methods

Preparation and incorporation of probe-labeled apoA-I for fluorescence resonance energy transfer studies of rHDL

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Abstract Apolipoprotein A-I (apoA-I), the major constituent of HDL, plays an essential role in regulating cholesterol metabolism, acting as the physiological activator of lecithin: cholesterol acyltransferase, which converts cholesterol to cholesterol ester. Thiol-reactive fluorescent probes attached to cysteine-containing apoA-I mutants are currently being used to investigate the "LCAT active" conformation of lipid-bound apoA-I. Herein, we report new methodologies allowing rapid expression, fluorescent labeling, and recombinant HDL (rHDL) preparation for use in apoA-I in fluorescence resonance energy transfer (FRET) studies. Cysteine-containing mutant forms of human apoA-I were cloned into the pTYB12 vector containing a T7 promoter, a modified self-splicing protein element (intein), and a small affinity tag [chitin binding domain (CBD)]. The fusion proteins were expressed in *Escherichia coli*, isolated from cell lysates, and bound to a chitin-affinity column. Release of mature human apoA-I was initiated by the addition of DTT, which induced self-cleavage at the COOH terminus of the intein-CBD fusion protein. ApoA-I was further purified by Q-sepharose and then used for fluorescent probe labeling. Discoidal rHDL were then prepared with donor and/or acceptor labeled apoA-I and characterized with respect to their size, composition and ability to activate LCAT.—Li, H-h., M. J. Thomas, W. Pan, E. Alexander, M. Samuel, and M. G. Sorci-Thomas. **Preparation and incorporation of probe-labeled apoA-I for fluorescence resonance energy transfer studies of rHDL. *J. Lipid Res.* 2001.** 42: **2084–2091.**

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Human apolipoprotein A-I (apoA-I) is secreted as pro-apoA-I from the liver and intestine and then rapidly processed to its mature 243-aminoacid form (1). Once in plasma, its main physiological function involves the transport of cholesterol from peripheral tissue sites to the liver. ApoA-I continuously stimulates the pathway referred to as "reverse cholesterol transport" through its unique ability to bind and organize phospholipid and to coactivate cholesterol esterification by lecithin:cholesterol acyltransferase (LCAT). It is through these processes that apoA-I/HDL is believed to protect the artery wall from the accumulation of cholesterol in atherogenesis (2–6).

ApoA-I's structure is composed of eight 22- and two 11-amino acid repeats, all possessing amphipathic α-helical character, an essential feature for both lipid binding and LCAT activation (7). Investigations of both naturally occurring human mutations and engineered mutations reveal that the apoA-I repeats 6 and 7 (amino acids 143–186) are essential for LCAT activation (8–15). However, the exact repeat conformation that supports LCAT activation remains unknown. Recent studies undertaken to elucidate the conformation of apoA-I on phospholipid discs have employed the technique of fluorescence resonance energy transfer (FRET) (15, 16), which can measure the intermolecular apoA-I repeat distances. Thiol-reactive fluorescent probes can be attached at selected cysteine substitution mutations within apoA-I repeats (17) for precise molecular measurements.

These type of structure:function studies of apoA-I routinely use milligram quantities of highly purified mature wild-type and mutant apoprotein. To address this need, we have adopted a new *Escherichia coli* expression system that does not involve modification of the protein and yields 10–15 mg/l of purified apoA-I after a single affinity column purification procedure.

Initially, *E. coli* was the first system used for expressing human apoA-I. However, due to the instability of pro-apoA-I, yields were low, and consequently other expression systems were developed (17–20). In 1987, human apoA-I was successfully expressed from Chinese hamster ovary cells at a concentration of 100 μg/ml (21) in condi-

Abbreviations: apoA-I, apolipoprotein A-I; DPPC, L-α-phosphatidylcholine dipalmitoyl; FRET, fluorescence resonance energy transfer; 5-IAF, 5-iodoacetamido-fluorescein; rHDL, recombinant HDL; 5-TMRIA, tetramethylrhodamine-5-iodoacetamide-dihydroiodide.

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tioned medium. This system provided a useful means of expressing wild-type and mutant forms of human apoA-I for LCAT activation studies (9). Higher yields of apoA-I expression were obtained by the use of baculovirus Sf-9 cell expression, which yielded 10–15 mg/l of purified apoA-I (22–25). However, this system was time consuming because of the lengthy experimental effort required for host cell maintenance and baculovirus titering.

