

Evidence for a Central Apolipoprotein A-I Domain Loosely Bound to Lipids in Discoidal Lipoproteins That Is Capable of Penetrating the Bilayer of Phospholipid Vesicles*

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Previous evidence indicated that discoidal reconstituted high density lipoproteins (rHDL) of apolipoprotein A-I (apoA-I) can interact with lipid membranes (Tricerri, M. A., Córscico, B., Toledo, J. D., Garda, H. A., and Brenner, R. R. (1998) *Biochim. Biophys. Acta* 1391, 67–78). With the aim of studying this interaction, photoactivable reagents and protein cleavage with CNBr and hydroxylamine were used. The generic hydrophobic reagent 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine gave information on the apoA-I regions in contact with the lipid phase in the rHDL discs. Two protein regions loosely bound to lipids were detected: a C-terminal domain and a central one located between residues 87 and 112. They consist of class Y amphipathic α -helices that have a different distribution of the charged residues in their polar faces by comparison with class A helices, which predominate in the rest of the apoA-I molecule. The phospholipid analog 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-[¹²⁵I]iodo-4-(trifluoro-methyl-3-H-diazirin-3-yl)benzyl]oxy]carbonyl]nonanoyl]-*sn*-glycero-3-phosphocholine, which does not undergo significant exchange between membranes and lipoproteins, was used to identify the apoA-I domain directly involved in the interaction of rHDL discs with membranes. By incubating either rHDL or lipid-free apoA-I with lipid vesicles containing ¹²⁵I-TID-PC, only the 87–112 apoA-I segment becomes labeled after photoactivation. These results indicate that the central domain formed by two type Y helices swings away from lipid contact in the discoidal lipoproteins and is able to insert into membrane bilayers, a process that may be of great importance for the mechanism of cholesterol exchange between high density lipoproteins and cell membranes.

Apolipoprotein A-I (apoA-I)¹ is the major protein component

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¹ The abbreviations used are: apoA-I, apolipoprotein A-I; HDL, high density lipoproteins; FC, free cholesterol; PL, phospholipids; LCAT, lecithin-cholesterol acyl transferase; ¹²⁵I-TID, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine; ¹²⁵I-TID-PC, 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-[¹²⁵I]iodo-4-(trifluoromethyl-3-H-diazirin-3-yl)benzyl]oxy] carbonyl]nonanoyl]-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PC, phosphatidylcholine; LUV, large unilamellar

of the high density lipoproteins (HDL), which play a central role in "reverse cholesterol transport" (1, 2). The interaction of lipid-free apoA-I with cell membrane binding sites triggers free cholesterol (FC) mobilization from intracellular membranes and from cholesteryl ester stores toward the cell membrane as a result of signals mediated by protein kinase C (reviewed in Ref. 3). The sequential removal of phospholipids (PL) and FC (4–7) yields lipid-poor and discoidal HDLs of pre- β electrophoretic migration. Discoidal pre- β HDLs, which also are secreted by liver, are then transformed into spherical α -migrating HDL by lecithin-cholesterol acyl transferase (LCAT), which esterifies FC, forming a hydrophobic core of cholesteryl ester (1, 2, 6). Circulating α -HDL are modified by accepting other apoproteins, such as apolipoprotein A-II, and by lipid exchange with other serum lipoproteins (8). This lipid and apoprotein exchange can produce lipid-free or lipid-poor apoA-I, which can begin again the cycle of lipid removal from cells in the interstitial space of peripheral tissues (6, 9). Whether the interaction of apoA-I with cell membrane is mediated by a specific receptor and the mechanism of its loading with PL and FC is still a matter of debate (7). The recent discovery that a defective ATP-binding cassette transporter (ABCA1) leads to Tangier disease (10, 11) has prompted the study of the role of this protein as a candidate apoA-I receptor (12–17). A direct molecular interaction between ABCA1 and apoA-I is indicated by some of these studies (12, 13). However, other studies (15, 16) do not support this interaction and suggest a role for ABCA1 in modifying the lipid distribution in the membrane and generating the biophysical microenvironment required for the docking of apoA-I to the cell surface.

A high conformational flexibility in apoA-I is needed for its existence in different states: lipid-free, lipid-poor, and discoidal or spherical lipoproteins of different size. Mature human apoA-I contains 243 amino acid residues (18) with 11- and 22-mer homologous repeats (19) that are predicted to form amphipathic α -helices (20, 21) that interact with lipids through their hydrophobic face. The helices are linked by short and flexible β -turns usually containing a proline residue. Lipid-free apoA-I is thought to be a bundle of helices (22) in a molten globular-like state (23), and sedimentation velocity experiments indicate significant conformational heterogeneity (24), supporting a flexible structure. In the spherical α -HDL, it has been proposed (25) that the amphipathic helices are oriented parallel to the surface of the phospholipid monolayer with the hydrophobic faces embedded into the hydrocarbon region and with the hydrophilic faces interacting with the phospholipid

vesicles; rHDL, reconstituted high density lipoproteins; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.

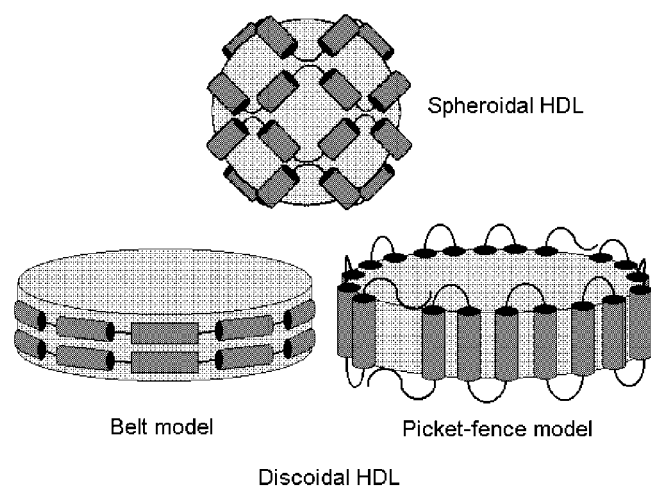


FIG. 1. Proposed models for apoA-I structure in spherical and discoidal HDL complexes.

polar groups and the aqueous phase. It is generally accepted that discoidal lipoproteins are composed of a lipid bilayer disc with the apoA-I at their edge, but the orientation of the helices with respect to the acyl chains is controversial (see Ref. 26 for a review; Fig. 1) The “picket fence” model (27–32) proposed eight antiparallel amphipathic α -helices connected by β -turns oriented parallel to the phospholipid acyl chains. However, recent experimental evidence supports the “belt” model (33–37), which proposes that apoA-I wraps around the edge of the disc with the axis of its α -helices oriented perpendicular to the acyl chains. The size of discoidal complexes is primarily determined by the number of apoA-I molecules per particle (38), but discs with the same number of apoA-I molecules and different amounts of lipids do also exist (39–41). A hinge domain, which may be excluded from the interaction with lipids in small discoidal complexes (39, 40, 42), has been proposed to be present in the central region of apoA-I (28, 43–45) to explain the ability of apoA-I to form different sized discoidal complexes.

