Molecular Cloning of the cDNA Encoding the Carboxy-Terminal Domain of Chimpanzee Apolipoprotein(a): An Asp57 → Asn Mutation in Kringle IV-10 Is Associated with Poor Fibrin Binding†

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ABSTRACT: Insight into the structural features of human lipoprotein(a) [Lp(a)] which underlie its functional implication in fibrinolysis may be gained from comparative studies of apo(a). Indeed, cloning of rhesus monkey apo(a) has shown that a Trp72 → Arg mutation in the lysine-binding site (LBS) of KIV-10 leads to loss of lysine-binding properties of the rhesus Lp(a) particle. Consequently, comparative studies of apo(a) sequences in different Old World monkey species should further our understanding of the molecular role of Lp(a) in the fibrinolytic process. In contrast to other Old World monkeys, including rhesus monkey, cynomolgus, and baboon, the chimpanzee exhibits an elevated level of Lp(a) and a distinct isoform distribution as compared to humans [Doucet et al. *J. Lipid Res.* (1994) *35*, 263−270]. Clearly then, the chimpanzee is an interesting animal model for study of the structure, function, and potential pathophysiological roles of Lp(a). We have cloned and sequenced the region of chimpanzee apo(a) cDNA spanning KIV-3 to the stop codon. The global organization of this region is similar to that of human apo(a) with the presence of KV, which is absent in rhesus monkey apo(a). Nucleotide sequence comparison indicates a variation of 1.4% between chimpanzee and man and 5.1% between chimpanzee and rhesus monkey. The differences concerned single base changes. An Asp57 → Asn mutation was detected in KIV-10; this residue is critical to the LBS of KIV-10 in human apo(a). To verify that the Asp57 → Asn substitution was specific to apo(a), we have also cloned the cDNA-encoding plasminogen, which exhibited an Asp at the corresponding position in kringle IV. Using an in vitro binding assay, we have demonstrated that chimpanzee Lp(a) exhibits poor lysine-specific interaction with both intact and plasmin-degraded fibrin as compared to its human counterpart. We propose that the Asn57 substitution in KIV-10 of chimpanzee apo(a) is responsible for this property. Chimpanzee Lp(a) therefore represents an appropriate particle with which to explore the potential effects of Lp(a) on the fibrinolytic system, such as the inhibition of plasminogen activation or inhibition of t-PA activity.

Lipoprotein(a) [Lp(a)] is a cholesteryl ester-rich particle closely resembling low-density lipoprotein (LDL). Lp(a) differs from LDL by the presence of a high molecular weight glycoprotein, apolipoprotein(a) [apo(a)], which is covalently linked to apolipoprotein B100 (apo B100) by a disulfide bond (*1*−*3*).

The physiological role of Lp(a) remains unknown. Epidemiological studies have demonstrated that elevated plasma concentrations of Lp(a) are associated with an increased prevalence of coronary heart disease (see ref *4* for review). In addition, accumulation of apo(a) has been demonstrated in atherosclerotic plaques (*5*, *6*). Furthermore, transgenic apo(a) mice develop atherosclerosis when fed on a lipid-rich diet (*7*).

The cloning and molecular characterization of the human apo(a) cDNA (*8*) provided clues as to the possibility that Lp(a) may possess additional pathological functions, particularly with respect to its potential interference in the fibrinolytic process. Indeed, the amino acid sequence of apo(a) exhibits a marked homology with that of plasminogen, the zymogen form of plasmin, and an enzyme involved in the fibrinolytic system. Apo(a) is composed of a variable number of repeats of kringle IV, followed by one copy of kringle V, and a protease-like domain; all of these domains are highly homologous to their corresponding counterparts in plasminogen. In apo(a), the number of copies of kringle IV is highly variable (12−51), and directly accounts for the size polymorphism of apo(a) (*9*).

The high degree of homology between apo(a) and plasminogen has led to the hypothesis that human Lp(a) may compete with plasminogen for binding to fibrin, thereby interfering with fibrin clot lysis. Several studies have demonstrated that Lp(a) binds to fibrin in vitro (*10*−*13*) and to the receptor for plasminogen on cell surfaces (*14*, *15*). These properties could lead to reduced plasmin activity with potentially pathophysiological consequences such as a decrease in fibrinolysis (*16*) and a decrease in TGFβ activation

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leading to an increase in smooth muscle cell proliferation (*17*). On the other hand, Lp(a) also binds with high affinity to extracellular matrix components (*18*), thereby favoring vascular accumulation of this cholesterol-rich particle. Thus, as a result of its resemblance to both LDL and plasminogen, Lp(a) constitutes a potential link between atherogenesis and thrombogenesis.

In addition to man, the presence of Lp(a) has been reported only in Old World monkeys (*19*) and, surprisingly, in the European hedgehog (*20*). The cDNA coding for hedgehog apo(a) was recently cloned and characterized. Hedgehog apo(a) is exclusively composed of a repetition of structures homologous to kringle III of plasminogen and is therefore clearly distinguished from human apo(a) (*21*). The structure of the carboxy-terminal domain of rhesus monkey apo(a) was also determined by cloning and sequencing of the corresponding cDNA (*22*). Interestingly, major differences were observed between the rhesus and human apo(a) sequences: the kringle V sequence was absent in rhesus monkey apo(a) whereas the 9 amino acids deleted in the protease-like domain of human apo(a) (as compared to the corresponding domain of plasminogen) were present. Substitutions in the catalytic triad of the protease-like domain were also observed in rhesus monkey apo(a). Finally, the lysine-binding site of KIV-10 was modified by the substitution of Trp 72 by an arginine, thereby rendering rhesus apo(a) ineffective in its interaction with lysine residues (*23*).

The Chimpanzee (*Pan troglodytes*) is the nonhuman primate most closely related to man. Indeed, Lp(a) in this species displays some remarkable characteristics. We observed that the mean plasma levels of Lp(a) and the distribution of apo(a) isoforms were distinct in chimpanzee as compared to those observed in man, cynomolgus, or baboon (*24*). Indeed, Lp(a) concentrations in chimpanzee are much higher than those in a healthy normolipidemic Caucasian population [mean Lp(a) level = 0.61 mg/mL vs 0.18 mg/mL]. Furthermore, the distribution of apo(a) isoforms is also distinct from that in man [mean apo(a) isoform size = 665 kDa vs 789 kDa]. In view of these contrasting characteristics, we compared the structure and function of chimpanzee Lp(a) to that of other species. The two major characteristics of Lp(a), i.e., the covalent linkage to apo B100 (*3*) and the kringle structures responsible for binding to fibrin (*25, 26*), are located in the carboxy-terminal domain of human apo(a). Therefore, we focused our attention on this domain. In the present study, we report the cloning by RT-PCR of the cDNA coding for the C-terminal domain of chimpanzee apo(a). The sequence was determined and compared to that in human and rhesus monkey apo(a). As in rhesus, an essential amino acid of the lysine-binding site (LBS) of kringle IV-10 was changed. In parallel, the fibrin-binding capacity of chimpanzee Lp(a) was significantly diminished as compared to that of human Lp(a).

MATERIALS AND METHODS

Samples. A chimpanzee liver sample (kindly provided by Pr. C. Bréchot) was obtained from a single female animal and was immediately frozen and stored at −70 °C.

Chimpanzee plasma samples were drawn onto EDTA and frozen; samples were obtained from five fasted Chimpanzees (two females and three males).

For fibrin-binding studies, the control subject was a healthy normolipidemic human subject with high Lp(a) level (0.5 mg/mL). After overnight fasting, a blood sample was taken on EDTA and the plasma frozen until required.