Recent advances in *E coli* expression systems offered more rapid expression and purification through the use of fusion proteins containing affinity tags, for example, glutathione S-transferase, β-galactosidase, and maltose binding protein or histidine tags (26–31). However, one disadvantage of these systems is the proteolytic cleavage step to remove tags from the fusion protein. This step requires additional purification steps to separate apoA-I from the affinity tags, protease, and nonspecifically cleaved protein fragments, an essential procedure if the protein is to be used for fluorescent probe attachment and incorporation into recombinant HDL (rHDL).

To overcome these problems, we have adapted an expression and purification system that uses the T7 promoter to generate high levels of the fusion protein from *E. coli*. Downstream of the promoter, human apoA-I was fused to an inducible self-cleaving intein to which a small affinity tag (chitin binding domain) was inserted in a loop region of the endonuclease domain of the intein, permitting affinity purification of the fusion protein (32–34). In this report, we demonstrate the use of this system for the rapid generation of cysteine-containing mutant apoA-I proteins, which are labeled with fluorescent probes and then used to make rHDL for FRET studies.

MATERIALS AND METHODS

Chemicals used were of reagent grade or the highest purity commercially available. DTT, aprotinin, leupeptin, and PMSF were purchased from Sigma Chemical Company. The mini-EDTA-free protease inhibitor tablets were purchased from Boehringer Mannheim. The plasmid cDNA containing human apoA-I was provided by Dr. Larry Chan. The expression vector pTYB12, *E. coli* strain ER2566, and chitin matrix were purchased from New England BioLabs. Restriction enzymes, T4 DNA ligase, DH5α, and isopropyl β-thiogalactopyranoside (IPTG) were from GIBCO BRL Life Technologies. 5-Iodoacetamido-fluorescein (5-IAF) and tetramethylrhodamine-5-iodoacetamide-dihydroiodide (5-TMRIA) were purchased from Molecular Probes, Inc. (Eugene, OR). Oligonucleotides were synthesized by International DNA Technologies, Inc. DNase I was purchased from Worthington Biochemical Corporation Inc., and cholesterol were purchased from Sigma Chemical Co. Q-sepharose and hiprep sephacryl S-200 columns (2.6 × 60 cm) were obtained from Pharmacia Biotech. Plasmid DNA was purified using the Wizard™ purification systems from Promega.

Construction of wild-type and cysteine-containing mutant apoA-I cDNA

Mutant forms of the human apoA-I cDNA were synthesized by PCR using the primers 5′-GCGGATCCCCCACGGCCCTT-3′ and 5′-CCTGCAGCCCACTTTGGAACG-3′, representing the 5′ and 3′ ends of the PCRed apoA-I cDNA, respectively (16). These primers embed unique restriction sites for *Bsm*I and *Pst*I, respectively, at the 5′ and 3′ ends, allowing directional in-frame cloning into the plasmid vector pTYB12 supplied as part of the IMPACT system from New England Biolabs (32–34).

Construction of Q132C and A154C apoA-I was accomplished using the mega-primer PCR method (34). For each cysteine-containing human apoA-I mutant, a different primer was used to introduce the change in nucleotide sequence. To introduce the Q132C and A154C apoA-I mutations into the wild-type human apoA-I cDNA, the following primers were used, respectively: 5′-CTCGTGCAGCTTACAGCGCGCGCCCTCTTG-3′ and 5′-CATC TCCTCACACCACTTCTT-3′. The above-mentioned 5′ and 3′ primers were used in conjunction with the mutant primers to generate each mutant apoA-I cDNA. Once the full-length fragment containing the apoA-I mutation was obtained, it was restricted and then ligated into the *Bsm*I *Pst*I site of the pTYB12 expression vector. Positive colonies were picked and sequenced to verify the mutation (16, 23).

Expression and purification of cysteine-containing mutant apoA-I

The ER2566 strain of *E. coli* was transformed with 100 ng of mutant apoA-I cDNA, and the next morning, the overnight culture was diluted 1:10 into LB medium containing 100 μg/ml ampicillin and grown at 37°C to an OD_{600} = 0.6–0.8. IPTG was added to a final concentration of 0.3 mM. The culture was then grown for 20 h at 16°C with shaking (250 rpm). The next day, cells were pelleted by centrifugation at 6,000 rpm for 15 min at 4°C. The pellet from a 1-liter culture was suspended in 35 ml PBS, pH 7.4, containing 3 mini-EDTA-free protease inhibitor tablets, and a final concentration of 30 μg/ml aprotinin, 20 μg/ml leupeptin, 40 μg/ml of pepstatin, and 50 μg/ml PMSF. In addition, DNase I and $MgCl_2$ were added to a final concentration of 40 μg/ml and 10 mM, respectively. The suspended bacteria cells were pre-chilled on ice and then lysed using a French pressure cell at a gauge pressure of 14,000–18,000 psi. After cell disruption, debris was pelleted by centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant containing the fusion protein was added to a chitin affinity column (2.5 × 20 cm) containing 30 ml of matrix pre-equilibrated with chitin affinity column buffer (CCB = 0.5 M NaCl, 30 mM HEPES, 0.1 mM EDTA, pH 8.0). The lysed cell supernatant was added to the chitin matrix-containing column and mixed using a rotator for 1 h at 4°C.