The conformational flexibility of apoA-I can also play a role in the interaction and lipid exchange of HDL with membranes. It was hypothesized (46, 47) that the interaction of HDL discs with membranes may involve helices of apoA-I that can swing away from lipid contact on the disc to insert into the phospholipid bilayer, thus facilitating cholesterol exchange. In spherical HDL, this apoA-I domain would be interacting with the particle lipids and would not be able to interact with membranes (47). According to this hypothesis the morphologic change from disc to sphere or the enrichment in cholesteryl ester would reduce the affinity of HDL particles for membranes, allowing the liberation of spherical α -HDLs to the circulation. The major problem of this hypothesis is that the morphologic change from disc to sphere requires LCAT whose activity is very low in lymph and interstitial fluid (48) but high only in plasma (6). In previous reports (49, 50) we suggested, as a possible alternative to this mechanism, that the increase in size or in the cholesterol content in the discs could cause, independently of LCAT activity, a conformational change in apoA-I that decreases the affinity for membranes. Then large cholesterol-rich discs would be liberated into circulation and transformed into α -HDL by LCAT in plasma. This alternative hypothesis is supported by the fact that the increase of size and cholesterol content in the discs modifies apoA-I conformation (45, 49) and decreases the interaction (49) and cholesterol exchange rate (50) with lipid vesicles. The interaction of discoidal complexes of apoA-I with lipid vesicles produces a transient leakage of the internal aqueous contents, which is faster compared with the leakage produced by lipid-free apoA-I (49),

and suggests the penetration of an apoA-I domain into the bilayer of the lipid vesicles. It was also shown that apoA-I discs are able to interact with lipid monolayers by increasing their lateral pressure (70). To gain further insight into the mechanism of this interaction, we have used two photoactivable reagents to obtain information on the lipid-bound regions of apoA-I in discoidal complexes and to identify the apoA-I region involved in the interaction of discoidal complexes with membranes.

EXPERIMENTAL PROCEDURES

Photoactivable Reagents—The reagents used were: 3-(trifluoromethyl)-3-(*m*- 125 I)iodophenyl)diazirine (125 I-TID) and 1-*O*-hexadecanoyl-2-*O*-(9-[[[2- 125 I]iodo-4-(trifluoromethyl)-3-*H*-diazirine-3-yl]benzyl]oxy]carbonyl]nonanoyl)-*sn*-glycero-3-phosphocholine (125 I-TID-PC) (Fig. 2). Upon photolysis, the trifluoromethyl diazirine group of these reagents is rapidly converted to carbene, which is capable of reacting with the full range of functional groups occurring in biomolecules, including paraffinic carbon-hydrogen bonds (51). In membranes or lipoproteins, the environment of carbene consists largely of acyl chains and protein segments inserted into or in contact with the hydrophobic region of the phospholipid. Membrane inserted domains of several proteins (52–56) were identified by using these reagents followed by chemical or enzymatic cleavage. These photoreagents exhibit very different monomer solubility in water, with one of the factors determining intermembrane exchange/transfer rates and the spontaneous incorporation in preformed membranes or lipoprotein structures (51, 52). 125 I-TID is easily incorporated into the lipid phase of preformed structures such as membranes or lipoproteins with a partition coefficient highly favorable to the lipid phase (39,000 bound ligands \times μ l external solution/free ligand \times mg of lipids) (57). It is supposed to be uniformly distributed across the hydrophobic region of the phospholipid bilayer, being a good candidate for the analysis of the apoA-I regions directly in contact with the acyl chains in discoidal complexes. On the other hand, phospholipid vesicles containing the phospholipid analog 125 I-TID-PC, which does not undergo significant intermembrane transfer (51) and should locate the active diazirine group very deep into the lipid bilayer, were used to identify the apoA-I region able to penetrate into the vesicle bilayer.

125 I-TID (10 μ Ci/nmol) was purchased from Amersham Pharmacia Biotech. 125 I-TID-PC was prepared by radioiodination of its nonradioactive tin-containing precursor 1-*O*-hexadecanoyl-2-*O*-(9-[[[2-(tributylstannyl)-4-(trifluoromethyl)-3-*H*-diazirine-3-yl]benzyl]oxy]carbonyl]nonanoyl)-*sn*-glycero-3-phosphocholine according to Weber and Brunner (51). The tin precursor was generously donated by Prof. J. Brunner from the Swiss Federal Institute of Technology (Zurich, Switzerland). The dried tin-containing precursor (\sim 20 nmol) was dissolved in 10 μ l of acetic acid in a 1-ml Reacti-Vial (Pierce). [125 I]NaI (1 mCi) was added, and the iodination was started by the addition of peracetic acid (2 μ l of a 32% solution in acetic acid). After 2 min at room temperature, the reaction was quenched with 50 μ l of 10% $\text{Na}_2\text{S}_2\text{O}_5$. Then 40 μ l of CHCl_3 /methanol (2:1) was added and vortexed. The organic phase was collected and concentrated using a charcoal filter to adsorb volatile radioactivity. The residue was dissolved in 20 μ l of methanol/ CHCl_3 /H₂O (9:1:1) and subjected to reverse-phase high pressure liquid chromatography using the same solvent and a 208HS54 C8 column (Vydac) in a Merck-Hitachi instrument with UV detection at 254 nm. The flow rate was 1 ml/min, and fractions of 0.5 ml were collected. 125 I-TID-PC eluted at \sim 20 min, whereas the excess of tin-containing precursor eluted at \sim 40 min. An aliquot (5 μ l) of each fraction in the elution region of 125 I-TID-PC was analyzed by TLC on silica gel plates (LK6D, 60 Å; Whatman, Clifton, NJ) and subjected to autoradiography. Fractions containing radioactivity were pooled and concentrated by co-evaporation with toluene/ethanol (1:1). 125 I-TID-PC was stored at -20°C and dissolved in ethanol/toluene (1:1) at \sim 1 mCi/ml.

Other Materials—1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol and sodium cholate were from Sigma. *L*- α -Dipalmitoyl-[2-palmitoyl-9,10- ^3H (N)]-phosphatidylcholine (^3H]PC), [^{14}C]cholesterol (^{14}C]FC), and [125 I]NaI were from PerkinElmer Life Sciences.

