# Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and A-I: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT

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**Abstract** Studies were carried out to determine whether apolipoprotein (apo) A-II, like apoA-I, can recruit phospholipid and cholesterol from cell membranes, thereby forming nascent apoA-II-specific HDL. ApoA-II and apoA-I were purified from plasma and each was incubated with CHO cells at a concentration of 10 $\mu$g/ml. Lipid-containing complexes were isolated from the medium in both cases; the composition of the apoA-II- and apoA-I-specific complexes were similar where percent protein, phospholipid, and cholesterol were 35 ± 3, 38 ± 2, and 25 ± 1 for apoA-II, respectively, and 40 ± 2, 35 ± 1, and 24 ± 2 for apoA-I, respectively. On a per mole of apolipoprotein basis, apoA-I recruited significantly more phospholipid and cholesterol than dimeric apoA-II suggesting that apoA-I with its greater number of alpha helices binds more lipid. By electron microscopy, nascent apoA-II- and apoA-I-specific particles were predominantly discoidal in morphology. ApoA-II complexes were unique in their nondenaturing polyacrylamide gradient gel size distribution as six distinct populations of particles with diameters of 8.1, 9.3, 10.4, 11.8, 13.1, and 14.6 nm were routinely noted, compared with apoA-I which formed only three major populations with diameters of 7.3, 9.2, and 11.0 nm. Nascent apoA-I complexes incubated with purified lecithin:cholesterol acyltransferase (LCAT) were transformed into predominantly 8.4 nm particles. The latter is similar in size to plasma $HDL_{3a}$, LpA-I particles, suggesting that extracellularly assembled apoA-I-lipid complexes can directly give rise to a major plasma LpA-I subpopulation upon interaction with LCAT. Unlike apoA-I, apoA-II–lipid complexes could not serve as substrates for LCAT and did not undergo transformation. This study also demonstrates, for the first time, that apoA-II and apoA-I show a preference in phospholipid recruitment from membranes. Although phosphatidylcholine is the major phospholipid removed by both apolipoproteins, apoA-II preferentially recruits phosphatidylethanolamine (PE) as its second most abundant phospholipid while apoA-I recruits sphingomyelin. As PE is usually associated with the inner leaflet of the membrane, it is likely that dimeric apoA-II, compared with apoA-I, can penetrate farther into the membrane and extract PE. This ability of apoA-II to insert more deeply into the lipid milieu may explain the known ability of apoA-II to resist dissociation from the mature HDL particle.—**Forte, T. M., J. K. Bielicki, R. Goth-Goldstein, J. Selmek, and M. R. McCall.** Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and A-I: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT. *J. Lipid Res.* 1995. **36:** 148–157.

**Supplementary key words** apolipoprotein A-I • apolipoprotein A-II • membrane interactions • phosphatidylcholine • phosphatidylethanolamine • sphingomyelin • nascent HDL • lecithin:cholesterol acyltransferase • Chinese hamster ovary cell

There is an inverse relationship between the concentration of plasma high-density lipoproteins (HDL) and risk for premature coronary artery disease. Plasma HDL possess two major apolipoproteins (apo), apoA-I and apoA-II, where the former constitutes approximately 70% of the total HDL protein and the latter, 20%. In plasma, HDL are composed of distinct apolipoprotein specific populations including apoA-I with apoA-II (LpAI/AII) and apoA-I without apoA-II (LpAI) (1). Recent reports also indicate that plasma contains some apoA-II without apoA-I (LpAII) HDL but this subpopulation accounts for less than 10% of total apoA-II (2, 3).

The in vivo site of assembly of nascent, i.e., discoidal, apolipoprotein-specific HDL has not been completely resolved. Potential mechanisms for assembly include (1) a

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; CHO, Chinese hamster ovary; LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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direct mechanism whereby cells producing apolipoproteins, e.g., hepatocytes, secrete the pre-assembled particles or (2) an indirect mechanism whereby particles are assembled extracellularly by interaction of free apolipoproteins with pre-existing lipoproteins or cell membranes. Early studies of Stein and Stein (4) indicated that apoHDL was able to stimulate removal of cholesterol and phospholipid from cell membranes. Previous reports on incubations of cholesterol-loaded macrophages (5), cholesterol-enriched fibroblasts (6), and endothelial and smooth muscle cells (7) with apoA-I and apoA-II suggested that both proteins can promote cholesterol efflux from cells. Lipidated complexes of apoA-I and apoA-II described in these studies contained both phospholipid and unesterified cholesterol; however, the physical-chemical properties of the complexes were not determined. These studies provide little insight into the nature of the nascent particles resulting from interaction of either apoA-I or apoA-II with cell membranes. We have recently shown that lipid-free, genetically engineered apoA-I can recruit phospholipid and cholesterol from Chinese hamster ovary (CHO) cell membranes and that, in so doing, is able to generate several discrete, nascent HDL subpopulations (8). The generation of nascent HDL resulting from the interaction of lipid-free apolipoproteins with cell membranes has important implications in reverse cholesterol transport because it provides a mechanism whereby excess cholesterol may be removed from cells unable to catabolize cholesterol.