The chitin affinity column was washed with 20 column volumes (600 ml) of 1 × CCB, pH 8.0, at a flow rate of 1–2 ml/min. After extensive washing to remove nonspecific proteins, the column was then quickly washed with 3 column volumes of 1 × CCB containing 50 mM DTT and protease inhibitors (30 μg/ml aprotinin, 20 μg/ml leupeptin, 40 μg/ml of pepstatin, and 50 μg/ml PMSF, final concentration). The column was then placed on a rotator for 20–22 h at 16°C to initiate cleavage of apoA-I at the intein cleavage site. Pure apoA-I was eluted from the chitin affinity matrix by washing with 3 column volumes of 1 × CCB, pH 8.0. The eluate was exhaustively dialyzed against 10 mM ammonium bicarbonate, pH 7.4, 3 μM EDTA, and 15 μM sodium azide, followed by lyophilization.

The lyophilized protein was dissolved in 9 ml of 200 mM Tris, pH 8.0, and 6 M urea and passed over a hiprep sephacryl S-200 column (2.6 × 60 cm) pre-equilibrated with 200 mM Tris, pH 8.0, and 6 M urea (4°C) at a flow rate of 0.7 ml/min. The first peak containing apoA-I was dialyzed exhaustively against 10 mM ammonium bicarbonate, pH 7.4, 3 μM EDTA, and 15 μM sodium azide overnight. This material was then purified by ion exchange chromatography on a Q-sepharose column pre-equilibrated with 2.5 mM Tris, pH 8.0, and 6 M urea at 4°C at a flow rate of 1–2 ml/min. Thirty milliliters of matrix slurry were washed extensively

with deionized water to remove traces of ethanol and then equilibrated with 150 ml of 2.5 mM Tris, pH 8.0, and 6 M urea and packed in a 2.5 × 20-cm column at a flow rate of 1–2 ml/min at 4°C. The dialyzed sample containing ~60 ml was adjusted to 6 M urea by adding 0.4 g of urea/ml. To decrease the conductivity of the sample for loading to <0.4 (using 2K scale), additional 6 M urea was added (about 120 ml). This entire mixture was loaded onto the column by gravity. The column was then washed with 2.5 mM Tris, pH 8.0, and 6 M urea at 4°C at a flow rate of 1–2 ml/min. Elution of apoA-I from the column was done by washing with 200 ml of 150 mM NaCl, 2.5 mM Tris, pH 8.0, and 6 M urea at a flow rate of 1–2 ml/min. The peak containing purified apoA-I was exhaustively dialyzed against 10 mM ammonium bicarbonate, pH 7.4, 3 μM EDTA, and 15 μM sodium azide and lyophilized. Purified apoA-I was then resuspended in 5–10 ml of 6 M guanidine HCl, dialyzed against 10 mM ammonium bicarbonate, pH 7.4, and then stored at −80°C. A Lowry assay (35) was performed to determine total protein concentration. Two micrograms of purified protein was analyzed in the presence and absence of 50 mM DTT (final concentration) using a Quattro II mass spectrometer (15, 29–31) to determine the final purity of each protein preparation.

Labeling of cysteine-containing mutant apoA-I with fluorescent probes

The purified apoA-I was labeled with either 5-IAF (donor) or 5-TMRIA (acceptor) probes, as previously described (16, 36). Briefly, stock solutions containing 2.5 mM 5-IAF in dimethylformamide and 5-TMRIA in dimethylsulfoxide were freshly prepared the day of the labeling. One milligram of apoA-I was adjusted to 10 mM sodium phosphate, pH 7.4, and 50 μM DTT by adding 144 μl of 100 mM sodium phosphate, pH 7.4, and 72 μl of 1 mM DTT at a final volume of 1.44 ml. To this mixture, a 5-fold molar excess of 5-IAF or 5-TMRIA (360 μl of 2.5 mM stock solution) over total thiols was added and incubated for 1 h at room temperature. The reaction tubes were covered by aluminum foil to protect from light. The final concentrations in each reaction tube were 625 μM 5-IAF or 5-TMRIA, 50 μM DTT, and 10 mM sodium phosphate, pH 7.4.

