Mapping of a Minimal Apolipoprotein(a) Interaction Motif Conserved in Fibrin(ogen) β- and γ-Chains*©

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Lipoprotein(a) (Lp(a)) is a major independent risk factor for atherothrombotic disease in humans. The physiological function(s) of Lp(a) as well as the precise mechanism(s) by which high plasma levels of Lp(a) increase risk are unknown. Binding of apolipoprotein(a) (apo(a)) to fibrinogen and other components of the blood clotting cascade has been demonstrated in vitro, but the domains in fibrinogen critical for interaction are undefined. We used apo(a) kringle IV subtypes to screen a human liver cDNA library by the yeast GAL4 two-hybrid interaction trap system. Among positive clones that emerged from the screen, clones were identified as fibrinogen β- and γ-chains. Peptide-based pull-down experiments confirmed that the emerging peptide motif, conserved in the carboxyl-terminal globular domains of the fibrinogen β and γ modules specifically interacts with apo(a)/Lp(a) in human plasma as well as in cell culture supernatants of HepG2 and Chinese hamster ovary cells, ectopically expressing apo(a)/Lp(a). The influence of lysine in the fibrinogen peptides and of lysine binding sites in apo(a) for the interaction was evaluated by binding experiments with apo(a) mutants and a mutated fibrinogen peptide. This confirmed the lysine binding sites in kringle IV type 10 of apo(a) as the major fibrinogen binding site but also demonstrated lysine-independent interactions.

Lipoprotein(a) (Lp(a))1 from human plasma is composed of a low density lipoprotein core and the highly polymorphic apo(a), covalently linked to apo B-100 by a single disulfide bridge (1, 2). apo(a) contains 10 distinct tandem repeats, named kringle IV type 1–10, closely resembling plg kringle IV followed by single plg kringle V-like and protease-like domains (3). The homology at the cDNA level between plg and apo(a) modules is 75–85% for the kringle IV domains and 94% for the protease domain (3). As a result of this similarity in the apo(a) gene, more than 30 different apo(a) isoforms have been found in human plasma, differing in the number of the kringle IV type 2 repeat (4–6).

Several epidemiological studies indicate that elevated Lp(a) levels are an independent risk factor for coronary heart disease (7). Lp(a) accumulates in atherosclerotic lesions of coronary bypass patients and can be cross-linked to the fibrin thrombus (8–10). Binding of Lp(a) to fibrinogen has been hypothesized to underlie a postulated role of Lp(a) in wound healing. Bound Lp(a) may protect the thrombus from premature digestion by plasmin and serve as an important source of phospholipids and cholesterol for membrane biogenesis and cell proliferation at the site of injury (11–13). Whereas the physiological role of Lp(a) remains unknown, several hypotheses have been proposed to account for the pathogenicity of Lp(a) (14). The high degree of homology between apo(a) and plg has been suggested to form the basis for the pathogenicity of Lp(a) as a modulator of fibrinolysis (15–18). Lp(a) effectively competes with plg for binding sites on fibrin and fibrinogen and reduces the generation of active plasmin (18–21). It has been demonstrated that Lp(a) increases smooth muscle cell migration and proliferation by inhibition of transforming growth factor-β activation by plasmin (16, 17, 22). In addition, Lp(a) competes with plg for binding to receptors present on endothelial cells and monocytes (23, 24).

The aim of the study was to identify critical motifs in fibrinogen (interacting with Lp(a)/apo(a) for the understanding of the functions and pathophysiological properties of Lp(a). Here we present fibrinogen(ogen) β- and γ-chain sequences interacting with apo(a) in the yeast two-hybrid system and the identification of a conserved 30-amino acid fibrinogen(ogen) minimal peptide motif that is sufficient for binding to apo(a)/Lp(a) in vitro.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The genotype of the Saccharomyces cerevisiae reporter strain H76c used for the two-hybrid screening, is MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers), CYC1-lacZ. The strain CG1945, used for the β-galactosidase assays, is characterized by an additional cycloheximide resistance. The genotype of SFYS26, used to test interactions between GAL4 DNA binding domain and GAL4 transcription activation domain fusions, is MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can¹, gal4-542, gal80-538, URA3::GAL1-lacZ (CLONTECH Laboratories, Inc, Palo Alto, CA). Strains were grown under standard conditions in rich or synthetic medium with appropriate supplements at 30 °C.