ApoA-I Purification and Preparation of Discoidal Reconstituted High Density Lipoproteins (rHDL)—ApoA-I was purified from the HDL fraction of human serum (kindly donated by the Banco de Sangre del Instituto de Hemoterapia de la Provincia de Buenos Aires, La Plata, Argentina), according to the procedure previously described (58) with some modifications (49, 50). The cholate dialysis method of Jonas *et al.* (39) was used to prepare the apoA-I rHDL complexes using initial

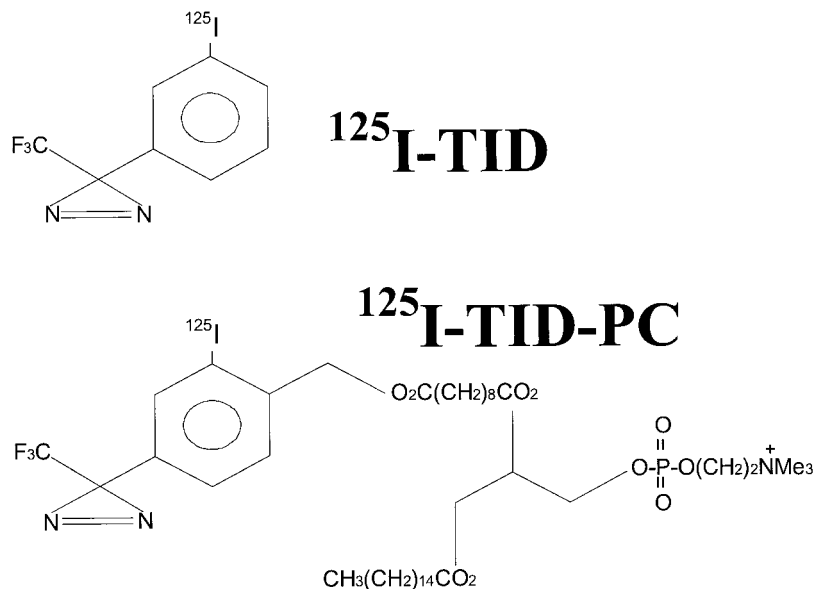


FIG. 1. Chemical structure of $^{125}\text{I-TID}$ and $^{125}\text{I-TID-PC}$.

mixtures of cholate/POPC/apoA-I in the molar ratio 250/100/1 or cholate/POPC/FC/apoA-I in the molar ratio 250/100/25/1. [^3H]PC and [^{14}C]FC were included to allow PL and FC quantification. Differently sized rHDL were separated by gel filtration fast protein liquid chromatography (49, 50) using two columns in tandem (Superose 6 and Superose 12 HR 10/30 from Amersham Pharmacia Biotech). The purity and size of the isolated rHDL were determined by nondenaturing gradient gel electrophoresis (41), and their composition was obtained by measuring the protein content by Lowry's method (59). PL and FC contents were measured by scintillation counting.

Preparation of Lipid Vesicles Containing $^{125}\text{I-TID}$ or $^{125}\text{I-TID-PC}$ —Large unilamellar vesicles (LUV) of POPC or POPC/FC (4/1 mol/mol) were prepared (0.5 mM in POPC) by extrusion through polycarbonate membranes with pore diameters of 100 nm (Avestin Inc., Ottawa, Canada). Lipids in CHCl_3 were mixed, dried under a stream of N_2 , and resuspended in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM N_3Na (buffer A) by vortexing. Then lipid suspensions were passed 11 times through the polycarbonate filters by using a Liposofast-extruder system (Avestin). To incorporate $^{125}\text{I-TID}$ into the LUV, 10 μCi of the photoactivable reagent in ethanol were added to 0.2 ml of the LUV suspension and incubated with stirring for at least 2 h at room temperature until use. On the other hand, to prepare the LUV containing $^{125}\text{I-TID-PC}$ (200 $\mu\text{Ci}/\mu\text{mol}$ of POPC), the photoreagent was mixed with the lipids in CHCl_3 previous to the LUV preparation.

Photolabeling and Delipidation of ApoA-I—The photoreagent-containing LUV (25 nmol of POPC) were incubated with the rHDL complexes or with lipid-free apoA-I (1 nmol of apoA-I) in buffer A containing 50 mM glutathione at room temperature. After the indicated times, mixtures (0.3 ml) in glass cuvettes were irradiated for 30 s with a Xenon lamp (450 Watts) at a distance of 25 cm. As a control, the photoreagent-containing LUV were also irradiated before mixing with apoA-I. $^{125}\text{I-TID}$ was directly incorporated into the rHDL complexes by adding the reagent (1.5 μCi) in ethanolic solution to the rHDL suspension (1 nmol of apoA-I) in buffer A with 50 mM glutathione and incubating for at least 4 h with stirring before the irradiation. After irradiation, 3 volumes of CHCl_3 /methanol (2:1) were added and vortexed, and the organic phase was discarded. ApoA-I was precipitated with 10% trichloroacetic acid and redissolved as indicated below for chemical cleavage or in 25 μl of sample buffer for direct analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

Chemical Cleavage of ApoA-I with CNBr and with Hydroxylamine—For cleavage with NH_2OH , samples were dissolved and incubated at 45 $^\circ\text{C}$ for 4 h in 50 μl of 2 M NH_2OH , 2 M urea, 0.2 M K_2CO_3 , pH 9.0. For cleavage with CNBr, samples were dissolved in 50 μl of 70% formic acid, and CNBr was added in a 30:1 ratio (CNBr: protein w/w) and incubated at 25 $^\circ\text{C}$ under a N_2 atmosphere for 24 h. Then the samples were dried in a Speed Vac system and redissolved in 20 μl of sample buffer for Tricine SDS-PAGE analysis.

Analysis by SDS-PAGE and Autoradiography—SDS-PAGE was carried out according to Schägger and von Jagow (60) in 16.5% acrylamide Tris-Tricine. After Coomassie Blue staining, gels were dried and exposed to X-Omat film (Kodak) at -80 $^\circ\text{C}$ for different times depending

on the amount of radioactivity.

N-terminal Sequencing of ApoA-I Fragments—Peptide fragments were transferred from SDS-polyacrylamide gels to polyvinylidene difluoride membranes using a Trans-Blot semi-dry transfer cell (Bio-Rad). N-terminal sequencing was performed on the bands in polyvinylidene difluoride membranes by the Laboratorio Nacional de Investigación y Servicio en Péptidos y Proteínas (Universidad de Buenos Aires-Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina) and by the Protein Structure Core Facility, University of Nebraska Medical Center (Omaha, NE).