Standard Molecular Biology Procedures. All the standard basic techniques of molecular biology were carried out as described (*27*). All DNA restriction and modification enzymes were obtained from New England Biolabs, except otherwise stated. Enzymatic reactions were performed in the buffers and under the conditions recommended by the manufacturer. The DH5α *Escherichia coli* strain [F^-,Φ80d*lac*ZΔ*M*15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rK^-, mK^+) *pho*A *sup*E44 l^- *thi*-1 *gyr*A96 *rel*A1] purchased from Gibco BRL was the host, and pBluescript KS^- (Stratagene) was the vector used for PCR cloning, subcloning, and sequencing. Liver tissues were powdered in liquid nitrogen and total RNA was extracted using RNA*B* (Bioprobe) and stored in ethanol at −20 °C.

Oligonucleotides. All the oligonucleotides used for PCR or sequencing were designed according to the human apo(a) cDNA sequence (*8*) or to the human plasminogen cDNA sequence (*28*). For reverse transcription, oligonucleotides used were RT (5′-TTTGTCAGTCAGACCTTAAAAGC-3′) and oligo dT (Boehringer Mannheim). For apo(a) sequence amplification, oligonucleotides were (A) 5′-CCGCTAGCATGCTGACACAATGCTCAGA-3′ (*Nhe*I site underlined), (B) 5′-TGTTCAGAAACAGCCGTGGAC-3′, (C) 5′-TCAGATGCAGAATGGACT-3′, (D) 5′-GAGGACACTCGATTCTGT-3′, (E) 5′-GTCCACGGCTGTTTCTGAACA-3′, (F) 5′-ATGGCCTGACAATGAACTA-3′, and (G) 5′-CCCTCGAGCACGTTTCAGCTTCTAAG-3′ (*Xho*I site underlined). For plasminogen sequence amplification, oligonucleotides were F, D, G, H [5′-CCTCTAGACCATGGAACATAAGGAAGTGG-3′ (*Xba*I site underlined)], and J (5′-ACCATGTCTGGACTGGAATG-3′).

Reverse Transcription. A total of 10 μg of total RNA extracted from the chimpanzee liver was mixed with 25 pmol of the reverse oligonucleotide in a volume of 30 μL and incubated for 10 min at 65 °C. After cooling to 30 °C, the reverse transcription reaction was carried out by adding the following components to the RNA/primer mixture: 10 mM DTT, 40 units of RNasin (Promega), 30 pmol of each dNTP, and 200 units of the Moloney's Murine Leukemia Virus reverse transcriptase (Gibco BRL) in 50 mM Tris-HCl, 75 mM KCl, and 3 mM $MgCl_2$ buffer at pH 8.3 in a 50 μL final volume. Incubation was performed at 37 °C for 60 min.

PCR Amplification, Cloning, and Sequencing. PCR amplifications were performed using the following oligonucleotide combinations: A + B, C + D, E + G, and F + G for apo(a) cDNA cloning and F + G, H + D, and J + G for plasminogen cDNA cloning.

The conditions for PCR were as follows: In a 50 μL final volume, 1 μL of the reverse transcription reaction used as template was mixed with 20 pmol of each primer, 10 pmol of each dNTP, and 5 units of *Taq* polymerase (Stratagene) in a buffer containing 10 mM Tris-HCl and 1.5 mM $MgCl_2$ at pH 8.8.

PCR reactions were carried out using the following program: denaturation at 95 °C for 30 s, annealing at 42 °C for 30 s (with the exception of 52 °C for the C + D couple), and elongation at 72 °C for 2 min (for couples F + G and

H + D) and for 7 min (for couples A + B, C + D, E + G, and J + G). Thirty cycles were carried out under these conditions after a first cycle for which the denaturation step was 5 min at 95 °C, in a Techne PHC-3 thermocycler. The bands of interest in the PCR products were extracted from agarose, purified by Gene Clean II (Bio101), and ligated to an *Eco*RV digested cloning vector (Bluescript). A progressive subcloning strategy was used to complete the sequence of the PCR products. The 5′ and 3′ extremities of PCR clones and subclones were sequenced by the dideoxy chain termination method of Sanger (*29*) with a Sequenase 2.0 kit (USB/Amersham) using T3 and T7 primers.

Lp(a) Preparation. Lp(a) was isolated from human and chimpanzee plasmas by sequential ultracentrifugation. To prevent degradation of lipoproteins, AEBSF (Pefablock SC; Interchim; 1 mM), gentalline (0.005%), NaN_3 (0.01%), and EDTA (0.01%) were first added to the plasma samples. The solvent density of the plasma samples (2−3 mL) was adjusted to $d = 1.040$ g/mL with KBr, and samples were centrifuged in a TL100 ultracentrifuge (Beckman) in a TLA 100.3 rotor at 100 000 rpm at 10 °C for 3 h. After removal of the top layer, the infranatant was adjusted to 1.100 g/mL with KBr and spun at 100 000 rpm at 15 °C for 5.5 h. The top layer, enriched in Lp(a), was concentrated against Aquacid II (Calbiochem) and extensively dialyzed against PBS (EDTA 0.01% and NaN_3 0.01%, pH 7.4). The purity of the preparation was evaluated by PAGE and revealed the presence of only Lp(a) and LDL particles. The Lp(a) concentration of this fraction was determined by an ELISA assay, as previously described (*24*). This assay was developed with an anti-human apo(a) polyclonal antibody as the "capture" antibody and a peroxidase-conjugated anti-human apo B100 polyclonal antibody as the detecting antibody.

Plasminogen Purification. Human plasminogen was purified from plasma without Lp(a) [Lp(a) < 0.05 mg/mL] as previously described (*30*).

Chimpanzee plasminogen was purified from the bottom fractions ($d = 1.040-1.100$ g/mL) of pooled chimpanzee plasmas. After dialysis against 100 mM Na_2HPO_4, 0.01% EDTA, and 0.01% NaN_3, pH 7.4 (buffer A), this fraction was passed through a lysine-Sepharose column in the same buffer. After extensive washing with buffer A containing NaCl 500 mM (buffer B), plasminogen was eluted with buffer B containing ϵ-amino caproic acid (ϵ-ACA 200 mM). Purified plasminogen was concentrated, dialyzed against PBS, aliquoted, and stored at −20 °C.

