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Functional Characterization of T7 and T8 of Human Apolipoprotein (a)

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Lipoprotein (a) [Lp(a)], a risk factor for coronary artery disease, is a LDL-like particle with apolipoprotein (a) [apo(a)] covalently linked to apolipoprotein B (apoB). Apo(a) has many repeats of kringle 4-like domain, classified as type 1 through type 10 (T1–T10). Deletion analysis was performed to define the functional modules of human apo(a). We found that T7 has an affinity for cell surfaces and is required for Lp(a) formation. Cell surface binding was inhibited by L-proline, K_I = 4.7 ± 3.6 mM (n=3). We also found that T8 has an affinity for subendothelial extracellular matrix (ECM). ECM binding was inhibited modestly by L-proline (K_I = 6.1 ± 1.9 mM, n=3), and more effectively by L-lysine (K_I = 2.7 ± 1.0 mM, n=3) and its analogue, 6-aminohexanoic acid (K_I = 0.35 ± 0.13 mM, n=3). These data point to T7 and T8 as important functional modules of apo(a).

Lp(a), a risk factor for coronary artery disease (1-3), is a LDL-like particle with a distinguishing glycoprotein, apo(a), covalently linked to apoB- the main protein component of LDL (4). Apo(a) is highly homologous to plasminogen; however, in contrast to plasminogen which has only five kringles, the cloned apo(a) has thirty-eight kringles (5). Except for the kringle 5-like domain, these kringles are repeats of the kringle 4-like domain and are classified as type 1 through type 10 (T1-T10) (6). Apo(a) and LDL are secreted independently by hepatocytes and are assembled into Lp(a) upon cellular surfaces (7-11). Lp(a) formation can be divided into two steps (12). Step 1 is the initial noncovalent interaction between T6 and possibly T7 of apo(a) and residues 3304-3317 of apoB, which brings LDL and apo(a) together forming an apo(a):LDL complex. This noncovalent interaction is the same as the extensively characterized noncovalent Lp(a):LDL interaction (13-16). Step 2 is the disulfide bond formation between T9 of apo(a) and apoB to form Lp(a). Step 2 is probably mediated by cell surface protein disulfide isomerase (11).

As pharmaceutic reagents known to affect LDL catabolism and anabolism have little affect on plasma Lp(a) levels, except high doses of niacin (17), it is logical to direct pharmaceutic intervention at the extracellular assembly of Lp(a). The almost complete absence of circulating apo(a) in abetalipoproteinemia (18), suggests that free apo(a) is cleared rapidly. Furthermore, there is *in vitro* evidence that apo(a) covalently linked to LDL is recalcitrant to protease degradation and/or cellular uptake (11). Therefore, administration of Lp(a) assembly inhibitors could reduce plasma Lp(a) concentration by increasing the clearance rate of apo(a). We report here that Lp(a) formation and cell binding are dependent on T7; and, extracellular matrix (ECM) binding is dependent on T8. These data point to these kringles as important functional modules of human apolipoprotein(a) and should be the focus of pharmaceutic intervention.

METHODS

Construction of cell lines expressing r-apo(a). The mini apo(a) cDNA (19) was digested with *Hin*dIII, and the 5′ fragment and the 3′ fragment were ligated to give Ha6 cDNA. Similarly, the mini apo(a) cDNA was digested with *Bam*HI, and the 5′ fragment and the 3′ fragment were ligated to give Ha3 cDNA. Deletion derivatives of Ha6 (D1-D4) were obtained by partial *Bam*HI digestion of the Ha6 cDNA. Due to fusion with adjacent kringles, the T7 of D3 has a $R_{58} \rightarrow S$ substitution and the T7 of D4 has a $R_{58} \rightarrow S$ and a $Q_{77} \rightarrow R$ substitutions. The numbering system is as in our previous publication (12). The cDNAs were subcloned into pTEJ-8 (20) giving rise to pTEJHa6, pTEJHa3, pTEJD1, pTEJD2, pTEJD3, and pTEJD4. The UbC promoter was used to drive r-apo(a) expression and the SV40 promoter was used to drive neomycin resistance expression. These plasmids were transfected into CHO-k1 using the Transfinity cal-

