methodology

A novel kringle-4 number-based recombinant apo[a] standard for human apo[a] phenotyping

E. Anglés-Cano,[1,*] S. Loyau,* G. Cardoso-Saldaña,† R. Couderc,§ and P. Gillery**

INSERM U.143,* Hôpital de Bicêtre, Paris, France; Departamento de Endocrinologia,† Instituto Nacional de Cardiologia de Mexico, Mexico City, Mexico; Laboratoire de Biochimie,§ Hôpital Tenon, Paris, France; and Laboratorie de Biochimie,** CHU de Reims, France

Abstract Apolipoprotein[a] phenotyping is a critically important method to explore the role of kringle-4 repeat number as a modulator of lipoprotein[a]-associated cardiovascular risk. The availability of a kringle-4 number-based reference standard is therefore necessary for a reliable and generally accepted classification of apo[a] phenotypes. We propose here a battery of recombinant apo[a] isoforms that may be used as the reference standard in various gel systems. Five plasmids encoding for r-apo[a] containing a known number (n = 9, 13, 17, 25, 33) of plasminogen-like kringle-4 copies were constructed, and transfected into the human embryonic kidney cell line 293. The electrophoretic mobility of the recombinant apo[a] isoforms expressed by these cells in a hollow-fiber bioreactor was determined after reduction by SDS-gel (agarose, acrylamide or a mixture of both) electrophoresis and immunoblotting using an antibody specific for human apo[a]. The equation of the linear relationship between log r-apo[a] kringle number and relative migration was used to determine the isoform size of apo[a] in normal human plasma. A very good correlation (r = 0.97) was found with the genotype (pulsed-field gel eletrophoresis of *kpn*I-digested restriction fragments of genomic DNA) and among electrophoretic methods. The proposed recombinant standard offers the possibility to identify apo[a] isoforms within a large range of molecular sizes, 9 to 33 kringle-4 copies, using simple electrophoretic techniques and a nomenclature based on its molecular structure, i.e., the number of kringle-4 repeats.—Anglés-Cano, E., S. Loyau, G. Cardoso-Saldaña, R. Couderc, and P. Gillery. **A novel kringle-4 number-based recombinant apo[a] standard for human apo[a] phenotyping.** ***J. Lipid Res.*** **1999.** 40: **354–359.**

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A number of epidemiological and clinical studies have now established that high plasma concentration of the lipoprotein Lp[a], a low density lipoprotein (LDL)-like particle discovered by Berg in 1963 (1), is a major and independent risk factor for atherosclerosis and cardiovascular disease (2–4). Lp[a] is a complex particle composed of a lipid core and two disulfide-linked apolipoproteins: apoB-100 and apo[a]. The lipid core and apoB-100 of Lp[a] are shared with LDL; in contrast, the apo[a] glycoprotein confers unique properties on Lp[a] and shows a high degree of homology with plasminogen, the precursor of plasmin the fibrinolytic enzyme. By amino acid sequence analysis (5) and cDNA cloning (6) it has been established that apo[a] consists of 10 different types of kringle 4 repeats, one of which, kringle 4 type 2, is present in variable numbers (7, 8). In addition, apo[a] shares single copies of plasminogen kringle 5 and an inactive serine-proteinase domain. The number of repeats of kringle 4 type 2 is genetically determined and gives rise to a series of apo[a] polymorphs, 34 apo[a] alleles (9) and glycoproteins (10), the size of which is inversely related to the plasma concentration of Lp[a] (11). It has been shown, however, that plasma levels of Lp[a] may vary widely within the same apo[a] isoform class (12, 13). Small apo[a] isoforms are associated with greater cardiovascular risk (14) and display the highest anti-fibrinolytic effect (15–17), thus suggesting that short apo[a] alleles may promote atherogenesis per se. It is therefore possible that in subjects heterozygous for the apo[a] trait (more than 90% of the population), the cardiovascular risk associated with Lp[a] may be subordinated to the relative concentration of the atherogenic apo[a] isoform. Taken altogether, these data stress the importance of apo[a] heterogeneity and the usefulness of isotype determination for the identification of subjects with cardiovascular risk associated with Lp[a]. However, a consensus reference standard for apo[a] phenotype determination is not as yet available. The purpose of the present work is to propose a series of recombinant apo[a] isoforms, of predefined kringle number covering

Abbreviations: apo[a], apolipoprotein[a]; Lp[a], lipoprotein[a]; LDL, low density lipoprotein; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; R_f, relative electrophoretic mobility.