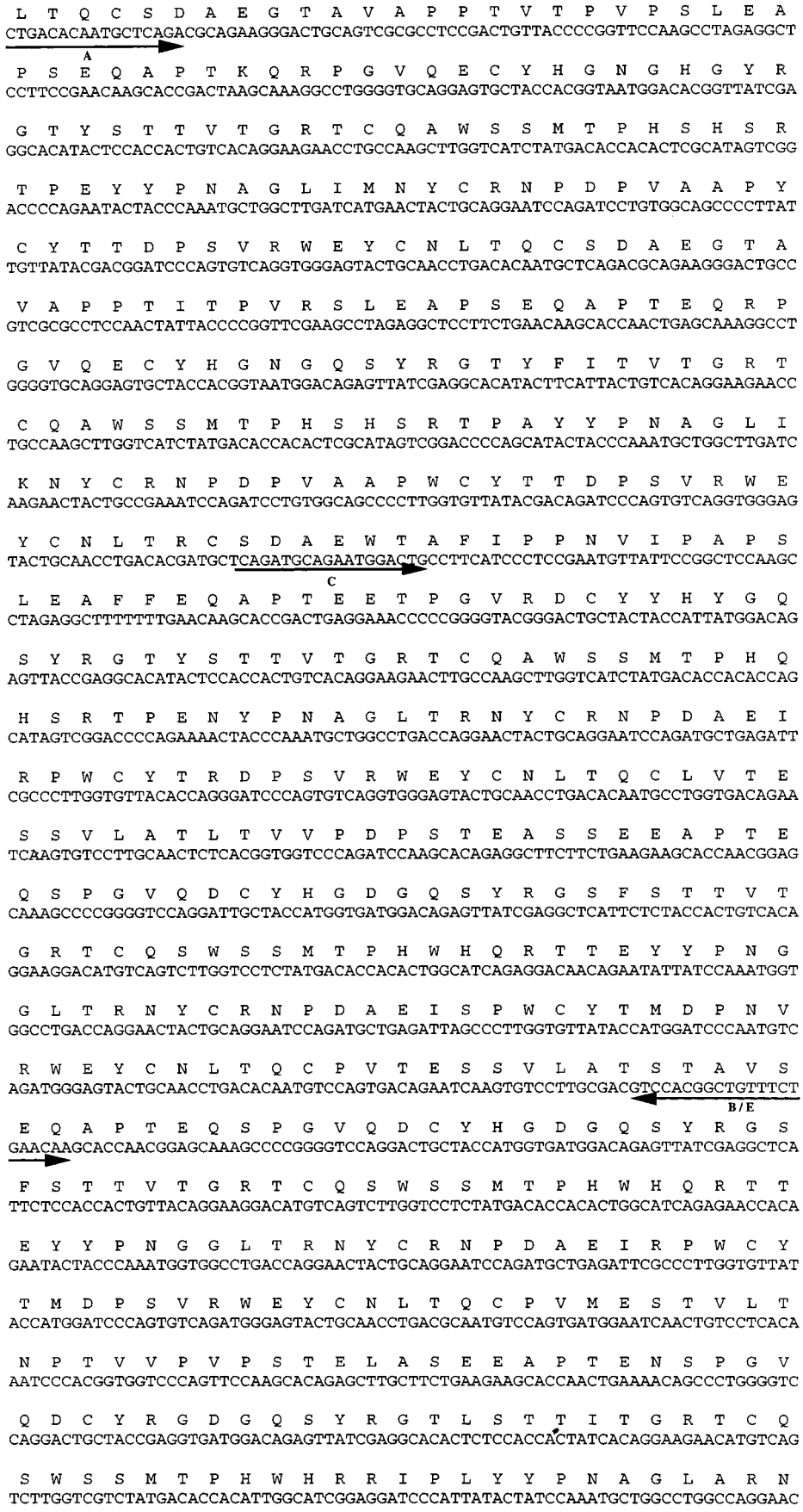
Fibrin-Binding Assays. The fibrin-binding properties of chimpanzee Lp(a) were investigated in an in vitro assay. Human fibrinogen (American Diagnostic, type L) was depleted of plasminogen by affinity chromatography on lysine-Sepharose and of fibronectin by affinity chromatography on Gelatin-Sepharose (Pharmacia) as previously described (*31*). Fibrinogen was then extensively dialyzed against 100 mM Na_2HPO_4, 300 mM NaCl, and 0.01% NaN_3, pH 7.4, and stored in aliquots (0.46 g/L) at −70 °C. Microtitration plates (Costar; EIA/RIA high binding) were coated with fibrinogen (100 μL containing 25 μg of protein/mL of 100 mM bicarbonate coating buffer, pH 9.5) and incubated overnight at 4 °C. The wells were washed four times in 50 mM Na_2HPO_4, 150 mM NaCl, 0.01% Tween 20, and 0.2% BSA, pH 7.4 (buffer I). Fibrinogen was then converted into fibrin by treatment with bovin thrombin (Sigma; 75 μL at 5 NIH units/mL) at 37 °C for 1.5 h in buffer I containing 1 mM $CaCl_2$. After three washes with buffer I, human plasmin (American Diagnostic; 50 μL at 25 nM for 30 min at 37 °C) was used to degrade fibrin surfaces. The wells were washed three times with buffer I and then incubated (2 × 15 min at room temperature) with 100 μL of buffer I containing the serine protease inhibitor AEBSF (0.5 mM) and ϵ-amino caproic acid (ϵ-ACA; 0.5 M) to respectively inhibit and remove plasmin from fibrin surfaces. In parallel, the buffer used to wash the wells coated with intact fibrin surfaces contained only AEBSF. Both intact and plasmin-degraded surfaces were then washed with buffer I and then with 50 mM Na_2HPO_4, 150 mM NaCl, 0.01% Tween 20, 0.4% BSA, and 2 mM EDTA, pH 6.8 (buffer II). Various amounts of Lp(a) diluted in buffer II containing 4% BSA, with or without 0.25 M ϵ-ACA, were added to the wells (50 μL) for 16 h at 4 °C. After washing with buffer II and then with buffer I, a polyclonal peroxidase-conjugated anti-apo(a) antibody was added (50 μL/well of a 1/5000 dilution in buffer I) (*32*). After a 2 h incubation at 37 °C, the wells were washed and 100 μL of the peroxidase substrate, *O*-Phenylenediamine dihydrochloride (Sigma; 1 mg/mL dissolved in 50 mM phosphate-citrate buffer containing 0.03% sodium perborate, pH 5.0) was added. The color development was followed at 405 nm with a microtiter plate reader (BIO TEK; kinetics reader EL 340). The results were obtained as the change in absorbance per minute.

Immunoreactivity of Antihuman Apo(a) Antibody. The immunoreactivity of the peroxidase-conjugated antihuman apo(a) antibody against Lp(a) and plasminogen was assessed by coating the Lp(a) enriched plasma density fractions of chimpanzee and human, or the chimpanzee and human plasminogen purified as described above, in serial dilutions into wells of microtitration plates (50 μL in 100 mM bicarbonate buffer, pH 9.5). After an overnight incubation, the detecting antibody was applied under the same conditions as for the fibrin-binding assay.

RESULTS

Cloning and Characterization of the Chimpanzee Apo(a) cDNA Sequence. The cDNA encoding the carboxy-terminal domain of chimpanzee apo(a) was obtained by the RT-PCR technique. A reverse transcription reaction was performed using chimpanzee liver RNA as template and the oligonucleotide RT, situated at the 3′-end of the apo(a) gene, as reverse primer. The single strand cDNA obtained was used as a template to amplify sequences corresponding to the carboxy-terminal end of apo(a) and to the region spanning the kringle IV-3 coding sequence to the stop codon of the apo(a) gene. We designed four different primer couples on the basis of the human apo(a) cDNA sequence (see Materials and Methods and Figure 1) in order to obtain different overlapping clones.