ApoA-I and apoA-II are both amphipathic proteins that possess several lipid binding helices. However, the properties of the helical repeats are clearly different for the two proteins where apoA-II contains class $A_2$ and apoA-I contains a class $A_1$ amphipathic helices (9). In the present investigation, we addressed the question whether apoA-I and apoA-II isolated from plasma are equally effective in binding and removing phospholipid and cholesterol from CHO cell membranes and whether apoA-II, like apoA-I, is able to form discrete nascent HDL subpopulations. As there are structural differences between these two proteins, we also evaluated whether they had the same or different preferences for membrane phospholipids and whether the nascent particles they assembled could function as substrates for the lecithin:cholesterol acyltransferase (LCAT) reaction.

## MATERIALS AND METHODS

### Isolation of apolipoproteins

Fresh human plasma was obtained from the blood bank and 1.0 mg/ml EDTA and 0.05 mg/ml gentamicin sulfate were added; the plasma was maintained at 4°C during processing. The HDL fraction was isolated at d 1.063–1.21 g/ml from approximately 1 l plasma. Washed HDL were treated with 3 M guanidine hydrochloride at 37°C for 3 h for isolation of apoA-I as previously described (10). ApoA-II was isolated from the apoA-I-depleted HDL using 6 M guanidine hydrochloride according to Blanche et al. (11). Isolated apoA-I was purified on a Sephacryl S-200 HR column and apoA-II on a DEAE-Sepharose CL 6B column. Eluted fractions were assayed for purity of apoA-I and apoA-II by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and dot blotting using monospecific anti-apoA-I and anti-apoA-II antibodies. In each case, only a single band of purified protein was seen on SDS-PAGE; apoA-II was electrophoresed in the absence of reducing agents and was present in the homodimeric form. The purified proteins were dialyzed into a buffer containing 150 mM NaCl, 20 mM Tris, and 0.27 mM EDTA, pH 8.0, and refrigerated.

### Cell incubations and isolation of apolipoprotein-lipid complexes

CHO Cl 9 cells were grown in 850 $cm^2$ roller bottles with DMEM/F12 (1:1) and 10% fetal bovine serum (FBS) as previously described (8). Prior to onset of incubations, cells were rinsed three times with Hank's balanced salt solution and incubated overnight with serum-free medium; this medium was discarded before incubations with purified apolipoproteins to insure that apolipoproteins in FBS were not carried over. Cells were then incubated for 24 h with serum-free medium containing either apoA-I (10 $\mu$g/ml) or apoA-II (10 $\mu$g/ml). Conditioned medium was subsequently collected and placed on ice. Gentamicin sulfate (0.05 mg/ml), EDTA (1 mg/ml), and PMSF (0.5 mM) were added and cell debris was removed by filtration.

The conditioned medium was concentrated by ultrafiltration (Amicon stirred cell, YM10 membranes) and apoA-I- or apoA-II-lipid complexes were isolated at $d < 1.235$ g/ml as previously described (7). These fractions were extensively dialyzed against saline-Tris-EDTA prior to further analyses.

### Electrophoretic analyses

The size distribution of particles was determined by nondenaturing polyacrylamide gradient (4–30%) gel electrophoresis according to previously described procedures (12). Peak positions of apolipoprotein-lipid complexes on gradient gels are based on the diameter of globular protein standards electrophoresed on the same gel. Standards used to calibrate the gels were thyroglobulin, apoferritin, lactate dehydrogenase, and albumin with Stokes diameters of 17.0 nm, 12.2 nm, 8.16 nm, and 7.1 nm, respectively.

SDS-polyacrylamide gel electrophoresis was carried out on 4–20% gels obtained from Schleicher and Schuell (Keene, NH) according to the procedure of Laemmli (13).

Agarose gel electrophoresis was carried out on Beckman Paragon gels according to the manufacturer's instructions. Proteins were transferred to nitrocellulose by diffusion and bands were probed with either antibody to apoA-I or apoA-II as previously described (14).



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### Electron microscopy

Samples examined by electron microscopy were first dialyzed against ammonium acetate buffer, pH 7.4, and then stained with 2% sodium phosphotungstate according to previously described procedures (15).

### Compositional analyses

Protein was determined by the method of Markwell et al. (16), phospholipid by the method of Chen, Toribara, and Warner (17) and cholesterol by an enzymatic method (18).

To analyze phospholipid subclasses, lipids from 125 μg of either apoA-I or apoA-II protein in the d<1.235 g/ml fractions were first partitioned into chloroform according to Bligh and Dyer (19). Phospholipids were separated by thin-layer chromatography using silica gel H plates with the following solvent system: chloroform-methanol-acetic acid-water 100:60:16:8. Phospholipid standards were visualized with iodine vapor. Subclasses were re-isolated from the silica gel by the method of Bamberger et al. (20). Recoveries of phospholipid standards from the silica gel were 88, 99, 93, and 100% for lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, respectively.

### LCAT transformation of nascent complexes

LCAT was isolated from human serum essentially as described by Chen and Albers (21) and characterized as previously described (22).