[1] To whom correspondence should be addressed.

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the range of the most common isoforms, that may be used as a reference standard for apo[a] kringle number determination in various gel systems.

MATERIALS AND METHODS

Production of recombinant apo[a]

The cloning of cDNA expression plasmid pCMV-A18 encoding a signal peptide, 17 kringle 4 repeats, one kringle 5 unit, and the protease domain of apo[a] has been previously described (18). Two internal *Bst*XI fragments in pCMV-A18 encode eight kringle 4 type 2 repeats. The plasmids pCMV-A10, -A14, -A26, and -A34, obtained by modification of the number of *Bst*XI fragments in pCMV-A18 by standard cloning methods (19), were stably transfected by electroporation into the human embryonic kidney cell line 293. The culture medium containing the different r-apo[a] was produced and obtained as described (S. Loyau and E. Anglés-Cano, unpublished results). Briefly, cells transfected with the different plasmids (pCMV −A10 to pCMV −A34) were grown in a hollow-fiber bioreactor installed according to the instructions of the manufacturer (Integra BioSciences). Approximately 5×10^8 cells were inoculated into a bioreactor cartridge having an extra-capillary space volume of 10 ml. The intracapillary medium consisted of RPMI 1640 supplemented with 10 mmol/l d-glucose, 2 mmol/l glutamine, 2% penicillin–streptomycin, 2% Geneticin®, 1% nonessential amino acids, 1% sodium pyruvate (Gibco BRL) and 20 k.i.u./ml aprotinin (Bayer). The medium in the extracapillary space was exchanged twice a week with 10 ml of fresh culture medium containing 10% plasminogen-free heat-inactivated fetal calf serum. The culture medium thus harvested was supplemented with serine–proteinase inhibitors (20 k.i.u./ml aprotinin, 0.5 mm aminoethyl-benzene-sulfonylfluoride, 2 mm EDTA, and 0.01% NaN_3, final concentrations) and conserved at −80°C until used.

Preparation of the recombinant apo[a] standard

The concentration of r-apo[a] in the culture medium was determined by electroimmunodiffusion (20) using an antiserum directed against human apo[a] (13) and purified r-apo[a] A18 as standard. Solutions containing the different recombinant proteins were prepared by mixing harvested culture medium of known r-apo[a] concentration in proportions able to produce protein bands of similar intensity after electrophoresis and immunoblotting. The final preparation was supplemented with 0.01% sodium azide and 0.1% SDS and was conserved at 4°C until used for electrophoresis.

Electrophoretic procedures

SDS-agarose (1.5%) gels. Electrophoresis and immunoblotting were performed according to the method of Kamboh, Ferrell, and Kottke (10) using a sheep antiserum against human apo[a], and an anti-sheep IgG from rabbit, Fc-specific, alkaline phosphatase-conjugated (Jackson ImmunoResearch, West Grove, PA). Vertical gel electrophoresis was also performed following modifications of the previous method as described (21); proteins were pressure-transferred for 2 h at 22°C to nitrocellulose membranes, immunolocalized (rabbit IgG against human apo[a]/goat IgG-PO against rabbit IgG) and stained with diaminobenzidine.

SDS-polyacrylamide/agarose (3.75%/0.75%) gels. Electrophoresis was performed in 3.75% polyacrylamide/0.75% agarose gels as previously described (22) using products from Immuno-Vienna. Electrophoretic transfer of protein from gel to nitrocellulose was performed for 18 h at 50 volts. Apo[a] protein bands were localized with a sheep antibody to human apo[a] followed by an alkaline phosphatase-conjugated rabbit antibody to sheep IgG.

SDS-polyacrylamide (6%) gels. SDS-PAGE was performed on 3.75% (C = 0.8%) stacking, 6% (C = 0.6%) separating gels of 0.75 mm thickness (8.3 cm × 10 cm) essentially as described by Laemmli (23) in a Bio-Rad Mini Protean II dual-slab gel system. A volume of 10 μl of plasma and 15 μl r-apo[a] was loaded on the gel after reduction and electrophoresis was performed at 4°C at 35 V for 30 min, at 65 V until the dye reached the bottom of the gel, and at 75 V for 90 min thereafter. Proteins were electroblotted for 40 min at 2 mAmp/cm² to nitrocellulose sheets with a graphite electroblotter system (Millipore, Bedford, MA). Apo[a] protein bands were localized with a sheep antibody to human apo[a] followed by a peroxidase-conjugated rabbit antibody to sheep IgG revealed with 4-chloro-1-naphthol.

