Mol Genet Genomics (2002) 267: 440–446
DOI 10.1007/s00438-002-0673-6

ORIGINAL PAPER

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Direct interaction of the extracellular matrix protein DANCE with apolipoprotein(a) mediated by the kringle IV-type 2 domain

Received: 21 January 2002 / Accepted: 21 March 2002 / Published online: 25 May 2002

Abstract Lipoprotein(a) [Lp(a)] consists of LDL and apolipoprotein(a), and has been shown to be a major, independent, risk factor for arteriosclerosis and thrombosis in humans. To further elucidate the (patho)physiological function(s) of Lp(a)/apo(a), we searched for new protein ligands, using the yeast two-hybrid system and employing the highly repetitive kringle IV type 2 (KIV-2) sequence from apo(a) as bait. The extracellular matrix protein DANCE [developmental arteries and neural crest epidermal growth factor (EGF)-like] recently implicated in atherogenesis was identified as an interactor. A direct physical interaction between DANCE and apo(a) was confirmed by co-purification of both recombinant proteins derived from culture supernatants of transiently transfected COS-1 cells. Furthermore, binding of human plasma-derived Lp(a) to recombinant DANCE was also observed. Finally, when monoclonal anti-apo(a) and polyclonal anti-DANCE antibodies were applied to tissue slices of atherosclerotic carotid artery, the two proteins were found to be co-localized in endothelial and smooth muscle cells, suggesting that they occur together in the arterial wall. However, as yet, the in vivo relevance and the possible functional role of this newly identified DANCE:Lp(a)/apo(a) interaction remains speculative.

Communicated by E. K. F. Bautz

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Keywords Lipoprotein(a) · Arteriosclerosis · Yeast-two hybrid system · Copurification · Histochemistry

Introduction

Lipoprotein(a) [Lp(a)] is a lipoprotein particle which contains LDL (low-density lipoproteins) as a major component. Lp(a) differs from LDL by the presence of a covalently linked, highly glycosylated protein of variable mass, termed apo(a). Variation in the number of the plasminogen-related kringle IV motifs in apo(a) explains the genetically determined size polymorphism of apo(a) (McLean et al. 1987; Utermann 1989). Plasma concentrations of Lp(a) vary markedly among individuals, from undetectable to 200 mg/dl, and are correlated with the number of kringle IV repeats in apo(a) (Lackner et al. 1991; Kraft et al. 1992). High Lp(a) has been recognized as an atherothrombogenic factor in several studies, thus linking the number of kringle IV repeats to atherosclerotic risk (Kraft et al. 1996). The pathogenetic mechanisms underlying this condition, and the physiological role of Lp(a), are, however, still unclear. Similarly, the reason for the increased Lp(a) plasma levels observed in patients with renal disease (Sato et al. 1993; Wanner et al. 1993; Mooser et al. 1996) remains unknown. Understanding the pathophysiology of Lp(a) could be crucial for the development of new strategies to counteract high plasma levels of Lp(a) and therefore reduce atherosclerotic risk in individuals with excessive Lp(a). Based on the homology of apo(a) to plasminogen, a variety of mechanisms have been proposed to explain how Lp(a) generates a prothrombotic state. These include binding to fibrinogen, competitive inhibition of fibrin-dependent activation of plasminogen to plasmin (De Rijke et al. 1992; Miles et al. 1995), plasmin-mediated activation of the cytokine TGF-β (transforming growth factor-β) (Grainger et al. 1994; Palabrica et al. 1995) and competition with plasminogen for uptake by receptors on endothelial cells and monocytes (Miles et al. 1989; Kojima et al. 1991). Increased susceptibility to

oxidative damage and uptake by the scavenger receptor (Hajjar et al. 1989), resulting in foam cell formation, has also been proposed.