All the PCR experiments were performed using Taq polymerase. Therefore, it was necessary to differentiate sequence variations between chimpanzee and man from artifactual mutations introduced during amplification cycles. Consequently, the cDNA sequences were characterized from two independently amplified PCR clones; when differences appeared between both clones, sequencing of a third clone was performed.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A
26 P S E Q A P T K Q R P G V Q E C Y H G N G H G Y R
76 CCTTCCGAACAAGCACCGACTAAGCAAAGGCCTGGGGTGCAGGAGTGCTACCACGGTAATGGACACGGTTATCGA
51 G T Y S T T V T G R T C Q A W S S M T P H S H S R
151 GGCACATACTCCACCACTGTCACAGGAAGAACCTGCCAAGCTTGGTCATCTATGACACCACACTCGCATAGTCGG
76 T P E Y Y P N A G L I M N Y C R N P D P V A A P Y
226 ACCCCAGAATACTACCCAAATGCTGGCTTGATCATGAACTACTGCAGGAATCCAGATCCTGTGGCAGCCCCTTAT
101 C Y T T D P S V R W E Y C N L T Q C S D A E G T A
301 TGTTATACGACGGATCCCAGTGTCAGGTGGGAGTACTGCAACCTGACACAATGCTCAGACGCAGAAGGGACTGCC
126 V A P P T I T P V R S L E A P S E Q A P T E Q R P
376 GTCGCGCCTCCAACTATTACCCCGGTTCGAAGCCTAGAGGCTCCTTCTGAACAAGCACCAACTGAGCAAAGGCCT
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451 GGGGTGCAGGAGTGCTACCACGGTAATGGACAGAGTTATCGAGGCACATACTTCATTACTGTCACAGGAAGAACC
176 C Q A W S S M T P H S H S R T P A Y Y P N A G L I
526 TGCCAAGCTTGGTCATCTATGACACCACACTCGCATAGTCGGACCCCAGCATACTACCCAAATGCTGGCTTGATC
201 K N Y C R N P D P V A A P W C Y T T D P S V R W E
601 AAGAACTACTGCCGAAATCCAGATCCTGTGGCAGCCCCTTGGTGTTATACGACAGATCCCAGTGTCAGGTGGGAG
226 Y C N L T R C S D A E W T A F I P P N V I P A P S
676 TACTGCAACCTGACACGATGCTCAGATGCAGAATGGACTGCCTTCATCCCTCCGAATGTTATTCCGGCTCCAAGC
C
251 L E A F F E Q A P T E E T P G V R D C Y Y H Y G Q
751 CTAGAGGCTTTTTTTGAACAAGCACCGACTGAGGAAACCCCCGGGGTACGGGACTGCTACTACCATTATGGACAG
276 S Y R G T Y S T T V T G R T C Q A W S S M T P H Q
826 AGTTACCGAGGCACATACTCCACCACTGTCACAGGAAGAACTTGCCAAGCTTGGTCATCTATGACACCACACCAG
301 H S R T P E N Y P N A G L T R N Y C R N P D A E I
901 CATAGTCGGACCCCAGAAAACTACCCAAATGCTGGCCTGACCAGGAACTACTGCAGGAATCCAGATGCTGAGATT
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976 CGCCCTTGGTGTTACACCAGGGATCCCAGTGTCAGGTGGGAGTACTGCAACCTGACACAATGCCTGGTGACAGAA
351 S S V L A T L T V V P D P S T E A S S E E A P T E
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376 Q S P G V Q D C Y H G D G Q S Y R G S F S T T V T
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426 G L T R N Y C R N P D A E I S P W C Y T M D P N V
1276 GGCCTGACCAGGAACTACTGCAGGAATCCAGATGCTGAGATTAGCCCTTGGTGTTATACCATGGATCCCAATGTC
451 R W E Y C N L T Q C P V T E S S V L A T S T A V S
1351 AGATGGGAGTACTGCAACCTGACACAATGTCCAGTGACAGAATCAAGTGTCCTTGCGACGTCCACGGCTGTTTCT
B/E
476 E Q A P T E Q S P G V Q D C Y H G D G Q S Y R G S
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501 F S T T V T G R T C Q S W S S M T P H W H Q R T T
1501 TTCTCCACCACTGTTACAGGAAGGACATGTCAGTCTTGGTCCTCTATGACACCACACTGGCATCAGAGAACCACA
526 E Y Y P N G G L T R N Y C R N P D A E I R P W C Y
1576 GAATACTACCCAAATGGTGGCCTGACCAGGAACTACTGCAGGAATCCAGATGCTGAGATTCGCCCTTGGTGTTAT
551 T M D P S V R W E Y C N L T Q C P V M E S T V L T
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576 N P T V V P V P S T E L A S E E A P T E N S P G V
1726 AATCCCACGGTGGTCCCAGTTCCAAGCACAGAGCTTGCTTCTGAAGAAGCACCAACTGAAAACAGCCCTGGGGTC
601 Q D C Y R G D G Q S Y R G T L S T T I T G R T C Q
1801 CAGGACTGCTACCGAGGTGATGGACAGAGTTATCGAGGCACACTCTCCACCACTATCACAGGAAGAACATGTCAG
626 S W S S M T P H W H R R I P L Y Y P N A G L A R N
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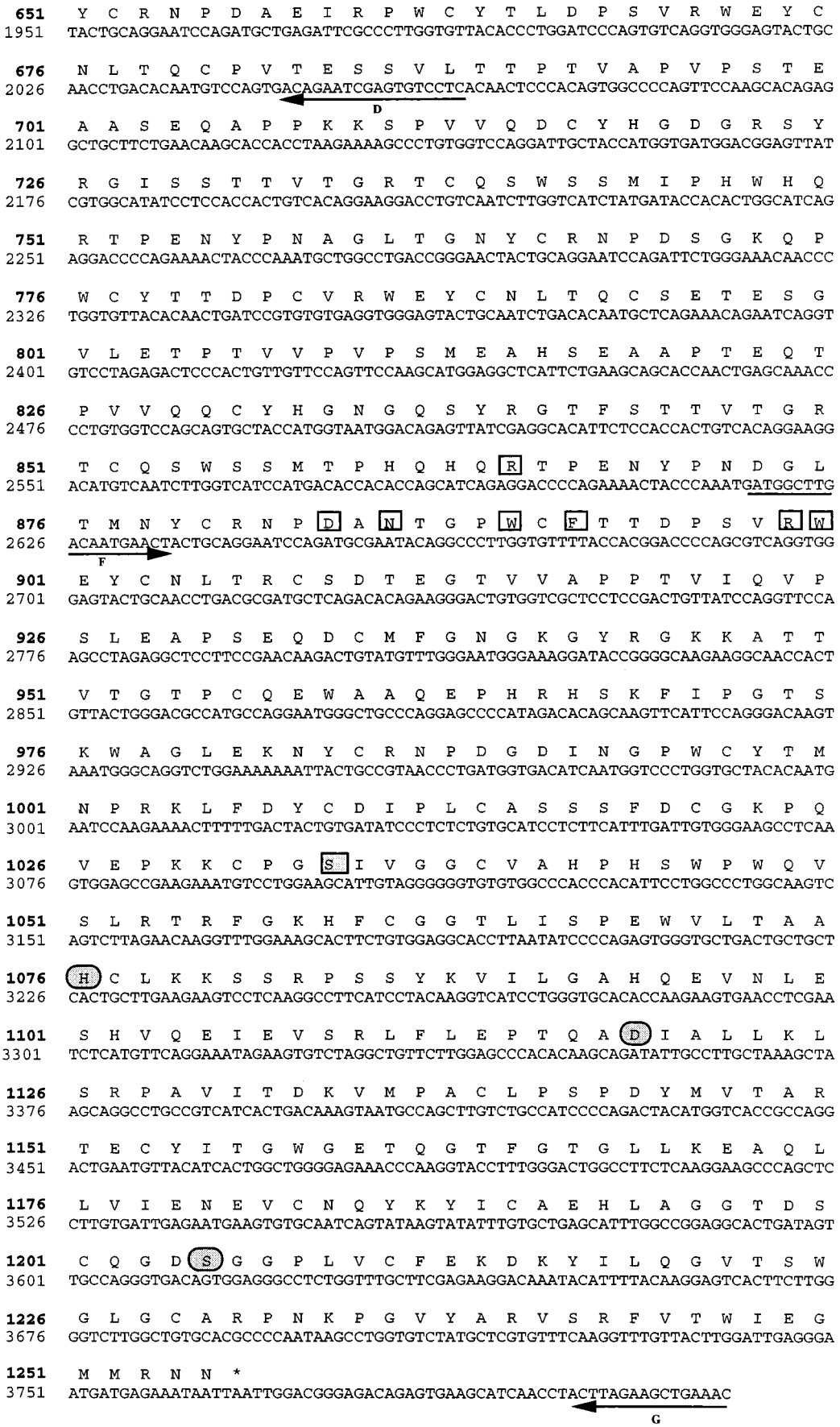

FIGURE 1: The 3′-end 3815 nucleotides of chimpanzee apo(a). The amino acids (top) and nucleotides (bottom) are numbered at the left of each line; the predicted protein sequence is shown above the DNA. (*) Stop codons. The arrows indicate the positions of the different oligonucleotides used for PCR amplification. (□) The amino acids involved in the lysine binding site of KIV-10 in apo(a). (Shaded oval) The amino acids involved in the catalytic center of the proteolytic domain.