RESULTS

Characterization of ApoA-I Discoidal rHDL Particles—Four kinds of discoidal lipoprotein complexes of apoA-I were used in this study. Two cholesterol-free (Lp2 and Lp4) and two cholesterol-containing (Lp2C and Lp4C) rHDL complexes were purified by gel filtration fast protein liquid chromatography after dialysis of the cholate-containing mixtures. They were pure as judged by nondenaturing gradient gel electrophoresis. Their size corresponded to that previously determined (49) for rHDL containing two (85 Å Stokes diameter for Lp2 and Lp2C) or four (150 Å for Lp4 and Lp4C) apoA-I molecules/rHDL particle. The determined molar compositions were as follows: 57/1 and 108/1 (POPC/apoA-I) for Lp2 and Lp4 and 59/4/1 and 101/18/1 (POPC/FC/apoA-I) for Lp2C and Lp4C, respectively.

Photolabeling of ApoA-I in the Lipid-free State or as Discoidal Complexes by Incubation with Lipid Vesicles Containing $^{125}\text{I-TID-PC}$ —The incubation of POPC LUV containing $^{125}\text{I-TID-PC}$ with apoA-I discoidal complexes or lipid-free apoA-I resulted in the labeling of the protein after photoactivation. The protein cleavage with CNBr and analysis by SDS-PAGE and autoradiography yielded the results shown in Fig. 3. ApoA-I contains three methionine residues at which cleavage with CNBr occurs: residues Met⁸⁶, Met¹¹², and Met¹⁴⁸ (see Fig. 7), and only four polypeptides should be obtained by complete cleavage. However, because of incomplete cleavage, a more complex pattern, which can be observed in Fig. 3A, is obtained. All of the peptide bands can be assigned as indicated in Fig. 3A by taking into account their molecular masses and the indicated N-terminal sequence. It must be noted that because of the lower Coomassie staining sensitivity to smaller peptides, the real cleavage extension is larger than the apparent one. Autoradiography analysis (Fig. 3B) shows that the radioactivity is located almost exclusively (more than 90%) in the smallest peptide band of a molecular mass similar to glucagon or the insulin β -chain (about 3 kDa). The N-terminal sequence (Ser-

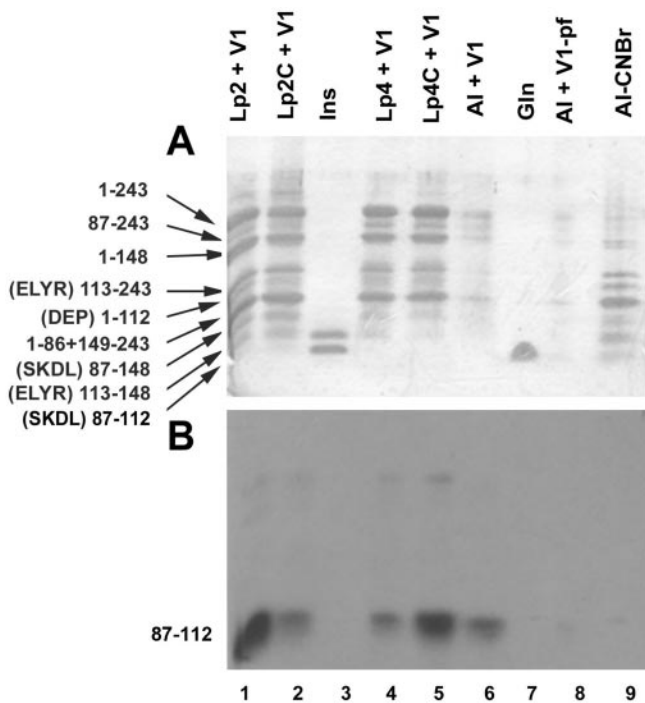


FIG. 3. Photolabeling of apoA-I in the lipid-free state or as discoidal complexes by incubation with lipid vesicles containing ^{125}I -TID-PC. POPC vesicles (25 nmol of PL) containing 5 μCi of ^{125}I -TID-PC (V1) were incubated with 1 nmol of apoA-I as Lp2 (lane 1), Lp2C (lane 2), Lp4 (lane 4), or Lp4C (lane 5) or in the lipid-free state (AI, lane 6) at room temperature for 1 min and photoactivated for 30 s. As control, 1 nmol of lipid-free apoA-I was mixed with previously photoactivated ^{125}I -TID-PC-containing vesicles (V1-pf, lane 8). After delipidation, samples were cleaved with CNBr and analyzed by SDS-PAGE with Coomassie blue staining (A) and autoradiography (B). Insulin (Ins, lane 3), glucagon (Gln, lane 7), and nonradiolabeled apoA-I cleaved with CNBr (AI-CNBr, lane 9) were used as standards. The assignment of the peptide fragments produced by apoA-I cleavage by CNBr (indicated at the left side) was made by taking into account the molecular mass and the determined N-terminal sequence.

Lys-Asp-Leu) indicates that this band corresponds to fragment 87–112. Fig. 3B (lane 8) shows that no labeling was found when the ^{125}I -TID-PC-containing POPC LUV were photoactivated previously to the incubation with lipid-free apoA-I. The same apoA-I fragment becomes almost exclusively labeled by the incubation of these ^{125}I -TID-PC-containing vesicles with the different discoidal complexes (Lp2, Lp2C, Lp4, or Lp4C) or even with lipid-free apoA-I, although a different level of labeling can be observed, which is possibly due to differences in affinity or the ability to interact as previously reported (49). The same apoA-I fragment is also labeled by photoactivation of mixtures of apoA-I discoidal complexes with POPC/FC (4/1 mol/mol) vesicles containing the phospholipid reagent ^{125}I -TID-PC (data not shown). By increasing the time of gel exposure to the autoradiographic films (not shown), it was possible to observe that the larger fragments that contain the 87–112 region (amino acids 1–243, 1–148, 1–112, and 87–148) become also labeled, although some unspecific label (less than 5%) is also observed in the band corresponding to fragments 1–87 and 149–243. These results strongly indicate that an apoA-I domain located between residues 87 and 112 is able to penetrate the bilayer of POPC or POPC/FC vesicles when either lipid-free apoA-I or discoidal complexes are used.