The labeled apoA-I mixture was applied to a Sephadex G-25 column (Pharmacia Biotech) pre-equilibrated in 10 mM sodium phosphate, pH 7.4, in order to remove unbound probe. Fractions containing the labeled apoA-I were pooled and dialyzed extensively against 10 mM ammonium bicarbonate, pH 7.4, 3 μM EDTA, and 15 μM sodium azide. Donor or acceptor labeled proteins were clarified by spinning for 15 min at 12,000 rpm at 4°C before making discs.

SDS gel electrophoresis

The extent of probe labeling was determined by both SDS-PAGE and electrospray mass spectrometry (16, 22). Twelve percent SDS-polyacrylamide gels were photographed on a UV light box before staining with Coomassie blue in order to visualize the protein fluorescence. Five micrograms of probe-labeled protein were analyzed by mass spectrometry in order to determine the extent of probe labeling.

Electrospray mass spectrometry

Proteins were analyzed on a Micromass Quattro II triple quadrupole mass spectrometer using MassLynx NT version 3.3 software. All analyses were performed at a flow rate of 5 μl/min provided by a Harvard Apparatus model 55-2111 syringe pump and a source temperature of 80°C. Typical values for capillary and cone voltages were 3.5 kV and 40 V, respectively. Data were recorded at 16 points/Dalton with a scan time of 3 s and a scan delay of 0.10 s. Samples were diluted to concentrations from 50 to 100 ng protein/μl in 1:1 acetonitrile:water (+2 μl formic acid) for analysis.

Preparation of probe-labeled apoA-I containing rHDL using L-α-phosphatidylcholine dipalmitoyl (DPPC)

Preparation of pure donor or acceptor probe-labeled rHDL was performed as previously described (16) except that in these studies, rHDL were made using DPPC. Briefly, 78 μl of 30 μg/μl DPPC stock solution in chloroform, 49 μl of a 1 μg/μl free cholesterol in ethanol, and 5 μl of [1,2-^{3}H]cholesterol (50 μCi/mmol; New England Nuclear) were added to a 4.5-ml polypropylene tube for a final molar concentration of 100:5:1 of DPPC:free cholesterol:labeled apoA-I, respectively. The mixture was lyophilized overnight, and then 88.8 μl of 30 μg/μl sodium cholate was added for a molar ratio of sodium cholate:DPPC of 2:1. This mixture was incubated at 37°C for 1 h to dissolve the phospholipid and then vortexed continuously for 30 min at 37°C using a VWR multi-tube vortexer to form uniform phospholipid vesicles. Then, 0.9 mg of probe-labeled apoA-I was added, and the mixture was adjusted to 0.6 μg/μl protein and incubated for 30 min at 37°C. Sodium cholate was removed by dialysis against 10 mM Tris, pH 7.4, 140 mM NaCl, 0.25 mM EDTA, and 0.15 mM sodium azide at 37°C. The rHDL were purified by fast performance liquid chromatography (FPLC) using three Superdex 200 HR 10/30 columns linked in tandem and run at 0.5 ml/min. The final phospholipid, cholesterol, and protein compositions were determined as previously described (16). The size and homogeneity of DPPC rHDL containing 5-IAF and 5-TMRIA apoA-I were determined by 4–30% nondenaturing gradient gel electrophoresis and then compared to proteins of known Stokes' diameter (15, 16, 22).