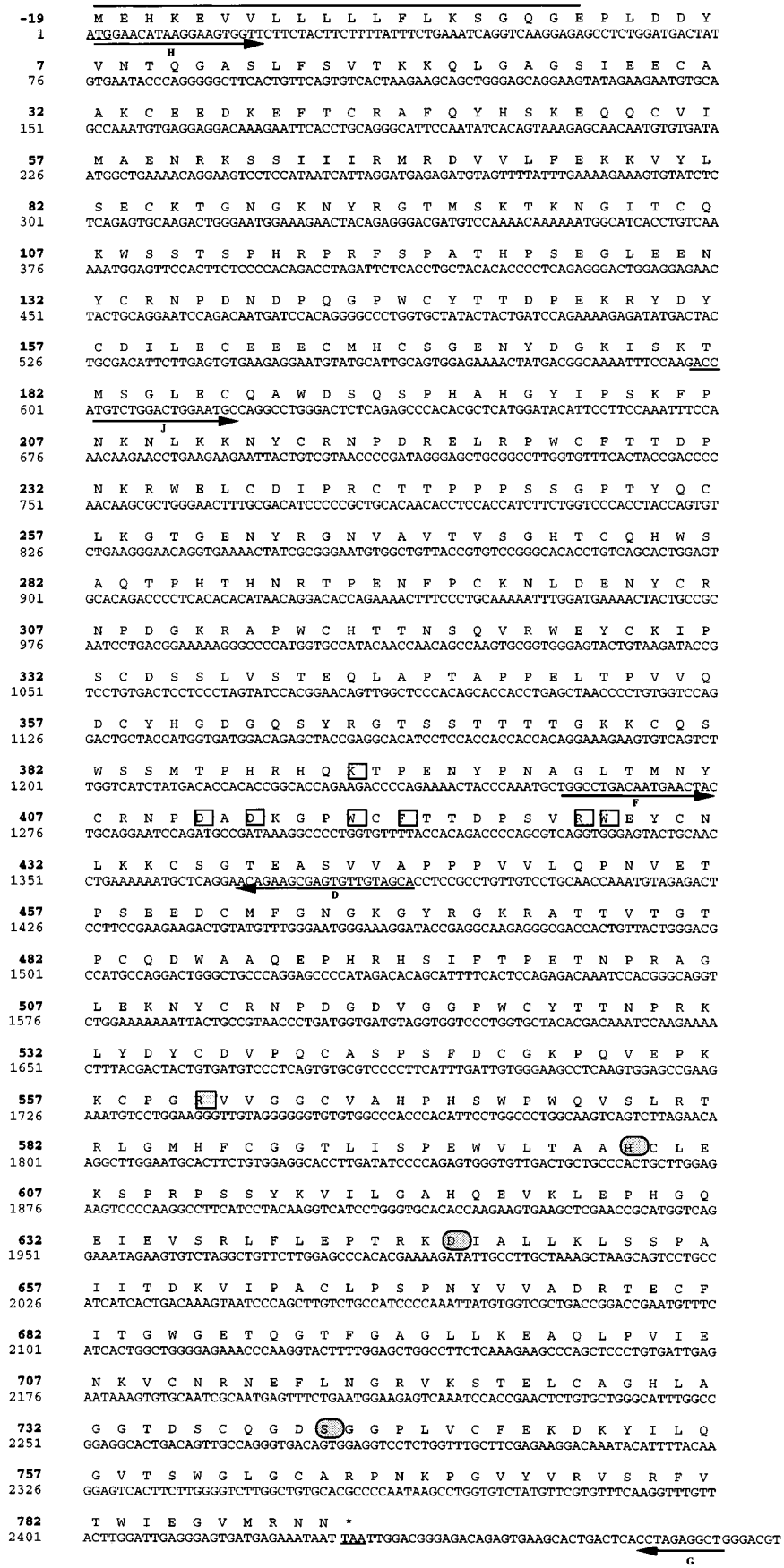

FIGURE 2: Structure and sequence of chimpanzee plasminogen. Amino acids (top) and nucleotides (bottom) are numbered at the left of each line; predicted protein sequence is shown above DNA. (*) Stop codons. Arrows indicate positions of different oligonucleotides used for PCR amplification. (□) Amino acids involved in the lysine binding site of KIV. (Shaded oval) Amino acids involved in the catalytic center of the proteolytic domain. (Shaded square) Amino acid in plasminogen which is cleaved to yield proteolytically active plasmin. For plasminogen, numbers of amino acids are negative 1−19 for the predicted signal peptide (overlined) and 1-end for the mature protein.

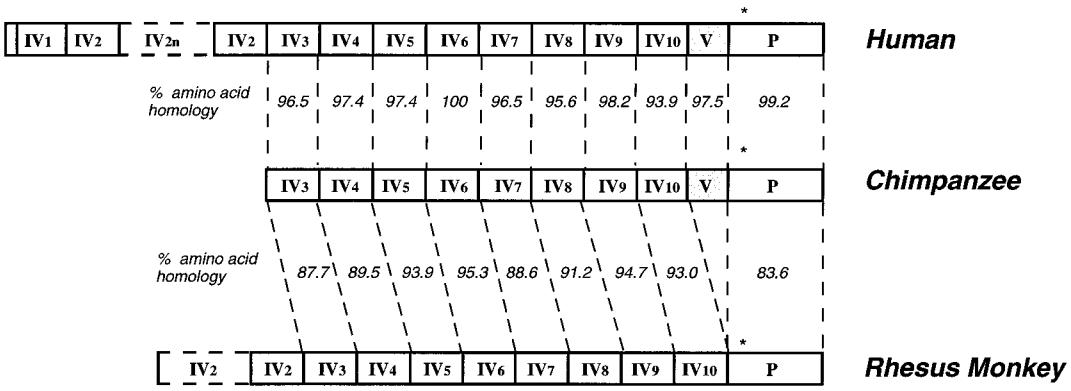

FIGURE 3: Protein sequence comparison of apo(a) from different species. From top to bottom: human, chimpanzee, and rhesus apo(a)s. Domain boundaries are based on sequence homologies. The percentage identity between homologous sequences is shown between each of the two proteins. (*) The mutated amino acid at the t-PA cleavage site.

The F + G and C + D primer couples generated fragments of 1208 and 1368 bp respectively; a 2408 bp fragment was obtained with the E + G primer couple. The A + B primer couple allowed the amplification of two fragments of 1431 and 1089 bp. An additional kringle IV coding sequence in the larger fragment was responsible for the 342 bp size difference between both fragments. From these fragments, the nucleotide sequence of 3815 bp of the chimpanzee apo(a) cDNA was determined and corresponded to 1255 amino acids of the C-terminal end of apo(a) (Figure 1).

Comparative Analysis of the Carboxy-Terminal Domains of Apo(a). A comparison of the amino acid sequences deduced from the cDNA encoding the carboxy-terminal end of the apo(a) of chimpanzee, man, and rhesus monkey is shown in Figure 3. The structural organization of apo(a) in the chimpanzee is identical to that in man: the C-terminal domain of chimpanzee apo(a) exhibits a repeat of kringles IV, a kringle V, and a protease-like domain.

Analysis of these sequences confirmed the high degree of conservation between chimpanzee and man and a more pronounced distance between chimpanzee and rhesus monkey. Indeed nucleotide sequence comparison indicated a variation of 1.4% between chimpanzee and man and 5.1% between chimpanzee and rhesus monkey. The deduced amino acid sequence encoded by the chimpanzee cDNA revealed a variation of 2.7% with man and 5.9% with rhesus monkey.

Comparison of the protease domains revealed a strong homology between chimpanzee and human apo(a) sequences (99.2%). The same nine amino acids are deleted in protease domains of human and chimpanzee apo(a) as compared to that of plasminogen from the corresponding species. By contrast, the putative catalytic triad in the protease domain is intact (His, Asp, and Ser residues are located at the positions numbered 1076, 1119, and 1205, respectively in Figure 1), and the Arg residue at the t-PA cleavage site (position 1034) is replaced by a Ser residue. These modifications are in contrast with those observed in the protease domain of rhesus monkey apo(a), in which no deletion was observed, but in which two amino acids of the catalytic triad are changed (*22*). The additional cysteine present at position 783 in kringle IV-9 and involved in covalent binding to apo B100 is conserved in all three species. The stop codons are identical in human and chimpanzee apo(a).