Plasmids—apo(a)KIV-2 and apo(a)KIV-6 were cloned as described in Ref. 25. apo(a)KIV-5, -7, -8, -9, and -10 were cloned likewise using polymerase chain reaction primers (apo(a)KIV-5: 5′-CCAAGGCAATTCCGGTGGCGGTAGTCCGGCTACTGCTAGGAGAAAAACCC-3′ and 5′-GCTTTGCTGACTCATCATCTTCCTACGAGAAACCGCTCTGTGTTTGATT-3′; apo(a)KIV-7: 5′-CTAGAGGCTGCTGGTGGTGACGTAACCAAGCCGAGGACCCGTTGC-3′).
CA-3’ and 5'-GGTTGTTGTCATCCATTCTAGCGAAAGGC-3’; apo(a)KIV-8: 5'-CCAAAGGAATTCGGTGGCGG-3’; apo(a)KIV-9: 5'-GGTCGACTGACCACTGACAGAAAGCGCTTGTGCTG-3’ and 5’-GGTTGTTGTCATCCATTCTAGCGAAAGGC-3’; apo(a)KIV-type-10: 5’-CCAAAGGAATTCGGTGGCGG-3’; apo(a)KIV-10: 5’-GGTCGACTGACCACTGACAGAAAGCGCTTGTGCTG-3’ and 5’-ATTTCTGGCAGTCT-ATATGTGGCAAGGGGCTTATAG-3’.

Plasmids pCMV-A18 (A18 wt), pCMV-A18VP (A18V-P), pCMV-A18/474Arg (A18-Arg), and pCMV-A18L32–35 (A18 5’–6’) are described in Ref. 26. Plasmid pCMV-A18KIV-9 was described in Ref. 30.

Two-hybrid Screening and cDNA Isolation—For the yeast two-hybrid screening, apo(a)KIV-6 was cotransformed with the human liver cDNA Matchmaker library in the pGAD10 vector (CLONTECH Laboratories, Inc., Palo Alto, CA) into the HF7c yeast strain, as described by the manufacturer, and the transformants were plated to selective dropout medium lacking leucine, histidine, and tryptophan but containing 5 μg 3-amino-1,2,4-triazole. The plates were incubated at 30 °C for up to 7 days.

β-Galactosidase Reporter Activity—His+ colonies were assayed for β-galactosidase activity by transferring individual colonies on filters placed on selection medium. The plates were incubated for 2 days at 30 °C, and the filters lifted and immersed in liquid nitrogen for 10 s. After thawing out, at room temperature, the filters were washed three times with 0.2 ml 10 mM 3-β-galactopyranoside in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 30 mM β-mercaptoethanol) in a Petri dish (permeabilized cells up and incubated overnight as indicated. To verify the significance of the interaction a liquid culture assay using o-nitrophenyl β-D-galactopyranoside as substrate was performed as described by the manufacturer (CLONTECH Laboratories, Inc., Palo Alto, CA). At least six individual cotransformants were assayed in the background of two different yeast strains. Only interactions of cotransformants in both yeast strains were assumed to be significant. The results are presented as the means ± S.D.