Photolabeling of ApoA-I in Discoidal Complexes by Direct Incubation with ^{125}I -TID or by Incubation with Lipid Vesicles Containing ^{125}I -TID—Fig. 4 (lanes 1 and 2) shows that the photoactivation of mixtures of Lp2 complexes with ^{125}I -TID-containing POPC/FC (4/1 mol/mol) vesicles also results in the

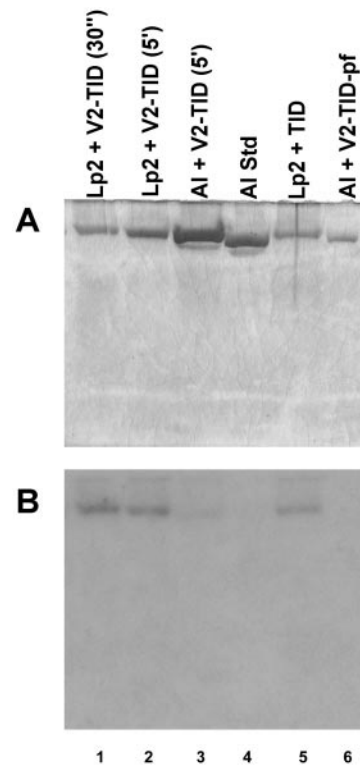
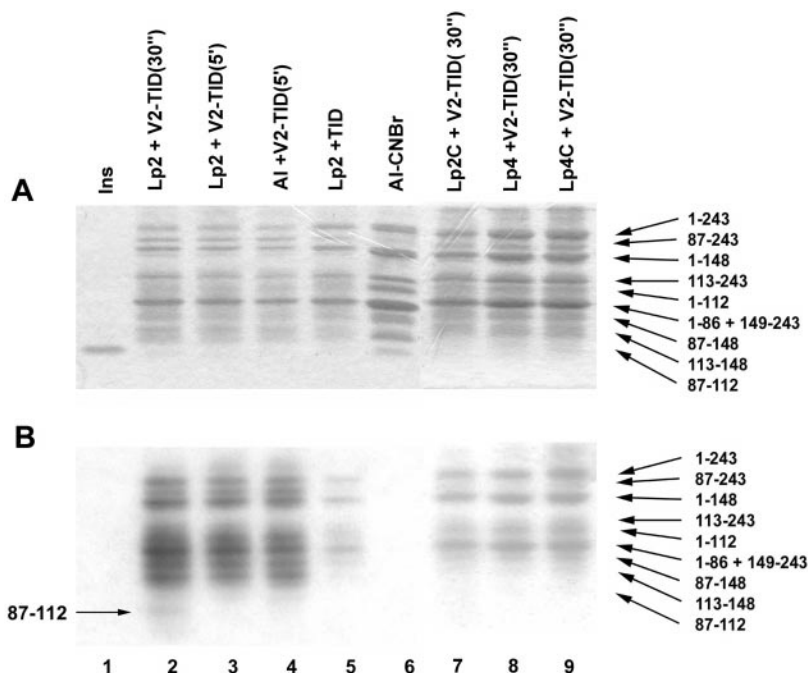


FIG. 4. Photolabeling of apoA-I in its lipid-free state or in discoidal complexes Lp2 by incubation with POPC/FC (4/1) vesicles containing ^{125}I -TID or by direct incubation with this photoagent. POPC/FC (4/1 mol/mol) vesicles (25 nmol of PL) containing 2.5 μCi of ^{125}I -TID (V2-TID), were incubated with 1 nmol of apoA-I as Lp2 for 0.5 (lane 1) and 5 (lane 2) min or with 1 nmol of lipid-free apoA-I for 5 min (lane 3) at room temperature and photoactivated for 30 s. 1 nmol of apoA-I as Lp2 was also incubated directly with 0.5 μCi of ^{125}I -TID for 8 h at room temperature and photoactivated for 30 s (Lp2 + TID, lane 5). As a control, 1 nmol of lipid-free apoA-I was also mixed with previously photoactivated ^{125}I -TID-containing vesicles (AI + V1-TID-pf, lane 6). After delipidation, the samples were analyzed by SDS-PAGE with Coomassie Blue staining (A) and autoradiography (B). Nonradiolabeled apoA-I (AI Std, lane 4) was used as standard.

labeling of apoA-I. No appreciable difference in the level of labeling is found when Lp2 complexes are preincubated for 0.5 or 5 min with the vesicles before the photoactivation. If lipid-free apoA-I is incubated with these vesicles, the protein becomes also labeled but at a level that is significantly lower than that found for the Lp2 complexes (lane 3). ApoA-I is also labeled if ^{125}I -TID is directly added to the Lp2 complexes (lane 5); in this case we added only 0.5 μCi of ^{125}I -TID to the rHDL sample to keep the same ^{125}I -TID/POPC ratio as in the incubation mixtures containing Lp2 and vesicles. On the other hand, no labeling of apoA-I is found when the ^{125}I -TID-containing LUV are photoactivated before their incubation with lipid-free apoA-I (lane 6).

Samples photoactivated as in Fig. 4 were cleaved with CNBr, and the corresponding SDS-PAGE and autoradiography analyses are shown in Fig. 5. Lane 5 shows the labeling pattern obtained by photoactivation after the direct addition of ^{125}I -TID to Lp2 discs. With this experimental approach, the apoA-I regions that are in contact with the lipid bilayer of the discs should be labeled, although it cannot be totally discarded that some hydrophobic domains not in contact with the bilayer could also be labeled. It is remarkable that the 87–112 fragment (which was almost exclusively labeled by ^{125}I -TID-PC-containing vesicles) does not become labeled by the direct addition of ^{125}I -TID to Lp2 discs. This indicates that the 87–112 region, although is able to penetrate into the lipid bilayer of vesicles, is loosely bound to the disc lipid bilayer.

FIG. 5. Chemical cleavage with CNBr of apoA-I photolabeled with ^{125}I -TID. Samples treated as in Fig. 4 were delipidated, digested with CNBr, and analyzed by SDS-PAGE with Coomassie Blue staining (A) and autoradiography (B). Lane 1, insulin standard (*Ins*). Lanes 2 and 3, Lp2 incubated for 0.5 and 5 min respectively with ^{125}I -TID-containing POPC/FC vesicles (V2). Lane 4, lipid-free apoA-I incubated for 5 min with the same vesicles. Lane 5, Lp2 incubated directly with ^{125}I -TID. Lane 6, nonradiolabeled apoA-I digested with CNBr. Lanes 7, 8, and 9, Lp2C, Lp4, and Lp4C respectively, incubated for 0.5 min with V2 vesicles.