Table 1: Amino Acid Substitutions in Chimpanzee Apo(a) as Compared to Human and Rhesus Monkey Apo(a) Sequences[a]

	amino acid			
position	chimpanzee	human	rhesus	domain
33	Lys	Glu	Glu	KIV-3
47	His	Gln	Gln	KIV-3
48	Gly	Ser	Ser	KIV-3
104	Thr	Arg	Met	KIV-3
134	Val	Ileu	Val	KIV-3
135	Arg	Pro	Pro	KIV-3
164	Arg	Glu	Arg	KIV-4
241	Ileu	Val	Met	KIV-4
247	Pro	Leu	Pro	KIV-4
259	Pro	Leu	Pro	KIV-5
267	Arg	Gln	Gln	KIV-5
332	Arg	Met	Met	KIV-5
485	Gly	Thr	Gly	KIV-7
573	Val	Leu	Val	KIV-7
576	Asn	Thr	Thr	KIV-7
588	Ala	Pro	Pro	KIV-7
598	Pro	Thr	Pro	KIV-8
648	Ala	Thr	Thr	KIV-8
666	Leu	Met	Met	KIV-8
679	Gln	Arg	Gln	KIV-8
702	Ala	Pro	Pro	KIV-8
709	Lys	Glu	Glu	KIV-9
763	Gly	Glu	Arg	KIV-9
829	Gln	Arg	Gln	KIV-10
862	Gln	Arg	Gln	KIV-10
886	Asn	Asp	Asp	KIV-10
894	Thr	Met	Met	KIV-10
898	Val	Ileu	Val	KIV-10
928	Glu	Gly	Glu	KIV-10
929	Ala	Pro	Ala	KIV-10
969	Lys	Thr		KV
975	Ser	Asn		KV
1185	Gln	His	His	PROT
1196	Gly	Arg	Gly	PROT

[a] Numbering is the same as in Figure 1. PROT indicates the protease domain.

The differences identified in human and chimpanzee apo(a) sequences concerned only single base changes; 66% of them led to an amino acid substitution (Table 1). Of the 34 differences in amino acids, 14 resulted in the presence of the same residue in chimpanzee as in rhesus monkey apo(a), while the residue was different at 15 positions from that present in both human and rhesus monkey. At three other positions, the amino acid was distinct in the three species, the two remaining substitutions occurring in the kringle V sequence which is absent in rhesus monkey. It is notable

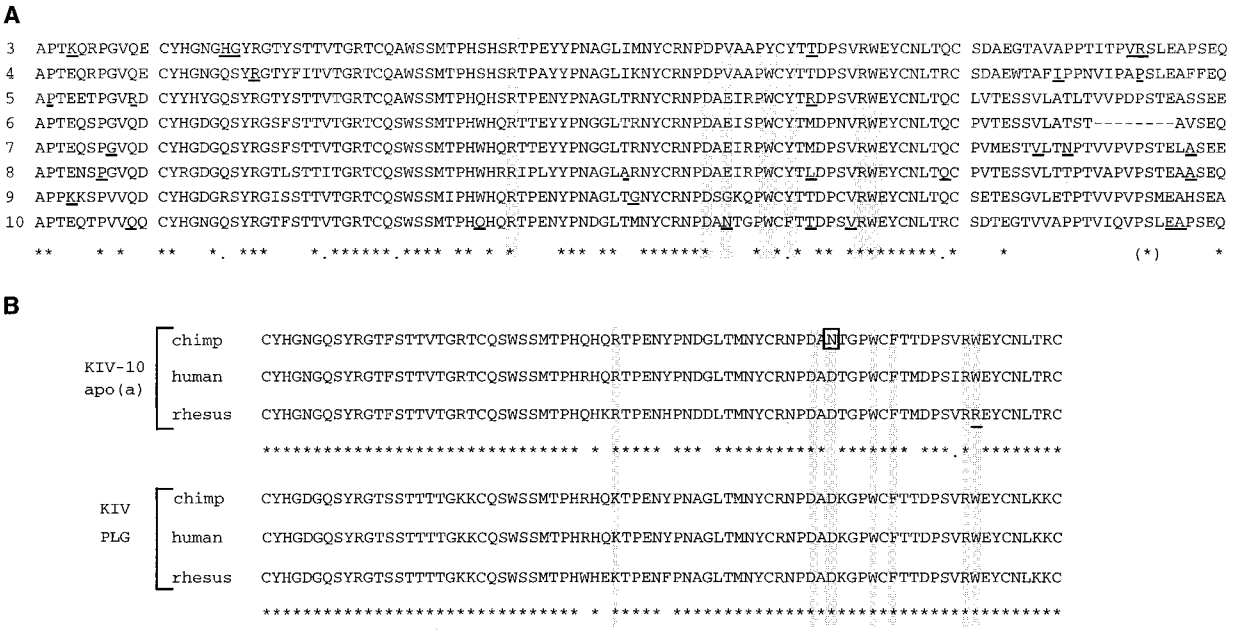

FIGURE 4: Comparison of kringle IV sequences. (A) Alignment of the amino acid sequences of the kringles of chimpanzee apo(a). Interkringle regions comprise: the first 11 residues at left of each sequence and the at right, the amino acids following the last cys residue. The amino acids in chimpanzee apo(a) which differ from their human counterparts are underlined. (*) Strict homology. (.) A conservative change. (B) Alignment of the amino acid sequences of the kringle IV-10 of apo(a) and kringle IV of plasminogen from chimpanzee with their human and rhesus monkey counterpart. The positions of the amino acids which are implicated in the lysine binding are indicated by vertical shading.

that no mutation was observed in KIV-6, which could be implicated in the process of assembly of Lp(a) (*26*, *33*, *34*). Also, it should be noted that the amino acids involved in the putative LBS of kringles IV-3 to IV-9 are unchanged as compared to human apo(a). Approximately 50% of the amino acid variations in the kringles [versus human apo(a)] are located in the interkringle regions (Figure 4). Accessible sites for cleavage by thermolysin or elastase are located in the interkringle region KIV-4/KIV-5 of human apo(a) at the Ala 3532−Phe 3533 bond (for thermolysin) (*13*) and at the Ile 3520−Leu 3521 bond (for elastase) (*35*). The thermolysin site is conserved, whereas the leucine residue of the elastase cleavage site is replaced by a proline residue in chimpanzee apo(a).

Kringle IV-10 in chimpanzee apo(a) is subject to a greater degree of variation in amino acid sequence (5.1%) than the other kringles IV (0−3.8%), kringle V (2.5%), and the protease-like domain (0.8%). A Met/Thr polymorphism has been described at position 66 of kringle IV-10 in human apo(a) (*36*). A Thr residue is present at this position in the cDNA sequence of chimpanzee apo(a) (Figure 1). We screened six different chimpanzee DNA samples at this position, and a Thr residue was found in all 12 alleles (data not shown). In contrast, a Met residue was present at this position in the only reported cDNA sequence of rhesus monkey apo(a). KIV-10 in chimpanzee apo(a) was also substituted at a position which could have functional implications. A G/A substitution at position 2656 in the cDNA sequence of the apo(a) C-terminal end induced the replacement of Asp 57 in human apo(a) by an Asn residue in its chimpanzee counterpart (Figure 5). Asp 57 is predicted to be one of the residues constituting the lysine-binding pocket of KIV-10 (*37*).