Peptide Synthesis and Characterization—Biotinylated peptides FbG200–225 and FbK212–235, were purchased from NeoSystem (Strasbourg, France). Biotinylated peptides FbG200–225 and FbK212–235 were prepared on an automated synthesizer ABI 421A (Applied Biosystems Inc.) using standard Boc/Bzl strategy. Biotin was coupled to the peptide-resin via N-hydroxy-succinimidyl-6-(biotinamido)-hexanoate in dimethyl formamide for 16 h at room temperature (28). The biotinylated peptide-resin was then washed twice with ter-amylalcohol, acetic acid, and diethylether. The peptides were cleaved from the vacuum-dried resins and simultaneously deprotected according to published procedures (29) to high hydrogen fluoride procedure (29). The peptides were purified and characterized by reversed phase high pressure liquid chromatography, capillary electrophoresis, and amino acid analysis. The biotinylated control peptide derived from protein kinase Cα (RFARKGSLRQKVNY) was from Genosys (Cambridge, UK).

Immuno blotting of Gel Binding Domain Fusion Bait in Yeast Extracts—A total of 5 μl of transformed yeast cells grown overnight in selective medium lacking tryptophan were used to inoculate 15 ml of yeast extract peptone dextrose medium. At an A600 of 0.5, the cells were pelleted, washed, resuspended at 5 × 106 cells/ml in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF) and frozen at −20 °C. Samples were analyzed by SDS-PAGE (10%) and transferred to polyvinylidene fluoride membrane (Millipore, Vienna, Austria), and fusion proteins were detected using a GAL4 DNA binding domain specific antibody (Santa Cruz Inc, Santa Cruz, CA), followed by a rabbit antinme IgG-peroxidase conjugate and a chemiluminescence detection kit (ECL reagent; Amersham Pharmacia Biotech).

Tissue Culture and Transient Transfection—The human hepatocarcinoma cell line HepG2 (30) and CHO cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended by the American Type Culture Collection. Transient transfection of cells was achieved by liposome-mediated gene transfer with the LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. After overnight incubation the transfection medium was replaced by 2 ml of serum-free medium of 48 h, cell culture supernatants were harvested, treated with proteinase inhibitors (1 mM PMSE, 5 μg/ml of each aprotinin and leupeptin), and centrifuged for 10 min each at 3000 and 4000 × g to remove cells and cell debris, respectively. The Lp(a) content was assayed as described elsewhere (31, 32).

Human Plasma Samples and Preparation of Lp(a)—Venous blood samples from healthy donors were collected in EDTA tubes, treated with proteinease inhibitors (1 mM PMSE, 5 μg/ml of each aprotinin and leupeptin), and centrifuged for 10 min at 300 × g to remove cells. Following enzyme-linked immunosorbent assay determination of Lp(a) plasma levels and prior to diluting for the pull-down experiments, the plasma apo(a) was analyzed by SDS-PAGE and immunoblotting. The Lp(a) isoforms used consisted of 18 apo(a) kringle units for plasma pull-down experiments and 21 apo(a) kringle units for pull-downs of purified Lp(a). Preparation of Lp(a) was performed by density centrifugation as described in Ref. 6.

Peptide Pull-down—Diluted Lp(a) preparations, diluted plasma samples, and cell culture supernatants were used for the library screening because of its low self-activation rate. To determine whether activation of the GAL4-dependent His+ phenotype was occurring, the peptide pull-down was performed using apo(a)-specific monoclonal antibody 1A2 (33) as described (1).
yeast strain in the absence or presence of the GAL4 kringle IV type 6 bait expression plasmid. Additionally, we transformed each of the putative ligand expression plasmids with an expression plasmid with the GAL4 DNA binding domain fused to a nonspecific protein (p53), which is not expected to interact with proteins that bind to apo(a). Four of the 25 clones showed specific interaction, activating β-galactosidase expression exclusively in the presence of the GAL4 kringle IV type 6 bait. Sequence analysis of these positive clones using primers in the pGAD10-flanking sequence revealed two cDNA sequences of so far unknown identity and two cDNA sequences showing a 100% match to fragments of the human fibrinogen β- and γ-chain, respectively. The fibrinogen β-chain clone (K6/β) extended from residues β1–310, and the fibrinogen γ-chain clone (K6/γ) extended from residues γ189–295 (Fig. 1).