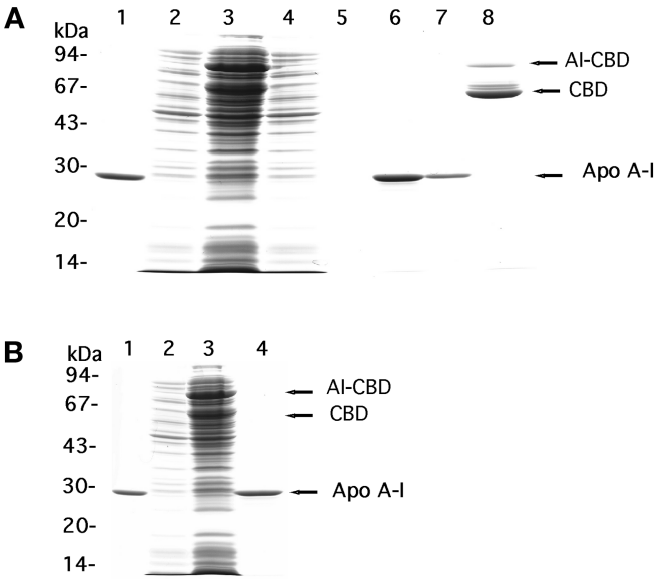

Fig. 1. Coomassie blue-stained 12% SDS-polyacrylamide gels showing sequential steps in the purification of wild-type (A) and Q132C apoA-I (B) using the pTYB12 expression system. A: Lane 1, purified human plasma apoA-I; lane 2, uninduced cell extract; lane 3, IPTG-induced cell extract; lane 4, flow through from chitin affinity column; lane 5, flow through after equilibration of column with 50 μM DTT; lanes 6 and 7, eluate after 20- and 40-h incubation, respectively, in the presence of 50 mM DTT at 16°C; lane 8, eluate from chitin affinity matrix after treatment with 1% SDS. Molecular weights were assigned by comparison to low molecular weight standards. B: Lane 1, purified human plasma apoA-I; lane 2, uninduced cell extract; lane 3, IPTG-induced cell extract; lane 4, eluate after 20-h incubation in the presence of 50 mM DTT at 16°C. AI-CBD, chitin binding domain–intein-apoA-I fusion protein (83 kDa); CBD, chitin binding domain (55 kDa); A-I, cleaved mature human apoA-I (28 kDa).

Determination of LCAT reactivity

LCAT reactivity was measured on purified DPPC rHDL containing either probe-labeled Q132C apoA-I, probe-labeled A154C apoA-I, or recombinant wild-type apoA-I. The rHDL assayed for LCAT reactivity was prepared and purified essentially as described in the preceding section. Measurements of the LCAT reactivity of each preparation of probe containing rHDL were carried out as previously described (15, 16), by measuring the conversion of [^{3}H]cholesterol to [^{3}H]cholesterol ester at a set substrate concentration (0.12 μg cholesterol), purified enzyme concentration (50 ng), and incubation time (15 min).

RESULTS

Expression of chitin binding domain-apoA-I fusion protein

Twelve percent SDS-PAGE was performed on samples taken at various steps during expression and purification to determine optimal conditions for the expression of wild-type and mutant forms of human apoA-I, as shown in **Fig. 1A** and **1B**. IPTG induction of the apoA-I-intein-chitin binding domain fusion protein (AI-CBD) (Fig. 1A, lane 3) shows a predominant band at 83 kDa in the crude bacterial cell extract that was not present in cells grown in the absence of IPTG (Fig. 1A, lane 2). The IPTG-induced cell extract (AI-CBD) was passed over a chitin affinity column, after which little AI-CBD was left in the eluate (Fig. 1A, lane 4). On-column cleavage via the intein self-cleavage site was initiated by rapid equilibration of the column with 50 mM DTT (Fig. 1A, lane 5); this 5–10 min procedure caused no apparent loss of apoA-I from the column. After equilibration with DTT, the column was incubated for 20 h at 16°C with gentle mixing. After this incubation, apoA-I was eluted from the column (Fig. 1A, lane 6); further DTT treatment for an additional 20 h resulted in the release of remaining apoA-I (Fig. 1A, lane 7). Finally, washing the chitin affinity column with 1% SDS-containing buffer showed that <5% of the apoA-I-intein-CBD was retained on the column after elution (Fig. 1A, lane 8).

Cysteine-containing apoA-I mutants were characterized with respect to their expression and purification, as shown in Fig. 1B. The IPTG induction of cells expressing Q132C apoA-I (Fig. 1B, lane 3) was compared with uninduced cells (Fig. 1B, lane 2). On-column cleavage with 50 mM DTT showed the release of mature human apoA-I (Fig. 1B, lane 4). In each case, the expression and purification of mutant A154C apoA-I were similar to Q132C apoA-I (data not shown). Overall, the yield of either wild-type or mu-