Apo[a] genotype determination

The apo[a] genotype of lymphocyte samples obtained from healthy volunteers was determined by pulsed-field gel electrophoresis and genomic blotting of *Kpn1*-digested restriction fragments containing the kringle-4 encoding sequences of the apo[a] gene, according to Lackner et al. (9) as previously described (24).

Plasma samples

Venous blood was drawn from the forearm into sterile polypropylene tubes containing 4 mm EDTA final concentration. Plasma was separated by centrifugation at 2000 *g* for 20 min at 4°C and supplemented with 0.01% NaN_3 and proteolytic inhibitors (aprotinin 100 k.i.u./ml, d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone 1 μmol/l, aminoethylbenzenesulfonyl fluoride 0.5 mmol/l) and stored at −80°C until used. Plasma Lp[a] was measured by an immunonephelometric assay (Beckman) (25).

Statistical analysis

Standard curves were generated by plotting the relative migration of the r-apo[a] bands against the log of their kringle number using least-square regression analysis; the number of kringles of the plasma apo[a] isoforms was calculated using the corresponding equation. Analyses were performed using the Kalleidagraph statistical software package (Synergy Software, Reading PA).

RESULTS

Characteristics of the r-apo[a] preparations

The embryonic human kidney cell line 293 was used to produce r-apo[a] of different sizes. Conditions chosen for development of the transfected cell lines allowed inhibition of the activity of proteases to which apo[a] may be sensitive and harvesting from the hollow-fiber bioreactor of culture medium containing high concentrations of intact unmodified r-apo[a] (**Table 1**). The absence of proteolysis was controlled by amino terminal analysis of r-apo[a] purified from the culture medium by affinity chromatography on an immobilized monoclonal antibody directed against apo[a]; the sequence of 10 N-terminal amino acid residues of the purified r-apo[a] was similar to the known deduced sequence of human apo[a] (T. Miyata, National Cardiovascular Center Research Institute, Osaka, Japan, personal communication). The variation in size of the secreted r-apo[a] isoforms corresponded to the kringle 4 repeat number in the transfected apo[a] plasmids (**Fig. 1**). The presence of an intact r-apo[a] preparation in the standard ensures the accuracy of apo[a]

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TABLE 1. Kringle composition of the recombinant apo[a] reference standard

Recombinant Apo[a]	Kringle-4 Number: Type 2	Kringle-4 Number: Types 1 to 10[a]	Total Number of Kringles[b]	r-apo[α] (μg/ml)[c]: Culture Medium	r-apo[α] (μg/ml)[c]: Standard
A10	0	9	10	737	184
A14	4	13	14	1531	115
A18	8	17	18	1368	125
A26	16	25	26	330	110
A34	24	33	34	683	171

[a]Including single copies of kringle 4 types 1 and 3 to 10, plus n copies of kringle 4 type 2.
[b]Including all kringle-4 repeats plus the kringle-5 domain.
[c]Electroimmunodiffusion assay (20).

kringle number determination and the absence of background noise in the blots due to proteolytic degradation. The characteristics of the rapo[a] preparations used for the construction of the reference standard are indicated in Table 1. The mean total protein concentration varied from 4 to 8 g/l; the concentration of the r-apo[a] in the culture medium was usually higher for isoforms having a low number of kringles. The proportion of each preparation in the standard was defined by the concentration that produced uniform bands of similar intensity after electrophoresis and immunoblotting.

Determination of r-apo[a] band migration

The relative migration of the different r-apo[a] of the standard was determined on SDS-agarose, SDS-PAGE, or SDS-agarose acrylamide gels. **Figure 2** shows composite photographs representing the migration in four different gel systems of the r-apo[a] standard and five plasma samples selected from those shown in **Table 2**. The mobility of r-apo[a] isoforms was determined as follows. The relative mobility (R_f) was calculated for each r-apo[a] standard and apo[a] plasma isoform by dividing the protein band migration distance by the distance reached by the dye front. In the systems where the dye was allowed to run off the gel, the distance migrated by the protein band was divided by the length from the origin to the bottom of the gel. Standard curves were generated by plotting the R_f of the r-apo[a] in the standard against the number of kringles or its log transformation using least-square regression analysis (**Fig. 3**).

