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RESEARCH COMMUNICATION

Structural requirements of apo-a for the lipoprotein-a assembly

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Lipoprotein-a [Lp(a)], one of the most atherogenic lipoproteins, is composed of a low-density lipoprotein (LDL) core in addition to an apo-a of variable size which is linked to apoB by a disulphide bridge. Lp(a) synthesized *in vitro* by incubation of recombinant apo-a (r-apo-a) with LDL is physico-chemically indistinguishable from native Lp(a). The synthesis of Lp(a) *in vitro* proceeds in two steps. In the first step, one of the unique kringle-IVs (K-IVs) in apo-a binds to a Lys residue of apoB; in the second step, Cys-4057 of K-IV type-9 (T-9) forms a disulphide bridge with Cys-3734 of LDL. Here we have produced r-apo-a with different combinations of unique K-IVs and shown that K-IV T-6 is required for the first step of Lp(a) assembly. For the second step not only is K-IV T-9 essential, but also the distance between T-6 and T-9 requires a length of two K-IVs. These findings give additional insight into the mode of Lp(a) assembly and are of relevance in the search for apo-a mutants influencing Lp(a) levels and for the development of Lp(a)-lowering medications.

INTRODUCTION

Lipoprotein-a [Lp(a)] is a highly atherogenic lipoprotein of human plasma with unknown physiological function [reviewed in 1–3]. It consists of a low-density lipoprotein (LDL) core-particle plus the characteristic antigen apo-a. Apo-a and apoB-100 are linked by a disulphide bridge at positions 4057 and 3734 respectively [4–6]. Apo-a shares extensive sequence identities with plasminogen (Plg) which include sequences homologous with kringle (K)-IV, K-V and the protease domain of Plg [7]. There exist probably more than 50 alleles in humans which code for proteins of variable size and plasma concentrations [8,9]. These size differences are due to variations of the number of K-IV repeats. The cDNA clone originally sequenced by McLean et al. [7] contained 37 K-IV repeats consisting of the repetitive kringles 2–29 in addition to the unique kringles 1 and 30–37. According to the nomenclature of Morrisett et al. [10] the K-$IV_{1\text{-}37}$ are denominated as types (T) 1–10 where K-IV_1 is T-1, K-$IV_{2\text{-}29}$ are T-2, K-$IV_{30}$is T-3 ... and K-IV_{37} is T-10 (Figure 1). The significance of the small variations in the sequence of K-IV remains to be established.

Lp(a) is biosynthesized in the liver. It has been demonstrated that plasma Lp(a) concentration is determined by the rate of synthesis and not by the catabolic rate [11,12]. Details of the assembly of native Lp(a) from LDL and apo-a emerge from studies of cultured baboon hepatocytes [13], from transfection of apo-a cDNA to mammalian cells [4,5,14,15] and from mice containing human apo-a and apoB transgenes [16].

By compilation of all previous publications, the following model of Lp(a) biosynthesis can be drawn. Apo-a is synthesized and secreted from the liver; outside the cell it assembles with LDL in a two-step process. First, Lys-rich epitopes of LDL bind

Figure 1 Apo(a) cDNA constructs used for transferrinfection

The constructions are compared with the apo-a cDNA structure published by McLean et al. [7] with the numbering of K-$IV_{1\text{-}37}$ on top. A- and B-kringles have an identical amino acid composition yet show slight differences in their DNA sequences. The numbers 1–10 denote the K-IV types proposed by Morrisett et al. [10]. S, Signal sequence; P, protease domain. The numbers 2.7, 2.9 etc. denote the length of the cDNA clones in kbs. F indicates the fusion kringles; the amino acid content of these fusion kringles is shown in Table 1.

Abbreviations used: ε-AHA, ε-amino hexoic acid; d.e.l.f.i.a., dissociation-enhanced lanthanide fluorescence immunoassay; DMEM, Dulbecco's modified Eagle's medium; LDL, low-density lipoprotein; Lp(a), lipoprotein-a; r-apo-a, recombinant apo-a; Plg, plasminogen; K-IV, kringle-IV; T, type.

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to K-IV motifs of apo-a forming a complex which is dissociable by ϵ-amino hexoic acid (ϵ-AHA). Secondly, a disulphide bridge is formed between amino acid 4057 of apo-a and 3734 of apoB. It appears that no specific enzyme is required for this process [14,15].