To assess the ability of apoA-I- and apoA-II-lipid complexes to serve as substrates for LCAT, apoA-I- and apoA-II-lipid complexes were first labeled with radioactive cholesterol by equilibration with [$^{14}$C]cholesterol-albumin according to Albers, Chen, and Lacko (23). Complexes were incubated with the labeled cholesterol for at least 18 h at 4°C and then for an additional 30 min at 37°C prior to LCAT exposure. Previously labeled apoA-I and apoA-II complexes (13 μg of unesterified cholesterol per incubation) were incubated at 37°C with purified LCAT, 1.5% human serum albumin, 5 mM mercaptoethanol, and 50 μg/ml gentamicin for up to 24 h. The final reaction volume was 250 μl and contained sufficient purified LCAT to esterify 3.5 nmol cholesterol per h. Reactions were terminated by the addition of 250 μl absolute ethanol. Cholesterol and cholesteryl ester were extracted from reaction mixtures by repeated additions of hexane (23). Hexane extracts were subsequently pooled, evaporated to dryness, and dissolved in chloroform (90 μl). Aliquots (10 μl) of the chloroform-solubilized lipids were spotted on polysilicic acid-impregnated glass fiber sheets and developed in toluene. Bands corresponding to unesterified and esterified cholesterol were visualized with iodine and cut into liquid scintillation vials for counting. Radioactivity was corrected for quench and LCAT activity was calculated as the percent conversion of [$^{14}$C]cholesterol to cholesteryl ester.

To evaluate LCAT-induced changes in size distribution of apoA-I-lipid complexes, incubations were performed without isotopically labeled substrate as previously described for HepG2 nascent HDL (22). Incubations were terminated at the times indicated by addition of 3.5 mM *p*-hydroxymercuriphenylsulfonic acid. Complexes were isolated at d<1.235 g/ml by ultracentrifugation and size distribution was determined by nondenaturing gradient gel electrophoresis.

### Statistical analysis

Pair-wise comparisons were made using the Student's *t*-test.

## RESULTS

### ApoA-I- and apoA-II-lipid complex formation

Purified plasma apoA-I or apoA-II incubated with CHO cells for 24 h formed apolipoprotein-lipid complexes that floated at d<1.235 g/ml. However, for both apoA-I and apoA-II, only 11-12% of the total incubated protein was recovered at this density and thus was associated with lipid. In both cases, the isolated apolipoprotein-lipid complexes contained almost exclusively the apolipoprotein used in the incubation medium indicating that CHO cell-derived proteins were absent in the d<1.235 g/ml fraction (**Fig. 1**). The apoA-II as-

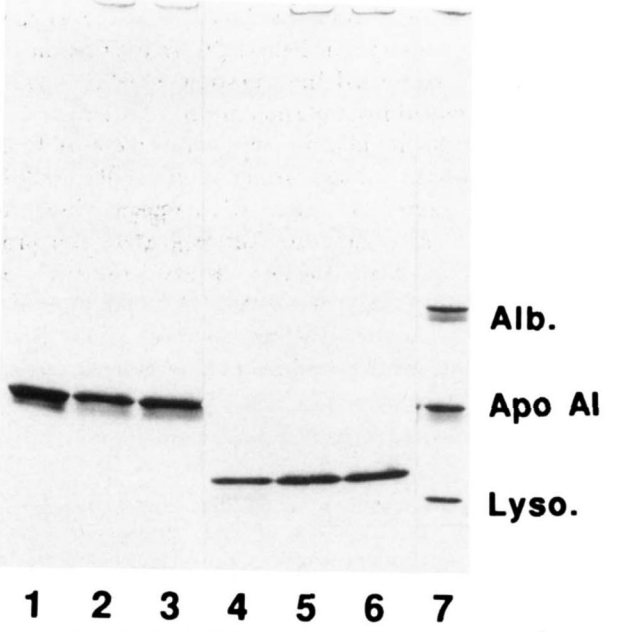


**Fig. 1.** SDS-PAGE (4-20% gel) analysis of ultracentrifugally isolated (d<1.235 g/ml) complexes formed by incubating apoA-I (10 μg/ml) or apoA-II (10 μg/ml) with CHO cells. Lane (1) purified apoA-I; (2 and 3) apoA-I-lipid complexes; (4) purified apoA-II; (5 and 6) apoA-II-lipid complexes; (7) standards consist of albumin (67,000 Da), apoA-I (28,000 Da), and lysozyme (14,500 Da).



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sociated with lipid complexes was a dimer indicating that it had not undergone reduction or degradation during cell incubation.

### Electron microscopic structure of nascent apoA-I- and apoA-II-lipid complexes

The electron microscopic images of ultracentrifugally isolated apoA-I and apoA-II assembly products are shown in **Fig. 2A and 2B,** respectively. It is evident that the floating complexes in each case contain heterogeneous sized discoidal particles. The mean particle diameter for the discoidal complexes was 13.6 ± 2.9 nm for apoA-I and 14.1 ± 4.0 nm for apoA-II. In addition to the discoidal structures, some large vesicular particles (33–70 nm diameter) and en face discs can also be discerned.