In addition to the known ligands for apo(a), e.g. fibrinogen (Harpel et al. 1989; Rouy et al. 1991), plasminogen receptors (Gonzales-Gronow et al. 1989; Hajjar et al. 1989; Miles et al. 1995), the extracellular matrix protein fibronectin (Salonen et al. 1989) and β2-glycoprotein I (Köchl et al. 1997), there may exist as yet unknown interaction partners for apo(a), which could be crucial for the understanding of the (patho)physiological role of Lp(a). Considering the highly repetitive nature of the kringle IV type 2 (KIV-2) domain in apo(a) and its possible position on the outer surface of the lipoprotein, KIV-2 may act as an anchor to attach Lp(a) to critical protein ligands. Therefore, in our attempt to identify novel apo(a) ligands, we used a single apo(a) KIV-2 motif as bait to screen a human kidney cDNA library in the yeast two-hybrid system. A kidney library was chosen because several lines of evidence have implicated the kidney as an organ involved in the removal of Lp(a) from plasma.

Among the candidate apo(a) binding partners identified by this technique was the recently described extracellular matrix and atherogenesis-related protein DANCE (Nakamura et al. 1999). Human DANCE [developmental arteries and neural crest epidermal growth factor (EGF)-like] was purified by affinity chromatography from human urine, and subsequently cloned at the Weizmann Institute in 1998 (Y. Shaul et al., unpublished results). Northern analysis by Nakamura et al. (1999) revealed that DANCE mRNA is expressed mainly in heart, ovary and colon, but also in kidney and pancreas. In addition, DANCE is expressed in developing arteries, neural crest cell derivatives and endocardial cushion tissue, as well as in balloon-injured vessels and atherosclerotic lesions, notably in intimal vascular smooth muscle cells and endothelial cells. Furthermore, DANCE is thought to promote adhesion of endothelial cells through interaction with integrins via its RGD motif (Nakamura et al. 1999). Therefore, DANCE appears to be an interesting apo(a)/Lp(a) ligand.

Materials and methods

Yeast strains and media

The genotype of the *Saccharomyces cerevisiae* reporter strain CG-1945 used for the two-hybrid screen and for β-galactosidase assays, is *MAT*α, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3,112*, *gal4-542*, *gal80-538*, *cyh*^{r}*2* *LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3*, *URA3::GAL4*$_{17mers\ (\times 3)}$*-CYC1*$_{TATA}$*-lacZ* (Clontech Laboratories). Strains were grown under standard conditions in rich or synthetic medium with appropriate supplements at 30°C.

Plasmids

The GAL4-BD plasmid pGBT9/apo(a)KIV-2 was cloned as described previously (Köchl et al. 1997). The human kidney cDNA library (cloned in the GAL4-AD vector pACT2) was obtained from Clontech. The full-length DANCE cDNA was tagged at the 3′ end with RGS(H)$_6$, and cloned into the *Not*I site of the mammalian expression vector pEFneo by PCR using the primers 5′-GCGCGCGCGGCCGCTCATTAATGGTGATGATGGTGATGGGATCCCCGGTCGACGAATGGGTACTGCGACACATATATCC-3′ and 5′-GCGCGCGCGGCCGCCATGCCAGGAATAAAAAGGATACTCACT-3′. Plasmids pCMV-A18 [18× kringle apo(a)], encoding eight copies of the KIV-2 motif, and pCMV-A11 [11× kringle apo(a)], including one copy of KIV-2, were described previously (Utermann et al. 1987).

Two-hybrid screening and cDNA isolation

For the yeast two-hybrid screen, apo(a)KIV-2 in the vector pGBT9 was cotransformed with the human kidney cDNA Matchmaker library constructed in pACT2 (Clontech) into the yeast strain CG-1945, as described by the manufacturer, and the transformants were plated on synthetic dropout medium lacking leucine, tryptophan and histidine but containing 5 mM 3-amino-1,2,4-triazole. The plates were incubated at 30°C for up to 10 days.