In a previous study we presented evidence that the addition of freshly prepared LDL to cell medium containing r-apo-a and incubation for 24 h, produced an Lp(a) lipoprotein virtually identical to native Lp(a) with respect to density, electrophoretic migration and morphology [14]. The apo-a–apoB complexes were stabilized by disulphide bridges, whether or not the assembly was performed in the presence of cells. In the former study, r-apo-a with 9, 15 and 18 K-IV repeats were incubated with LDL and it was found that under the chosen conditions 50–70 % of the r-apo-a assembled with LDL. All these r-apo-a constructs contained one copy of K-IV T-1–10, each with a variable number of the repetitive K-IV of T-2. A 3.0 kb construct lacking the unique K-IV of T-3–T-8 (Figure 1) was unable to assemble with LDL. The 'Cys-4057 mutation', in which Cys-4057 in K-IV T-9 was replaced by Arg, only formed loose complexes with LDL which were dissociable by ϵ-AHA [14].

We show here that for the first step in Lp(a) assembly, the binding of apo-a to apoB-100 K-IV T-6, is absolutely essential.

EXPERIMENTAL

Materials

Cell lines were obtained from American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM) and fetal lipoprotein free calf serum were obtained from Böhringer-Mannheim; replication-defective biotinylated adenovirus, streptavidin–polylysine conjugate and human transferrin–polylysine conjugate were produced at the Institute of Molecular Pathology, Vienna by E. Wagner [17]; and apo-a cDNA clones were obtained from R. Lawn [7]. All antibodies were prepared in our laboratory [18]. Biochemicals were purchased from Sigma. Human serum was obtained from healthy, fasting volunteers.

Construction of apo-a expression vectors

The apo-a expression plasmids containing DNA sequences coding for various K-IV types in addition to K-V and the protease-like domain were assembled using standard recombinant DNA technology [19]. A detailed description of the construction and cloning of apo-a cDNA has been described previously [14,15]. Some of the cDNA fragments were ligated in such a way that so-called fusion kringles (F) were obtained containing part of the amino acid sequence from one and the remaining amino acids from another K-IV (Figure 1). It is worth noting that all K-IVs in apo-a contain 114 amino acids except for T-6 which contains only 106 amino acids. Specific apo(a) cDNA constructs were ligated into the *Eco*RI site of pSG5 as described previously [14,15]. This expression vector contains the simian-virus-40 early promotor, rabbit β-globin intron II and the simian-virus-40 poly-A signal. For transfection experiments, plasmid DNA was purified by the alkaline lysis method followed by CsCl gradient ultracentrifugation [19].

Expression vectors coding for eight different apo-as were constructed (Figure 1) and transfected into Cos-7 cells by transferrinfection, a receptor-mediated gene delivery system [20]. Transiently transfected cells were incubated in DMEM. After 48 h incubation the medium contained 0.4–0.8 mg/ml of r-apo-a.

Association (assembly) of r-apo-a with LDL

LDL was purified by single-spin density-gradient ultracentrifugation in a VT-65 rotor (Beckmann) according to the following method. Freshly drawn serum (2 ml) was mixed with 546 mg of NaBr and overlayed with 4 ml of 0.15 mol/l NaCl. Tubes were sealed and spun for 90 min at 350000 ***g***. LDL, which floated as a distinct narrow band in the middle of the tube, separated well from very-high-density lipoprotein and high-density liprotein, was aspirated by a syringe and washed once under identical conditions. For the removal of contaminating Lp(a), LDL was passed over an anti-apo-a sepharose column. To avoid LDL oxidation all steps of preparation were performed under nitrogen and in the presence of 0.5 mg/ml EDTA. A 5 mg portion of LDL (measured as protein) was mixed with 0.5 nM r-apo-a in 1 ml of cell medium and incubated for 24 h at 37 °C in the presence of aprotinin, leupeptin and phenylmethanesulphonyl fluoride protease inhibitors. This condition was shown previously to yield maximal r-apo-a–LDL associations [14,15].