Further Characterization of the Binding Site—The matched sequences of both clones are located within the carboxy-terminal globular domain of fibrinogen. These carboxy-terminal sections of the fibrinogen β- and γ-chains are characterized by high sequence and structural homology (35). A carboxy-terminal fragment of K6/β containing the sequence overlapping with K6/γ was cloned in fusion with the GAL4 activation domain, and the resulting plasmid K6/β1 (Fig. 1) was assayed in yeast cells for interaction with the GAL4 DNA binding domain-apo(a) kringle IV type 6 fusion protein to localize the region of the fibrinogen β-chain clone that mediated binding to apo(a) kringle IV type 6. The positive result in the two-hybrid assay showed that the 64 carboxy-terminal residues of fibrinogen β-chain that correspond by sequence alignment to residues 189–246 of fibrinogen γ-chain are sufficient for binding to apo(a) kringle IV type 6.

Moreover, we performed a two-hybrid assay with the fibrinogen clones K6/β1 and K6/γ using the apo(a) kringle IV types 2, 5, 6, 7, 8, 9, and 10 as baits. Kringle subtypes 5, 7, and 10 showed specific interaction with the fibrinogen β- and γ-chain in both strains, despite some quantitative differences observed. However, kringle IV types 2, 6, and 9 did not interact (Fig. 2). The expression of the bait and prey proteins has been tested by analyzing the extracted yeast proteins after SDS-PAGE and Western blot by GAL4 fusion domain-specific antibody. Expressed proteins of the expected molecular weights have been detected in the corresponding yeast protein extracts (not shown).

![Fig. 1. Interaction of fibrinogen clones with apo(a) kringle IV type 6 bait in the two-hybrid screening.](image1.png)
containing distinct apo(a)/Lp(a) derivatives were obtained after transient transfection of HepG2 cells with apo(a) expression vector constructs A18 wt, DKV-P, and DkIV 8-P representing wt apo(a) with 18 kringle units and two 39 deletions of different length (as outlined in Fig. 5). Equal amounts of apo(a)/Lp(a) (as measured by enzyme-linked immunosorbent assay) were used for pull-down experiments with FibG207–235 and immunoprecipitation. As a result, A18 wt Lp(a) as well as DKV-P Lp(a) showed a strong interaction with FibG207–235. In contrast, only marginal binding of DkIV 8-P apo(a) to FibG 207–235 was observed (Fig. 6, A and B). The three distinct forms of apo(a)/Lp(a) were expressed equally as verified by immunoprecipitation (Fig. 6C).

Influence of Lysines or LBS on the Interaction of Fibrinogen Peptides with apo(a)/Lp(a) from Supernatants of Transfected HepG2 Cells—HepG2 cells were transfected with the apo(a) expression vector constructs A18 wt and the mutant A18-Arg (Fig. 5). The latter is A18 apo(a) with a Trp-4174 to Arg substitution in the LBS of kringle IV type 10, which renders A18-Arg Lp(a) unable to bind to lysine-Sepharose (26). Equal amounts of apo(a)/Lp(a) (as measured by enzyme-linked immunosorbent assay) from the resulting supernatants were used for pull-down experiments with FibG207–235 and the mutant peptide FibKA207–235. In contrast, only marginal binding of DkIV 8-P apo(a) to FibG 207–235 was observed (Fig. 6, A and B). The three distinct forms of apo(a)/Lp(a) were expressed equally as verified by immunoprecipitation (Fig. 6C).

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FIG. 3. Pull-down of Lp(a) with fibrinogen peptides from human plasma. A, evaluation of a representative experiment was determined by densitometric quantification. The maximal binding was set at 100% for calculation purposes. Statistical evaluation of five pull-down experiments expressed as the means ± S.E. is presented below. Control, 15.4% ± 1.7%; FibG 190–235, 80.7% ± 21.2%; FibKA 190–235, 97.7% ± 31.9%; FibG207–235, 100% ± 33.0%; FibKA207–235, 32.0% ± 4.2%. B, interacting complexes were formed in plasma by using four different fibrinogen peptides and a control peptide as indicated. Immunodetection for the presence of apo(a) was performed using anti-apo(a) mAb IA2. A representative experiment is shown.