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Abbreviations used: ECM, extracellular matrix; Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); r-apo(a), recombinant apolipoprotein(a); apoB, apolipoprotein B; LDL, low density lipoprotein (d= 1.006-1.063 g/mL); and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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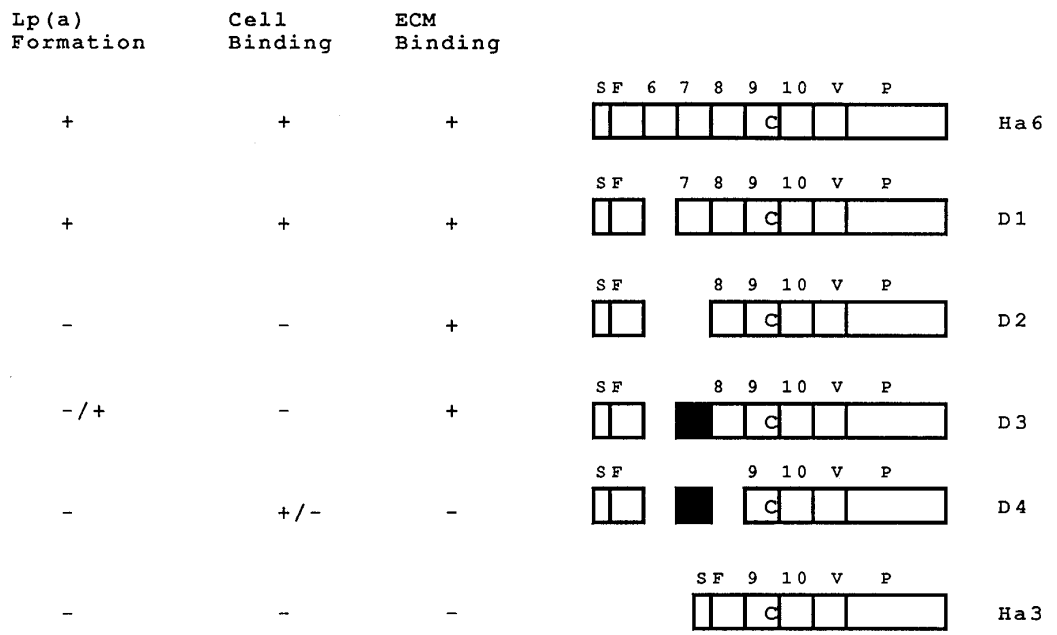

FIG. 1. Structures of r-apo(a). The structures of Ha6, D1, D2, D3, D4, and Ha3 are shown. The kringle-4 repeats are numbered according to the accepted nomenclature (6). Signal peptide (S), kringle V (V), and protease domain (P) are shown. F indicates fusion between T1 and T8 in Ha3 and T1 and T5 in Ha6 and D1-D4. The filled boxes indicate the T7 with a $R_{58} \rightarrow S$ substitution of D3 and the T7 with a $R_{58} \rightarrow S$ and a $R_{77} \rightarrow Q$ substitution of D4. C indicates the free cysteine of T9. Lp(a) formation activity, cell binding activity, and ECM binding activity of each apo(a) variant are also indicated (+ = positive, +/− = intermediate, and − = negative).

cium phosphate transfection kit (Gibco-BRL, Gaithersburg, MD). Selection by the antibiotic G-418 at a final concentration of 1.0 mg/mL gave more than 50 neomycin resistance colonies per plasmid. The transfected cells were pooled and named CHO-pTEJHa6, CHO-pTEJHa3, CHO-pTEJD1, CHO-pTEJD2, CHO-pTEJD3, and CHO-pTEJD4 according to the plasmid used for transfection. Expression levels were equivalent among these cell lines.

