The Central Helices of ApoA-I Can Promote ATP-binding Cassette Transporter A1 (ABCA1)-mediated Lipid Efflux

AMINO ACID RESIDUES 220–231 OF THE WILD-TYPE ApoA-I ARE REQUIRED FOR LIPID EFFLUX IN VITRO AND HIGH DENSITY LIPOPROTEIN FORMATION IN VIVO*

We have mapped the domains of lipid-free apoA-I that promote cAMP-dependent and cAMP-independent cholesterol and phospholipid efflux. The cAMP-dependent lipid efflux in J774 mouse macrophages was decreased by ∼80–90% by apoA-I(1–41) or apoA-I(Δ(1–59)), and was restored to 75–80% of the wild-type apoA-I control value by double deletion mutants apoA-I(Δ(1–41)Δ(185–243)) and apoA-I(Δ(1–59)Δ(185–243)). Similar results were obtained in HEK293 cells transfected with an ATP-binding cassette transporter A1 (ABCA1) expression plasmid. The double deletion mutant of apoA-I had reduced thermal and chemical stability compared with wild-type apoA-I. Sequential carboxyl-terminal deletions showed that cAMP-dependent cholesterol efflux was diminished in all the mutants tested, except the apoA-I(Δ(232–243)) which had normal cholesterol efflux. In cAMP-untreated or in mock-transfected cells, cholesterol efflux was not affected by the amino-terminal deletions, but decreased by 30–40% and 50–65% by the carboxyl-terminal and double deletions, respectively. After adenovirus-mediated gene transfer in apoA-I-deficient mice, wild-type apoA-I and apoA-I(Δ(1–41)) formed spherical high density lipoprotein (HDL) particles, whereas apoA-I(Δ(1–41)Δ(185–243)) formed discoidal HDL. The findings suggest that although the central helices of apoA-I alone can promote ABCA1-mediated lipid efflux, residues 220–231 are necessary to allow functional interactions between the full-length apoA-I and ABCA1 that are required for lipid efflux and HDL biogenesis.

Apolipoprotein A-I (apoA-I)

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Apoe 3′-5′-cyclic monophosphate; FBS, fetal bovine serum; HDL, high density lipoproteins; HER293 cells, human embryonic kidney 293 cells; Hisα-apoA-I, apoA-I with a histidine tag; Hisα-apoA-I, apoA-I without a histidine tag; rTEV protease, recombinant Tobacco Etched Viral protease; WT, wild-type; DMPC, dimyristoyl-L α-phosphatidylcholine; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay.
changes the membrane lipid microenvironment so that apoA-I can associate with the cell membrane and acquire phospholipids by binding in the proximity of ABCA1 (20). Analysis of five naturally occurring ABCA1 mutants, in the first and fourth extracellular domains, that are defective in cholesterol efflux showed that four of the five mutants failed to cross-link to apoA-I. The findings suggest that either direct binding of apoA-I to ABCA1 or insertion of apoA-I in the plasma membrane in close proximity to ABCA1 is necessary but not sufficient to promote cholesterol efflux (31). It has also been shown that ABCA1-mediated binding and lipid efflux are not specific for apoA-I but may also occur with other apolipoproteins that contain multiple amphipathic helical domains (27).

Previously, deletion mutants of apoA-I have been analyzed to unravel the structural requirements of apoA-I for inducing cholesterol efflux. Deletion of the carboxyl-terminal helices 8–10 in apoA-I (apoA-I[Δ187–243]) or apoA-I[Δ(210–243)] reduced the ability of the mutant protein to bind to macrophages and to promote efflux of cholesterol and phospholipid (32, 33). Likewise, deletion of amino acid residues 211–243 and 222–243 impaired the ability of apoA-I to promote cholesterol efflux from cultures of hepatocytes and fibroblasts (13, 34), whereas deletion of several helices in apoA-I (apoA-I[Δ(100–143)], apoA-I[Δ(122–165)], apoA-I[Δ(144–186)], apoA-I[Δ(135–243)], apoA-I[Δ(150–243)], and apoA-I[Δ(187–243)]) did not affect cholesterol efflux from the same cells (13, 32, 34).

In the present study we have analyzed the ability of various recombinant apoA-I mutants lacking amino- and carboxyl-terminal domains to promote efflux of cellular cholesterol and phospholipid from J774 mouse macrophages in the presence of a cAMP analog (i.e. ABCA1-catalyzed) or in the absence of a cAMP analog (i.e. at least partially independent of ABCA1). We have performed similar analysis in human embryonic kidney (HEK) 293 cells following transient transfection with an ABCA1 expression plasmid. Thereby we found evidence that the ABCA1-mediated cholesterol efflux by the full-length apoA-I requires a carboxyl-terminal domain in apoA-I, which encompasses amino acid residues 220–231. The analysis of apoA-I mutants containing deletions in the amino-terminal region, carboxyl-terminal region, or both domains suggests that although there is ligand specificity of the full-length apoA-I, the central helices of apoA-I alone are capable of promoting ABCA1-mediated lipid efflux and the formation of discoidal HDL in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials

DNA-modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Oligonucleotides for PCR, DH10Bac competent cells, cell culture reagents, recombinant Tobacco Etched Viral (rTEV) protease, and LipofectAMINE-2000 were purchased from Invitrogen. 1,2-[3H]Cholesterol (1 mCi/ml, specific activity range 40–60 Ci/mmol), methyl[3H]choline chloride (1 mCi/ml, specific activity range 60–90 Ci/mmol), and materials for PCR were obtained from PerkinElmer Life Sciences. Nickel-nitrotriacetic acid resin was purchased from Qiagen, Inc. (Valencia, CA). HiTrap Q HP columns, HiPrep Sepharyl S-200 column, Sepharose 6 FC column, and Q-Sepharose high performance resin were from Amersham Biosciences. 8-4 Chlorophenylthio/adenosine 3′-5′-cyclic monophosphate (cT-cAMP) and bovine serum albumin (BSA) were from Sigma. All other reagents were purchased from Sigma, Bio-Rad, Fisher, or other standard commercial sources.

Generation and Isolation of Recombinant Wild-type ApoA-I or ApoA-I Mutants in the Baculovirus System

To generate baculoviruses expressing wild-type (WT) and mutant apoA-I forms, a BamHI-SalI fragment containing human apoA-I cDNA was cloned into the polylinker region of pFASTBAC donor plasmid that contains the ampicillin and gentamycin resistance genes (Invitrogen). This recombinant plasmid also contains a histidine (His) tag and the rTEV protease cleavage site. The apoA-I containing plasmid was used to transform DH10Bac Escherichia coli cells. These cells had been transformed previously with the baculovirus genome containing the lacZ and kanamycin resistance genes, along with a helper plasmid containing the tetracycline resistance gene and the transposase genes. Transposition of the apoA-I gene into the lacZ gene disrupts the expression of the lacZ and provides a recombinant plasmid named bacmid.

The generation of the donor plasmid expressing WT apoA-I and of plasmids expressing apoA-I[Δ(1–59)], apoA-I[Δ(185–243)], and apoA-I[Δ(1–59)/Δ(185–243)] mutants was described previously (35, 36). The other mutant forms of apoA-I were produced in a similar manner. Briefly, the human apoA-I cDNA was tagged by PCR, using a set of flanking universal primers containing the restriction sites BamHI and SfiI, using the pBluescript-AI cDNA plasmid as template. The set of specific primers used in mutagenesis, and the set of universal primers 5′(AIWC-5) and 3′(AIWC-3) are shown in Table I. Specifically, we used 5′(AIM1–5) and 3′(AIM1–3) primers for the generation of apoA-I[Δ(1–41)], and 5′(AIM1–5) and 3′(AIM3–3) primers for the generation of apoA-I[Δ(1–41)/Δ(185–243)]. The DNA fragment containing the mutation of interest was digested with BamHI and SfiI, and cloned into the corresponding sites of pFASTBAC donor plasmid. Cells containing recombinant bacmids were selected by kanamycin, tetracycline, and gentamycin resistance as white colonies due to the disruption of lacZ sequence in the recombinant bacmid. Recombinant bacmid DNA was isolated from miniprep and used to transfect a monolayer of SF-9 insect cells (38–40). Recombinant viruses were isolated, amplified, titrated, and used to infect larger SF-9 cell cultures grown in suspension at 27 °C. SF-9 cells were pelleted and resuspended in a lysis buffer. The supernatant was used for the purification of apoA-I fusion proteins (His6- apoA-I) using a nickel-nitrotriacetic acid affinity column (41, 42). The pure apoA-I without the His tag (His- apoA-I), when needed, was obtained by cleavage with rTEV protease and purified by using again the nickel-nitrotriacetic acid affinity chromatography.