β-Galactosidase reporter activity

His$^+$ colonies were assayed for β-galactosidase activity by transferring individual colonies onto filters placed on selection medium. The plates were incubated for 2 days at 30°C, then the filters were removed and immersed in liquid nitrogen for 10 s to permeabilize the cells. After thawing at room temperature, the filters were placed (colony side up) on filter paper saturated with 0.2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 30 mM β-mercaptoethanol) in a petri dish, 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 recommended by the manufacturer (Clontech). At least 12 individual cotransformants were assayed in the background of two different yeast strains. Only interactions that were observed in both yeast strains were assumed to be significant. The enzyme activities are presented as means ± standard deviation.

Transient transfection of COS-1 cells

The cell line COS-1, which is derived from African green monkey kidney cells transformed with SV40, was obtained from the American Type Culture Collection (ATCC, Rockville, Md.) and cultured as recommended by the supplier. For electroporation, cells were washed twice with OPTIMEM (Gibco). Aliquots (400 µl) of cells in OPTIMEM were mixed with 20-µg samples of plasmid DNA in electroporation tubes (Equibo). Electroporation was performed at 500 V, 1500 F, and 48 Ω, using 13-ms pulses, in an electro cell manipulator. Finally cells were seeded at a density of 2×10^6 cells/well in 10-cm petri dishes in DMEM supplemented with 10% fetal calf serum at 37°C. Cell culture supernatants were harvested 48 hours after transfection, and protease inhibitors were added (1 mM PMSF, and aprotinin and leupeptin each at 50 µg/ml).

Human plasma samples and preparation of Lp(a)

Venous blood samples from healthy donors were collected in EDTA tubes, treated with proteinase inhibitors (1 mM PMSF, aprotinin and leupeptin each at 5 µg/ml) and centrifuged for 10 min at 300×*g* to remove cells. Following determination of Lp(a) plasma levels by ELISA and prior to diluting for the binding assays, the integrity of plasma apo(a) was analyzed by SDS-PAGE and immunoblotting. Preparation of Lp(a) was performed by density centrifugation as described previously (Brunner et al. 1993).

Immunoprecipitation and immunoblotting

An affinity-purified polyclonal anti-DANCE antibody (anti-D3′) directed against the C-terminal 15 amino acids of the DANCE protein was obtained from Davids Biotechnology. Ni-NTA magnetic beads for the affinity purification of His-tagged proteins (based on the high affinity of the chelated Ni^{++} for histidine residues) and anti-His antibody for detection of these proteins were obtained from Qiagen. Purification of His-tagged proteins via affinity chromatography on Ni-NTA agarose was performed as recommended by the manufacturer. The anti-D3′ antibody was bound to IgG attached to magnetic beads (Pierce) and pulldown assays were performed as recommended. Polyclonal anti-DANCE was raised against the full-length DANCE protein (Y. Shaul, unpublished results). The apo(a)-specific monoclonal antibody 1A2 (Brunner et al. 1993) and the procedures used for immunoblot analysis were described previously (Dieplinger et al. 1995).

Immunohistochemical staining

The monoclonal anti-apo(a) antibodies 1A2 and 5A5 were described previously; anti-DANCE antiserum and the corresponding pre-immune serum were obtained as described above. Slides bearing frozen tissue sections (4 μm thick) were air-dried for 30–60 min at room temperature. Endogenous peroxidase activity was quenched by incubating the slides for 30 min in TRIS-buffered saline (TBS, pH 7.4)-0.1 M NaN_3, containing 50 μl of H_2O_2 per 40 ml of solution. Non-specific antibody binding was reduced by incubation with 10% normal human serum (heat inactivated at 56°C for 30 min) in TBS for 15 min. Excess serum was blotted off and the primary antibodies diluted in TBS (rabbit-anti-DANCE 1:10, mouse-anti-apo(a) 1A2: 1:20 and mouse-anti-apo(a) 5A5 9:10) were applied directly without any further washing, and the sections were incubated for 60 min. Optimal dilutions for all antibodies were determined in pilot studies. Incubations were carried out in a humidified chamber at room temperature. The sections were then rinsed three times in TBS, the secondary antibodies [horseradish peroxidase-conjugated goat-anti-mouse immunoglobulin and biotin-conjugated swine-anti-rabbit immunoglobulin, both at a dilution of 1:100 (DAKO)] were applied and the sections were incubated for another 30 min. The slides were rinsed as described above, and incubation with streptavidin-coupled alkaline phosphatase followed, at room temperature for 20 min. After three further changes of TBS, visualization with Fast Red Naphthol and DAB (both from Sigma) was performed. Slides were counterstained with hematoxylin and finally mounted in Kayser's glycerol-gelatin (both from Merck).