To confirm this striking difference between human and chimpanzee apo(a) and in order to avoid the amplification

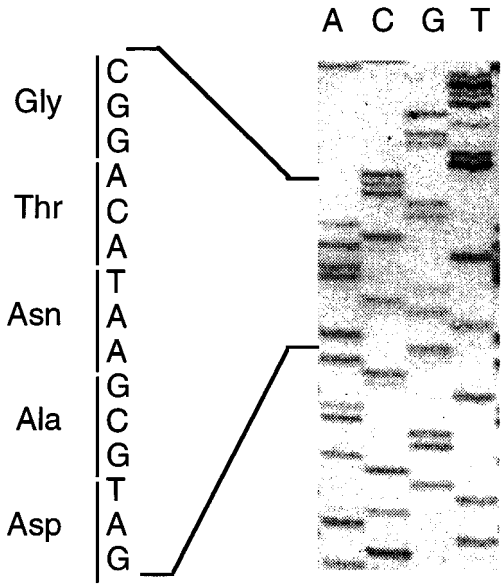

FIGURE 5: DNA sequencing gel and derived amino acid sequence of a PCR-amplified segment of a genomic DNA isolated from chimpanzee.

of contaminating kringles, we amplified a genomic DNA fragment spanning exon 2 of kringle 37, the intronic sequence, and exon 1 of kringle V from the DNA of three chimpanzees. The direct sequencing of the PCR products obtained confirmed the homozygosity (Asn/Asn) of this substitution in each animal studied (data not shown). The other residues of kringle IV-10 apo(a) predicted to participate in lysine binding are conserved and, in particular, the Trp residue at position 72, which is replaced by an Arg residue in rhesus monkey apo(a) (*22*).

Cloning and Characterization of the cDNA Encoding Chimpanzee Plasminogen. To verify that the Asp 57 → Asn

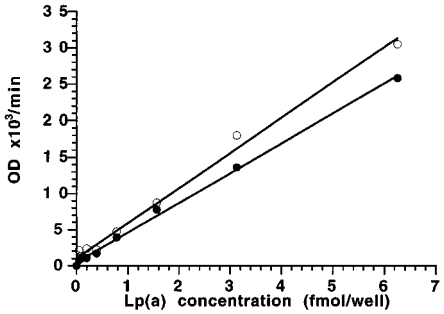

FIGURE 6: Comparison of the immunoreactivity of the peroxidase-conjugated polyclonal anti-human apo(a) antibody against human (○) and chimpanzee (●) Lp(a) (see Materials and Methods).

substitution was specific to apo(a), we cloned and sequenced the cDNA encoding plasminogen in chimpanzee.

The cDNA encoding chimpanzee plasminogen was also cloned by RT-PCR. Oligo-dT was used to reverse transcribe chimpanzee liver RNA. PCR products which allowed the determination of the sequence of plasminogen were essentially subproducts of PCR amplifications performed in order to obtain apo(a) clones. These products were obtained with the primer couples H + D, F + G, and J + G (Figure 2). The comparison with human and rhesus plasminogen revealed a stronger homology between chimpanzee and human (0.8% variations in the nucleotide sequence, 1.2% in the amino acid sequence), than between chimpanzee and rhesus monkey (5.1 and 7.7%, respectively). A total of 60% of the amino acid substitutions are located in the protease domain, the others occurring in the preactivation peptide and in the interkringle regions between kringles III, IV, and V. No amino acid variation was observed within the kringles. The catalytic triad (His 603, Asp 646, and Ser 741 residues) and the cleavage motif for t-PA are conserved. The cleavage site of the preactivation peptide (Lys 77−Lys 78) and the N- and O-linked glycosylation sites (Asn 289 and Thr 346) are also conserved.

It is noteworthy that all the residues predicted to be involved in the lysine binding site of human plasminogen kringle IV (particularly the Asp residue) are present (Figure 1), indicating that the observed Asp 57 → Asn substitution in KIV-10 is specific for chimpanzee apo(a). To assess the possible functional consequences of this substitution, we studied the activity of chimpanzee Lp(a) in binding to fibrin in an in vitro assay.

Fibrin Binding. The fact that chimpanzee Lp(a) does not bind to lysine-Sepharose (Helmohld and Thillet, unpublished observation) and the results of the sequence determination led us to investigate the fibrin-binding properties of chimpanzee Lp(a). For this purpose, we isolated fractions enriched in Lp(a) by a sequential ultracentrifugation procedure (see Materials and Methods). It was not possible to perform lysine-Sepharose affinity column chromatography after ultracentrifugation, since the chimpanzee Lp(a) was not retained on this resin. Plasmas from five chimpanzees were tested independently. Lp(a)s isolated from these plasmas exhibited a total of four different apo(a) isoforms ranging in size from 500 to 800 kDa. We confirmed that the antihuman apo(a) antibody exhibited the same reactivity against chimpanzee as for human Lp(a) (Figure 6). It has been previously shown that treatment of fibrinogen with

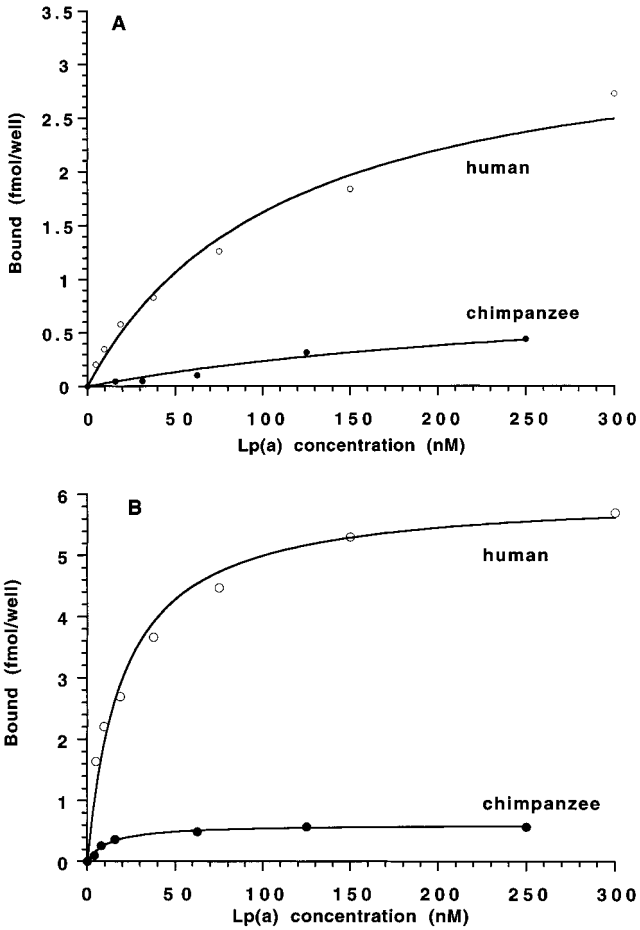

FIGURE 7: Binding of human (○) and chimpanzee (●) Lp(a) to intact fibrin (A) and to plasmin-degraded human fibrin (B). The fibrin surfaces were prepared as indicated in the Materials and Methods. Lysine-specific binding was obtained by subtracting the binding in the presence of ϵ-ACA from total binding. The absolute values of nonspecific binding are within the same range for human and chimpanzee Lp(a).

plasmin results in an increased number of binding sites for Lp(a) (*10*). We therefore measured the binding of chimpanzee Lp(a) either to fibrin or to plasmin-degraded fibrin. Human Lp(a), purified under the same conditions as chimpanzee Lp(a), was used as a control.

The binding of chimpanzee Lp(a) to fibrin (Figure 7A) was almost undetectable. The binding to degraded fibrin was also weak (Figure 7B), but it was possible to calculate a $K_{d,app} = 15 \pm 6$ nM and a $B_{max} = 0.8 \pm 0.3$ fmol/well (mean values for all animals tested). The $K_{d,app}$ was similar to that obtained for human Lp(a) in the same experiments ($K_{d,app} = 20$ nM) which is within the normal range (*10, 38*). On the contrary, the B_{max} was approximately 10 times lower for chimpanzee than for human Lp(a) (6 fmol/well). No significant difference was observed according to isoform size. To confirm the validity of this finding, we purified plasminogen from chimpanzee plasmas and tested its activity in binding to human fibrin or to degraded fibrin. The binding of chimpanzee plasminogen to human plasmin-degraded fibrin ($K_{d,app} = 660$ nM) was similar to that of human plasminogen (data not shown) (*39*). These data suggest that the absence of binding of chimpanzee Lp(a) to fibrin in this in vitro assay does not arise from a lack of recognition between the human and chimpanzee proteins or from a weak

immunoreactivity of the antihuman apo(a) antibody against chimpanzee Lp(a).