### Size distribution of apoA-I- and apoA-II-lipid complexes

Nondenaturing gradient gel electrophoretic analysis of the apolipoprotein specific assembly products revealed that several subpopulations of particles were generated for each apolipoprotein after 24 h incubation with cells (**Fig. 3**). Purified plasma apoA-I forms three major populations of nascent particles with peaks at 7.3 ± 0.05, 9.3 ± 0.08, and 11.1 ± 0.09 nm (n = 3); additionally, minor components banding at 14.2 ± 0.16 and 16.3 ± 0.12 nm (n = 3)

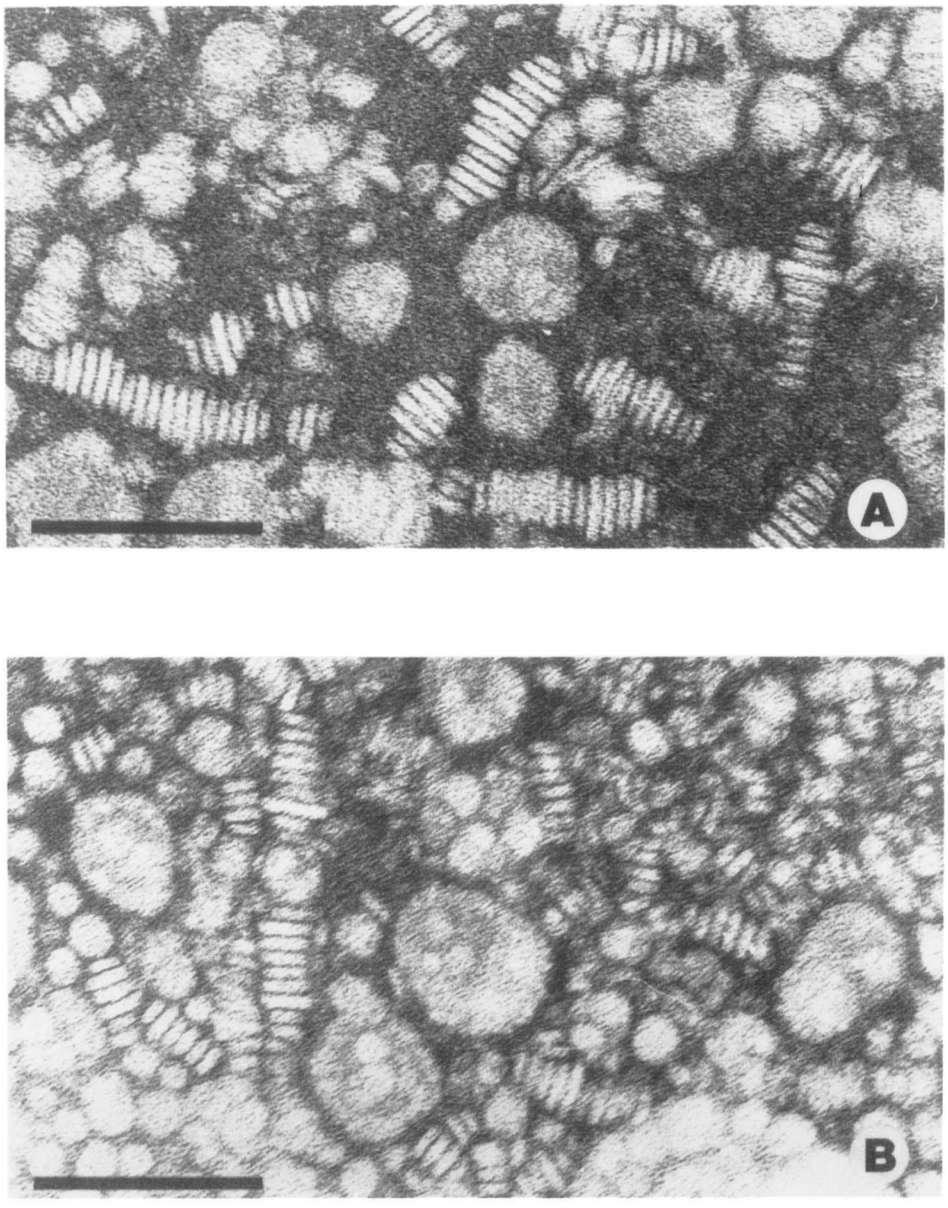


**Fig. 2.** Electron micrographs of (A) apoA-I-lipid complexes isolated from 24 h CHO cell conditioned medium, and (B) apoA-II-lipid complexes isolated from 24 h CHO cell conditioned medium. The bar marker represents 100 nm.



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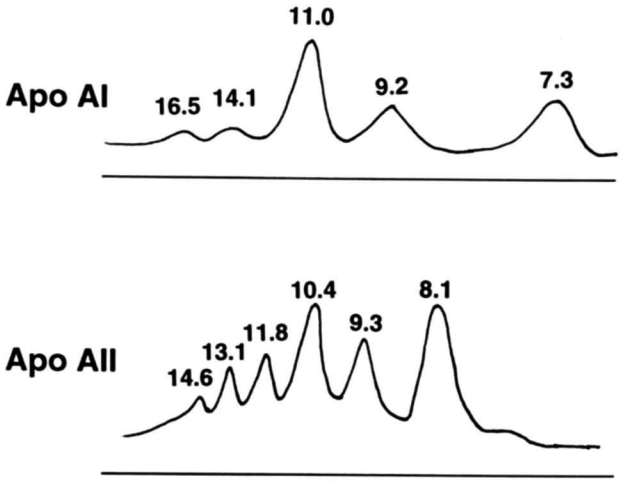