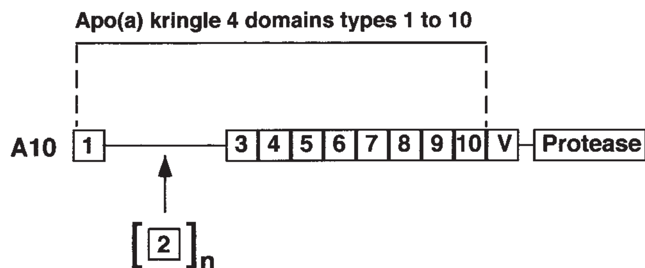

Fig. 1. Schematic representation of the domain structure of the recombinant apo[a] isoforms used to construct the standard. The number of kringle 4 type 2 repeats is as follows: A10, n = 0; A14, n = 4; A18, n = 8; A26, n = 16; A34, n = 24. Detailed composition is shown in Table 1.

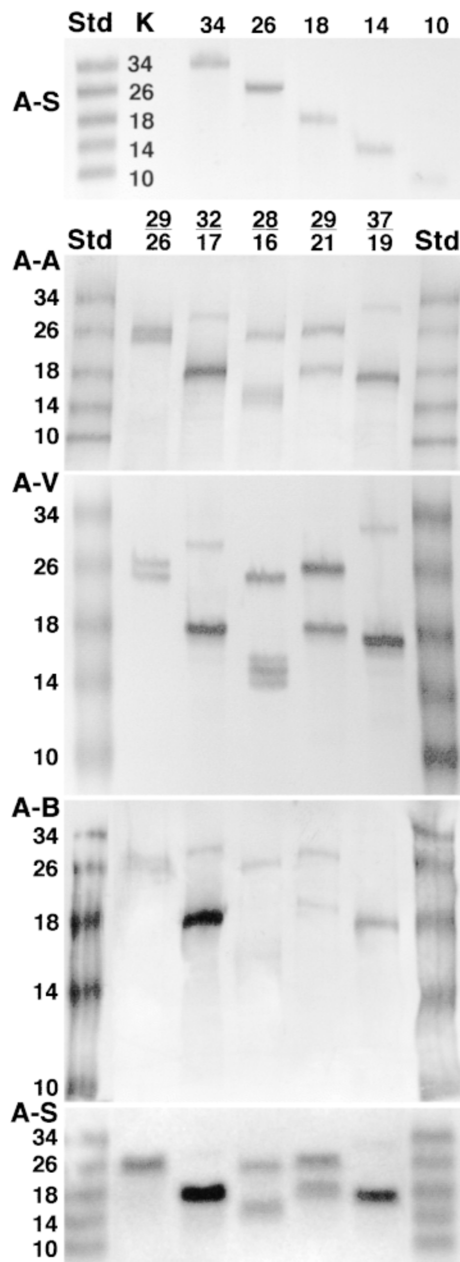

Fig. 2. Electrophoretic patterns of the r-apo[a] standard and of plasma apo[a] isoforms. Five plasmas were phenotyped using different electrophoretic supports: A-S, 1.5% agarose-SDS submarine gel; A-A, 3.75% acrylamide/0.75% agarose-SDS gel; A-V, 1.5% agarose-SDS vertical electrophoresis gel; A-B, 5% acrylamide/0.6% bisacrylamide-SDS gel. A-S upper panel: migration of r-apo[a] isoforms in the standard (Std) and of single r-apo[a] A10, A14, A18, A26, and A34 expressed by transfected 293 cells cultured in a hollow-fiber bioreactor. The genotype of each subject tested is indicated in A-A; the corresponding number of kringles determined by reference to the genetically engineered recombinant apo[a] isoforms (n = 10, 14, 18, 26, 34) is given in Table 2. The linear regression of the number of kringles versus the migration of the r-apo[a] isoforms of the standard in each gel is indicated in Fig. 3.

