form three disulfide bonds in order to obtain the correct conformation (10–12). Different sized apo(a) isoforms may, therefore, experience varying degrees of difficulty in moving out of the ER. To determine whether the efficiency of transport of apo(a) out of the ER contributes to the inverse correlation between apo(a) size and plasma level, hepatocytes and sera were examined from two baboons, each of which expressed two apo(a) isoforms in their plasma (Fig. 4). The apo(a) isoform phenotypes of these animals were AI (animal 1) and AL (animal 2). The relative levels of the large and small isoforms in serum and culture medium were compared by immunoblotting (Fig. 4A). For each animal, the small isoform was present at a higher level than the large isoform, and the ratio of the small to large isoform was approximately equal in the serum and hepatocyte culture medium (Fig. 4A). This is consistent with the inverse relationship between apo(a) size and plasma Lp(a) level (7, 8, 12) and confirms that plasma levels of Lp(a) are determined by the rate of apo(a) secretion (21). In addition, this result confirms the suitability of the hepatocyte culture system to study characteristics of Lp(a) observed in vivo.

To compare the relative intracellular level of each isoform, hepatocytes from both animals were labeled to steady state, and apo(a) was immunoprecipitated from the cell lysates and culture media. For each animal, the small isoform was again present at a higher level in the culture medium than the large isoform (Fig. 4B). ApoB co-immunoprecipitated with apo(a) in the culture media, consistent with the extracellular association of these proteins (28). Examination of mature apo(a) proteins in cell lysates showed the same preponderance of the smaller isoform as was detected in the serum and culture medium. In sharp contrast, the immature forms were present in approximately equal proportions. This suggests that the difference in the level of secretion of the different sized isoforms is not due to a difference in their rate of synthesis, but rather to a difference in their ability to undergo post-translational processing.

To examine this issue further, the kinetics for intracellular maturation of large and small apo(a) isoforms were compared in pulse-chase experiments. Hepatocytes were analyzed from three animals, each of which expressed two isoforms of apo(a) in their plasma. Such that the experiments were internally controlled (Fig. 5). Animal A had an AI apo(a) phenotype (Animal 1 from Fig. 4), animal B had an AL phenotype (Animal 2 from Fig. 4), and animal C was a second animal with an AL phenotype. Hepatocytes were labeled for 10 min and chased for 0.5, 1, 2, or 5 h, and apo(a) was immunoprecipitated from the cell lysates. At the 0.5-h time point, only the immature form of each isoform could be seen inside the cells from each animal. In each case, the amount of the small and large isoforms labeled at this time point were similar, suggesting comparable rates of synthesis. At the 1-h chase time point, 23% of the I isoform from animal A (Fig. 5A), 31% of the L isoform from animal B (Fig. 5B), and 25% of the I isoform from animal C (Fig. 5C), were present in their mature forms. The mature form of the A isoform for each animal, however, did not appear until the 2-h chase time point. These data demonstrate that the larger apo(a) isoforms require a longer period of time to exit the ER than the small isoforms, consistent with the more extensive post-translational processing required for the large proteins.

**Fig. 5D** represents a summary of data compiled from 16 pulse-chase experiments involving hepatocytes from seven different animals expressing apo(a) isoforms of four different size classes, A, B, I, and L. The values shown represent the mean percentage of apo(a) (± S.D.) in the mature form at 1 h of chase and were 2 ± 4.9, 6 ± 7.9, 20 ± 4.2, and 29 ± 7.9% for the A, B, I, and L isoforms, respectively. Standard deviations indicate the between-experiment variation for multiple experiments involving hepatocytes from the same donor, hepatocytes from different animals expressing the same apo(a) allele, and hepatocytes from unrelated animals expressing apo(a) isoforms of indistinguishable size. The data conclusively demonstrate an inverse relationship between apo(a) size and maturation rate (Fig. 5D).

Preliminary data suggest that a portion of apo(a) is degraded in the ER and that the proportion degraded correlates with the residence time of apo(a) in the ER (data not shown). The ability of apo(a) to exit the ER may, therefore, account not only for transcript positive null apo(a) phenotypes but also for the inverse relationship between apo(a) glycoprotein size and plasma Lp(a) level.