Monitoring of the efficiency of Lp(a) assembly

The efficiency of the assembly of r-apo-a with LDL was monitored by Western blotting and by dissociation-enhanced lanthanide fluorescence immunoassay (d.e.l.f.i.a.). For Western blotting, the samples were heated for 5 min in 2 % SDS in the absence of reducing agents and subjected for 12 h to SDS agarose gel electrophoresis [22]. Proteins were then transferred to nitrocellulose membranes by electroblotting and incubated for 3 h with rabbit anti-apo-a at a dilution of 1:1000. For the detection of apoB, a monospecific polyclonal antibody was used [11,18]. After extensive washing, the blots were incubated with horseradish peroxidase-labelled protein A and stained with 4-chloro-1-naphthol. D.e.l.f.i.a. is an e.l.i.s.a.-like technique where 96 well-containing plates are coated with a 'capture' antibody followed by incubation with the samples to be analysed. After extensive washing, Eu-labelled 'detection' antibodies are added and the fluorescence of the complexed Eu is measured [21]. Two pairs of antibodies were applied. As capture antibody, affinity-purified rabbit anti-apo-a was used. The detection antibodies (both from sheep) for quantification of total apo-a consisted of anti-apo-a, while anti-apoB was used for the quantification of apo-a–apoB complexes. As standards, highly purified LDL, Lp(a) and r-apo-a were used. In order to measure apo-a–apoB complexes which are not stabilized by disulphide bridges, the d.e.l.f.i.a. was carried out in the presence and absence of 50 mM ϵ-AHA. This amount was sufficient to block completely the assembly of Lp(a) if added to the medium together with LDL. Here ϵ-AHA was added after the incubation, just before the d.e.l.f.i.a. was performed. ϵ-AHA did not influence the immunochemical reaction as determined in previous experiments. The d.e.l.f.i.a. was linear between 2 and 200 ng of apo-a per well. With this combination of d.e.l.f.i.a.s we first determined the concentration of total apo-a in the incubate followed by the determination of apoB-bound apo-a.

RESULTS

The previous work performed in our laboratory as well as that published by others [4–6,14,15] does not provide any detailed information concerning the protein structures in apo-a necessary for the first step in Lp(a) assembly. In order to study this process in more detail, additional recombinant apo-a segments with various combinations of the unique K-IVs (Figure 1 and Table

Table 1 Composition of the cDNA constructs with respect to K-IV

The ligation of the cDNA fragments yielded so-called fusion kringles which contained some of the amino acid sequences from one and the remaining amino acids from another K-IV. Amino acid numbering according to Morrisett et al. [10].

cDNA construct	Kringle-IV sequences	Amino acids in the fusion kringle
2.7 kb	F(T-1:T-7)*; T-8; T-9; T-10	T-1: 1–75; T-7: 76–114
2.9 kb	F(T-1:T-6); T-7; T-8; T-9; T-10	T-1: 1–75; T-6: 76–106
3.0 kb	T-1; T-2; F(T-2:T-8); T-9; T-10	T-2: 1–75; T-8: 76–114
3.2 kb	T-1; F(T-2:T-6); T-7; T-8; T-9; T-10	T-2: 1–6; T-6: 8–106†
3.2Δ kb	T-1; F(T-2:T-6); F(T-7:T-8); T-9; T-10	T-2: 1–6; T-6: 8–106† T-7: 1–75; T-8: 76–114
4.0 kb	T-1; F(T-2:T-3); T-4; T-5; T-6; T-7 T-8; T-9; T-10	T-2: 1–98; T-3: 99–114

* F(T-*x*:T-*y*) denotes the fusion kringles containing part of the amino acids from K-IV type-*x* and part from type-*y*.

† The amino acid number 7 of K-IV T-2 is missing in this construct.

Table 2 Efficiency of the assembly of various r-apo-as with LDL

r-Apo-a (0.5 nM) in 1 ml cell medium derived from c-DNAs of different length (Figure 1) was incubated for 24 h with apo-a-free LDL and the assembly was studied by d.e.l.f.i.a. D.e.l.f.i.a. was performed in the presence and absence of 50 mM ϵ-AHA. The results are expressed in percent of r-apo-a in the medium complexed to LDL. Values are means ($\pm$S.D.) of three experiments.