Apo[a] kringle number determination

In all the gel systems the relationship between log r-apo[a] kringle number and R_f produced linear relationships that allowed us to calculate the number of kringles

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TABLE 2. Apo[a] glycoprotein kringle number determination using the r-apo[a] standard as compared to genotyped kringle 4-encoding sequence number

Sample Lp[a] (g/l)[a]	Apo[a] Kringle Number (SDS-Electrophoresis and Immunoblot)[b]				Genotype Determination PFGE[c]
	Agarose 1.5%		Agarose 0.75% Acrylamide 3.75%	Acrylamide 6% (C = 0.6)	
	Vertical	Submarine			
0.027	26	26	26	26	27/25
0.238	23/21	23	ND	25/22	24/21
0.326	27/23	23	26/22	28/26	29/24
0.158	28	27	25	29/26	31/30
0.04	29/26	29	26/23	28/24	33/29
0.044	29/24	30	32/26	28/26	33/26
0.111	28/25	28/25	33/26	27/25	30/26
0.973	20	20	20	23/19	22/20
0.239	26/24	26	27/25	28/26	29/26
1.51	30/18	30/18	29/19	30/18	32/17
0.298	24/15	25/16	26/16	27/16	28/16
0.093	26/18	26/19	27/19	29/21	29/21
0.498	33/17	33/18	33/18	>34/18	37/19

ND, none determined.
[a] Nephelometric assay.
[b] Digits indicate the total number of kringles of each apo[a] protein band.
[c] Pulsed-field gel electrophoresis. Digits indicate the number of kringle-4-encoding sequences.

of each plasma apo[a] isoform using the equation of the linear regression. Alternatively, analysis of scanned gel images was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/), and the number of kringles was calculated using an ad hoc program (26). Table 2 represents the results obtained for all the plasma tested using the rapo[a] standard and four different electrophoretic gel systems as compared to the genotype. The reproducibility of the plasma apo[a] kringle number determination by reference to the rapo[a] standard in the different gel systems was striking. There was an excellent agreement with the genotype determination and among electrophoretic methods. The size of the *kpn*Idigested restriction fragments, which contain all of the sequences encoding the kringle 4 repeat, was correlated with the number of kringles of the apo[a] proteins (**Fig. 4**). This strong correlation indicates that the r-apo[a] standard allows accurate determination of the number of kringles of a given apo[a] isoform independent of the nature of the electrophoretic support, provided that it is sensitive enough to allow physical detection of the apo[a] band.

DISCUSSION

The role of high concentrations of Lp[a] as a cardiovascular risk factor has been documented by several indepen-

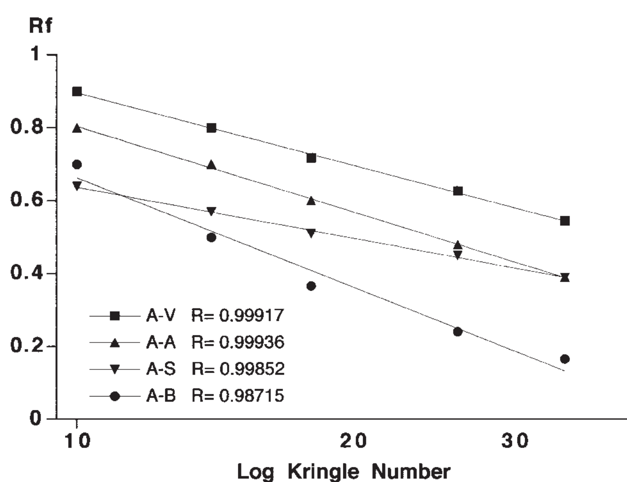

Fig. 3. Representation of the linear correlation between log of kringle number and the relative mobility (R_f) of the r-apo[a] isoforms of the standard in each gel; (■) 1.5% agarose/vertical electrophoresis; (▼) 1.5% agarose/submarine electrophoresis; (▲) 3.75% acrylamide/0.75% agarose; (●) 5% acrylamide (C = 0.6%).