**DISCUSSION**

Despite the well documented association between high plasma Lp(a) levels and the incidence of cardiovascular disease
the absence of detectable levels of transcription or the presence of an extremely unstable mRNA product explains the null phenotype. For the transcript positive nulls, however, a different mechanism must be responsible for reduced levels of Lp(a) production. In the present study, we have identified the molecular basis for the null phenotype associated with three transcript positive null alleles. In each case an apo(a) protein was synthesized but was unable to exit the ER. The results also clearly demonstrate the presence of more than one transcript positive null allele in this limited population of baboons since proteins of different sizes were produced for each of these alleles. Further studies will be required to determine whether all transcript positive nulls have the same intracellular phenotype.

The reason for the retention of null apo(a) proteins in the ER is currently unclear. Only correctly assembled and folded proteins are permitted to exit the ER (36). The apo(a) protein consists of a large number of repeats of a plasminogen kringle 4-like domain (9). Each of these domains requires the formation of three disulfide bonds in order to reach the correct conformation. In addition, each of the kringle domains in apo(a) has an N-linked glycosylation site (9). N-Linked glycosylation is initiated in the ER and may also affect the ability of the protein to fold correctly. A reasonable hypothesis would be that a null protein results from the presence of a mutation in one or more of the kringle domains of apo(a), which prevents correct folding and results in retention in the ER. The apo(a) gene is believed to have arisen relatively recently by a combination of gene duplication and homologous recombination. The within-species polymorphic nature of the protein suggests that contraction and expansion of the apo(a) locus is still occurring (9). During this process a “rogue” kringle domain may have arisen by mutation that is unable to fold correctly, and this kringle may have become incorporated into a number of apo(a) alleles encoding different size isoforms. Alternatively, the same mutation, or different mutations resulting in the same null phenotype may have arisen independently in different apo(a) genes. These theories are, however, purely speculative, and extensive future studies will be required to determine the molecular basis for the ER retention of null apo(a) proteins.

A number of clinical disorders have been described in which the disease phenotype is due to a defect in secretion of a particular protein (for review, see Ref. 37). Abetalipoproteinemia is caused by a defect in apoB secretion. The homozygous state is characterized by fat malabsorption, acanthocytosis, spinocerebellar degeneration, and retinopathy (38). Another example is the Z variant of α1-antitrypsin. This protein is unable to exit the ER, resulting in lung disease and liver cirrhosis (39). In the case of homozygous null apo(a) phenotypes, no detrimental effects have been described.

Results from the current studies also demonstrate that the efficiency of post-translational processing of apo(a) contributes to the inverse relationship between plasma Lp(a) concentrations and apo(a) isoform size. A variation in the ability of apo(a) to move out of the ER resulted in the different levels of secretion of large and small isoforms. This most likely reflects the greater extent of post-translational processing required for large isoforms. Preliminary studies suggest that a portion of apo(a) is not secreted but is degraded inside the ER and that the proportion degraded correlates with the ER residence time of the protein (data not shown). Larger apo(a) proteins may have more trouble adopting a correct conformation, and, perhaps, a larger proportion of these proteins are unable to reach their final conformation and be secreted.

Clearly, there are exceptions to the inverse relationship between the size of apo(a) and plasma Lp(a) level (7, 8, 12, 19). These exceptions may represent other sequence variations at
the apo(a) locus that affect the ability of apo(a) to reach a correct conformation. Alternatively, the exceptions may reflect the multiple levels of regulation of apo(a) gene expression, such that the rate of production of a particular protein is determined by the efficiency of transcription, mRNA stability, translation efficiency, and efficiency of post-translational processing. Sequence variations independent of apo(a) size may influence efficiency, and efficiency of post-translational processing. Se-

In summary, we have demonstrated that the efficiency of post-translational processing of apo(a) is a major determinant of plasma Lp(a) concentrations. We conclude that Lp(a) levels are controlled by a combination of transcriptional and post-translational regulation. The transcript negative null and transcript positive null alleles represent the extremes in each case, whereas a continuum of regulation at both levels accounts for the >1000-fold range in plasma Lp(a) levels.

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REFERENCES