Results and discussion

Identification of DANCE as an apo(a) KIV-2 binding protein by two-hybrid screening of a human kidney cDNA library

By screening a human kidney cDNA library using the KIV-2 motif of apo(a) as a bait, several candidate ligands for apo(a) were identified (data not shown). One particularly interesting clone obtained by this interaction trap approach, encoded the extracellular matrix protein DANCE. Human DANCE is a secreted protein comprising six EGF-like domains following the signal peptide, the first of which contains an RGD integrin binding motif. DNA sequence analysis demonstrated that the selected DANCE prey plasmid 371 contained only a partial sequence, spanning amino acids 350–448 of the protein and thus representing the complete C-terminal segment plus a very small part of the last EGF-like motif. The C-terminal segment shows no homology to proteins in the databases (Fig. 1). Nevertheless, this region of DANCE was able reproducibly to interact with the KIV-2 motif of apo(a), inducing the *lacZ* reporter gene in the yeast two-hybrid system to levels well above those seen with prey and well bait controls (Fig. 2).

Expression and detection of recombinant DANCE protein

Next, the DANCE ORF, including a sequence encoding a C-terminal $RGS(H)_6$ tag, was cloned and used for transient transfection of COS-1 cells. Recombinant DANCE could be readily detected by the anti-His tag

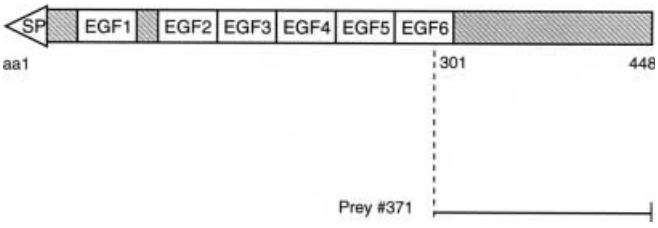


Fig. 1 Schematic representation of the DANCE protein. The C-terminal region of DANCE (prey #371) that interacts with the KIV-2 motif in the yeast two-hybrid system is indicated. SP, signal peptide; EGF, calcium-binding Epidermal Growth Factor-like domain

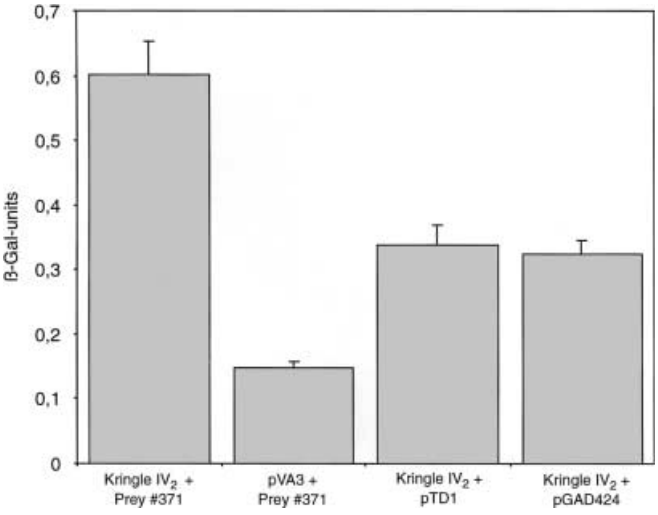


Fig. 2 Direct physical interaction of C-terminal DANCE (prey #371) with the KIV-2 motif of apo(a) in the yeast two-hybrid system, measured by a β-galactosidase reporter assay. Reporter gene activity (expressed in β-galactosidase units) serves as a measure of the interaction between the bait and prey proteins. The inert protein controls, pVA3 (p53) and pTD1 (SV40 large T antigen), show background interaction with KIV-2 and with DANCE #371. The empty prey vector control (pGAD424) indicates the level of self-activation of the KIV-2 bait in this assay. The results shown were obtained using different preparations of expression plasmids and represent the means ($\pm$S.E.) of 12 experiments, each done in triplicate

antibody in the transfected, but not control, COS-1 cell culture supernatants (Fig. 3B).