Expression and Purification of Recombinant WT and Mutant Pro-apoA-I Isomers in C127 Cells

Construction of plasmids expressing WT pro-apoA-I and pro-apoA-I[Δ(188–243)] has been described previously (44, 45). The pro-apoA-I[Δ(209–243)] deletion mutant was constructed in a similar manner. Briefly, the fourth exon of the human apoA-I gene was amplified and mutagenized by PCR, using a set of specific mutagenic primers (209S and 209A) containing the mutation of interest (a stop codon at nucleotide 209) and a set of flanking universal primers (AI501 and AI5101) containing the restriction sites NotI and SalI. The sequences of the primers are shown in Table I. The pUC4A-St promoter vector, which contains a NotI site in intron 3 and a SalI site in the 3′-end of the apoA-I gene, was used as the template for the generation of pro-apoA-I. The DNA fragment containing the mutation of interest was digested with NotI and SalI and cloned into the NotI and SalI sites of the pBMSTX-AI vector.

Periplasmic cell lines in mice mammary tumor C127 cells expressing the WT pro-apoA-I and the carboxyl-terminal truncation pro-apoA-I[Δ(188–243)] and pro-apoA-I[Δ(209–243)] were generated as described previously (43). C127 cell clones overproducing the WT and various apoA-I forms were grown in roller bottles on collagen-coated lead microspheres (Verax Corp.), and the proteins were purified from the serum-free medium as described previously (43) with some modifications. Briefly, the medium collected from the roller bottles was dialyzed against 0.01 m Tris, pH 8, and filtered. Up to 350 ml of the medium were passed through a 5-mL HiTrap Q HP column or up to 1200 ml were passed through a 25-ml Q-Sepharose high performance column at a rate of 1 ml/min. Both columns had been equilibrated with the Tris buffer. The proteins were eluted with a linear gradient of 1 m NH4HCO3 in the Tris buffer (15 volumes of elution buffer for HiTrap Q column and 5 volumes for Q-Sepharose high performance column) at the same flow rate. The fractions were analyzed by SDS-PAGE, and those containing the apoA-I protein were pooled and further concentrated down to 2 ml, using a Centripur concentrator (Amicon) with a molecular weight cutoff of 10,000. The concentrated sample was applied to a gel filtration column HiPrep Sepharyl S-200 and eluted with 1 column volume (120 ml) of 0.15 m NH4HCO3 buffer at a rate of 0.4 ml/min. The purity of the apoA-I preparation was assessed by SDS-PAGE, and fractions greater than 95% pure were recovered.

Expression of WT and Mutant Pro-apoA-I Isomers in an Adenovirus System

The construction of recombinant adenoviruses carrying the genomic sequence for the WT pro-apoA-I and pro-apoA-I[Δ(220–243)] has been described before (45). The adenovirus expressing pro-apoA-I[Δ(232–
243)] was generated in a similar way. The DNA fragments containing the mutations of interest were produced as described in the previous paragraph, using the 220S and 220A primers for the mutagenesis of pro-apoA-I[1(220–243)] and the 2325 and 232A primers for pro-apoA-I[1(232–243)] (Table I). The recombinant adenoviruses expressing pro-apoA-I[1(1–41)] and pro-apoA-I[1(1–41)Δ185–243)] were generated using cDNAs for apoA-I deletion mutants. The human apoA-I cDNA was mutagenized by PCR, using a set of primers containing the restriction sites BamHI and SaI and using the pBluescript-AI cDNA plasmid as template. The set of specific primers used in mutagenesis and the set of primers containing the restriction sites BamHI and SaI, designated AI–1(309F) and AI–1(309R), are shown in Table I. Specifically, we used AI–1(309F) and AI–1(309R) primers for the generation of pro-apoA-I[1(1–41)] and AI185F and AI185R primers for generating the pro-apoA-I[1(185–243)] form. For the generation of pro-apoA-I[1(1–41)Δ185–243)], AI–1(41)DF and AI–1(41)DR primers were used to further mutate the apoA-I cDNA using pBluescript-AI [1(185–243)] as template. Following amplification and mutagenesis, the DNA fragments were digested with BamHI and SaI and subcloned into the pCA13 vector under the control of the cytomegalovirus promoter (Microbix Biosystems Inc.). Recombinant adenovirus constructs carrying this cDNA were prepared following co-transfection of E1 transformed 293 cells with the plasmid pJM17 (Microbix Biosystems Inc.) and the appropriate pCA13 plasmid (45). Human HTB13 cells (SW 1783, human astrocytoma grown to 80% confluence in Leibovitz’s L-15 medium containing 10% (v/v) fetal bovine serum (FBS) in roller bottles) were infected with adenoviruses expressing WT pro-apo-A-I, pro-apo-A-I[1(220–243)], and pro-apo-A-I[1(232–243)] at a multiplicity of infection of 20. After 24 h of infection, the cells were washed twice with serum-free medium and preincubated in serum-free medium for 30 min, and fresh serum-free medium was added. After 24 h the medium was harvested, and fresh serum-free medium was added to the cells. The harvests were repeated 8–10 times.

The proteins were purified from the serum-free medium by ion exchange chromatography followed by gel filtration, as described above for C127 cells.

### Table I

| Name          | Sequence | Location and other features |
|---------------|----------|----------------------------|
| AIWC-5        | 5'-ACT GAA GCC TCC 3' | ACTCAA + BamHI site followed by nt 73–99 (sense) (amino acids +1 to +9) |
| AIWC-3        | 5'-ACT GAA GCC TCC 3' | ACTCAA + SaI site followed by nt 864–832 (antisense) (poly(A) region) |
| AIM-5         | 5'-AGC GAA GCC TCC 3' | AGC+ + BamHI site followed by nt 196–225 (sense) (amino acids +42 to +51) |
| AIM-3         | 5'-AGC GTC GAC 3' | AGC+ + SaI site followed by nt 864–832 (antisense) (poly(A) region) |
| AINoI        | 5'-CAG GTC GAC TCA 3' | AGC+ + SaI site + stop codon followed by nt 624–598 (antisense) (amino acids +184 to +176) |
| AIXhO1        | 5'-TCC TCT AGA GTC GAC CCT TGC TCG AGC CCC TTT 3' | nt 216–2203 of pUC-A1-1, vector, 3' end of apoA-I gene |
| 209S         | 5'-AAG GCC TAA GGC TCT GAC GAC 3' | nt 658–691 (sense) (amino acids +206 to +213) |
| 209A         | 5'-GTC GTC GAC GAG TTA CTC GCT TCT 3' | nt 691–688 (antisense) (amino acids +213 to +206) |
| 220S         | 5'-CAG GTC GAC TGT GTC TCT GAG AGC 3' | nt 716–744 (sense) (amino acids +216 to +224) |
| 220A         | 5'-CCT CTC GAC CAC TGT CAG GCC TTC 3' | nt 744–718 (antisense) (amino acids +224 to +216) |
| 232S         | 5'-AGC TCT GCT AGA TCA CCT CCT GAC 3' | nt 754–780 (sense) (amino acids +228 to +236) |
| 232A         | 5'-GTA CTC GCT CCT GAC TCA AGA GCT CAA 3' | nt 780–754 (antisense) (amino acids +236 to +228) |
| AI1(1–59)F   | 5'-TATT ATT GTC GAC ATG ACA GGT GCT CTT ACC TGC 3' | TATATT + SaI site followed by nt 2–24 (sense) (amino acids +24 to +18) (antisense) (amino acids +243 to +237) |
| AI1(1–59)R   | 5'-ATT CTT AGA GGC TCC TGA GGT GTT CCT 3' | AATTCTCTAGA + BamHI site followed by nt 804–780 (antisense) (amino acids +243 to +237) |
| AI1(41)DF    | 5'-GGC GTC CTC TCC TGG CAA CTA AAT CAG TCT 3' | nt 52–72 and 196–216 (sense) (amino acids −7 to −1 and +42 to +49) |
| AI1(41)DR    | 5'-GTC GAG GAG CCT TCC GTT TCT CCA GAA ATG 3' | nt 215–196 and 72–52 (antisense) (amino acids +49 to +24 and −1 to −7) |
| AI185F       | 5'-CTC AAG GAG TCT GGC GCC AGA CGT 3' | nt 613–639 (sense) (amino acids +181 to +189) |
| AI185R       | 5'-CAT CAG GCC TCC GTT GCT CTT GAG 3' | nt 639–613 (antisense) (amino acids +189 to +181) |

* The restriction enzyme recognition sites are marked in boldface type.
* nt., nucleotide number of the published apoA-I cDNA sequence (37), oligonucleotide position (+) relative to the transcription initial site (i.e., +1 from ATG).
* Amino acid position (+) relative to the mature apoA-I (i.e., +1 from mature apoA-I protein).
* The stop codon is underlined.

### ABCA1 Expression Plasmid

The expression plasmid of human ABCA1 was a generous gift of Dr. M. W. Freeman and was constructed as described (46).