**Fig. 3.** Size distribution of extracellular assembly products resulting from incubations of CHO cells with apoA-I or apoA-II; representative scans of apoA-I- and apoA-II-lipid complexes electrophoresed on 4-30% nondenaturing gradient gels are shown. The numbers over the peaks indicate relative particle diameter (nm).

are also present. Typically after 24 h incubation, an apoA-I-lipid complex at 11.0 nm was the predominant peak. There are six distinct populations of apoA-II complexes with peaks at 8.0 ± 0.07, 9.2 ± 0.11, 10.3 ± 0.08, 11.8 ± 0.05, 13.2 ± 0.04, and 14.6 ± 0.08 nm (n = 3); consistently, the two dominant apoA-II populations are those at 8.0 nm and 10.3 nm. It should be noted that there is very little overlap between the particle size distribution of the apoA-I and apoA-II assembly products (compare scans in Fig. 3). Comparison of the mean disc diameters determined by electron microscopy with the particle sizes estimated on gradient gels suggests that nondenaturing gradient gels underestimate the diameter of the discoidal complexes. This is due, in part, because the gradient gel is calibrated with globular proteins that are thought to possess spherical geometry while the discoidal particles have a long and short axis. Discoidal particles have been shown to migrate a greater distance into the gel than spherical particles of the same diameter (10).

### Agarose electrophoretic migration of apoA-I- and apoA-II-lipid complexes

The electrophoretic migration of apoA-I- and apoA-II-lipid complexes compared with the migration of plasma HDL is shown in **Fig. 4.** Purified apoA-I used in the CHO cell incubation medium has pre-beta mobility (Fig. 4, lane 2). The apoA-I-lipid complexes, however, contain three distinct populations of particles based on charge (Fig. 4, lane 3); one with pre-beta mobility which suggests the presence of lipid-free apoA-I, one that migrates in the alpha position, and a band that migrates considerably faster than plasma HDL in the pre-alpha region. Both conditioned medium and the d>1.235 g/ml fraction contain pre-beta and alpha migrating apoA-I.

Purified apoA-II also migrates in the pre-beta position (Fig. 4, lane 7). Although the apoA-II-lipid complex isolated from CHO cell medium migrates further than the purified protein, it does not reach the alpha position noted for plasma HDL (Fig. 4, lane 8). Unlike apoA-I, the apoA-II-conditioned medium and d>1.235 g/ml fractions possess mainly pre-beta mobility.

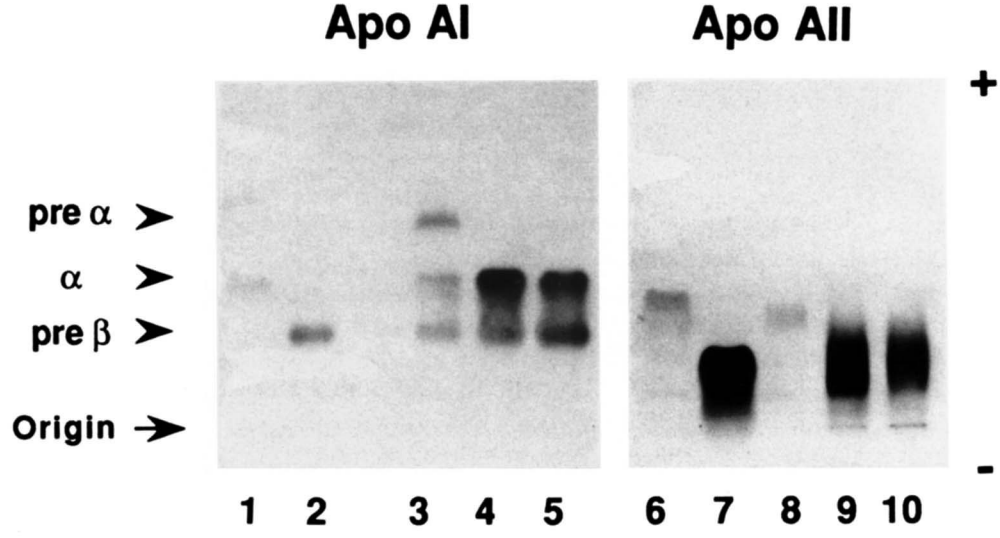


**Fig. 4.** Agarose electrophoretic mobility of apoA-I- and apoA-II-lipid complexes. Samples were electrophoresed on Beckman Paragon gels and then transferred to nitrocellulose. The NC paper was probed with antibody to apoA-I or apoA-II as indicated. (1) Plasma HDL; (2) purified apoA-I; (3) apoA-I-lipid complexes (d<1.235 g/ml); (4) concentrated conditioned medium before isolation of complexes; (5) d>1.235 g/ml fraction from conditioned medium; (6) plasma HDL; (7) purified apoA-II; (8) apoA-II-lipid complexes (d<1.235 g/ml); (9) concentrated conditioned medium; and (10) d>1.235 g/ml fraction from conditioned medium.



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### Composition of apoA-I- and apoA-II-lipid complexes

The weight percent compositions of complexes formed during incubation of apoA-I and apoA-II with CHO cells were similar where the percentage distribution of protein, phospholipid, and cholesterol was 40 ± 2, 35 ± 1, and 24 ± 2, respectively, for apoA-I complexes and 35 ± 3, 38 ± 2 and 25 ± 1, for apoA-II complexes, respectively. In both cases, the particles were relatively rich in phospholipid and unesterified cholesterol. Both triglyceride and cholesteryl ester were below the level of detection, hence the particles possess little or no core lipid which is consistent with their discoidal morphology.