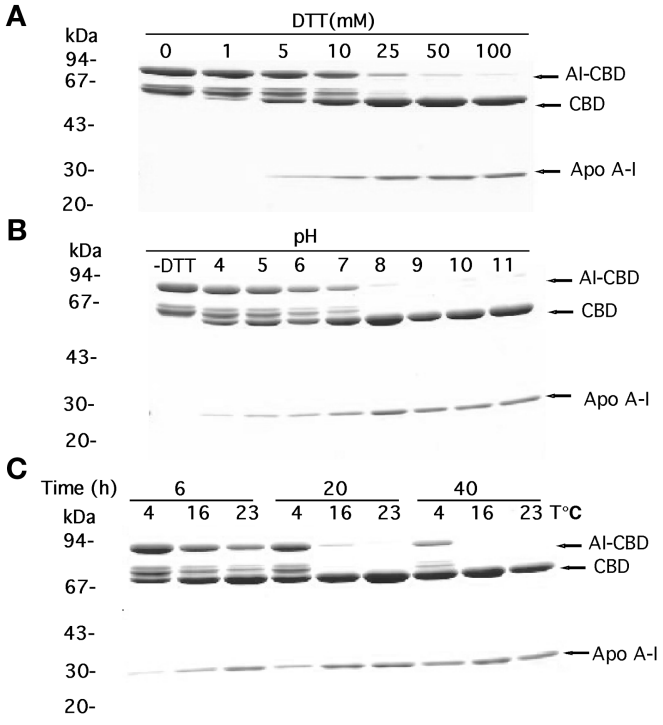

Fig. 2. Coomassie blue-stained 12% SDS-polyacrylamide gels showing DTT-induced cleavage efficiency at the intein COOH terminus. Cleavage of mature apoA-I from the chitin binding domain fusion protein (AI-CBD = 83 kDa) was induced by incubation with DTT to generate release of cleaved mature human apoA-I (AI = 28 kDa) from the CBD (CBD = 55 kDa). A: The effect of various final concentrations of DTT (0–100 mM) in 0.5 M NaCl, 30 mM HEPES, 0.1 mM EDTA, pH 8.0, for 20 h at 16°C on cleavage efficiency. B: The effects of pH on intein cleavage efficiency. The CBD-AI fusion protein was incubated with 50 mM of DTT at various pH (pH 4.0–11.0) for 20 h at 16°C. C: The effect of time and temperature on cleavage efficiency. The CBD-AI fusion protein was treated with 50 mM DTT in 0.5 M NaCl, 30 mM HEPES, and 0.1 mM EDTA, pH 8.0, at 4, 16, and 23°C for 6, 20, and 40 h, respectively.

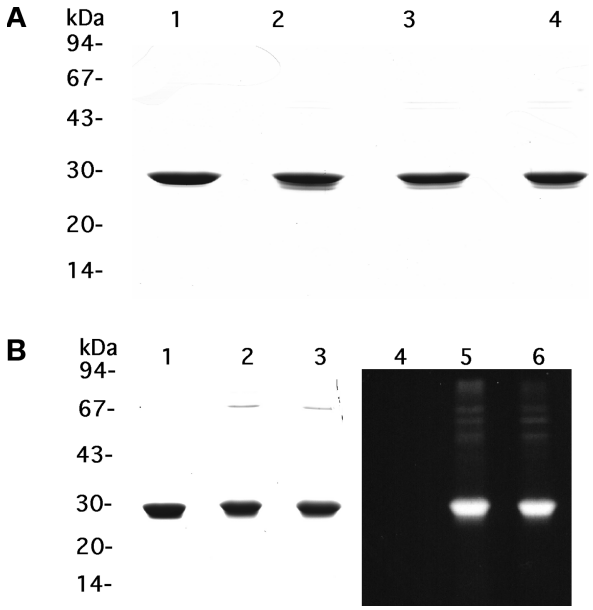

Fig. 3. Coomassie blue-stained 12% SDS-polyacrylamide gels showing purified apoA-I before and after labeling with donor and acceptor probes. In A and B, each lane represents the equivalent of 6 μg of protein. A: Lane 1, purified human plasma apoA-I; lane 2, purified recombinant wild-type apoA-I; lane 3, purified Q132C apoA-I; lane 4, purified A154C apoA-I. B: Lanes 1 and 4 represent unlabeled Q132C apoA-I stained with Coomassie blue and UV illuminated to show fluorescence staining, respectively; lanes 2 and 5 represent acceptor (5-TMRIA)-labeled Q132C apoA-I stained with Coomassie blue and UV illuminated, respectively; lanes 3 and 6 represent donor (5-IAF)-labeled A154C apoA-I stained with Coomassie blue and UV illuminated, respectively.

tant forms of apoA-I were between 10 and 15 mg/l of bacterial culture.

Intein cleavage efficiency and release of mature apoA-I

Cleavage of mature apoA-I using the intein contained within the apoA-I-CBD fusion protein was studied at DTT concentrations ranging from 0 to 100 mM, as shown in **Fig. 2A**. Optimal conditions employed from 50 to 100 mM DTT with 20-h incubation at 16°C. Cleavage efficiency at the apoA-I-intein junction was affected by pH, as shown in Fig. 2B. The optimal pH for the cleavage reaction was from 8.0 to 11.0. Cleavage temperatures of 4, 16, and 23°C were tested at 6, 20, and 40 h of incubation, at pH 8.0, 50 mM DTT, as shown in Fig. 2C. These results demonstrated that cleavage at 16°C for 20 h released >95% of apoA-I from the apoA-I-intein-CBD fusion protein.