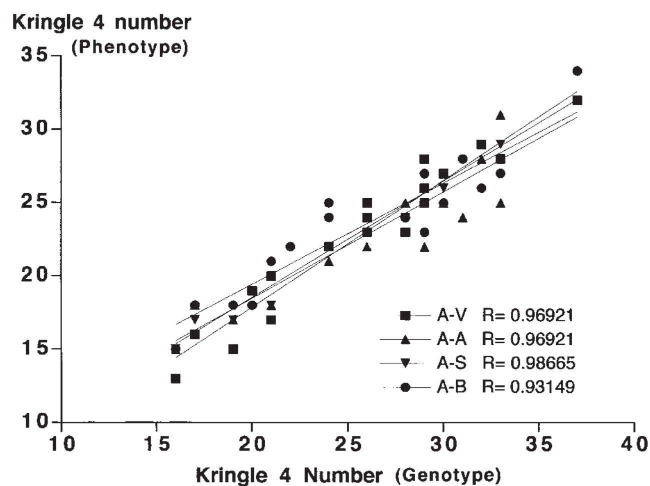

Fig. 4. Representation of the linear correlation between the number of kringles as determined by apo[a] phenotyping using the r-apo[a] standard, and the number of kringle 4 encoding sequences determined by genotyping; (■) 1.5% agarose/vertical electrophoresis; (▼) 1.5% agarose/submarine electrophoresis; (▲) 3.75% acrylamide/0.75% agarose; (●) 5% acrylamide (C = 0.6%).

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dent groups (2, 4). In contrast, the role of apo[a] structural heterogeneity as a modulator of the risk represented by Lp[a] is still a matter of debate. Recent studies have indicated, however, that small size apo[a] isoforms are more frequently found in patients with cardiovascular disease (15), and that Lp[a] isoforms display heterogeneity with regard to fibrin and cell binding (13, 14). It is therefore possible that the effect of high concentrations of Lp[a] may be subordinated to the relative concentration of Lp[a] bearing the pathogenic isoform. Apo[a] isoform phenotyping may thus constitute a useful tool for the identification of subjects at cardiovascular risk.

The analysis of apo[a] isoforms at the protein level is, however, complicated by the nature of the apo[a] protein itself and by the absence of a stable, reliable, and generally accepted reference standard for apo[a] phenotyping. Apo[a] isolated from plasma Lp[a] and recombinant apo[a] expressed in HepG2 cells (19) or 293 cells (present study) are highly glycosylated (23 wt%) proteins (27) and bind lower amounts of SDS. This lower charge-to-mass ratio distorts its electrophoretic mobility (28) and questions the use of external standards (apoB-100, phosphorylase B, haptoglobulin polymers) in determining the molecular weight of apo[a]. In such a case the results may underestimate or overestimate the size of apo[a]. As the particularity of apo[a] resides in its singular composition, a variable number of kringle-4 repeats, the right solution will be to identify apo[a] isoforms with regard to the number of kringles (29).The appropriateness of such a solution is further stressed by the fact that the number of kringle 4 copies is inversely related to the anti-fibrinolytic effect of Lp[a] (12). Furthermore, in agreement with these data, it has been widely documented that apo[a] isoforms of low molecular size are more frequently found in patients with cardiovascular diseases (15).

Apo[a] isoforms that may be of pathophysiological relevance because of their ability to inhibit plasminogen functions do so at circulating concentrations that are always detected by most of the electrophoretic and immunoblotting systems available. Therefore, provided that plasma samples are obtained under conditions that avoid proteolytic degradation, the use of a reliable and permanent reference standard for protein phenotyping will ensure correct identification of subjects at cardiovascular risk. The right choice will probably be to calibrate a set of apo[a] glycoproteins whose size will encompass the whole range of apo[a] isoforms described up to now (9, 10, 22, 30, 31). A commercial product (Immuno-Vienna) containing five different apo[a] polymorphs prepared by mixing human plasmas, and named after the nomenclature introduced by Utermann et al. (32), has been in use for such a purpose. We propose here, as an alternative choice, to compare the electrophoretic migration of apo[a] from plasma to reference apo[a] glycoproteins of known structure and composition, e.g., a genetically engineered battery of r-apo[a] preparations containing a predefined number of kringle-4 repeats. Indeed, limitations in the use of the proposed recombinant standard are those of the gel electrophoresis system used. The acrylamide/agarose and the vertical agarose electrophoresis gel systems seem to provide the best resolution as estimated from the slope of the linear correlation between the relative migration of the recombinant apo[a] and the kringle number (Fig. 3).

An important advantage of the use of this r-apo[a] standard is that the size of apo[a] polymorphs can be accurately reported in kringle numbers whatever the gel system used for electrophoresis. Indeed, a very good correlation (r = 0.93, 0.97, and 0.98) was found between the number of kringle 4 encoding sequences determined by genotyping and the number of kringle 4 repeats of plasma apo[a] as estimated with the r-apo[a] standard in the four-gel electrophoretic system used. Finally, we would like to stress the fact that the availability of these recombinant apo[a] proteins expressed by stable transfected cell lines ensures the permanence and solidity of the standard in terms of durability, composition, and electrophoretic behavior.