Apo-a type (kb of the cDNA)	Assembly (%)	
	$-\epsilon$-AHA	$+\epsilon$-AHA
2.7	4.6 (0.2)	3.8 (0.2)
2.9	24.3 (1.4)	10.4 (0.7)
3.0	1.9 (0.1)	0.3 (0.004)
3.2	**91.1 (6.4)**	**60.0 (3.3)**
3.2 Δ	18.9 (1.4)	6.2 (0.5)
4.0	72.0 (4.8)	59.7 (5.0)

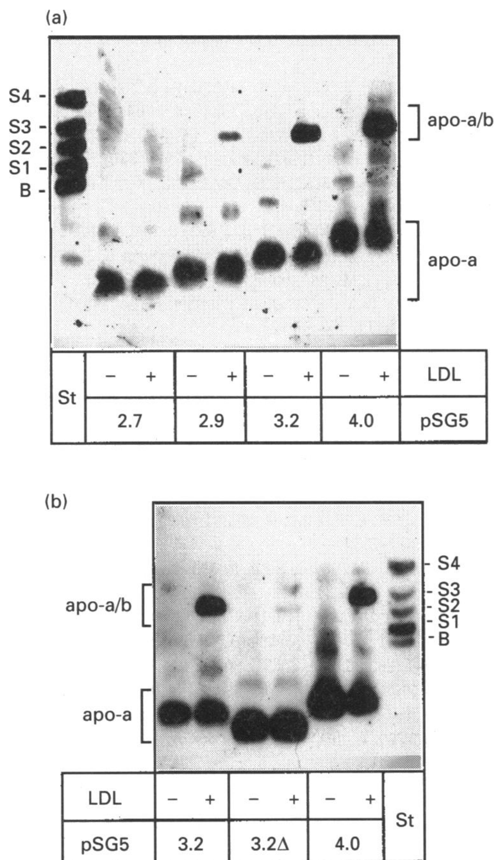

Figure 2 Western blot of r-apo-a and complexes thereof with LDL

Cos-7 cells were transfected with pSG5-constructs shown in Figure 1 and incubated in DMEM for 24 h–48 h. The medium was harvested, adjusted to an apo-a concentration of 0.5 nM and incubated for 24 h with 5 mg/ml of LDL (protein) followed by SDS/agarose gel electrophoresis under non-reducing conditions and Western blotting [22]. St, Apo-a standard from Immuno A. G., Vienna with apo-a isoforms named according to Utermann et al. [8]. A, pSG5 constructs 2.7, 2.9, 3.2 and 4.0. B, pSG5 constructs 3.2, 3.2Δ and 4.0.

1) were prepared and studied with respect to their ability to assemble with LDL.

Figure 2 exhibits a non-reducing SDS/agarose gel electrophoresis of various r-apo-as and of five standard apo-a isoforms from human serum (St) before and after incubation with LDL. Despite some aggregates, r-apo-as migrate in agarose according to their size, forming one major band. The addition of LDL to the r-apo-a followed by 24 h incubation at 37 °C led to the formation of apo-a–apoB heterodimers only with r-apo-a 2.9, 3.2, 4.0 and very little with 3.2Δ. The heterodimers reacted with anti-apo-a and with anti-apoB. It should be noted here that step-I complexes (see below) dissociate under these conditions. Thus, only stable step-II apo-a–apoB100 complexes linked by disulphide bridges are seen.

In order to obtain quantitative data on the efficiency of the assembly, the amount of apo-a bound to apoB was measured by d.e.l.f.i.a. as outlined in the Experimental section (Table 2). The r-apo-a 4.0, containing all the unique K-IVs, which was used in previous studies to investigate the assembly of Lp(a) [14], yielded 72% assembly with LDL under these experimental conditions. The addition of ϵ-AHA dissociated 18% of the complex, indicating that some 60% of the 'artificial Lp(a)' contained a disulphide bridge. When 50 mM ϵ-AHA was added to the incubation mixture from the beginning, $< 1\%$ of disulphide bridges were formed (results not shown). We know already from previous work that the r-apo-a 3.0, lacking the unique K-IV of T-3–T-7, gives no stable complexes with LDL. Several new constructs were therefore tested in this study. The results summarized in Table 2 demonstrate that the r-apo-a 3.2, containing 99 amino acids from K-IV T-6, in addition to the complete K-IV of T-1 and T-7–T-10, assembled with LDL to 91% with the highest efficiency. The addition of ϵ-AHA dissociated 34% of this complex. The construct 2.9 with only 31 amino acids from K-IV T-6 yielded 24.3% and 10.4% assembly in the presence and absence of ϵ-AHA respectively. Taking these data together we conclude that K-IV T-6 is absolutely necessary for an efficient Lp(a) assembly.