Cell culture. All cell culture reagents were obtained from Life Technologies (Gaithersburg, MD). CHO-k1 and HepG2 were obtained from the American Type Culture Collection (Rockville, MD) and maintained on CHO-k1 media [DMEM/F-12 (1/1, v/v) supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin/streptomycin] and HepG2 media [EMEM supplemented with 10% FBS, glutamine, penicillin/streptomycin, sodium pyruvate, and nonessential amino acids], respectively. By immunoblotting, apo(a) was not detected in the conditioned media of either CHO-k1 or HepG2; and, apoB was not detected in the conditioned media of CHO-k1. Cocultures of apo(a) producing cell lines and HepG2 were established to assay for Lp(a) formation. Approximately 5×10^5 HepG2 cells were plated per 60 × 15 mm Falcon plate (Becton Dickinson and Company, Plymouth, England) and allowed to attach, reaching 50% confluency overnight in HepG2 media. The same number of apo(a) producing cells was added the next day and allowed to attach overnight in CHO-k1 media. The cocultures were washed once with serum free CHO-k1 media and then grown in 4 mL of the same media. After 24 hours, the media were collected and analyzed for Lp(a) formation. Lone cultures of HepG2 cells and apo(a) producing cells established simultaneously were similarly processed and analyzed. For cell surface binding, these cultures were allowed to grow for 48 hours in the serum free CHO-k1 media, incubated with 4 mL of fresh media for 10 minutes at 22°C, and then 1 mL of the same media, supplemented with 0.2 M L-proline, for an additional 10 minutes at 22°C. The 48 hours conditioned media and the 0.2 M L-proline containing media were immunoblotted using anti-r-apo(a). SDS-gradient polyacrylamide gel electrophoresis (2.5-10%) (SDS-PAGE) and immunoblotting using sheep anti-r-apo(a) were performed as previously described (11,12). The apo(a) immunoreactivities were quantiated using the Optimas Video Imaging system (BioScan, Inc., Edmonds, WA) coupled to the VGA quantitation program (BioMed Instruments, Inc., Fullerton, CA).

Binding of apo(a) to subendothelial extracellular matrix (ECM). Cultures of bovine corneal endothelial cells were established from steer eyes as previously described (21) and maintained in DMEM supplemented with 15% FBS and penicillin/streptomycin. Partially purified brain-derived bFGF (100 ng/mL) was added every other day. When confluent, cells were dissociated with Trysin/Versene and plated onto 96-well plates at an initial density of 1×10^6 cells/well. Cells were maintained as above in the presence of 5% dextran T-40, which improved the thickness of the ECM. Six to eight days after confluency, the subendothelial ECM was exposed by dissolving (3 min, 22°C) the cell layer with a solution of 0.5% Triton X-100 and 20 mM NH_4OH in PBS, followed by three washes in PBS. The ECM remained intact, firmly attached to the entire area of the well and free of nuclear or cellular debris. Nonspecific binding sites were blocked with 3% (w/v) of BSA in PBS for 2 hours at 22°C. The ECM plates were washed three times, incubated with apo(a) conditioned media for 2 hours at 22°C, and washed thrice with PBS. The bound apo(a) was detected using sheep anti-r-apo(a) and peroxidase labeled anti-sheep IgG as in a typical ELISA assay. OD_{405} was read using BioRad EIA reader (BioRad, Hercules, CA).

RESULTS

Lp(a) Formation Requires the Presence of T7

In a coculture of HepG2 and CHO-pTEJHa6, LDL and Ha6 (an apo(a) missing T1 through T5) (Figure 1), were covalently linked by a disulfide bond following their secretion by HepG2 and CHO-pTEJHa6, respectively (Figure 2). The Lp(a) complex was dissociated by

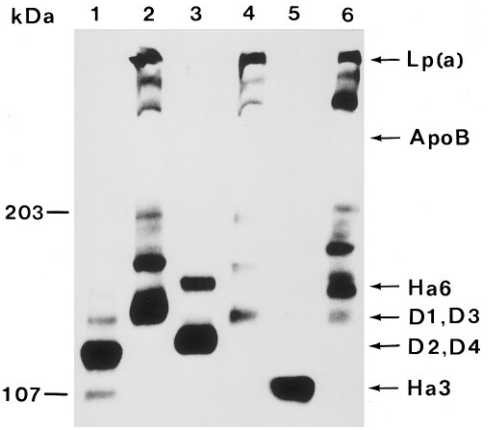

FIG. 2. Lp(a) formation requires the presence of T7. Media (60 μL) from cocultures of HepG2 and CHO-pTEJD4 (1), CHO-pTEJD3 (2), CHO-pTEJD2 (3), CHO-pTEJD1 (4), CHO-pTEJHa3 (5), or CHO-pTEJHa6 (6) were separated on a 3-15% SDS-PAGE and immunoblotted using anti r-apo(a). Lp(a), Ha6, Ha3, and D1-D4 are indicated by arrows. The locations of apoB and the 203 and 107 kDa molecular weight standards are also indicated. This figure is representative of three experiments.