### Cell Cultures and Lipids Efflux Assays

Studies in J774 Mouse Macrophages—J774 mouse macrophages were grown in RPMI 1640 with 10% (v/v) FBS and antibiotics. J774 macrophages were plated in 12-well plates at density of 5 × 10⁵ cells/well. The following day, cells were labeled with 1 ml of labeling medium (6 μCi/ml 1.23[H]cholesterol or 1 μCi of methyl[3H]choline chloride dissolved in 0.1% (v/v) ethanol as the final concentration and dispersed into RPMI 1640 supplemented with 0.2% (w/v) BSA) for 24 h. Following 24 h of labeling, cells were washed twice with serum-free medium and equilibrated for 24 h with or without 0.3 mM cpt-cAMP in 1 ml of RPMI 1640 supplemented with 0.2% (w/v) BSA. At the end of the treatment period with cpt-cAMP, cells were washed twice with serum-free medium and incubated with 1 ml of RPMI 1640 supplemented with 0.2% (w/v) BSA. After 24 h of incubation, the media were collected and clarified by centrifugation in a microcentrifuge for 2 min. The radioactivity in 40 μl of the supernatant was determined by liquid scintillation counting. For phospholipid efflux experiments, after 4 h of incubation, the media were collected and clarified by centrifugation in a microcentrifuge for 2 min. A 100-μl aliquot of the supernatant was first extracted with chloroform and methanol (47), and then the radioactivity of the chloroform phase was determined by liquid scintillation counting. At the end of the incubation, cells were lysed by 800 μl of lysis buffer (PBS containing 1% (v/v) Triton X-100) for 30 min at room temperature. For cholesterol efflux experiments, the radioactivity in 40 μl of each cell lysate was determined by scintillation counting. For phospholipid efflux experiments, the phospholipids in 100 μl of each lysate were extracted with chloroform and methanol (47), and the radioactivity was determined by scintillation counting. The percentage of secreted [3H]lipid was calculated by dividing the radioactivity determined in the medium by the radioactivity found in the supernatant.
cells was subtracted from the lipid efflux of the cells treated with apoA-IV.

Studies in HEK293 Cells following Transfection with an ABCA1 Expression Plasmid—HEK293-EBNA cells were cultured in DMEM (high glucose) with 10% (v/v) FBS and antibiotics (31, 46). One day before transfection, the cells were plated on 24-well plates coated with poly-l-lysine at density of 2 × 10⁴ cells/well in the same medium without antibiotics. The next day, cells at about 95% confluence were transfected with LipofectAMINE 2000 and ABCA1 or pcDNA1 (mock) (Invitrogen) plasmids for 16 h. The DNA-LipofectAMINE 2000 complexes were formed in serum-free Opti-MEM I. After this period of time, cells were labeled with 0.5 μCi/ml 1,2-3H[Cholesterol in DMEM (high glucose) supplemented with 10% (v/v) FBS. Following 24 h of labeling, cells were washed twice with PBS and incubated for 1 h with DMEM (high glucose) supplemented with 0.2% (w/v) fatty acid-free BSA. At the end of this period, cells were incubated with 0.5 ml of DMEM (high glucose) and supplemented with 0.2% (w/v) fatty acid-free BSA with or without 1 μl WT apoA-I or mutant forms at 37°C for 4 h. The media were collected and clarified by centrifugation in a microcentrifuge for 2 min, and the radioactivity in 300 μl of the supernatant was determined by liquid scintillation counting. The cells were lysed with 0.1 ml NaOH, and the radioactivity in a 300-μl aliquot of the cell lysate was determined by scintillation counting. [3H]Cholesterol efflux was expressed as the percentage of the radioactivity released in the medium relative to the total radioactivity in cells and medium. To calculate the net ABCA1-mediated cholesterol efflux, the cholesterol efflux of the cells transfected with the control plasmid (mock) was subtracted from the cholesterol efflux of the cells transfected with the ABCA1 plasmid.

Animal Studies

Male apoA-I-deficient mice (apoA-I−/−;apoA-I−/−) C57BL/6J mice (48) were purchased from The Jackson Laboratories (Bar Harbor, ME). The mice were maintained on a 12-h light/dark cycle and standard rodent chow. The mice were 6–8 weeks old at the time of injection of the adenoviruses. All procedures performed on the mice were in accordance with the National Institute of Health and institutional guidelines. The A1−/− mice were injected via the tail vein with 1 × 10⁶ plaque-forming units of recombinant adenovirus per animal, and the animals were sacrificed 4 days post-injection following a 4-h fast. The plasma of the mice was adjusted to 1 mM phenylmethylsulfonyl fluoride, 0.2% (w/v) aprotinin, 0.1% (w/v) EDTA, and 0.002% (w/v) sodium azide prior to analysis of lipids, lipoproteins, and apoA-I.

Cholesterol, Triglycerides, and Plasma ApoA-I Levels and Steady-state Hepatic Human ApoA mRNA Levels

The concentration of cholesterol and triglycerides in plasma was determined using the INFINITY triglyceride reagent (Sigma) according to the manufacturer (51). The concentration of cholesterol and triglycerides in plasma was determined using the INFINITY cholesterol reagent (Sigma) and 10% (v/v) FBS and antibiotics (31, 46). One day before transfection, the cells were transfected with the ABCA1 plasmid. The next day, cells at about 95% confluence were transfected with LipofectAMINE 2000 and ABCA1 or pcDNA1 (mock) (Invitrogen) plasmids for 16 h. The DNA-LipofectAMINE 2000 complexes were formed in serum-free Opti-MEM I. After this period of time, cells were labeled with 0.5 μCi/ml 1,2-3H[Cholesterol in DMEM (high glucose) supplemented with 10% (v/v) FBS. Following 24 h of labeling, cells were washed twice with PBS and incubated for 1 h with DMEM (high glucose) supplemented with 0.2% (w/v) fatty acid-free BSA. At the end of this period, cells were incubated with 0.5 ml of DMEM (high glucose) and supplemented with 0.2% (w/v) fatty acid-free BSA with or without 1 μl WT apoA-I or mutant forms at 37°C for 4 h. The media were collected and clarified by centrifugation in a microcentrifuge for 2 min, and the radioactivity in 300 μl of the supernatant was determined by liquid scintillation counting. The cells were lysed with 0.1 ml NaOH, and the radioactivity in a 300-μl aliquot of the cell lysate was determined by scintillation counting. [3H]Cholesterol efflux was expressed as the percentage of the radioactivity released in the medium relative to the total radioactivity in cells and medium. To calculate the net ABCA1-mediated cholesterol efflux, the cholesterol efflux of the cells transfected with the control plasmid (mock) was subtracted from the cholesterol efflux of the cells transfected with the ABCA1 plasmid.

Expression Plasmid cpt-cAMP.

Cells transfected with the ABCA1 plasmid. The next day, cells at about 95% confluence were transfected with LipofectAMINE 2000 and ABCA1 or pcDNA1 (mock) (Invitrogen) plasmids for 16 h. The DNA-LipofectAMINE 2000 complexes were formed in serum-free Opti-MEM I. After this period of time, cells were labeled with 0.5 μCi/ml 1,2-3H[Cholesterol in DMEM (high glucose) supplemented with 10% (v/v) FBS. Following 24 h of labeling, cells were washed twice with PBS and incubated for 1 h with DMEM (high glucose) supplemented with 0.2% (w/v) fatty acid-free BSA. At the end of this period, cells were incubated with 0.5 ml of DMEM (high glucose) and supplemented with 0.2% (w/v) fatty acid-free BSA with or without 1 μl WT apoA-I or mutant forms at 37°C for 4 h. The media were collected and clarified by centrifugation in a microcentrifuge for 2 min, and the radioactivity in 300 μl of the supernatant was determined by liquid scintillation counting. The cells were lysed with 0.1 ml NaOH, and the radioactivity in a 300-μl aliquot of the cell lysate was determined by scintillation counting. [3H]Cholesterol efflux was expressed as the percentage of the radioactivity released in the medium relative to the total radioactivity in cells and medium. To calculate the net ABCA1-mediated cholesterol efflux, the cholesterol efflux of the cells transfected with the control plasmid (mock) was subtracted from the cholesterol efflux of the cells transfected with the ABCA1 plasmid.