The incubation of Lp2 discs with ^{125}I -TID-containing vesicles during a short period of time (30 s) before the photoactivation, results in a detectable labeling of the 87–112 fragment (lane 2). However, if the preincubation time is extended up to 5 min (lane 3), the label in the 87–112 fragment is no longer detected, and a similar labeling pattern to that obtained by the direct addition of the reagent to the discs is obtained. These results indicate that ^{125}I -TID, as expected from its higher monomer solubility in water and its intermembrane exchange/transfer rate as compared with ^{125}I -TID-PC (51), diffuses rapidly from the lipid bilayer of the vesicles toward the bilayer of the discs, where it should label the apoA-I regions in contact with the disc lipids. Only at a short time after mixing the ^{125}I -TID-containing vesicles with the rHDL discs should the reagent concentration in the vesicles be high enough to produce a detectable labeling of the 87–112 apoA-I region. With the time course after mixing, the reagent concentration decreases in the vesicles and increases in the discs until the equilibrium is reached. By assuming a similar partition of ^{125}I -TID between both vesicle and disc lipid bilayer, it can be calculated that in the conditions used here, the reagent concentration in the vesicles would decrease at the equilibrium more than 3- or 5-fold when incubated with Lp2 or Lp4, respectively. Moreover, it must be also considered that at any time, only a small proportion of the discs (depending on the membrane affinity of each kind of rHDL disc) are interacting with vesicles through the 87–112 apoA-I region, whereas most of the discs would be free and thus with the apoA-I 87–112 region inaccessible to be labeled by the reagent located in the vesicles. On the contrary, 100% of the apoA-I molecules are interacting with the disc lipid bilayer and thus are accessible to be labeled in their contact regions by the reagent located there. This fact makes difficult the detection of label in the membrane inserted domain when a significant amount of reagent is transferred from the vesicles toward the disc bilayer.

Even by using a short incubation time before the photoactivation, no detectable labeling of the 87–112 fragment was observed when ^{125}I -TID-containing vesicles were incubated with large (Lp4) or FC-containing (Lp2C or Lp4C) rHDL discs (Fig. 5, lanes 7–9). Because the 87–112 fragment was labeled when these discs were incubated with vesicles containing the

nondiffusible reagent ^{125}I -TID-PC (Fig. 3), it must be concluded that these large and FC-containing discs interact with vesicles in a way similar to that of Lp2 through the membrane insertion of a central domain in the 87–112 region. The lack of labeling of this apoA-I region by ^{125}I -TID-containing vesicles in Lp2C, Lp4, and Lp4C is very likely to be due to their lower ability to interact with lipid membranes in comparison with Lp2, as it was previously shown (49).

Although ^{125}I -TID was not useful in detecting the membrane inserted domain, because of its higher intermembrane transfer rate, it was very helpful to detect the apoA-I regions loosely bound to the disc lipid bilayer. Incomplete CNBr cleavage of apoA-I allows us to conclude that, in addition to the central 87–112 region, a C-terminal apoA-I domain is also not in contact with the lipid bilayer of discoidal complexes. As already mentioned the complete cleavage should produce four peptides: 1–86, 87–112, 113–148, and 149–243. In contrast to the 87–112 fragment, the 113–148 peptide becomes labeled by the ^{125}I -TID, and it should be in contact with the lipid bilayer of the discs (Fig. 5). The N- and C-terminal fragments 1–86 and 149–243 co-migrate on SDS-PAGE, giving a unique band that contains a high level of radioactivity. Co-migration of these peptides does not allow us to conclude whether both or only one of them becomes labeled. However, the radioactivity distribution in peptides 1–112 and 113–243, which are produced by incomplete cleavage, indicates that the C-terminal region becomes labeled at a very low level. Fragment 1–112 contains a higher level of radioactivity than the 113–243 fragment, despite its lower mass as indicated by the intensity of Coomassie staining. Moreover, fragment 1–112 contains the 87–112 region, which is not labeled, and fragment 113–243 contains the 113–148 region, which is labeled. For all of these reasons we conclude that the C-terminal region of apoA-I is poorly labeled and that it should be loosely bound to the lipid bilayer of discs. To confirm this, a second cleavage strategy was used. Hydroxylamine cleaves apoA-I at its unique Asn¹⁸⁴–Gly¹⁸⁵ bond (Fig. 6), giving a large N-terminal polypeptide (1–184) and a shorter C-terminal fragment (185–243) as can be observed in Fig. 6A. Autoradiography shows that only the large 1–184 fragment becomes labeled by photoactivation of mixtures of Lp2 or lipid-free apoA-I with vesicles containing ^{125}I -TID (Fig. 6B). No

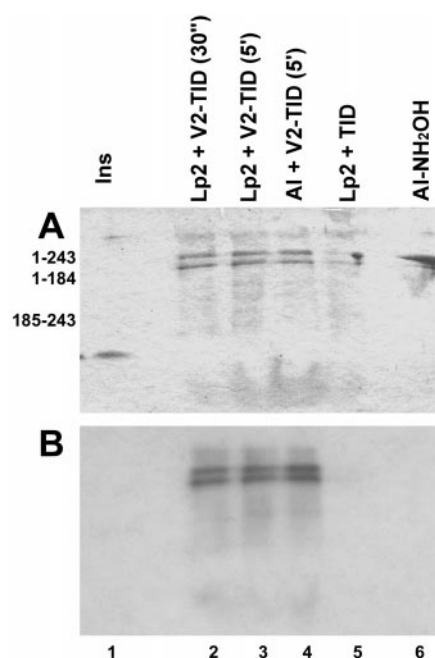


FIG. 6. Chemical cleavage with hydroxylamine of apoA-I photolabeled with ^{125}I -TID. Samples treated as in described in the legends of Figs. 3 and 4 were delipidated, digested with hydroxylamine, and analyzed by SDS-PAGE with Coomassie Blue staining (A) and autoradiography (B). Lane 1, insulin standard (*Ins*). Lanes 2 and 3, Lp2 incubated for 0.5 and 5 min, respectively, with ^{125}I -TID-containing POPC/FC vesicles. Lane 4, lipid-free apoA-I incubated for 5 min with the same vesicles. Lane 5, Lp2 incubated directly with ^{125}I -TID. Lane 6, nonradiolabeled apoA-I digested with hydroxylamine ($\text{AI-NH}_2\text{OH}$).

appreciable radioactivity was found in the shorter C-terminal 185–243 fragment, indicating that this apoA-I region has a low contact with the lipid bilayer of the discs.

DISCUSSION

The results obtained with the ^{125}I -TID reagent indicate that two apoA-I regions have little if any contact with the lipid bilayer of the discoidal lipoprotein complexes: a C-terminal region and a central one located between the residues 87–112. Fig. 7 shows the amino acid sequence of apoA-I and the amphipathic α -helices, which are predicted to be present in this protein (21, 25, 26). Segrest and colleagues (21) and others (15) proposed that the α -helices of apolipoproteins are not all equivalent in their affinities toward lipids. The differences in affinity do not appear to be only related to their hydrophobic moment but also to the distribution of charged residues along the axis of the helix. Amphipathic helices are classified into seven major and distinct classes (A, H, L, G, K, C, and M) based upon a detailed analysis of their physico-chemical and structural properties (21). The most frequent helix class found in exchangeable apolipoproteins is class A, which is characterized by a unique clustering of positively charged amino acid residues at the polar-nonpolar interface and negatively charged residues at the center of the polar face. As shown in Fig. 7, six class A helices have been identified in apoA-I (25, 26): helices 44–65, 66–87, 121–142, 143–164, 166–186, and 187–208. In addition, two other helix types were also identified: class G* (helix 8–33) and class Y (helices 88–98, 99–120, 209–219, and 220–241). Class G* helices are distinguished by a random radial arrangement of positive and negative residues, which is similar but not identical to that of the class G helices found in globular proteins. The basic features of the class Y motif are two negative residue clusters on the polar face separating the two arms and the base of the Y motif formed by three positive residue clusters (25). Interestingly, the two apoA-I regions not labeled with