In conclusion, the results of this study indicate that the use of a proper and reliable standard based on recombinant apo[a] isoforms having a predefined number of kringle-4 repeats is suitable for size determination of apo[a] independent of the gel electrophoresis system used. Thus, the combination of apo[a] protein phenotyping with the determination of Lp[a] levels will provide complete information on the association of Lp[a] with cardiovascular risk.

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REFERENCES

1. Berg, K. 1963. A new serum type system in man: the Lp system. *Acta Pathol. Microbiol. Scand.* **59:** 369–382.
2. Dahlen, G. H., J. R. Guyton, M. Attar, J. A. Farmer, J. A. Kautz, and A. M. Gotto. 1986. Association of levels of lipoprotein Lp[a], plasma lipids and other lipoproteins with coronary artery disease documented by angiography. *Circulation.* **74:** 758–765.
3. Sandkamp, M., H. Funke, H. Schulte, E. Kohler, and G. Assmann. 1990. Lipoprotein[a] is an independent risk factor for myocardial infarction at a young age. *Clin. Chem.* **36:** 20–23.
4. Schreiner, P. J., J. D. Morrisett, A. R. Sharrett, W. Patsch, H. A. Tyroler, K. Wu, and G. Heiss. 1993. Lipoprotein[a] as a risk factor for preclinical atherosclerosis. *Arterioscler. Thromb.* **13:** 826–833.
5. Mc Lean, J. W., J. E. Tomlinson, W. J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein[a] is homologous to plasminogen. *Nature.* **330:** 132–137.
6. Eaton, D. L., G. M. Fless, W. J. Kohr, J. W. Mc Lean, Q. T. Xu, C. G. Miller, R. M. Lawn, and A. M. Scanu. 1987. Partial amino acid sequence of apolipoprotein[a] shows that it is homologous to plasminogen. *Proc. Natl. Acad. Sci. USA.* **84:** 3224–3228.
7. van der Hoek, Y. Y., M. E. Wittekoek, U. Beisiegel, J. J. P. Kastelein, and M. L. Koschinsky. 1993. The apolipoprotein[a] kringle IV re-