The concentration of cholesterol and triglycerides in plasma was determined using the INFINITY cholesterol reagent (Sigma) and 10% (v/v) FBS and antibiotics (31, 46). One day before transfection, the cells were transfected with the ABCA1 plasmid. The next day, cells at about 95% confluence were transfected with LipofectAMINE 2000 and ABCA1 or pcDNA1 (mock) (Invitrogen) plasmids for 16 h. The DNA-LipofectAMINE 2000 complexes were formed in serum-free Opti-MEM I. After this period of time, cells were labeled with 0.5 μCi/ml 1,2-3H[Cholesterol in DMEM (high glucose) supplemented with 10% (v/v) FBS. Following 24 h of labeling, cells were washed twice with PBS and incubated for 1 h with DMEM (high glucose) supplemented with 0.2% (w/v) fatty acid-free BSA. At the end of this period, cells were incubated with 0.5 ml of DMEM (high glucose) and supplemented with 0.2% (w/v) fatty acid-free BSA with or without 1 μl WT apoA-I or mutant forms at 37°C for 4 h. The media were collected and clarified by centrifugation in a microcentrifuge for 2 min, and the radioactivity in 300 μl of the supernatant was determined by liquid scintillation counting. The cells were lysed with 0.1 ml NaOH, and the radioactivity in a 300-μl aliquot of the cell lysate was determined by scintillation counting. [3H]Cholesterol efflux was expressed as the percentage of the radioactivity released in the medium relative to the total radioactivity in cells and medium. To calculate the net ABCA1-mediated cholesterol efflux, the cholesterol efflux of the cells transfected with the control plasmid (mock) was subtracted from the cholesterol efflux of the cells transfected with the ABCA1 plasmid.

Electron Microscopy

Aliquots of the fractions from equilibrium density gradient centrifugation after dialysis against ammonium acetate and carbonate buffer were stained with sodium phosphotungstate, visualized in the Phillips CM-129 electron microscope (Phillips Electron Optics, Eindhoven, Netherlands), and photographed as described previously (50). The photomicrographs were taken at ×75,000 magnification and enlarged 3 times.

Preparation and Electron Microscopy Analysis of rHDL Containing POPC:Cholesterol:Apo-I

rHDL particles were prepared by the sodium cholate dialysis method (51), using POPC:cholesterol:apoA-I:sodium cholate in a molar ratio of 100:10:1:100, as described previously (52). The lipid-free apoA-I that was not incorporated in the lipid-protein complexes was removed by dialysis overnight against PBS buffer at 4°C with at least 3 changes of 4 liters each, using dialysis tubing of 50,000 daltons molecular weight cut-off. The rHDL samples were adjusted either by dilution or by concentration to a final protein concentration of 1 mg/ml. A 5-μl aliquot of each sample was used for electron microscopy as described above. The photomicrographs were taken at ×45,000 magnification and enlarged 2.5 times.

CD Spectroscopy

CD measurements were performed on an AVIV 62 DS spectropolarimeter (AVIV Associates, Inc.), equipped with a thermoelectric temperature control and calibrated with d-10-camphorsulfonic acid in 5-, 2-, or 1-mm path length quartz cuvettes, at protein concentration 20–100 μg/ml. Far-UV CD spectra were recorded as described previously (44, 53). The spectra were normalized to molar residue ellipticity using a molar residue weight of 115.0 for WT apoA-I, 115.3 for A-I32→K11 (41), and 116.7 for A-I32→K11Δ (155–243). The complete superimposition of the normalized spectra obtained at various protein concentrations for each recombinant apoA-I is consistent with the absence of protein self-association within the range of protein concentrations used in our experiments. The α-helix content was estimated from the molar residue ellipticity at 222 nm [θ222] (54).

Thermal and Chemical Unfolding Monitored by CD

Monitoring of thermal and chemical unfolding of apoA-I by ellipticity at 222 nm was performed at protein concentrations 25–80 μg/ml as described previously (44, 53). The midpoint (melting temperature), Tm, and van’t Hoff enthalpy, ΔHv, of the temperature-induced transitions were determined from van’t Hoff plots (55). In Keq versus 1/β, where Keq is the apparent equilibrium constant, and β is temperature in degrees Kelvin. The equilibrium constant was calculated as Keq (T) = (θH − θobs)/(θH − θc), where θobs is the observed ellipticity, and θH and θc are base lines for the protein folding and unfolding states determined by
Lipid Efflux and HDL Formation by ApoA-I Mutants

6723

and then incubated in serum-free culture medium for 24 h in the presence or absence of 0.3 μM of the cAMP analog cpt-cAMP. cAMP analogs have been reported to enhance the expression of ABCA1 (18, 58, 59). Cholesterol efflux was monitored as a function of time in the presence of 1 μM lipid-free WT or mutant forms of apoA-I, as described under “Experimental Procedures.” The apoA-I concentration used is higher than that required for saturable cholesterol or phospholipid efflux in untreated or cpt-cAMP-treated J774 macrophages (60) and in transiently or stably transfected cells expressing the ABCA1 transporter (27, 28).

The time course of cholesterol efflux from untreated (cAMP-independent cholesterol efflux) and cpt-cAMP-treated (total cholesterol efflux) cells, respectively, using apoA-I forms lacking the amino-terminal 1–41 or 1–59 segments or the carboxyl-terminal 185–243 segment or both amino- and carboxyl-terminal segments, was monitored over a 6-h period. The time course of the net cpt-cAMP-dependent cholesterol efflux was obtained by subtracting the efflux curves of the untreated cells from the treated cells. This analysis indicated that the efflux curves (total, cpt-cAMP-independent [data not shown] and cpt-cAMP-dependent [Fig. 2A]) are almost linear over a 4-h period. To facilitate the analysis of the cholesterol efflux by the different apoA-I forms, we tabulated total, cpt-cAMP-independent, and cpt-cAMP-dependent efflux after 4 h of incubation with 1 μM WT apoA-I and mutants at 37 °C, setting the efflux values for WT apoA-I to 100% (Fig. 2B). (Similar values were obtained using the efflux results determined after 2 h of incubation.)

The cpt-cAMP-dependent efflux of cellular cholesterol was not affected by amino-terminal deletions (apoA-I[Δ(1–41)] and apoA-I[Δ(1–59)]) but was decreased by ~30% by the carboxyl-terminal truncation of apoA-I (apoA-I[Δ(185–243)]). Removal of both amino-terminal and carboxyl-terminal domains in apoA-I (apoA-I[Δ(1–41)Δ(185–243)]) and apoA-I[Δ(1–59)Δ(185–243)]) decreased the cpt-cAMP-independent cholesterol efflux by 50–65% (Fig. 2B, white bars). The cpt-cAMP-dependent cholesterol efflux was reduced by 80% compared with WT apoA-I when the carboxyl-terminal apoA-I[Δ(185–243)] deletion mutant was used as the cholesterol acceptor. Much less pronounced impairments of cholesterol efflux were seen in the presence of apoA-I variants with amino-terminal deletions since removal of the amino terminus decreased cholesterol efflux by 15%. Unexpectedly, cpt-cAMP-dependent cholesterol efflux was restored to 78% of WT apoA-I when the cholesterol efflux assay was performed in the presence of double deletion mutants lacking both the amino- and carboxyl-terminal domains (Fig. 2B, shaded bars). These findings indicate that the various apoA-I mutants affect differently cpt-cAMP-dependent and -independent cholesterol efflux.