In order to assess the efficiency with which apoA-I and apoA-II can recruit membrane lipids, we calculated the moles of phospholipid and cholesterol per mole of apolipoprotein assuming, as our SDS-PAGE data suggests, that all apoA-II was in the homodimer form. These data are summarized in **Table 1.** Even though the unesterified cholesterol:phospholipid molar ratios are similar for apoA-I and apoA-II complexes, the number of phospholipid and cholesterol molecules recruited by apoA-I is significantly greater ($P < 0.05$) than that for apoA-II.

The major phospholipid recruited by both apoA-I and apoA-II is phosphatidylcholine as indicated in **Table 2.** Differences are noted, however, in the recruitment of the minor phospholipids where apoA-I preferentially binds sphingomyelin and apoA-II preferentially binds phosphatidylethanolamine. Lysophosphatidylcholine was either not detected or was present at concentrations <0.5%.

### Transformation of apolipoprotein-lipid complexes by LCAT

To determine whether the phospholipid and unesterified cholesterol-rich complexes are substrates for LCAT, both apoA-I- and apoA-II-lipid complexes labeled with [$^{14}$C]cholesterol were incubated with purified LCAT. As shown in **Fig. 5,** there is a linear increase in [$^{14}$C]cholesteryl ester formation in apoA-I complexes over a 24-h incubation period. There is little conversion of labeled cholesterol to cholesteryl ester in the apoA-II complexes supporting previous reports that apoA-II functions as a weak activator of LCAT (24). To evaluate whether increases in cholesteryl ester formation in apoA-I complexes were associated with changes in particle size distribution, unlabeled apoA-I-lipid complexes were incubated with purified LCAT and subjected to nondenaturing gradient gel electrophoresis after various incubation periods. Changes in particle size distribution as a function of LCAT incubation time are shown in **Fig. 6.** After 2 h incubation, a major new peak is seen at 8.4 nm; the appearance of this particle is associated with the disappearance of both larger and smaller components noted in the control sample (0 h). With increasing time of incubation there is a progressive increase in the 8.3–8.4 nm peak at the expense of other assembly complexes and, at 8 h, LCAT-induced particle transformation appears to be complete.

TABLE 1. Comparison of the moles of phospholipid (PL) and unesterified cholesterol (UC) bound by apoA-I and apoA-II

| Apolipoprotein | Phospholipid | Unesterified Cholesterol | UC/PL Mole Ratio |
|---|---|---|---|
| | *mol/mol apolipoprotein* | | |
| A-I | 32.0 ± 2.0 | 43.6 ± 4.5 | 1.36 |
| A-II[a] | 24.5 ± 3.3[b] | 32.0 ± 4.7[b] | 1.30 |

Values represent mean ± SD of three experiments.
[a] The homodimer form of apoA-II (17,000 Da) was used in the calculation.
[b] $P < 0.05$, comparison between apoA-II and apoA-I complexes.

TABLE 2. Phospholipid composition of nascent apoA-I- and apoA-II-lipid complexes isolated after incubation of apolipoproteins with CHO cells

| | Percent Distribution of Phospholipid Subclasses | | |
|---|---|---|---|
| Apolipoprotein | PC | SM | PE |
| A-I | 79.3 ± 2.0 | 15.1 ± 0.9 | 5.3 ± 1.6 |
| A-II | 78.2 ± 1.8 | 5.4 ± 5.3 | 16.4 ± 3.6 |

PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine. Values are the mean ± SD of three experiments.

## DISCUSSION

High density lipoproteins have a putative role in the process of reverse cholesterol transport. Several studies,

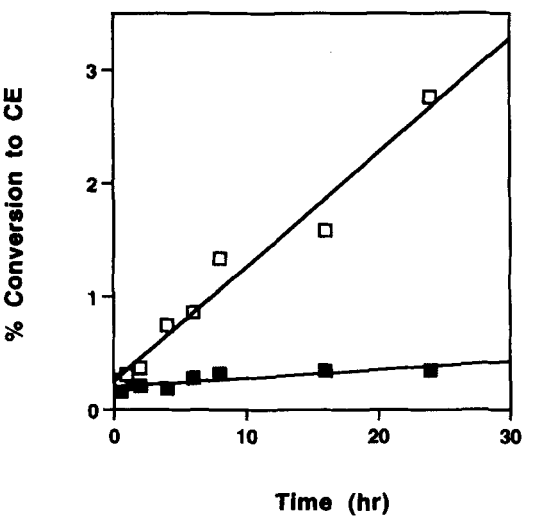


**Fig. 5.** Effect of LCAT on conversion of cholesterol to cholesteryl ester in apoA-I-lipid complexes (open squares) and in apoA-II-lipid complexes (closed squares). To assess the ability of apoA-I- and apoA-II-lipid complexes to serve as substrates for LCAT, complexes were first labeled with [$^{14}$C]cholesterol and incubated as outlined in Methods. The data is expressed as the percent conversion of radiolabeled cholesterol to cholesteryl ester.