Fluorescent probe labeling of cysteine-containing apoA-I

After apoA-I purification by chitin affinity and Q-sepharose chromatography, the final purity was assessed by SDS-PAGE. **Figure 3A**, lanes 2, 3, and 4 shows that *E. coli* produced wild-type, Q132C, and A154C apoA-I, respectively, and migrated to a similar molecular size as plasma-derived apoA-I (Fig. 3A, lane 1).

Next, the purified Q132C and A154C apoA-I were labeled with either donor probe, 5-IAF, or acceptor probe, 5-TMRIA, and the noncovalently bound probe removed by gel filtration on sephadex G-25 columns. Probe-labeled Q132C and A154C apoA-I, shown in Fig. 3B, lanes 2 and 3, respectively, were analyzed by 12% SDS-PAGE and had the same molecular size as human plasma apoA-I (Fig. 3B, lane 1). UV illumination of the gel showed the probe comigrated with Q132C apoA-I (acceptor probe) and A154C apoA-I (donor probe), as shown in Fig. 3B, lanes 5 and 6, respectively.

To determine the extent of probe attachment, electrospray mass spectometry was performed on purified probe-labeled apoA-I. **Figure 4** shows that the experimentally derived mass for each protein analyzed was identical with the calculated mass (Fig. 4A, DTT-treated A154C apoA-I; Fig. 4B, 5-IAF-labeled A154C apoA-I; Fig. 4C, DTT-treated Q132C apoA-I; Fig. 4D, 5-TMRIA-labeled Q132C apoA-I).