The baculovirus system produces a fusion of apoA-I-(1–243) with a 28-residue amino-terminal segment with the sequence MSYHHHHHHHHYDDPIFTNFLVFQAGMS. This segment contains 6 His residues, a spacer region, and the TEV protease cleavage site. Removal of the His tag by rTEV protease generates a His6-apoA-I, which still contains an additional 5 amino-terminal sequence (AGMS). To assess the potential interference of the His tag on the acceptor properties of apoA-I, control cholesterol efflux experiments were performed for 4 h using 1 μM His6 and His6-apoA-I forms (WT, apoA-I[Δ(1–41)], apoA-I[Δ(1–59)], apoA-I[Δ(185–243)], apoA-I[Δ(1–41)Δ(185–243)], and apoA-I[Δ(1–59)Δ(185–243)]). This analysis showed that the His tag does not affect the cpt-cAMP-dependent and cpt-cAMP-independent cholesterol efflux properties of WT apoA-I and the carboxyl-terminal deletion mutant (data not shown). However, the presence of His tag reduced by 38–41 and 25–32% the cpt-cAMP-dependent cholesterol efflux of the amino-terminal deletion mutants and the double deletion mutants.
FIG. 2. Lipid efflux from J774 mouse macrophages in the presence of lipid-free WT and mutant forms of apoA-I. A, time course of the cpt-cAMP-dependent [3H]cholesterol efflux. Cells were labeled with 6 μCi/ml [3H]cholesterol for 24 h in RPMI 1640 containing 0.2% (w/v) BSA. Cells were then incubated for 24 h in the absence or presence of 0.3 mM cpt-cAMP followed by incubation with 1 μM WT and mutants apoA-I for up to 6 h, at 37°C. Aliquots of media were removed at the indicated time points, and the radioactivity in the media, as well as in the cell lysate at the 6th time point, was determined as described under “Experimental Procedures.” The percent of [3H]cholesterol efflux represents the amount of the radioactivity released in the medium divided by the total radioactivity present in the culture medium and the cell lysate. The percent of the net cpt-cAMP-dependent [3H]cholesterol efflux was calculated as the difference in percent of cholesterol efflux for every time point between treated and untreated cells. Values are the means ± S.D. of three to four independent experiments performed in duplicate. B, effect of amino- and carboxyl-terminal deletions or double deletions of both the amino- and carboxyl-terminal regions of apoA-I on cholesterol efflux from J774 mouse macrophages. Cholesterol efflux following 4 h of incubation at 37°C was determined as described in A. Black and white bars show the percent of [3H]cholesterol efflux from cpt-cAMP-treated and -untreated cells, respectively. Shaded bars show the percent of the net cpt-cAMP-dependent [3H]cholesterol efflux. The numbers on top of the bars represent the cholesterol efflux relative to the WT control set to 100%. Values are the means ± S.D. from three to four independent experiments performed in duplicate. C, effect of amino- and carboxyl-terminal deletions or double deletions of both the amino- and carboxyl-terminal regions of apoA-I on ABCA1-mediated efflux of cellular cholesterol in HEK293 cells transfected with an ABCA1 expression plasmid. Cells were transfected with empty vector (mock) or with ABCA1 plasmid, labeled with 0.5 μCi/ml [3H]cholesterol for 24 h in DMEM (high glucose) containing 10% (v/v) FBS, and then incubated with 1 μM WT and mutants apoA-I forms, in DMEM (high glucose) containing 0.2% (w/v) free fatty acid BSA for 4 h at 37°C. Media and cells were collected separately, and the radioactivity in the media as well as in the cell lysate was determined as described under “Experimental Procedures.” The percent of [3H]cholesterol efflux represents the amount of the radioactivity released in the medium divided by the total radioactivity present in the culture medium and the cell lysate. Black and white bars show the percent of the cholesterol efflux from ABCA1-transfected cells or mock-transfected cells, respectively. Shaded bars show the percent of the net ABCA1-mediated [3H]cholesterol efflux, calculated as the difference in percent of cholesterol efflux between ABCA1-transfected and mock-transfected cells. The numbers on top of the bars represent the cholesterol efflux relative to the WT control set to 100%. Values are the means ± S.D. from three independent experiments performed in duplicate. D, effect of carboxyl-terminal deletions of apoA-I on cholesterol efflux from J774 mouse macrophages. Cholesterol efflux following 4 h of incubation at 37°C was determined as described in A. Black, white, and shaded bars represent total, cpt-cAMP-independent, and cpt-cAMP-dependent efflux, respectively, obtained as described in B. WT apoA-I represents pro-apoA-I produced by stably transfected cells C127, and WTA apoA-I represents pro-apoA-I produced by HTB13 cells infected with adenovirus expressing the pro-apoA-I. The numbers on top of the bars represent the cholesterol efflux relative to the WT control set to 100%. Values are the means ± S.D. from three independent experiments performed in duplicate. E, effect of amino- and carboxyl-terminal deletions or double deletions of both the amino-, carboxyl-terminal regions of apoA-I on phospholipid efflux from J774 mouse macrophages. Cells were labeled with 1 μCi/ml [3H]choline for 24 h in RPMI 1640 containing 0.2% (w/v) BSA. Cells were then incubated for 24 h in the absence or presence...
Lipid Efflux and HDL Formation by ApoA-I Mutants

respectively, as compared with the corresponding His-apoA-I forms (data not shown). The presence of His tag also reduced 27–53% the cpt-cAMP-independent efflux capacities of amino-terminal deletion mutants but had no significant effect on the double mutants (data not shown). These observations led us to utilize the His-apoA-I forms in the preceding and the following experiments.

Effect of Amino-, Carboxyl-terminal and Double Deletion Mutants of ApoA-I on ABCA1-mediated Efflux of Cellular Cholesterol in HEK293 Cells Transfected with an ABCA1 Expression Plasmid—To confirm that cAMP-dependent cholesterol efflux of amino-, carboxyl-terminal and double deletion mutants of apoA-I was mediated by ABCA1, we compared the cAMP-dependent cholesterol efflux to the ABCA1-dependent efflux. HEK293-EBNA cells were transfected with empty vector or an ABCA1 expression plasmid for 16 h, as described under “Experimental Procedures.” Following transfection, the cells were labeled with [3H]cholesterol for 24 h and then incubated with or without 1 μM lipid-free WT or mutant forms of apoA-I for 4 h. The efflux values obtained for WT apoA-I were set as 100%.

The cholesterol efflux pattern in HEK293-EBNA cells transfected with an ABCA1 plasmid was similar to that of cAMP-dependent cholesterol efflux in J774 macrophages (compare Fig. 2, B and C). Compared with WT-apoA-I, ABCA1-mediated cholesterol efflux was 20% in the presence of apoA-I[Δ(185–243)] and 72 and 107% in the presence of apoA-I[Δ(1–41)] and apoA-I[Δ(1–59)], respectively. Similarly to the cAMP-dependent cholesterol efflux experiments, the deletion of both the amino-terminal and the carboxyl-terminal regions restored the ABCA1-mediated cholesterol efflux capacity of apoA-I to 76 and 97% of the WT control.

In mock-transfected cells, efflux of cellular cholesterol followed the same pattern as in cAMP-untreated cells (compare Fig. 2, B and C). Similar to the findings of Fig. 2B, the control experiment of Fig. 2C showed that cholesterol efflux was not affected by amino-terminal deletions (apoA-I[Δ(1–41)] and apoA-I[Δ(1–59)]), was decreased by ~40% by the carboxyl-terminal truncation of apoA-I (apoA-I[Δ(185–243)]), and was further decreased by 45–60% by the double amino- and carboxyl-terminal domain mutants (apoA-I[Δ(1–41)Δ(185–243)] and apoA-I[Δ(1–59)Δ(185–243)]) compared with the WT control.

Mapping the Carboxyl-terminal Domains Necessary for cAMP-dependent Cholesterol Efflux by the Full-length ApoA-I Molecule—For a more detailed localization of the cholesterol efflux promoting domain within the carboxyl-terminal domain of apoA-I, we studied pro-apoA-II[Δ(198–243)], pro-apoA-I[Δ(209–243)], apoA-I[Δ(220–243)], and apoA-I[Δ(232–243)] (Fig. 1) for their cholesterol efflux capacity.

The cpt-cAMP-independent cholesterol efflux values for the WT pro-apoA-I produced by either the permanent cell lines or the adenovirus system were much lower than the efflux values observed for the WT apoA-I produced with the baculovirus system (compare Fig. 2, B and D) but were at the same levels as the efflux values obtained with human apoA-I isolated from plasma (data not shown). By contrast to cpt-cAMP-independent, the cpt-cAMP-dependent cholesterol efflux values for all four WT apoA-I were similar (Fig. 2, B and D).

The cpt-cAMP-independent cholesterol efflux was very low for the WT pro-apoA-I and barely detectable in the pro-apoA-I mutants with carboxyl-terminal truncations (Fig. 2D, white bars). The cpt-cAMP-dependent cholesterol efflux was 20, 33, 9, and 99% of the WT control value for the mutants lacking the carboxyl-terminal domains 198–243, 209–243, 220–243, and 232–243, respectively (Fig. 2D, shaded bars). These data suggest that amino acid residues 220–231 of apoA-I form a domain that is important for the ability of the intact apoA-I to induce cpt-cAMP-dependent cholesterol efflux.

Effect of Amino-, Carboxyl-, and Double Deletion Mutants of ApoA-I on cAMP-dependent Efflux of Cellular Phospholipid from J774 Mouse Macrophages—To explore further whether the amino- and carboxyl-terminal domains of apoA-I affect the cholesterol and the phospholipid efflux to a similar degree, we performed phospholipid efflux studies in J774 mouse macrophages using WT apoA-I, apoA-I[Δ(1–41)], apoA-I[Δ(1–59)], apoA-I[Δ(185–243)], apoA-I[Δ(1–41)Δ(185–243)], and apoA-I[Δ(1–59)Δ(185–243)] as phospholipid acceptors. For these studies the cells were labeled with 3H]choline chloride for 24 h and then incubated in serum-free medium in the presence or absence of 0.3 mM cpt-cAMP for 24 h, and the efflux of 3H-phospholipid in the presence of 1 μM of the WT and the indicated apoA-I forms was determined following a 4-h incubation period. The efflux values obtained for WT apoA-I were set as 100%.

The analysis showed that the cpt-cAMP-dependent phospholipid efflux followed similar patterns to those described for cholesterol efflux. Compared with WT apoA-I, cpt-cAMP-dependent phospholipid efflux was 8% in the presence of apoA-I[Δ(185–243)] and 72 and 102% in the presence of apoA-I[Δ(1–41)] and apoA-I[Δ(1–59)], respectively. As in the cholesterol efflux experiments, the deletion of both the amino- and the carboxyl-terminal segments restored the phospholipid efflux capacity of apoA-I to 75% (compare Fig. 2, B and E).

The cpt-cAMP-independent phospholipid efflux was low for WT apoA-I, was decreased approximately by about 50% for the two amino-terminal deletion mutants, and was barely detectable in the other mutants.