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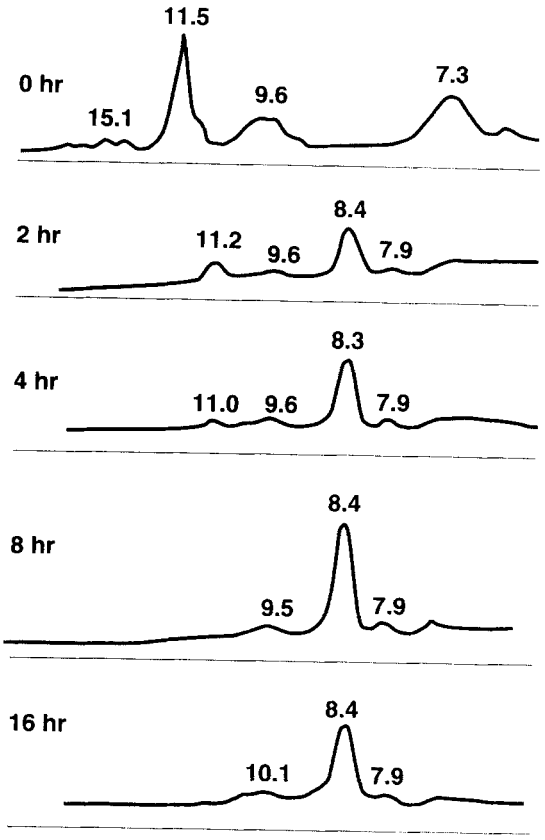


**Fig. 6.** LCAT-induced transformations of apoA-I–lipid complexes with time. Complexes were incubated with LCAT and albumin as described in Methods. After incubation the complexes were ultracentrifugally re-isolated from the incubation medium at d < 1.235 g/ml. Fractions were electrophoresed on 4–30% nondenaturing gradient gels, stained with Coomassie G250, and subsequently scanned. The numbers over the peaks indicate particle diameters (nm).

however, using apolipoprotein-specific populations of HDL including the LpA-I and LpA-I/A-II populations suggest that the LpA-I particles are more efficient in promoting cholesterol efflux from cells than the apoA-II-containing particles (25, 26). Although the majority of apoA-I and apoA-II in plasma and interstitial fluid is associated with lipoproteins, there is evidence that a small proportion (7–10%) of apoA-I is present in the lipid-free form (27). It has been suggested by ourselves and others that the lipid-free apoA-I may have a significant role in the removal of excess cholesterol from peripheral cells, particularly arterial foam cells (5–8) and may, thus, play a role in the reverse cholesterol transport process. Our previous investigations with genetically engineered apoA-I indicated that the process of cholesterol recruitment from cells was closely linked with phospholipid recruitment and that lipidation of apoA-I with phospholipid preceded the recruitment of cholesterol. The availability and/or quantity of lipid-free apoA-II in plasma and interstitial fluid is not known. However, it is likely that some free apoA-II is produced in plasma during lipolysis of triglyceride-rich particles. Incubation studies with VLDL, HDL, hepatic lipase, and cholesteryl ester transfer protein have suggested that both apoA-I and apoA-II are displaced from HDL particles during VLDL catabolism and appear in the lipid-free form in the d > 1.21 g/ml fraction (28). The latter process provides a mechanism whereby lipid-free apoA-II may be generated and therefore available, like apoA-I, to participate in phospholipid and cholesterol recruitment from cells.

In the present investigation, the apoA-I–lipid complexes isolated from 24 h CHO cell-conditioned medium were primarily discoidal particles with major subpopulations possessing diameters of 9 and 11 nm. We noted, moreover, that a subpopulation of the lipidated apoA-I had unique pre-alpha migration on agarose gels; pre-alpha mobility is most likely associated with the discoidal complexes as reconstituted apoA-I–egg yolk phosphatidylcholine complexes that are discoidal in shape have the same mobility (T. M. Forte and A. V. Nichols, unpublished observation). Small quantities of HDL particles with pre-alpha mobility have recently been described for dog prenodal lymph (29) and human plasma (30). In humans, pre-alpha HDL appear in more than one form and have been designated pre-alpha$_{1-4}$ where the estimated size by nondenaturing gradient gel is 11.1, 9.4, 8.4, and 7.7 nm for pre-alpha$_{1-4}$, respectively. Although the size of the 11.1 and 9.4 nm plasma pre-alpha HDL populations correspond with the major apoA-I–lipid complexes isolated from CHO cell medium in the present study, it is unclear whether they are the same in chemical composition and/or morphology.

Our results from LCAT incubation studies revealed that extracellular assembly products formed by apoA-I, but not apoA-II, serve as substrates for LCAT. The lack of LCAT activation by apoA-II is consistent with published observations that apoA-II is a poor activator of the enzyme (31). The transformation of apoA-I extracellular assembly products by LCAT results in the formation of an 8.4 nm particle that is similar in size to human adult plasma HDL$_{3a}$ LpA-I particles (32). These data suggest that when extracellularly assembled apoA-I–lipid complexes are formed in vivo they can directly give rise to a major plasma LpA-I subpopulation upon interaction with LCAT.