DISCUSSION

The present studies describe the cloning and the characterization of the nucleotide sequence of the cDNA encoding the C-terminal domain of chimpanzee apo(a), in addition to its fibrin-binding properties. The analysis of the deduced amino acid sequence demonstrates that chimpanzee apo(a) closely resembles human apo(a). However, a major difference occurred in kringle IV-10 in which Asp 57, an amino acid predicted to be involved in the lysine-binding site (LBS) of human apo(a), is substituted by an Asn. The fibrin-binding capacity of chimpanzee Lp(a) is considerably decreased, which we interpret as a consequence of this substitution. For comparative purposes, the cDNA encoding chimpanzee plasminogen was also cloned and characterized and revealed a marked homology with its human counterpart (98.8% of amino acid sequence homology).

To date, little information is available concerning the molecular characterization of nonhuman primate apo(a) proteins. Indeed, only the carboxy-terminal domain of rhesus monkey and the amino-terminal extremity of cynomolgus apo(a)s have been characterized (*22*, *40*). The available data indicate that the main properties of apo(a), i.e., its covalent attachment to apoB100 and its ability to bind to fibrin, principally involve the carboxy-terminal domain of the apo(a) protein (*1*, *3*, *13*, *26*). Therefore, we focused our attention on the cloning and characterization of the cDNA encoding the carboxy-terminal domain of chimpanzee apo(a), spanning the region from kringle IV-3 to kringle IV-10, kringle V, and the protease-like domain.

Sequence analysis revealed a markedly high degree of nucleotide conservation between chimpanzee and human apo(a) (1.4% of variation). Consequently, the C-terminal end of the chimpanzee apo(a) protein (1255 deduced amino acids) displayed an elevated degree of homology (97.3%) with its human counterpart. Sequence homology is less pronounced with rhesus apo(a), in which differences of 5.1% in nucleotide and 5.9% in amino acid have been observed. As previously mentioned with respect to the comparison of human and rhesus apo(a) (*41*), each kringle in the chimpanzee protein is more closely related to the corresponding kringle in its human counterpart than to the other kringles of the same species.

The structural organization of apo(a) in chimpanzee closely resembles that of human apo(a) (Figure 3), the repeated kringle IV motifs being contiguous with a single copy of kringle V and the protease-like domain. The organization of the key structural domains clearly differs therefore from rhesus apo(a), which does not possess the kringle V sequence and in which the catalytic triad of the protease-like domain is mutated (*22*). Nonetheless, rhesus and chimpanzee apo(a) share in common the mutation of a single amino acid in the LBS of KIV-10, although the precise position of the substituted residue differs between the two species. Thus, rhesus apo(a) is substituted at position 72, an Arg residue replacing a Trp, while chimpanzee apo(a) is substituted at position 57 where an Asn replaces an Asp (Figure 4).

It is established that human apo(a) of Lp(a), like plasminogen, binds to lysine residues in fibrinogen and in fibrin (*10*, *12*). The three-dimensional structure of the lysine-binding site (LBS) present in kringle IV of plasminogen has been defined (*42*, *43*). This kringle possesses an LBS structure which consists of a hydrophobic trough containing three aromatic amino acids (Trp 62, Phe 64, and Trp 72), an anionic center composed of two aspartic residues (Asp 55 and Asp 57), and a cationic center composed of a lysine and an arginine residue (Lys 35 and Arg 71). Since each type of kringle IV in apo(a) contains amino acid substitutions relative to kringle IV of plasminogen, the question has been raised as to whether the apo(a) kringles may be implicated in the lysine-binding affinity of Lp(a). Putative lysine-binding sites have been previously identified in kringles IV-5, -6, -7, -8, and -10 by modeling studies using plasminogen kringle IV for comparison (*37*). These kringles possess five of the seven residues constituting the LBS, with conservative substitutions at other positions; the possibility therefore arises that such kringles could possess lysine binding activity. Several studies have demonstrated that kringles IV-1, -2, -3, and -4 do not bind to lysine, in contrast to kringle IV-10 (*26*, *44*). However, the anionic center of the LBS is considerably modified with the absence of Asp 57 in each of these kringles. KIV-10 of human apo(a) contains the LBS which most closely resembles that of plasminogen kringle IV, with the exception of a Lys residue which is replaced by an Arg at position 35. Several studies have provided evidence that apo(a) kringle IV-10 is clearly involved in the lysine-binding properties of Lp(a) (*23*, *26*, *44*, *45*). Moreover, mutations at the KIV-10 LBS have important functional consequences. It has been shown that the Trp 72 → Arg substitution in KIV-10 results in a loss of binding of recombinant apo(a) to lysine-Sepharose (*26*). In addition, the same substitution in two human subjects resulted in the finding that Lp(a) particles containing this substituted apo(a) did not bind to lysine-Sepharose (*46*). Furthermore, recent data demonstrate that the substitution of Asp 55 and Asp 57 by an Ala residue in the LBS of kringle IV-10 of human recombinant apo(a) leads to the loss of 70% of the lysine-binding activity (*47*). Therefore, the replacement of the Asp 57 residue by an Asn in kringle IV-10 of chimpanzee apo(a) could affect the lysine-binding properties of Lp(a) from this species, which would be observed as a significant loss of the binding affinity of Lp(a) for fibrin. We presently evaluated the ability of chimpanzee Lp(a) to bind to fibrin in an in vitro assay (*13*). Our data demonstrate that the binding activity of Lp(a) to either intact or degraded fibrin was substantially decreased, irrespective of the preparation of chimpanzee Lp(a) tested (Figure 7).

Lp(a) has been colocalized with fibrin in human atherosclerotic lesions (*6*, *48*), but no such colocalization was detected either in the cynomolgus macaque (*49*) or in hypercholesterolemic rhesus (*23*, *50*). In rhesus, the absence of such a co-localization can be explained by the absence of a functional lysine-binding site.

In summary, high levels of Lp(a) are associated in man with an increased risk for cardiovascular disease (*51*−*54*). Some of the pathological functions of human Lp(a) may be mediated through the lysine-binding site(s). In man, kringle IV-10 plays a major role in the lysine-binding properties of Lp(a). Lp(a) may act as a competitive inhibitor of plasminogen at the surface of cells or fibrin clots. On the basis of the results of both the present sequence analysis and of fibrin-

binding assays, we consider that the striking distinction between chimpanzee and human apo(a), i.e., the substitution of the Asp 57 residue by an asparagine in kringle IV-10, could be responsible for the poor binding of chimpanzee Lp(a) to fibrin in in vitro experiments. Several studies suggest that Lp(a) could act by inhibiting t-PA activity or by forming complexes with plasminogen (*11*, *55*, *56*). The study of the biological activity of Lp(a) particles with reduced binding capacity to fibrin as compared to human Lp(a) will help to define more precisely the crucial step(s) at which Lp(a) might interfere in fibrinolysis. Moreover, study of such Lp(a) particles will further our understanding of the atherothrombogenic action of Lp(a). The loss of lysine binding may have major functional consequences on the reduction or the disappearance of certain pathological activities of Lp(a), despite the elevated plasma levels of Lp(a) in the chimpanzee. Consistent with this hypothesis, mutations of the LBS of KIV-10 are associated with a marked decrease in fatty streak development in the vascular wall of apo(a) transgenic mice fed on an atherogenic diet as compared to transgenic mice which express wild-type apo(a) (*57*).

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