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peats which differ from the major repeat kringle are present in variably sized isoforms. *Hum. Mol. Genet.* **2:** 361–366.
8. Lackner, C., J. C. Cohen, and H. H. Hobbs. 1993. Molecular definition of the extreme size polymorphism in apolipoprotein[a]. Hum. Mol. Genet. **2:** 933–940.
9. Lackner, C., E. Boerwinkle, C. C. Leffert, T. Rahmig, and H. H. Hobbs. 1991. Molecular basis of apolipoprotein[a] isoform size heterogeneity as revealed by pulsed-field gel electrophoresis. *J. Clin. Invest.* **87:** 2153–2161.
10. Kamboh, M. I., R. E. Ferrell, and B. A. Kottke. 1991. Expressed hypervariable polymorphism of apolipoprotein[a]. *Am. J. Hum. Genet.* **49:** 1063–1074.
11. Utermann, G., H. J. Menzel, H. G. Kraft, H. C. Duba, H. G. Kemmler, and C. Seitz. 1987. Lp[a] glycoprotein phenotypes: inheritance and relation to Lp[a]-lipoprotein concentrations in plasma. *J. Clin. Invest.* **80:** 458–465.
12. Rader, D. J., W. Cain, L. A. Zech, D. Usher, and H. B. Brewer. 1993. Variation in lipoprotein[a] concentrations among individuals with the same apolipoprotein[a] isoform is determined by the rate of lipoprotein[a] production. *J. Clin. Invest.* **91:** 443–447.
13. Perombelon, Y. F. N., A. K. Soutar, and B. L. Knight. 1994. Variation in lipoprotein[a] concentration associated with different apolipoprotein[a] alleles. *J. Clin. Invest.* **93:** 1481–1492.
14. Kraft, H. G., A. Lingenhel, S. Köchl, F. Hoppichler, F. Kronenberg, A. Abe, V. Mühlberger, D. Schönitzer, and G. Utermann. 1996. Apolipoprotein[a] kringle IV repeat number predicts risk for coronary heart disease. *Arterioscler. Thromb. Vasc. Biol.* **16:** 713–719.
15. Herivo, L., M. J. Chapman, J. Thillet, S. Loyasu, and R. Anglés-Cano. 1993. Does apolipoprotein[a] heterogeneity influence lipoprotein[a] effects on fibrinolysis? *Blood.* **82:** 392–397.
16. Hervio, L., V. Durlach, A. Girard-Globa, and E. Anglés-Cano. 1995. Multiple binding with identical linkage: a mechanism that explains the effect of lipoprotein[a] on fibrinolysis. *Biochemistry.* **34:** 13353–13358.
17. Kang, C., V. Durlach, T. Soulat, C. Fournier, and E. Anglés-Cano. 1997. Lipoprotien[a] isoforms display differences in affinity for plasminogen-like binding to human mononuclear cells. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2036–2043.
18. Brunner, C., H. G. Kraft, G. Utermann, and H. J. Müller. 1993. Cys(4057) of apolipoprotein[a] is essential for lipoprotein[a] assembly. *Proc. Natl. Acad. Sci. USA.* **90:** 11643–11647.
19. Brunner, C., E. M. Lobentanz, A. Pethö-Schramm, A. Ernst, C. Kang, H. Dieplinger, H. J. Müller, and G. Utermann. 1996. The number of identical kringle IV repeats in apolipoprotein[a] affects its processing and secretion of HepG2 cells. *J. Biol. Chem.* **271:** 32403–32410.
20. Laurell, C. B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel-containing antibodies. *Anal. Biochem.* **15:** 45–52.
21. Couderc, R., K. Valenti, K. Peoc'h, M. E. Diop, S. Bailleul, L. Kiénou, F. Ferrière, and J. Etienne. 1998. A simple electrophoretic method for phenotyping apo[a]: phenotype frequency in healthy subjects from Paris, France. *Clin. Chem.* **44:** 1047–1050.
22. Gaubatz, J. W., K. I. Ganem, J. Guevara, M. L. Nava, W. Patsch, and J. D. Morrisett. 1990. Polymorphic forms of human apolipoprotein[a]: inheritance and relationship of their molecular weights to plasma levels of lipoprotein[a]. *J. Lipid. Res.* **31:** 603–613.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
24. Valenti, K., E. Aveynier, F. Laporte, and A. J. Hadjian. 1997. Evaluation of the genotyping and phenotyping approaches in the investigation of apolipoprotein[a] size polymorphism. *Clin. Chim. Acta.* **263:** 249–260.
25. Gillery, P., P. Arthuis, C. Cuperlier, and R. Circaud. 1993. Rate nephelometric assay of serum lipoprotein[a]. *Clin. Chem.* **39:** 503–508.
26. Seebacher, T., and E. G. Bade. 1996. Quick and easy weight determination with Macintosh computers and public domain image analysis software. *Electrophoresis.* **17:** 1573–1574.
27. Fless, G. M., M. L. Snyder, J. W. Furbee, M. T. Garcia-Hedo, and R. Mora. 1994. Subunit composition of lipoprotein[a] protein. *Biochemistry.* **33:** 13492–13501.
28. Segrest, J. P., R. L. Jackson, E. P. Andrews, and V. T. Marchesi. 1971. Human erythrocyte membrane glycoproteins: a re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* **44:** 390–395.
29. Kraft, H. G., A. Lingenhel, G. Bader, G. M. Kostner, and G. Utermann. 1996. The relative electrophoretic mobility of apo[a] isoforms depends on the gel system: proposal of a nomenclature for apo[a] phenotypes. *Atherosclerosis.* **125:** 53–61.
30. Marcovina, S. M., Z. H. Zhang, V. P. Gauer, and J. J. Albers. 1993. Identification of 34 apolipoprotein[a] isoforms: differential expression of apolipoprotein[a] alleles between American blacks and whites. *Biochem. Biophys. Res. Commun.* **191:** 1192–1196.
31. Kraft, H. G., S. Köchl, H. J. Menzel, C. Sandholzer, and G. Utermann. 1992. The apolipoprotein[a] gene: a transcribed hypervariable locus controlling plasma lipoprotein[a] concentration. *Hum. Genet.* **90:** 220–230.
32. Utermann, G., H. J. Menzel, H. G. Kraft, H. C. Duba, H. G. Kemmler, and C. Seitz. 1987. Lp[a] glycoprotein phenotypes. *J. Clin. Invest.* **80:** 458–465.

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