^{125}I -TID, indicating little contact with the lipid bilayer of discoidal complexes, are rich in the class Y motif. In both regions, a short helix of 10–11 residues is followed by a long one of 21 residues. This fact would agree with the hypothesis that lipid affinity correlates with the extent to which a helix domain fits to class A motif (25, 61). However, a synthetic peptide containing the 220–241 apoA-I has been shown to have the highest affinity toward lipids among the other apoA-I helices (62). These discrepancies could be due to different affinity toward the edge of a lipid bilayer disc compared with the affinity toward the surface monolayer of vesicles or spherical particles and due to the fact that other factors such as interhelix interactions are playing an important role.

The central region 87–112, although loosely bound to the lipids of the discoidal complexes, is able to penetrate the bilayer of lipid vesicles as indicated by the results obtained with the ^{125}I -TID-PC reagent. It is remarkable that the same 87–112 segment of apoA-I becomes labeled when ^{125}I -TID-PC-containing vesicles are mixed either with discoidal complexes or with lipid-free apoA-I. This fact suggests that apoA-I could be liberated from the discs and that it then could interact with the vesicle bilayer as lipid-free apoA-I. This possibility, however, is not likely because it was shown (49) that discoidal complexes induce leakage of the vesicle internal aqueous space much faster than lipid-free apoA-I. Also, the lateral pressure increase in lipid monolayers occurs more rapidly with discoidal complexes than with lipid-free apoA-I (70).

The interaction between apoA-I discs and lipid vesicles results also in resonance energy transfer from tryptophan fluorescence to a nonexchangeable fluorescent phospholipid (diphenylhexatrienyl-phosphatidylcholine) in the vesicles (70). However, tryptophan fluorescence is not quenched when the vesicles contain phospholipid analogs (doxyl-phosphatidylcholines) having a collisional quencher group in different positions.² These observations indicate that no tryptophan residue of apoA-I penetrates the lipid bilayer of the vesicles so that it could be quenched by collision with doxyl-phosphatidylcholines, although at least one tryptophan residue would lie at a distance short enough to produce an energy transfer to the fluorescent phospholipid. The 87–112 apoA-I fragment that becomes labeled with ^{125}I -TID-PC contains a tryptophan residue at the 108 position. This residue would not be inserted into the bilayer of the lipid vesicles but would be located very near the interface, so that it would be responsible for the resonance energy transfer to the fluorescent phospholipid.

Fig. 8 shows the Edmundson wheel representation of the type Y amphipathic α -helices of this apoA-I segment. It contains the short 88–98 helix and the first half (99–112) of the long 99–120 helix. Because the hydrophobic faces of these helices are narrower than the hydrophilic ones, an interaction between both helices through their hydrophobic faces would expose only the hydrophilic residues, so that this domain could avoid contact with the lipid bilayer in the discoidal complexes, independently of which model is considered. To penetrate the membrane bilayer, rotation of the helices should occur to expose the nonpolar residues to the hydrophobic lipid phase environment. There are at least two possibilities for the membrane penetration by amphipathic helices (Fig. 9): (a) a vertical penetration with the helix axis parallel to the hydrocarbon phospholipid chains, which would require two or more helices interacting through their hydrophilic faces to expose the hydrophobic faces to the lipid environment, and b) a horizontal interaction with the helix axis parallel to the membrane surface, which requires the insertion of the nonpolar face into the

² J. D. Toledo and H. A. Garda, unpublished results.