Biochemical confirmation of the DANCE-apo(a) interaction by copurification of the two proteins

For copurification studies, COS-1 cells were cotransfected with the tagged DANCE gene and the coding sequence for either an apo(a) 18 isoform (containing eight copies of KIV-2) or an apo(a) 11 isoform (containing only one KIV-2 domain), respectively (Fig. 3). In agreement with the physical interaction detected in the yeast two-hybrid screen, both apo(a) isoforms could interact with DANCE, bound via its His tag to Ni-NTA agarose beads (Fig. 3A). Importantly, however, no apo(a) interacted with the Ni-NTA agarose beads in the absence of ectopic DANCE expression (Fig. 3A and B, lanes 1 and 3), indicating that the interaction between these two proteins was specific. A truncated DANCE mutant lacking the N-terminal end and all EGF-like domains also interacted with recombinant apo(a) from COS-1 cell supernatants (data not shown), suggesting that the EGF domains of DANCE do not play any role in this protein-protein interaction. This is consistent with the presence of only an incomplete EGF sequence in the DANCE prey clone 371 originally identified in the two-hybrid screen.

COS-1 cell-derived recombinant DANCE, bound to Ni-NTA agarose beads [via its $RGS(H)_6$ tag] was also shown to interact with native Lp(a) from human plasma (Fig. 4). This also implies that KIV-2 domains are exposed at the outer rim of the Lp(a) particle, thus allowing free access for the potential binding partner DANCE. Nonspecific binding of Lp(a) to the matrix can be excluded based on the results with the Ni-NTA agarose beads alone, emphasizing the potential physiological relevance of this interaction. Furthermore, native DANCE could be readily immunodetected in human plasma using a polyclonal anti-DANCE antibody, and COS-1 derived recombinant DANCE as an antigen control (Fig. 5). Finally, a newly generated anti-DANCE peptide antibody (directed against the C-terminal 15 amino acids of DANCE) was able to coimmunoprecipitate recombinant apo(a) with DANCE from COS-1 cell culture supernatants (Fig. 6). Taken together, these biochemical experiments confirm the interaction between DANCE and apo(a) initially suggested by the results of the yeast two-hybrid screen, and demonstrate that DANCE also interacts with the Lp(a) particle.