An important observation in the present study is that the composition of the apoA-I–lipid complexes resulting from incubation of apoA-I with CHO cells is especially rich in unesterified cholesterol compared with in vitro reconstituted HDL. Several laboratories have shown that reconstituted discoidal HDL formed by cholate dialysis of phospholipid, cholesterol, and apoA-I have particles in the same size range as those isolated from CHO cell culture medium; however, the cholesterol:phospholipid molar ratio in these reconstituted HDL ranged from 0.08 to 0.3 (33–36) compared with 1.3 in the present study. The high



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cholesterol:phospholipid molar ratio in the present study suggests that a biological system using cell membranes as a source of phospholipid may be more favorable for increased incorporation of cholesterol into the disc bilayer than incorporation by means of cholate dialysis. In support of this suggestion, our extracellular assembly complexes are remarkably similar in their unesterified cholesterol:phospholipid molar ratio to that found in discoidal HDL isolated from HepG2 medium (14) and LCAT-deficient plasma (37). The exact role, however, played by different phospholipid species and their fatty acid composition, as well as the contribution of apoA-I conformation, on extracellular assembly still remains to be elucidated.

In the present study we have shown that apoA-II also recruits phospholipid and unesterified cholesterol from membranes, thereby generating nascent apoA-II-specific HDL. Formation of apoA-II-specific nascent HDL may be physiologically important, not only as a mechanism for removal of excess cholesterol from cells, but also as a mechanism for HDL lipid transport in genetic disorders where apoA-I synthesis is impaired. It has recently been demonstrated in subjects with apoA-I deficiency that LpA-II particles are present in the plasma (2, 3). Such particles are usually associated with apoCs and/or apoE which may assist in their catabolism.

Although our investigation shows that apoA-II extracellular assembly products have the same unesterified cholesterol:phospholipid molar ratio as apoA-I complexes, we have calculated that, on a per mole of apolipoprotein basis, less phospholipid and cholesterol are recruited by dimeric apoA-II than by apoA-I (Table 1). This difference is not surprising as apoA-I possesses a greater number of amphipathic helices than apoA-II (9). ApoA-II extracellular assembly complexes are uniquely different in size from those of apoA-I; unlike apoA-I complexes, the six components that are consistently seen following incubations with apoA-II occur at regular, step-wise intervals where peaks are separated by a distance of 1.3 ± 0.14 nm. This change in size is also associated with a constant 4.0 nm increment in the circumference of the discs. The incremental change in size of apoA-II-containing particles suggests regular quantized increases that result in thermodynamically stable particles. The quantized increment may be the result of accrual of additional phospholipid and cholesterol with or without addition of apoA-II. Previous studies from our laboratory, examining reconstituted particles formed during association of dimeric apoA-II with egg yolk phosphatidylcholine, showed that complexes ranging from 8 to 11 nm diameter possessed three apoA-IIs while particles 11–15 nm diameter possessed four apoA-IIs (11). Extrapolating these data to the present suggests that the 8.1, 9.3, and 10.4 nm cell-derived dimeric apoA-II–lipid complexes are likely to contain three molecules of apoA-II while the 11.8, 13.1, and 14.6 nm species contain four apoA-IIs. The regular 1.3 nm increment within the putative three and four apoA-II-containing particles suggests that the protein conformation is flexible and can accommodate additional lipids without adding additional apolipoprotein. The regular increase in diameter of the apoA-II–lipid complexes is consistent with the addition of one layer of phospholipid and cholesterol to the edge of the discs assuming that the width of the phospholipid head group is approximately 0.5 nm.

It has been reported that reconstituted apoA-II HDL complexes formed by cholate dialysis of apoA-II, palmitoyloleoyl phosphatidylcholine, and cholesterol (38) have particle diameters of 9.4 and 10.1 nm, sizes analogous to two (9.3 and 10.4 nm) of the six complexes formed by incubation of apoA-II with CHO cells. The cholate reconstituted apoA-II particles, unlike the extracellular assembly products, transported only small quantities of cholesterol relative to phospholipid. Although speculative, specific phospholipid subclasses, such as those found in CHO cell membranes, may be important determinants of the apoA-II assembly product composition and distribution found in the present study.

The present study demonstrates, for the first time, that apoA-II and apoA-I show selectivity in recruitment of phospholipid subclasses from cell membranes. Although the major phospholipid for both apoA-I and apoA-II complexes is phosphatidylcholine, apoA-I preferentially recruits sphingomyelin as the second most abundant phospholipid, whereas apoA-II recruits phosphatidylethanolamine. The relatively high content of phosphatidylethanolamine (a phospholipid associated with the inner leaflet of plasma membranes) in apoA-II complexes suggests that dimeric apoA-II, compared to apoA-I, can penetrate farther into the membrane bilayer thus extracting this phospholipid. ApoA-II α-helices are unique because their basic residues consist entirely of lysines and thus the helices have a well defined class $A_2$ structure (9). This structure allows "snorkeling" of interfacial lysine residues to the aqueous surface and extends the nonpolar backbone of the α-helix more deeply into the hydrophobic region of the phospholipid than that of the class $A_1$ structure of apoA-I which contains both lysine and arginine residues (9, 39). The ability of apoA-II to insert more deeply into the phospholipid milieu, either membrane or lipoprotein, may help to explain the well-known ability of apoA-II to displace apoA-I from HDL (40) and the relative resistivity of apoA-II, compared with apoA-I, to dissociation from HDL particles (41).



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