Physicochemical Analysis of the Amino-terminal Deletion ApoA-I[Δ(1–41)] and Double Deletion ApoA-I[Δ(1–41)Δ(185–243)] Mutants—The unexpected observation that the double deletion mutants restored the lipid efflux activity that was lost when only the carboxyl-terminal domain of apoA-I was deleted prompted us to study the conformation and the thermal and chemical stability of this unusual mutant.

The α-helical content of the WT apoA-I, the amino-terminal deletion apoA-I[Δ(1–41)], and the double deletion apoA-I[Δ(1–41)Δ(185–243)] mutants was estimated from the normalized far-UV CD spectra. It was found that compared with the WT apoA-I, the 41-amino acid amino-terminal truncation Δ(1–41) of apoA-I does not change the α-helical content significantly. In contrast, the 100-amino acid double truncation Δ(1–41)Δ(185–243) of apoA-I leads to an ~7% reduction in the α-helical content of the mutant protein (Table II).

The thermal unfolding curves monitored by the ellipticity at 222 nm, [θ]222(T), are shown in Fig. 3A; the corresponding van’t Hoff plots, ln K_m/°C, are shown in the inset; and the values of the melting temperature, T_m, and the effective enthalpy, ΔH_m, determined by the van’t Hoff analysis are given in Table II. This analysis did not show a significant effect of the Δ(1–41) truncation on the melting temperature (T_m) but did show an ~9 °C reduction in T_m as a result of the double truncation Δ(1–41)Δ(185–243), indicating a significant destabiliza-
Lipid Efflux and HDL Formation by ApoA-I Mutants

| Protein                | No. residues | α-Helix% | In protein | In helix% | Tm°C | ΔHf kcal/mol | ΔGf ΔHf kcal/mol | m °C | D1/2μ |
|------------------------|--------------|----------|------------|-----------|------|--------------|-----------------|------|-------|
| WT apoA-I              | 58           | 248      | ~144       | 59 ± 1    | 40 ± 1 | 2.5 ± 0.1    | 2.4 ± 0.1       | 1.0 ± 0.05 |
| ApoA-I(Δ(1–41))        | 57           | 207      | ~118       | 58 ± 2    | 28 ± 4  | 1.4 ± 0.1Vi | 1.8 ± 0.2      | 0.8 ± 0.07 |
| ApoA-I(Δ(1–41)Δ(185–243)) | 51f        | 148      | ~75        | 50 ± 1f   | 29 ± 2f | 1.4 ± 0.3f  | 2.1 ± 0.3       | 0.7 ± 0.03f |
| WT apoA-I              | 58           | 249      | ~144       | 58 ± 1    | 41 ± 1  | 2.3 ± 0.1    | 2.1 ± 0.15      | 1.1 ± 0.06 |
| ApoA-I(Δ(185–243))     | 59           | 190      | ~112       | 57 ± 1    | 39 ± 0.4 | 2.2 ± 0.1   | 2.0 ± 0.15      | 1.1 ± 0.1  |

* Estimated from the value [θ222°] at 25 °C according to Ref. 54; systematic error of the estimation is ±3%, statistical error is within ±1–3%.

* Determined by multiplying the number of residues in the protein by its α-helical content.

* Melting temperature (Tm) and effective enthalpy ΔHf of thermal unfolding were determined from van’t Hoff analysis of the melting curves monitored by ellipticity at 222 nm.

* Conformational stability, ΔGf, midpoint of chemical denaturation, D1/2, and m values were determined by the linear extrapolation method from CD-monitored chemical unfolding curves.

* WT apoA-I, apoA-I(Δ(1–41)) and apoA-I(Δ(1–41)Δ(185–243)) were produced by the baculovirus system.

* Significance of differences from the value for WT apoA-I is p < 0.05.

* Significance of differences from the value for WT apoA-I is p < 0.01.

* Data from Ref. 44, WT apoA-I and apoA-I(Δ(185–243)) were produced by stably transfected C127.

### Table II

| α-Helical content and thermodynamic parameters of lipid-free WT or truncated forms of apoA-I determined by far-UV CD |
|----------------------------------------------------------------------------------------------------------------|
| Values are the means ± S.D. from three to six experiments for two or three independent preparations of each protein. |

### Fig. 3

**Thermal unfolding (A) and denaturant-induced unfolding (B) of WT and truncated forms of apoA-I monitored by the ellipticity at 222 nm.** The symbols indicate the WT apoA-I ( • ), the apoA-I[Δ(1–41)] truncation (○), and the apoA-I[Δ(1–41)Δ(185–243)] double truncation ( △ ). The inset shows the van’t Hoff plots (ln Kd versus 1/T) derived from the corresponding thermal unfolding curves.

### Kinetics of Binding of ApoA-I to DMPC Liposomes—The kinetics of association of lipid-free WT apoA-I, amino-terminal deletion and double deletion mutants of apoA-I with DMPC multilamellar vesicles was followed by the decrease in the turbidity at 325 nm, which reflects the formation of DMPC-apoA-I complexes. These data for WT and each mutant apoA-I are shown in Fig. 4A. It was found that both the apoA-I[Δ(1–41)] mutant and the double deletion mutant apoA-I[Δ(1–41)Δ(185–243)] have a much lower rate of association with DMPC liposomes as compared with the WT apoA-I (Fig. 4A).**

### Formation of Discoidal POPC-ApoA-I Particles by the WT ApoA-I and the Truncated ApoA-I Form ApoA-I[Δ(1–41)]Δ(185–243)—The sodium cholate dialysis method allowed the formation of discoidal particles with the WT and the double-truncated apoA-I form (Fig. 4, B and C). Under the negative staining conditions used, the electron micrographs showed that these particles form the typical "rouleaux," indicating that they are discoidal and have the thickness of a phospholipid bilayer. The number of rouleaux observed depends on the concentration of the sample on the carbon grid. In less concentrated samples, a large number of round particles that lie flat on the grid were also observed. The findings indicate that the double truncation...
of apoA-I did not affect the ability of the central helices to form discoidal particles in vitro.

Analysis of the Ability of the Amino-terminal Deletion and Double Deletion Mutant of apoA-I to Form HDL in Vivo—The ability of the WT and truncated mutants of apoA-I to form HDL in vivo was tested by adenovirus-mediated gene transfer in apoA-I−/− mice. Four days post-infection, plasma was collected and analyzed for plasma lipid and apoA-I levels and the presence of HDL particles. Livers were also collected and analyzed for human apoA-I mRNA levels to assess the expression of the apoA-I. Table III shows the plasma cholesterol, triglycerides, and apoA-I levels and the hepatic apoA-I mRNA levels of the mice 4 days post-infection. The apoA-I concentration was estimated by three different approaches as described under “Experimental Procedures,” and the results are shown in Table III. These analyses showed that after normalization for the mRNA levels, there is still an ~15-fold difference between the observed and expected plasma concentration of apoA-I between the WT and the double deletion mutant. A similar difference is also observed between the WT and the amino-terminal deletion mutant. It appears that the low levels of the double deletion mutant may be the result of both faster catabolism and lower hepatic expression of the recombinant adenovirus. On the other hand, the low plasma levels of apoA-II[Δ(1–41)] can only be attributed to the faster catabolism because the hepatic apoA-II mRNA levels of this mutant are high.

Analysis of the apoA-I distribution following equilibrium density gradient centrifugation of plasma showed that the WT apoA-I and the apoA-II[Δ(1–41)] mutant formed predominantly in the HDL2 and HDL3 region (Fig. 5A), whereas the double deletion mutant apoA-II[Δ(1–41)Δ(185–243)] was shifted more toward the HDL2 region (Fig. 5A). Similar results were obtained by analysis of the apoA-I distribution in the gel filtration fractions of plasma (data not shown).

Analysis of the nature of the lipoprotein particles formed by electron microscopy showed that WT and apoA-II[Δ(1–41)] mutant formed spherical particles, whereas apoA-II[Δ(1–41)Δ(185–243)] mutant formed discoidal particles, and apoA-I−/− mice had few spherical particles (Fig. 5, B–E). Fig. 5, A–E, indicates that WT apoA-I and apoA-II[Δ(1–41)] form mature HDL. apoA-II[Δ(1–41)Δ(185–243)] forms discoidal HDL in vivo. The findings of Figs. 4C and 5D establish that the central helices 3–7 of apoA-I retain their ability to form discoidal HDL particles in vitro and in vivo.