FIG. 4. Pull-down of Lp(a) purified from human plasma with fibrinogen peptides and control. A, statistical evaluation of the experiment was determined by densitometric quantification. The maximal binding was set at 100% for calculation purposes, and data are expressed as the means ± S.E. (n = 5). B, interacting complexes were formed in a solution of purified human Lp(a) by using four different fibrinogen peptides and a control peptide as indicated. The double band represents a PAGE artifact. Immunodetection for the presence of apo(a) was performed using anti-apo(a) mAb IA2.

FIG. 5. Schematic representation of wt and mutant apo(a) encoded by cDNA expression plasmids. Kringle IV types are numbered from 1 to 10. The W4174R mutation in pCMV A18-Arg and the premature stop codon in pCMV ΔKIV 5–8 are indicated. SP, signal peptide; V, kringle V; PD, protease domain. The cDNA is flanked at the 5’ end by the cytomegalovirus enhancer/promotor and a heterologous intron and at the 3’ end by a SV40 polyadenylation site (1, 26).

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binding efficiency compared with the wt FibG207–235 peptide. Binding of FibG207–235 to A18 wt Lp(a) was significantly reduced after preventing lysine-dependent interactions by addition of EACA. Binding of both fibrinogen peptides to the A18-Arg mutant Lp(a) was greatly reduced when compared with the binding to A18 wt Lp(a). Again further reduction in binding of both peptides was observed in the presence of EACA (Fig. 7).

Interaction of FibG207–235 with apo(a) from Supernatants of Transfected CHO Cells—To address the question whether the low to absent binding of FibG207–235 to AKIV 8-P apo(a) is due to the presence of this mutant form as free apo(a) exclusively,
we transfected apoB100 negative CHO cells with the apo(a) expression vector constructs and performed pull-down experiments with equal amounts of apo(a) from the cell supernatants. We observed strong binding of A18 wt apo(a) and reduced binding of ΔKV-P apo(a) to FibG_{207-235} (Fig. 8, A and B). Binding to ΔKIV 8-P was below the value obtained by the control peptide. Equal expression of the three apo(a) forms was controlled by immunoprecipitation (Fig. 8C).

Moreover, we tested binding of the mutant apo(a) ΔKIV 5–8 to FibG_{207-235}. This mutant which lacks the kringle IV types 5–8 and does not form Lp(a) particles (26) also showed interaction with the fibrinogen peptide FibG_{207-235} at low concentrations (not shown).

**DISCUSSION**

Fibrinolysis is a surface-controlled process leading to the plasmin-catalyzed proteolysis of fibrin. The adsorption of plg to fibrin and the surface dynamics of fibrin and plg transformation during this process have been well characterized (37). The plg paralogue apo(a) also binds to fibrinogen. However so far there is little information about the apo(a) fibrinogen interaction at the molecular level. We have confirmed direct physical interaction between apo(a) and fibrinogen by the yeast two-hybrid system. With the apo(a) unique kringle IV type 6 as bait, we have isolated two of the three fibrinogen subunits (fibrinogen β- and γ-chain) from a human liver cDNA library, and we were able to localize the apo(a) binding sites within these two individual fibrinogen subunits. The strongest similarities between the fibrinogen β- and γ-chain subunits are located in their carboxyl termini with stretches of about 250 amino acids sharing marked homology. The two fibrinogen sequences isolated from the positive two-hybrid clones revealed 64 overlapping amino acids that have been shown to be sufficient for interaction with kringle IV type 6 by the two-hybrid β-galactosidase filter assay. In cross-linked fibrin this interacting amino acid sequence is a constituent of fragment D. It is noteworthy that also the plg binding site of fibrin has been localized to the carboxyl-terminal fragment D (38). In the intact fibrin polymer the two proposed interacting carboxyl-terminal domains are located in close proximity within the two distal globular domains of the (αβ)-fibrinogen dimer (36). These globular domains at the surface of fibrin seem to be easily accessible for large molecules as Lp(a), and it has indeed been demonstrated that Lp(a) blocks specifically carboxyl-terminal lysine residues on the surface of fibrin (19). Moreover, binding leads to a large conformational change that may prevent other molecules from interacting with fibrinogen (39).