The results of our previous report were compatible with a two-step model for the Lp(a) assembly [14]. In the first step, apo-a forms a loose complex by the interaction of Lys groups from LDL with one or several K-IVs, followed by the stabilization by a disulphide bridge located in K-IV T-9. Since we found here that only one specific K-IV (T-6) mediates the first binding step, we hypothesized that for steric reasons an efficient assembly might only occur if the distance between kringles T-6 and T-9 is appropriate. To test this hypothesis the assembly of LDL with r-apo-a 3.2Δ, a construct which misses one K-IV between T-6 and

T-9, was studied (Figure 2b). Instead of full-length T-7 and T-8, r-apo-a 3.2Δ contained a fusion kringle with 75 amino acids from T-7 and 39 amino acids from T-8. Indeed, r-apo-a 3.2Δ assembled with LDL in step I only to 19%, and only 6.2% of LDL–apo-a complexes were stabilized by a disulphide bond (Table 2).

DISCUSSION

The plasma concentration of the atherogenic lipoprotein Lp(a) is determined to a major extent by the rate of biosynthesis [11,12]. There are two major post-translational regulatory events which influence plasma Lp(a) levels. One is the rate of apo-a secretion from liver cells, which depends on apo-a size; the larger the size the lower the efficiency of secretion [23]. The other event relates to the extracellular assembly of apo-a with LDL, a two-step reaction in which apo-a is first loosely bound to apo-B 100 by a K-IV–Lys interaction, followed by the stabilization of the heterodimer via a disulphide bond [4–6,14,15]. In a previous report we have shown that in addition to the structural requirements of apo-a, an intact LDL structure is also required for the assembly. Individuals suffering from lecithin:cholesterol acyltransferase deficiency, whose LDLs lack core lipids and are structurally altered, neither have detectable amounts of circulating Lp(a) in their plasma nor is their LDL capable of assembly to Lp(a) with r-apo-a *in vitro* [15]. All previously published work reported that some or all of the unique K-IVs in apo-a are required for the assembly, whereas the repetitive K-IVs are not [14]. The K-IV absolutely required is T-9 because it carries the only free -SH group in apo-a which is necessary for the formation of a stable complex. With respect to the other unique K-IVs little information has been available so far.

In this study we produced a series of cDNA clones from cDNA fragments obtained by R. Lawn, Genetech [7] with various combinations of the unique K-IVs (Figure 1, Table 1). The shortest fragment which gave high-yield assembly with LDL (derived from 3.2 kb cDNA) contained the majority of K-IV T-6 plus T-7–T-10. This r-apo-a 3.2 was even more efficient in forming the first-step complex with LDL than the r-apo-a 4.0, the latter containing all unique K-IVs T-1–T-10. The amount of stable complexes formed with r-apo-a 3.2 and 4.0, however, were almost identical (Table 2). Since the unique K-IVs T-1, T-7, T-8 and T-10 are present in the 2.7, 2.9 and 3.0 r-apo-as in various combinations, constructs which exhibited either none or only little assembly with LDL, it can be concluded that for an efficient Lp(a) assembly, besides kringle T-9, the only one required is T-6. The deletion of 114 amino acids in r-apo-a 3.2Δ corresponding to a whole K-IV (part of T-7 and part of T-8) reduced the efficiency to form a stable Lp(a) complex by almost 90%, despite the fact that K-IV T-8 by itself is not required for step I in the Lp(a) assembly (the construct 2.7 kb containing a full T-8 K-IV exhibits almost no binding to LDL). It is possible, though not very likely, that K-IV T-7 has an enhancing effect in the Lp(a) assembly: the 2.7 kb construct which exhibited $< 5\%$ assembly contained only part of T-7 (amino acid 76–114) and all other constructs with high-efficient assembly contained K-IV T-7 together with K-IV T-6.

In summary, we have shown that two unique K-IVs in apo-a are required to form an Lp(a) *in vitro*: T-6 and T-9; T-6, the only K-IV with 106 amino acids (all other K-IVs consist of 114 amino acids) is necessary for the first step and T-9 for the second step of the assembly. There has to be a defined stretch of amino acids between these two kringles in order to match complementary structures in LDL. Epidemiological studies revealed that the size of apo-a heavily reflects the plasma Lp(a) concentration *in vivo*, yet large racial and interindividual differences are found [24,25]. It will be interesting to see whether some of these differences are due to mutations in the region of K-IV T-6–T-9.

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