FIG. 7. Amino acid sequence and amphipathic α -helices in apolipoprotein A-I. Amino acid residues in predicted amphipathic helices (from residue 15) are highlighted: class G* helix (8–33) is marked in *black*, class A helices (44–65, 67–87, 122–142, 144–164, 166–186, and 187–208) are in *light gray*, and class Y helices (88–98, 100–120, 209–219, and 221–241) are in *dark gray*. The three sites for cleavage with CNBr (*/*), the unique site for cleavage with hydroxylamine (*|*), and the 87–112 region that is labeled with ^{125}I -TID-PC (*double underlined*) are also indicated.

```

001  ASP GLU PRO PRO GLN SER PRO TRP-ASP-ARG - VAL-LYS-ASP-LEU-ALA-THR-VAL-TYR-VAL-ASP-
021  -VAL-LEU-LYS-ASP-SER-GLY-ARG-ASP-TYR-VAL - SER-GLN-PHE GLU GLY SER ALA LEU GLY LYS
041  GLN LEU ASN LEU-LYS-LEU-LEU-ASP-ASN-TRP - ASP-SER-VAL-THR-SER-THR-PHE-SER-LYS-LEU-
061  -ARG-GLU-GLN-LEU-GLY PRO VAL-THR-GLN-GLU - PHE-TRP-ASP-ASN-LEU-GLU-LYS-GLU-THR-GLU-
081  -GLY-LEU-ARG-GLN-GLU-MET/SER LYS-ASP-LEU - GLU-GLU-VAL-LYS-ALA-LYS-VAL-GLN PRO TYR-
101  -LEU-ASP-ASP-PHE-GLN-LYS-LYS-TRP-GLN-GLU - GLU-MET/GLU-LEU-TYR-ARG-GLN-LYS-VAL-GLU
121  PRO LEU-ARG-ALA-GLU-LEU-GLN-GLU-GLY-ALA - ARG-GLN-LYS-LEU-HIS-GLU-LEU-GLN-GLU-LYS-
141  -LEU-SER PRO LEU-GLY-GLU-GLU-MET/ARG-ASP - ARG-ALA-ARG-ALA-HIS-VAL-ASP-ALA-LEU-ARG-
161  -THR-HIS-LEU-ALA PRO TYR-SER-ASP-GLU-LEU - ARG-GLN-ARG-LEU-ALA-ALA-ARG-LEU-GLU-ALA-
181  -LEU-LYS-GLU-ASN GLY-GLY ALA-ARG-LEU-ALA - GLU-TYR-HIS-ALA-LYS-ALA-THR-GLU-HIS-LEU-
201  -SER-THR-LEU-SER-GLU-LYS-ALA-LYS PRO ALA - LEU-GLU-ASP-LEU-ARG-GLN-GLY-LEU-LEU PRO
221  VAL-LEU-GLU-SER-PHE-LYS-VAL-SER-PHE-LEU - SER-ALA-LEU-GLU-GLU-TYR-THR-LYS-LYS-LEU-
241  -ASN THR GLN

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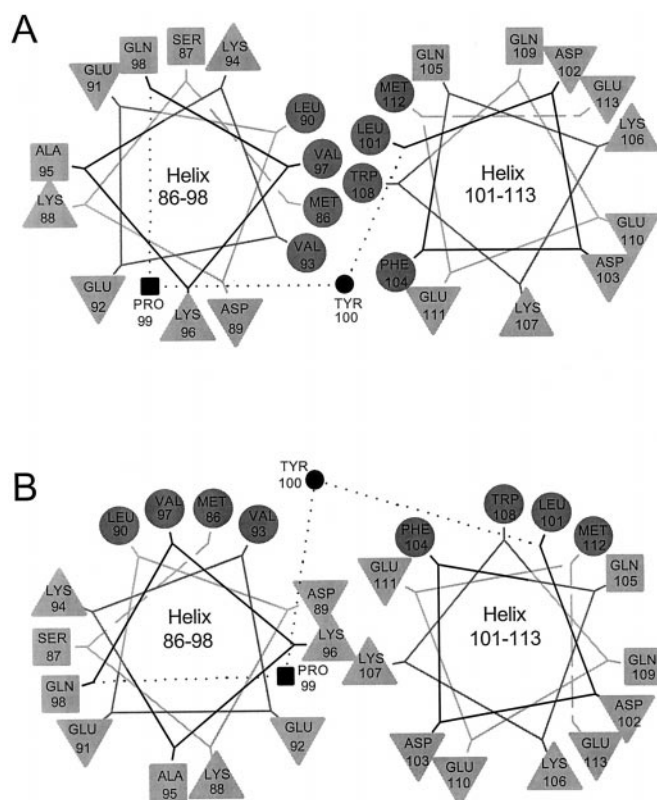


FIG. 8. Edmunson wheel diagram of the class Y amphipathic α -helices in the central apoA-I domain. Helix 86–98 and the first part (101–113) of the 99–120 helix are represented as antiparallel helices interacting through their hydrophobic faces (A) or exposing these faces (B) to interact with lipids. Hydrophobic, positive, negative, and neutral amino acid residues are shown with *circles*, *triangles*, *inverted triangles*, and *squares*, respectively. Viewed from *top*, the first (*solid black line*), second (*solid dark gray line*), third (*solid light gray line*), and fourth (*dashed light gray line*) helix turns are distinguished. Proline 99 and tyrosine 100 residues are shown out of the helical regions linked by a *dotted black line*.

hydrophobic interior of the external phospholipid monolayer; the hydrophilic face remains exposed to the aqueous phase and/or interacts with the phospholipid polar groups.

The vertical membrane penetration of only these two helices interacting by their polar faces seems to be very unlikely. Although the charged residue distribution at both sides of proline 99 would make the interaction possible through salt bridges between both helices, the hydrophilic faces are too wide, a fact that would result in the exposure of polar residues

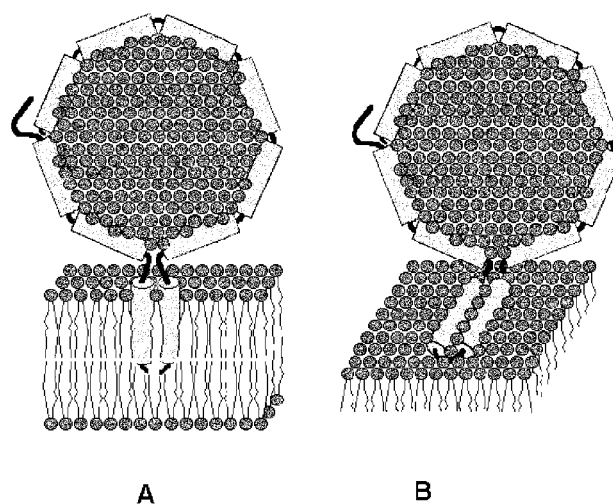


FIG. 9. Two possible models for the membrane insertion of the central apoA-I domain located in the 87–1123 region. A, vertical insertion. B, horizontal insertion.

to the lipid environment. Moreover, this would require a large conformational change because a 180° rotation of each helix is needed to switch from the lipid-unbound state in the discoidal complexes to the membrane inserted state. Another possibility is suggested by the analogy found with the three-dimensional PSSM program (63) of this apoA-I central region with ectatomin. This is a toxic peptide of 7.9 kDa that inserts into cellular and artificial membranes to form ionic channels (64, 65) and whose spatial structure in aqueous solution was resolved by ^1H NMR (66). Ectatomin contains two homologous polypeptide chains (37 and 34 residues) of similar conformation linked by disulfide bridges. Each chain consists of two α -helices and a hinge region of four residues giving an overall structure of a four-helix bundle (66). On insertion into artificial membrane bilayers, two ectatomin molecules rearrange with considerable movement of their helical parts (64) to form an ion pore. Thus, it should be possible for the two helices of the 87–112 region of apoA-I to interact with the same region of another apoA-I molecule to vertically penetrate the vesicle bilayer in a way similar to ectatomin. As recently determined (37), positions 132 of each apoA-I monomer in Lp2 discs are in close proximity, suggesting the possibility that the 87–112 regions are also near each other. Moreover, it is also possible that central domains of apoA-I molecules belonging to different discoidal particles can interact in a way similar to that of ectatomin to be inserted into membranes. Other possibility to be considered is that helix

content could change during the membrane insertion of the central domain. Although the crystal structure of an apoA-I fragment lacking the 43 N-terminal amino acids confirmed that the 87–112 region has a helical conformation (33), the helical content has shown to change among the lipid-free and different lipid-bound states (23, 39, 40, 67)

Although more experimental evidence would be required to determine how this central apoA-I domain is inserted into the bilayer of lipid vesicles, the horizontal insertion parallel to the membrane surface seems to be more likely. This type of interaction was postulated for apolipoprotein insertion into the surface phospholipid monolayer of spherical lipoproteins (25, 61), and it is known as the “snorkel” model.

From the reports of Segrest *et al.* (25) and Palgunachari *et al.* (62), a relatively low affinity for the membrane bilayer of the 87–112 region of apoA-I could be expected. Cholesterol exchange between HDL and membranes or other lipoproteins can occur by free diffusion through the aqueous medium (68, 69), but there is also evidence (47, 50) that it can be facilitated by the binding of HDL to membranes. The low affinity of the central apoA-I domain in the 87–112 region could be important for the functional need of a reversible and transient anchoring of HDL discs to cell membranes.

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