Because of the high sequence and structural homology in the binding region of the β- and γ-chain of fibrinogen defined by
two-hybrid interaction with apo(a) kringle IV type 6, we restricted the further investigation of the binding site in a more physiological environment to the γ-chain that does not contain the insertion and extra disulfide bridge found in the corresponding β-chain B1-loop region (35). The shorter peptide FibG207–235 contains the two helices flanking the loop, whereas FibG190–235 contains an additional third helix preceding the loop. We reproducibly demonstrated interaction of wt fibrinogen peptides (FibG) and corresponding mutated peptides (FibKA) with Lp(a) from different sources indicating a specific interaction. The minor quantitative differences observed between pull-downs (as in Figs. 3 and 4) are most likely due to different experimental setups e.g. buffer composition or state of native versus purified Lp(a)/apo(a). Taken together the four fibrinogen peptides significantly interacted with Lp(a)/apo(a).

However, our study does not exclude additional binding sites for apo(a)/Lp(a) in the globular domain of fibrin(ogen). The occurrence of more than one binding site has been demonstrated for other fibrin ligands. FibG190–235 overlaps with part of the MAC-1 binding site characterized by Tang et al. (40). A subsequent report described a second binding motif for MAC-1 (Fig. 6). The binding may also be context-dependent and influenced by the overall conformation of apo(a) in the particle. This might be a reason for the marginal binding of the splice site mutant KIV 8-P apo(a) to FibG207–235. This mutant apo(a) contains kringle types 1–7 and only 34 amino acids of kringle IV type 8 that are likely to be misfolded, probably influencing the conformation of the mutant molecule.

Importantly, however, we observed only minimal residual binding of FibG207–235 to the mutant A18-Arg Lp(a), suggesting a major role of the LBS in apo(a) kringle IV type 10 for the FibG207–235 and apo(a)/Lp(a) interaction (Fig. 7). Mutations of lysine residues in the fibrinogen peptide FibG207–235 the W4174R mutation in the apo(a) KIV-10 LBS and the presence of EACA all resulted in a reduced interaction of the fibrinogen peptide with apo(a)/Lp(a). This clearly demonstrates the LBSs and particularly the LBS in apo(a) kringle IV type 10 is involved in the interaction. However, EACA alone did not totally block the interaction of wt FibG207–235 with wt apo(a)/Lp(a). On the other hand EACA reduced binding even when both the LBS in apo(a) kringle IV type 10 and the lysine residues in the fibrinogen peptide were mutated. This suggests a more complex interaction, which in addition to the LBS in kringle IV type 10 involves other sites. There is one publication reporting a lysine-insensitive component of the interaction of isolated apo(a) kringle IV type 10 and plasmin modified fibrinogen (51). It further suggests an effect of EACA on apo(a)/Lp(a) beyond the inhibition of lysine binding. This might be due to changes in Lp(a) conformation promoted by EACA as demonstrated by Fless et al. (52). Taken together, our data for the first time clearly identify an apo(a)/Lp(a) binding site in fibrin(ogen).
They also confirm the LBS in apo(a) kringle IV type 10 as one fibrinogen binding site in apo(a)/Lp(a).

The interaction of Lp(a) and fibrinogen is well established, but so far no binding site in fibrinogen had been identified. Here we present the FibG207–235 sequence as a novel and sufficient binding site for Lp(a)/apo(a) in vitro. Our finding may have practical consequences because the FibG207–235 sequence may represent a pharmacological target site to interfere with the pathological interaction of Lp(a)/apo(a) and fibrinogen. However, more work has to be done to elucidate the full functional relevance of this interaction in vivo.

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