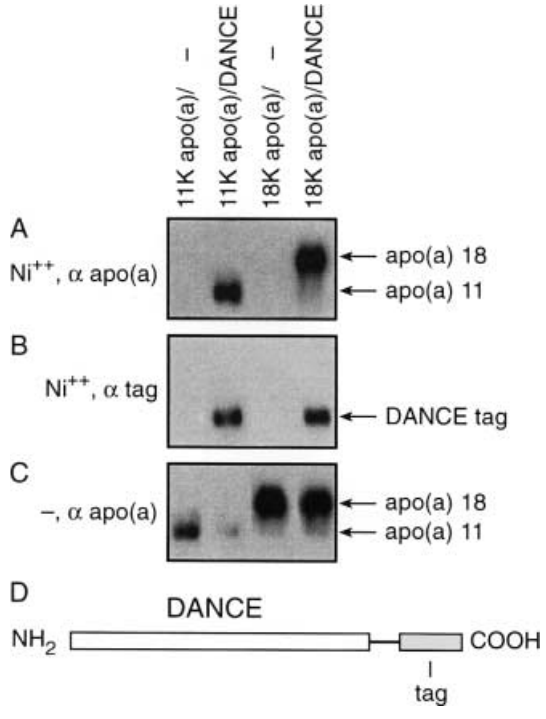


Fig. 3A–D Copurification of recombinant apo(a) from COS-1 cell supernatants with 3′ $RGS(H)_6$ tagged DANCE. COS-1 cells were cotransfected with 11× kringle apo(a) (containing just one KIV-2 motif) and pEFneo (lane 1), 11× kringle apo(a) and tagged DANCE (lane 2), 18× kringle apo(a) (containing eight copies of KIV-2) and pEFneo (lane 3), 18× kringle apo(a) and tagged DANCE (lane 4). Recombinant DANCE was isolated from cell culture supernatants by binding to Ni^{++}-NTA agarose via the His tag. After electrophoresis of the bound protein fraction on a reducing SDS-PA gel, recombinant apo(a) and the tagged DANCE were detected by Western analysis with (**A**) α-apo(a) (1A2) and (**B**) α-His tag (αRGSHis) antibodies, respectively. **C** Concentrated aliquots of COS-1 cell culture supernatants (that had not been incubated with Ni-NTA agarose) probed with α-apo(a) to determine the relative abundance of recombinant apo(a). **D** Schematic diagram of the tagged DANCE, used for pull down assays

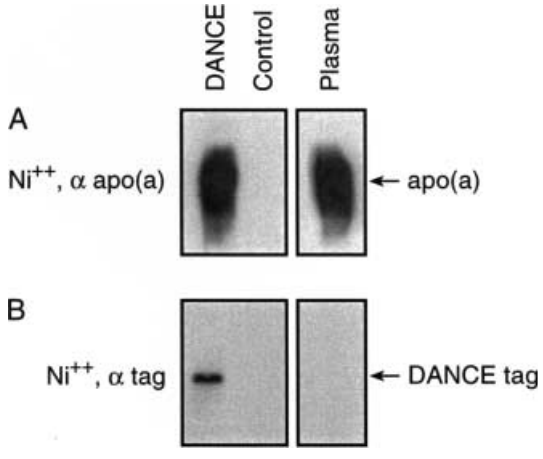


Fig. 4A, B Pulldown of Lp(a) from human plasma using tagged DANCE bound to Ni-NTA agarose. COS-1 cells were transiently transfected with an expression vector encoding tagged DANCE or an empty vector control. Recombinant DANCE secreted into the supernatant was then captured on Ni-NTA agarose beads, and the beads were then incubated with human plasma. After gel electrophoresis of the bound protein fraction (together with an aliquot of plasma), immunoblot analysis was performed by successive application of α-apo(a) (**A**) and α-DANCE tag (**B**). Levels of native apo(a) in plasma are indicated in the lane on the *right* (Plasma)

Immunohistochemical staining of DANCE and Lp(a)/apo(a) in tissue slices of arterial wall

Since both apo(a)/Lp(a) and DANCE have been shown to be present in atherosclerotic plaques, we went on to ask whether the two proteins colocalize in vessel walls. Tissue slices of highly atherosclerotic carotid artery were stained with anti-apo(a) (mAb 1A2, IgG subclass 1), and the polyclonal antibody raised against the whole DANCE protein. The resulting images (Fig. 7A) show that Lp(a)/apo(a) is present in the stratum of smooth muscle cells, while DANCE is found in the endothelial cells layer at the luminal surface of the vessel wall. Interestingly, however, no significant signals were detected in a healthy vessel control (Fig. 7C). IgG1 antibodies and pre-immune serum were used as controls (Fig. 7B, D). At higher resolution it became apparent that both proteins are also present within the shift of luminal endothelial cells as shown in Fig. 7E. Using another anti-apo(a) antibody (mAb 5A5), raised against a different epitope of apo(a), identical results were obtained (not shown). Thus this spatial proximity of DANCE and apo(a) in the atherosclerotic vessel wall, including the regions of proposed Lp(a)/apo(a) localization within the inner layer of endothelial cells and also within the tunica media (Lawn et al. 1992; Sangrar et al. 1997), provides further evidence for a physiological interaction of these two proteins. As a working hypothesis, the Lp(a)/apo(a)-DANCE interaction, once upregulated in situations of hemodynamic stress or in endothelial cells overlying atherosclerotic plaques in vessel walls (Nakamura et al. 1999), might act as a trigger for atherosclerotic plaque formation: DANCE could eventually contribute to the deposition