β-mercaptoethanol into apo(a) and apoB. The formation of Lp(a) from the independently secreted Ha6 and LDL supports the contentions that Lp(a) formation occurs extracellularly (7-11) and that T1-5 are not essential for Lp(a) formation (22).

Lp(a) formation was also examined using the Ha6 deletions shown in Figure 1. T6-8 deletion (Ha3) is Lp(a) formation incompetent; T6-7 deletion (D2) is also Lp(a) formation incompetent; however, T6 deletion (D1) is Lp(a) formation competent (Figure 2). This suggested that Lp(a) formation required the presence of T7. The T8 deletion in D4 rendered it Lp(a) formation incompetent (Figure 2). This is consistent with T8 being necessary for the correct spacing between the noncovalent (T7) and covalent binding sites (T9) on apo(a) (22). Surprisingly, a $R_{58} \rightarrow S$ substitution in T7 (D3) was Lp(a) formation competent.

Cell Surface Binding Requires the Presence of T7

When a washed confluent culture of CHO-pTEJHa6 was treated with 0.2 M L-proline for 10 minutes at room temperature, Ha6 was eluted (Figure 3). Ha6 elution by L-proline is dose dependent; Ha6 was not eluted when the culture was treated with serum-free media. The K_I was calculated to be 4.7 $\pm$ 3.6 mM (n=3).

Cell surface binding was also examined using the Ha6 deletions (Figure 3). When the apo(a) bands were scanned, quantitated, and normalized against Ha6, the expression levels of Ha6 and its deletions were statistically equivalent (Figure 3, panel M). The T6-8 deletion (Ha3), the T6-7 deletion (D2), and the T6 deletion (D1) have 0%, 10.1 $\pm$ 3.9 %, and 64.6 $\pm$ 8.1 % binding, respectively (n=3) (Figure 3, panel P). This suggested that binding requires the presence of T7. Consistently, mutations introduced into T7 reduced cell surface binding significantly. D3, T7 with a $R_{58} \rightarrow S$ substitution, has 17.6 $\pm$ 8.0% (n=3) binding which is significantly lower than D1 (p=0.002) (Figure 3). D4, T7 with a $R_{58} \rightarrow S$ and a $Q_{77} \rightarrow R$, has a slightly restored binding activity of 40.6 $\pm$ 9.8 % (Figure 3).

ECM Binding Requires the Presence of T8

When conditioned media from CHO-pTEJHa6 was incubated with ECM, Ha6 was bound to the matrix. Saturable binding was detected within 10 minutes and reached half maximal at 36 minutes. Binding was not inhibited by glycine but was inhibited by L-lysine, L-proline, and 6-aminohexanoic acid, with K_Is of 6.1 $\pm$ 1.9 mM, 2.7 $\pm$ 1.0 mM, and 0.35 $\pm$ 0.13 mM, respectively (n=3). L-lysine was a slightly better inhibitor than L-proline (p= 0.0522); and, 6-aminohexanoic acid was a much better inhibitor than either L-proline (p= 0.0065) or L-lysine (p= 0.0156).

ECM binding was not affected by T6 deletion (D1), T6-7 deletion (D2), or T6 deletion with a $R_{58} \rightarrow S$ substitution in T7 (D3) (Figure 4). However, the T6-8 deletion (Ha3) was binding incompetent, suggesting that the ECM binding domain is located within T8 which is present in D1-D3 and is absent in Ha3 (Figure 4). Consistently, deletion of T8 in D4 significantly decreased ECM binding (Figure 4).