DISCUSSION

Lipid-free apoA-I has been shown to induce efflux of phospholipid and cholesterol from cellular plasma membranes by two processes, one independent of cellular proteins (i.e. “microsolubilization”) and one dependent on ABCA1 (12–17). In this study we performed systematic deletions of helices of apoA-I to localize domains that are required for the fulfillment of these two lipid efflux functions. To differentiate ABCA1-mediated and ABCA1-independent cholesterol efflux mechanisms, we performed the experiments in the presence and absence of a cAMP analog that was shown previously to be a potent inducer of ABCA1 gene expression and activity (18, 58, 59, 61). To prove that cAMP-dependent lipid efflux represents ABCA1-mediated lipid efflux, we also investigated the ability of apoA-I variants to induce cholesterol efflux from cells that are transfected to overexpress ABCA1. For the interpretation of the data on ABCA1-independent lipid efflux it is, however, important to bear in mind that in the absence of cAMP, J774 cells have some ABCA1 activity. Thus cAMP-independent lipid efflux probably includes some ABCA1-mediated processes.

The present study shows that in cAMP-stimulated cells and ABCA1-transfected cells, cholesterol and phospholipid efflux was strongly impaired in the presence of several apoA-I variants with a carboxyl-terminal deletion, such as apoA-II[Δ(185–243)] and apoA-II[Δ(220–243)], but was either not affected or affected to a lesser extent by amino-terminal deletions (apoA-II[Δ(1–41)] and apoA-II[Δ(1–59)]). These findings are consistent with a previous study, which also showed that truncation of a carboxyl-terminal (apoA-II[Δ(210–243)]) but not of the amino-terminal region (apoA-II[Δ(1–43)] and apoA-II[Δ(1–65)]) of apoA-I interferes with cAMP-induced cholesterol efflux from macrophages (33). The objective of the present study was to map the domain(s) of apoA-I that are required for cholesterol efflux. For this purpose we analyzed several additional apoA-I mutants containing deletions in the carboxyl-terminal region. This analysis showed that the cAMP-dependent cholesterol efflux capacity of several apoA-I variants truncated in the carboxyl-terminal region remained decreased for all the mutants that lacked the 220–231 amino acid sequence of apoA-I. However, efflux was restored to normal levels when the 232–243 region of apoA-I was deleted. This analysis indicated that the region 220–231 of the intact apoA-I molecule is required for the ABCA1-mediated cholesterol efflux. Interestingly, this is a domain within apoA-I that shows a much higher degree of evolutionary conservation than other domains of apoA-I (62). It has also been shown that substitution of the hydrophobic amino acids L222K, F225K, and F229K of apoA-I in this region diminishes the ability of apoA-I to associate with HDL and phospholipids in vitro (43). Furthermore, expression of this mutant in apoA-I-deficient mice by adenovirus gene transfer prevents the maturation of HDL in vivo and results in the accumulation of discoidal HDL particles in plasma (45). Similarly, it has been shown that expression of a carboxyl-terminal apoA-II[Δ(220–243)] mutant, which lacks the 220–231 region in
plasma obtained from apoA-I−/− mice prior to or after infection with 1 × 10^9 plaque-forming units of adenoviruses expressing WT apoA-I or the indicated truncated forms of apoA-I, 4 days post-infection, were analyzed for cholesterol and triglyceride levels, as well as apoA-I levels (determined by ELISA using human serum or purified lipid-free WT and mutant apoA-I forms or by immunoblotting) as described under “Experimental Procedures.” Liver human apoA-I mRNA levels were determined by Northern blot analysis as described under “Experimental Procedures.” The mRNA levels are expressed relative to that in mice infected with WT apoA-I virus. Values are the means ± S.D. (n = 3).

| ApoA-I | Cholesterol mg/dl | Triglycerides mg/dl | Determined by ELISA using human serum as standard | Determined by ELISA using WT and mutant forms of apoA-I mg/dl as standards | Determined by immunoblotting | ApoA-I mRNA % |
|--------|-------------------|---------------------|-----------------------------------------------|-------------------------------------------------|-----------------------------|---------------|
| WT apoA-I | 200 ± 28 | 200 ± 9 | 404 ± 86 | 186 ± 39 | 514 ± 27 | 100 |
| ApoA-I[Δ(1–41)] | 138 ± 25 | 121 ± 15 | 24 ± 3 | 34 ± 4 | 90 ± 4 | 149 ± 22 |
| ApoA-I[Δ(1–41)Δ(185–243)] | 92 ± 3 | 37 ± 5 | 4.2 ± 0.3 | 1.6 ± 0.1 | 1.7 ± 0.4 | 15 ± 1 |
| apoA-I−/− | 30 ± 5 | 35 ± 3 | | | | |

A.

B.

C.

D.

E.

Fig. 5. Analysis of the ability of the amino-terminal deletion and double deletion mutant of apoA-I to form HDL in vivo. A. distribution of apoA-I in fractions obtained by equilibrium density gradient centrifugation of plasma of apoA-I−/− mice following adenovirus-mediated gene transfer as described under “Experimental Procedures.” An aliquot of 250 μl of plasma obtained from apoA-I−/− mice infected with WT apoA-I, apoA-I[Δ(1–41)], or apoA-I[Δ(1–41)Δ(185–243)] expressing adenoviruses was adjusted to 2 ml with PBS, and the lipoproteins were separated on 10–20% sodium bromide gradients. The apoA-I in the fractions was analyzed by Western blot, as described under “Experimental Procedures.” B–E, electron photomicrographs of apoA-I-containing fractions. The indicated fractions obtained by equilibrium density gradient centrifugation shown in A, which contain the highest concentration of apoA-I, were examined by electron microscopy as described under “Experimental Procedures.” The photomicrographs were taken at ×75,000 magnification and enlarged 3 times. The fractions from the plasma of mice expressing WT apoA-I were diluted 3 times.

apoA-I deficient mice, produced only small numbers of discoidal HDL particles in vivo (45). These data are also in agreement with the virtual HDL deficiency of a patient in whom a frameshift mutation resulted in the synthesis and secretion of a truncated apoA-I mutant with a changed sequence of amino acids 203–229 (63). Surprisingly however, the lipid efflux defect of apoA-I was restored when, in addition to the carboxyl terminus, the amino terminus of apoA-I was also deleted. A possible explanation of these data could be that conformational constraints brought about by the deletion of the carboxyl-terminal region diminish the functional interactions between apoA-I and ABCA1. Further deletion of both the amino- and carboxyl-terminal regions may relieve these conformational constraints to allow the double deletion mutant of apoA-I to interact functionally with the ABCA1 and thus promote cholesterol efflux from cell cultures.

To gain further insight into the relationship between the structure and function of apoA-I, we studied the physicochemical properties of the double deletion mutant of apoA-I and compared them with those of WT apoA-I and the amino- or the carboxyl-terminal deletion mutants. Structural analysis showed that neither deletions of amino-terminal amino acid residues 1–41 performed in this study nor of the carboxyl-terminal amino acid residues 185–243 studied previously (44) changed significantly the α-helical content. The carboxyl-terminal truncation did not interfere with the stability of the mutant protein (44). On the other hand, based on chemical denaturation data the amino-terminal truncation destabilizes the protein.

The functional studies showed that the carboxyl-terminal deletion diminished greatly the ABCA1-mediated lipid efflux, as well as the ability to solubilize multilamellar DMPC phospholipid vesicles (43). In contrast, the amino-terminal deletion has only small effect on lipid efflux and solubilizes with a slower rate multilamellar DMPC phospholipid vesicles. These data indicate that the carboxyl-terminal domain of apoA-I is required to enable the WT apoA-I to induce cAMP- and hence ABCA1-dependent lipid efflux from cells and to solubilize multilamellar DMPC phospholipid vesicles, whereas the amino-terminal domain has a smaller effect on both processes.

The current study also showed that the apoA-I[Δ(1–41)Δ(185–243)] mutant which lacks both the amino- and the carboxyl-terminal domain shows a ~7% reduction in its α-helical content and has greatly reduced chemical and thermal stability and cooperativity of unfolding as compared with WT apoA-I. When tested in vitro and in vivo, the double deletion mutant has the ability to form discoidal HDL particles. Physicochemical analysis of rHDL particles containing the apoA-I[Δ(1–41)Δ(185–243)] mutant showed that the lipid-bound form of this mutant has 0.4 kcal/mol increased chemical stability compared with the lipid-free form (data not shown). Thus the lipid-bound form of this mutant appears to be thermodynamically more favorable than the lipid-free form. This may explain the partial ability of this mutant to solubilize multilamellar DMPC vesicles and to promote ABCA1-mediated lipid...
Lipid Efflux and HDL Formation by ApoA-I Mutants

efflux. Furthermore, the α-helical content of the double deletion mutant apoA-I[Δ(1–41)Δ(185–243)] increases from 51% in the lipid-free form to 78% in the rHDL particles, whereas the α-helical content of the WT apoA-I increases from 58% in the lipid-free form to 67% in the rHDL particles (data not shown). This indicates that compared with WT apoA-I a greater percentage of amino acids of the double deletion mutant organizes into α-helical structure when this mutant associates with phospholipids. It is possible that similar conformational changes that increase the α-helical content of the apoA-I[Δ(1–41)Δ(185–243)] mutant may occur as a result of functional interactions between the lipid-free form of the double deletion mutant and the ABCA1. Although we have no evidence to support direct protein-protein interactions between the double deletion mutant and ABCA1, it has to be noted that this mutant lacks the carboxyl-terminal helices 8–10 which might promote the association of apoA-I with lipid bilayers.