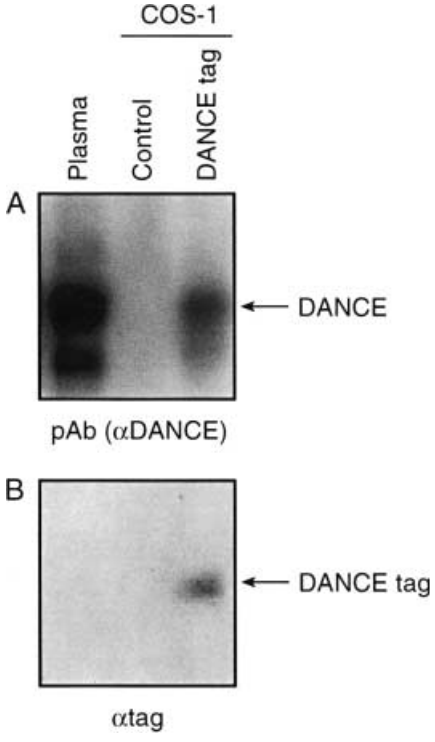
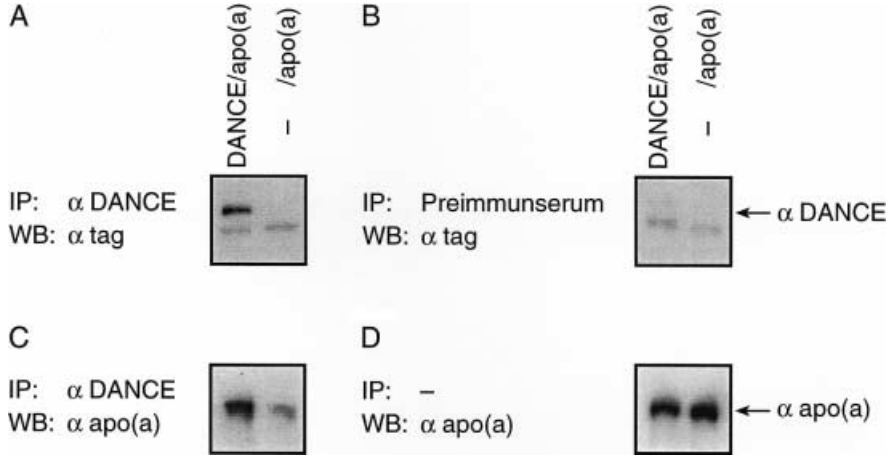


Fig. 5A, B Immunoblot analysis of recombinant and native DANCE. COS-1 cells were transiently transfected with an expression vector encoding tagged DANCE or with an empty vector control. Serum-free supernatants from COS-1 cell cultures transfected with the DANCE or the control vector were incubated with Ni-NTA agarose beads, and the bound protein fraction was electrophoresed on a reducing SDS-PA gel, together with an aliquot of human plasma. Immunoblot analysis was done with polyclonal α-DANCE (**A**) and α-His tag (**B**) antibodies

Fig. 6A–D Coimmunoprecipitation of apo(a)/Lp(a) with recombinant DANCE from COS-1 cell supernatant using an anti-DANCE peptide antibody. COS-1 cells were transiently cotransfected with expression vectors encoding tagged DANCE, apo(a) or empty vector control, respectively. Proteins were immunoprecipitated (IP) from COS-1 cell culture supernatants, using an α-DANCE peptide antibody directed against the last 15 C-terminal amino acids sequence of DANCE (**A**). Preimmune serum was used for immunoprecipitation of recombinant DANCE as a negative control (**B**). The precipitates were then fractionated by electrophoresis, and subjected to Western analysis (WB) using α-His tag (**A, B**) or α-apo(a) (**C**) antibodies. A nonspecific band of lower molecular weight was also detectable when this polyclonal anti-DANCE antibody was used for Western analysis (**A, B**). To determine the abundance of recombinant apo(a) in the transfected COS-1 cell culture supernatants, concentrated aliquots were probed with α-apo(a) antibody (**D**). The weak apo(a) band seen in lane 2 in panel **C** [-/apo(a)] is due to minor nonspecific binding of recombinant apo(a) to the anti-DANCE antibody