DISCUSSION

In this communication, we have demonstrated the requirement of T7 in Lp(a) formation. This reaffirmed

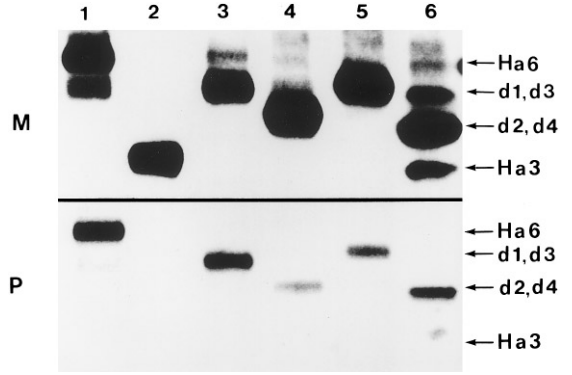

FIG. 3. Cell surface binding requires the presence of T7. Conditioned media (60 μL) (M) and 0.2 M L-proline wash (P) (60 μL) from confluent cultures of CHO-pTEJHa6 (1), CHO-pTEJHa3 (2), CHO-pTEJD1 (3), CHO-pTEJD2 (4), CHO-pTEJD3 (5), and CHO-pTEJD4 (6) were separated on a 3-15% SDS-PAGE and immunoblotted using anti r-apo(a). These cells are secreting Ha6, Ha3, and D1, D2, D3, and D4, respectively. The location of these apo(a) derivative are indicated by arrows. This figure is representative of three experiments.

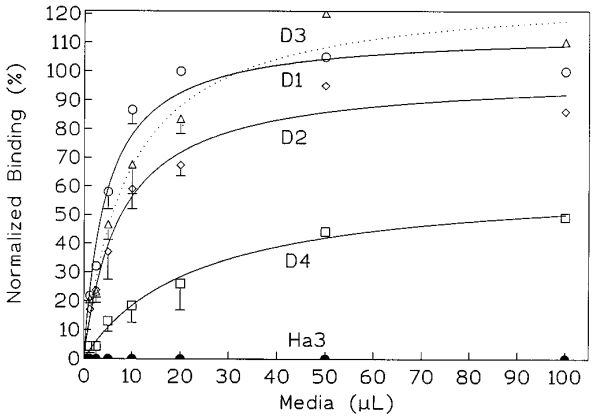

FIG. 4. ECM binding requires T8. Different amounts of conditioned media from CHO-pTEJD1 (circle), CHO-pTEJD2 (diamond), CHO-pTEJD3 (triangle, dotted line), CHO-pTEJD4 (square), and CHO-pTEJHa3 (filled circle) were incubated with the ECM for 2 hours, washed with PBS and developed using anti r-apo(a). The data were normalized against CHO-pTEJD1 which has the same binding as CHO-pTEJHa6. Except for the data points at 50 and 100 μL, these data were compiled from three experiments.

the two-step model for Lp(a) formation proposed earlier (12) and identified T7 as the kringle involved in Step 1. However, as T6 and T7 are 98% homologous, T6 and T7 probably could function interchangeably, consistent with the partial reduction in LDL binding (12) and cell surface binding following inactivation of T6. LDL binding is probably necessary to bring apo(a) and LDL together and cell surface binding is probably necessary to assemble the apo(a):LDL complex on cell surface where protein disulfide isomerase can catalyze the disulfide bond formation. It is interesting to note that D3, a T7 mutant with no cell surface binding activity, was Lp(a) formation competent. This suggests that cell surface binding is not absolutely necessary for Lp(a) formation.

We have also shown that T8 mediates the binding of apo(a) to the ECM and is inhibited by L-proline, and L-lysine and its analogue, 6-aminohexanoic acid. T8 is probably the lysine binding site II (LBS II) identified by Ernst et al. (25). As LBS II on apo(a) is masked in Lp(a) (25), the binding of T8 to the ECM could explain the higher atherogenic potential of apo(a) versus Lp(a). Binding and accumulation of apo(a) in the vascular wall should increase the local concentration of apo(a) above that of plasminogen, creating a condition favorable for inhibition of plasmingen activation (26). L-proline, an inhibitor of ECM binding, cell surface binding, and LDL binding, while having no effect on plasminogen kringle-4, has the potential of becoming a specific inhibitor of Lp(a) atherogenic potential.

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