Previous studies (19, 22) have indicated that ABCA1 is not directly involved in the efflux of free cholesterol. Other groups have proposed that the interaction of apoA-I with ABCA1 or a partner protein stimulates translocation of intracellular cholesterol and phospholipids from the Golgi apparatus to the plasma membrane ABCA1, where it associates with apoA-I to form the precursors HDL particles, which subsequently mature to spherical HDL (61, 64). One study has suggested that apoA-I-ABCA1 complexes may recycle between plasma membrane and intracellular lipid pools where apoA-I may be lipidated by the action of ABCA1 and subsequently exocytosed (61, 64, 65). Our data show that the ABCA1-dependent cholesterol and phospholipid effluxes follow similar patterns, suggesting that the two processes are kinetically indistinguishable.

It was observed previously (13, 27, 66) that water-soluble apolipoproteins as well as artificial peptides with amphipathic α-helices induce cholesterol efflux from cells. Because the number of amphipathic α-helices was correlated roughly with the efficacy of cholesterol efflux, it was believed that apoA-I does not contain stringent structural domains that are important for this process. The impaired ABCA1-mediated cholesterol efflux of apoA-I variants with carboxyl-terminal deletions does not seem to be a consequence of alterations in the structure of apoA-I. Thus, it is reasonable to assume that stringent structure-function relationships between apoA-I and ABCA1 are required to enable apoA-I to induce cAMP- and hence ABCA1-dependent lipid efflux from cells. The present study indicates that these specific functional interactions between the full-length apoA-I and ABCA1 require the carboxyl-terminal residues 220–231 of apoA-I.

Carboxyl-terminal truncation interfered much less with the ability of apoA-I to induce cAMP-independent efflux (Fig. 2B). Moreover, and in contrast to the cAMP-dependent lipid efflux, further removal of the amino terminus did not restore the defect in the double deletion mutant but rather reduced further the cAMP-independent cholesterol efflux. This ABCA1-independent cholesterol efflux pathway, i.e. microsolvulization, appears to be largely a function of the apolipoprotein content of amphipathic α-helices rather than of a specific domain. Because the number of amphipathic α-helices is a major determinant of the phospholipid binding capacity of apoA-I, these data also indirectly support the previous notion that physicochemical interactions between apoA-I and plasma membrane phospholipids rather than biological ligand/receptor interactions may underlie “microsolvulization” (12, 13).

Our in vivo data suggest that the specific, i.e. cAMP- and ABCA1-dependent lipid efflux pathway, and the nonspecific, i.e. cAMP- and ABCA1-independent lipid “microsolvulization” pathway, have different impacts on HDL biogenesis. WT apoA-I or apoA-I mutants with a preserved carboxyl terminus such as apoA-I[Δ(1–41)] maintain their capacity to induce ABCA1-mediated cholesterol efflux and to form spherical HDL in vivo. Previous studies also showed that the apoA-I[Δ(7–43)] mutant forms spherical particles and few discoidal particles, whereas the apoA-I[Δ(7–65)] mutant and various internal deletion mutants form discoidal HDL particles (33, 67). By contrast, apoA-I variants with carboxyl-terminal truncations such as apoA-I[Δ(220–243)], which lacks the 220–231 domain, interfere with ABCA1-mediated cholesterol efflux and, although not strongly interfering with ABCA1-independent cholesterol efflux, do not form HDL in vivo (45). The inability of the carboxyl-terminal truncated apoA-I variants to form HDL most likely reflects their limited capacity to promote cholesterol efflux and form pre-β-HDL precursors, as well as their reduced affinity for phospholipids and pre-formed HDL particles. Previous studies have shown that carboxyl-terminal truncated apoA-I forms fail to associate with preformed HDL particles following density gradient ultracentrifugation (43). Similarly, carboxyl-terminal truncated apoA-I forms, produced by proteolysis of HDL by metalloproteinases secreted by macrophage cultures, are found in lipid-free fractions after gel filtration (68). Thus, the lipid-poor particles or lipid-free mutant apoA-I forms generated by adenovirus-mediated gene transfer most likely are catabolized rapidly by the cubilin receptor or other mechanisms (69, 70). This may explain our findings that indicate that the steady-state apoA-I mRNA levels of the WT and mutant apoA-I forms do not correlate with the plasma apoA-I levels. The combined deletion of the carboxyl and amino terminal partially restores the inability of the carboxyl-terminal deletion variant to induce ABCA1-dependent cholesterol efflux and to form lipidated discoidal HDL precursors, despite the fact that this variant has an impaired ability to release cellular lipids by cAMP-independent mechanisms. We therefore conclude that not apolipoprotein-mediated lipid efflux per se, but rather the mechanism of lipid efflux which involves functional interactions between ABCA1 and apoA-I, is essential for the formation of HDL precursors which can mature to HDL. This process has at least two prerequisites, namely the cellular abundance of ABCA1 and the presence of a full-length apoA-I with an intact carboxyl-terminal domain.

Overall, our findings suggest that the central region of apoA-I alone, which contains helices 3–7, has the capacity to promote ABCA1-mediated lipid efflux. Following acceptance of cholesterol and phospholipids, the lipidated particles containing the central apoA-I helices 3–7 may proceed to form discoidal particles in vivo.

When carboxyl-terminal segments that contain residues 220–231 are deleted, the apoA-I cannot function in ABCA1-mediated cholesterol efflux. This may lead to the formation of unstable HDL precursors that may be catabolized rapidly by the kidney.

It is possible that deletion of the carboxyl-terminal domain hinders the functional interactions of the mutant apoA-I with ABCA1, whereas this hindrance may be limited when both the amino- and carboxyl-terminal domains are deleted.

The 220–231 domain of apoA-I has a smaller effect on the ability of apoA-I to induce ABCA1-independent efflux, i.e. microsolvulization, of cellular lipids.

The restoration of ABCA1 lipid efflux in the apoA-I[Δ(232–243)] mutant, which contains the 220–231 region, as well as in the double deletion mutant, which lacks both the amino- and the carboxyl-terminal domains, indicates that specific interactions of the 220–231 region with amino-terminal domain of apoA-I may be necessary to allow functional interactions be-
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REFERENCES

1. Zannis, V. I., Kardassis, D., and Zanni, E. E. (1993) Adv. Hum. Genet. 21, 145–139
2. Rothblat, G. H., de la Llera-Moya, M., Atger, V., Kellner-Weiher, G., Williams, D. L., and Phillips, M. C. (1999) J. Lipid Res. 40, 781–796
3. von Eckardstein, A., Nefer, J. R., and Assmann, G. (2001) Arterioscler. Thromb. Vasc. Biol. 21, 13–27
4. Gagescu, R., Demaurex, N., Parton, R. G., Hanzlik, W., Huber, L. A., and Groszner, J. (2000) Mol. Biol. Cell 11, 2775–2791
5. Fielding, C. J. (2001) Curr. Opin. Lipidol. 12, 281–287
6. Fielding, C. J., and Fielding, P. E. (2001) Biochem. Biophys. Res. Commun. 288, 104–108

7. Gu, X., Kozarsky, K., and Krieger, M. (2000) J. Biol. Chem. 275, 20992–20995
8. Stangl, H., Hyatt, M., and Hassel, H. H. (1999) J. Lipid Res. 40, 3269–32698
9. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
10. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
11. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
12. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
13. Gillotte, K. L., Zaiou, M., Lund-Katz, S., Anantharamaiah, G. M., Holvoet, P., Dhoest, A., Pulpanachari, M. N., Segrest, J. P., Weisgraber, K. H., Rothblat, G. H., and Phillips, M. C. (1999) J. Lipid Res. 40, 2999–3007
14. Lawn, R. M., Wade, D. P., and Uhr, N. (1999) J. Biol. Chem. 274, 10407–10412
15. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L. H., Roomp, K., van Dam, M. C., and McManus, D. C. (1999) J. Lipid Res. 40, 2999–3007
16.-desforges, M., Rassart, E., and Marcel, Y. L. (1999) J. Biol. Chem. 274, 10407–10412
17. Rust, S., Rosier, M., Funke, H., von Eckardstein, A., Pritchard, P. H., Karas, M., Albers, J. J., and Funke, H. (1999) J. Lipid Res. 40, 2999–3007
18. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
19. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
20. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
21. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
22. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
23. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
24. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
25. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
26. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
27. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
28. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
29. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
30. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
31. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
32. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
33. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
34. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
35. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
36. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
37. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
38. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
39. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
40. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
41. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
42. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
43. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Commun. 46, 1459–1468
44. Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) J. Biol. Chem. 274, 21149–21153
45. Fielding, C. J., Shore, V. G., and Fielding, P. E. (1972) Biochem. Biophys. Res. Comm