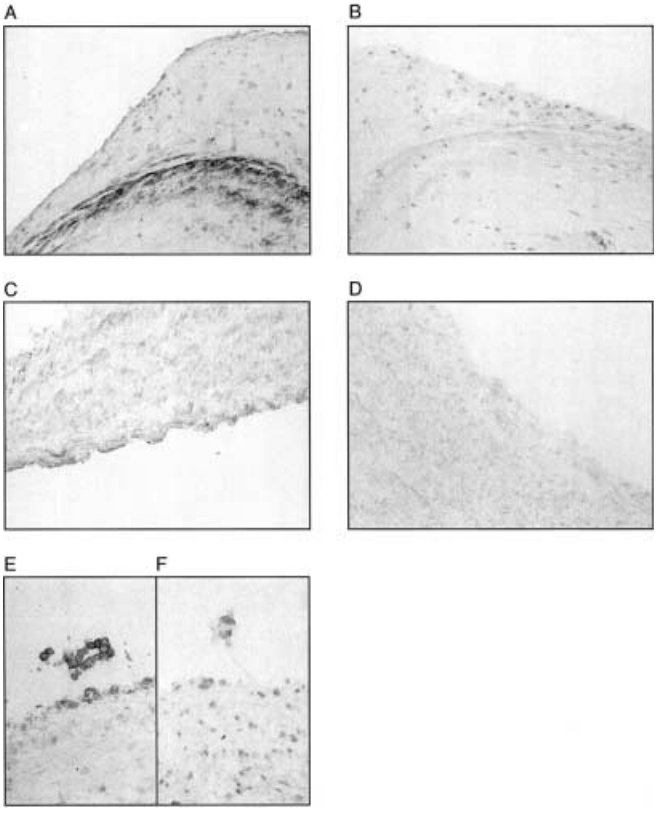


Fig. 7A–F Immunohistochemical staining of apo(a) and DANCE in human atherosclerotic arterial wall. Tissue slices of highly atherosclerotic carotid artery were stained with polyclonal α-DANCE (directed against the whole protein) and monoclonal α-apo(a) 1A2 antibodies (**A**), or IgG1 and preimmune serum (**B**) (magnification 100×). Interestingly, tissue slices obtained from a healthy 3-month-old nursling demonstrated no significant immunoreactivity with αapo(a) and αDANCE (**C**) or with IgG1 and preimmune serum (**D**). Panels **E** and **F**, respectively, show the luminal endothelial cell layer stained with α-apo(a) and α-DANCE, and with IgG1 and preimmune serum, at higher magnification (200×)

of cholesterol-loaded Lp(a) particles into newly forming atherosclerotic plaques, e.g. by capturing Lp(a) from the bloodstream.

Conclusion

In summary, the present studies identify the extracellular matrix protein DANCE (Nakamura et al. 1999) as a novel Lp(a)/apo(a) binding partner. However, the significance of this interaction remains speculative. As long as the physiological role of DANCE is undefined, the biochemical function of this newly identified DANCE/apo(a) interaction will remain the subject of further detailed investigations.

Acknowledgements We thank Dr. Walter Parson (Institute for Forensic Medicine, University of Innsbruck, Austria) for sequencing services, and Nina Krumboeck for excellent technical assistance.

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Note added in proof. By mouse gentic evidence, DANCE (also now known as fibulin-5), is an essential determinant of elastic fibre organization in the skin, lung and vasculature (Nakamura et al. 2002). Fibulin-5 thereby acts as a scaffold protein (by the RGD motif in fibulin-5, which binds to cell surface integrins, and the Ca^{++}-binding EGF repeats, which bind elastin) that organizes and links elastic fibres to cells (Yanagisawa et al. 2002).

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