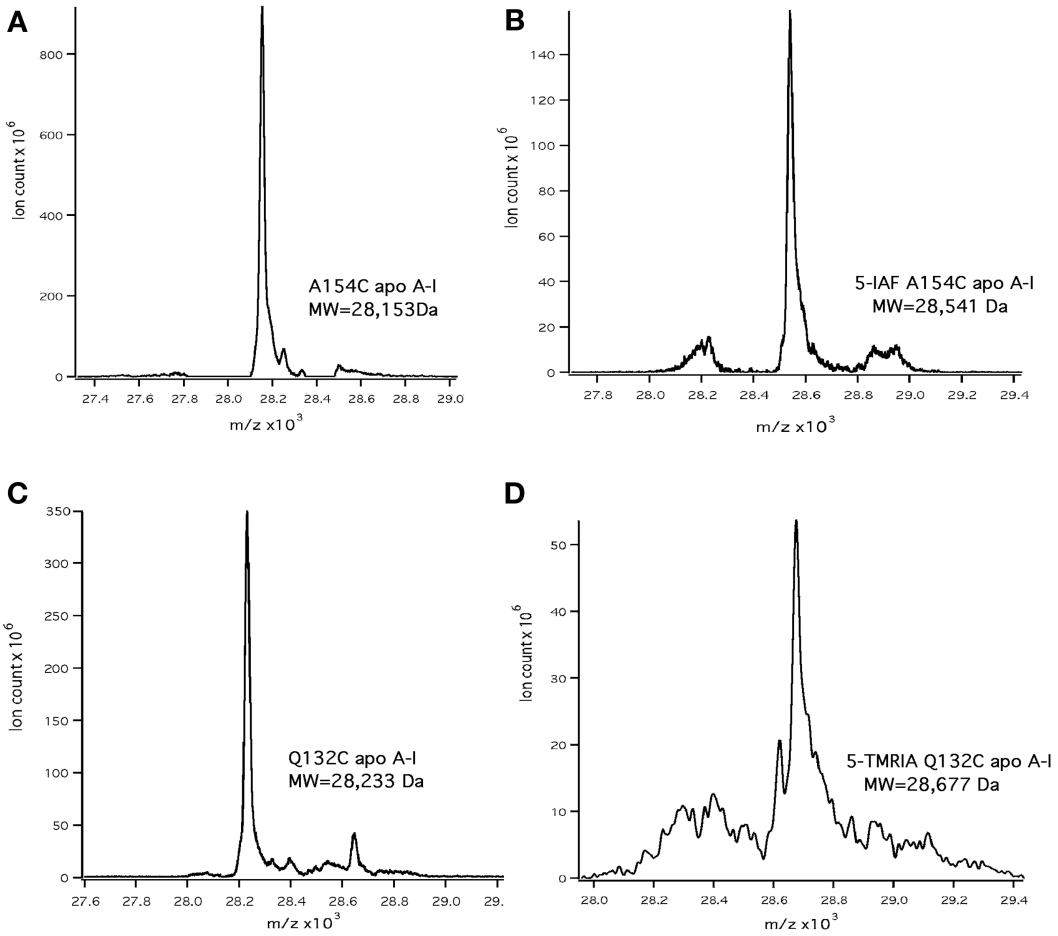

Fig. 4. Electrospray mass spectra of labeled and unlabeled Q132C and A154C apoA-I. A: A154C apoA-I treated with 50 mM DTT final concentration showing an experimental molecular mass of 28,153 Da; B: donor (5-IAF)-labeled A154C apoA-I with an experimental mass of 28,541 Da; C: Q132C apoA-I with an experimental mass of 28,233 Da; D: acceptor (5-TMRIA)-labeled Q132C apoA-I with an experimental mass of 28,677 Da. Each of the experimentally determined molecular weights was the same or similar to its corresponding theoretical molecular mass.

Preparation of fluorescent probe containing rHDL

Using either pure donor or acceptor probe-labeled apoA-I, rHDL complexes were prepared at a starting molar ratio of 100:5:1 DPPC:cholesterol:labeled apoprotein (14–16). The rHDL particles were then purified by FPLC and analyzed by 4–30% nondenaturing PAGE. **Figure 5** shows sizes of rHDL prepared with either pure donor- or acceptor-labeled apoA-I. Figure 5A, lane 1 shows DPPC rHDL containing donor-labeled Q132C apoA-I; lane 2 shows DPPC rHDL containing acceptor-labeled Q132C apoA-I; lane 3 shows DPPC rHDL containing donor-labeled A154C apoA-I; lane 4 shows DPPC rHDL containing acceptor-labeled A154C apoA-I; lane 5 shows DPPC rHDL containing human plasma apoA-I. Figure 5B shows the UV-illuminated 4–30% ND GGE for each of the described lanes in A, respectively. The calculated diameter for the series of DPPC rHDL shown in Fig. 5 was calculated by comparison to standards of known Stokes' diameter and was ~98 ± 2 Å. The final molar composition of all the rHDL shown in Fig. 5 (mean ± SD) was ~92 ± 5.2:6.4 ± 1.5:1 for DPPC:cholesterol:protein, respectively.

LCAT reactivity in probe-labeled rHDL

To determine if the presence of the acceptor or donor probe bound to apoA-I contained in rHDL functionally altered its ability to activate LCAT, we assayed each of the discoidal HDL preparations, as described in Materials and Methods. Pure acceptor and donor probe-labeled rHDL were used as substrate in LCAT reactivity assays, and the rate of cholesterol esterification determined for each preparation is shown in **Fig. 6**. As a control, rHDL containing wild-type apoA-I was also assayed using our standard conditions alongside the probe containing rHDL. No significant differences in LCAT reactivity were seen among the rHDL preparations, indicating that that the presence of fluorescent probes even within apoA-I repeat 6 had little or no effect on the apoA-I's ability to activate LCAT.

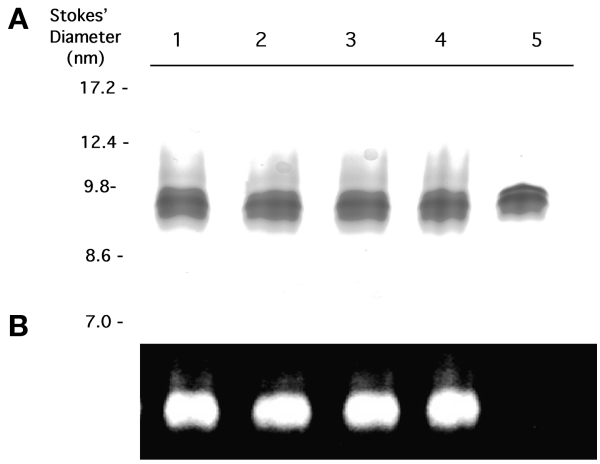

Fig. 5. Four to 30% nondenaturing polyacrylamide gradient gel of discoidal HDL prepared with either pure donor- or acceptor-labeled apoA-I. A: Lane 1, DPPC rHDL containing donor-labeled Q132C apoA-I; lane 2, DPPC rHDL containing acceptor-labeled Q132C apoA-I; lane 3, DPPC rHDL containing donor-labeled A154C apoA-I; lane 4, DPPC rHDL containing acceptor-labeled A154C apoA-I; lane 5, DPPC rHDL containing human plasma apoA-I. B: The UV-illuminated 4–30% non-denaturing gradient gel electrophoresis (ND GGE) for each of the described lanes in A, respectively. All DPPC rHDL complexes were prepared as described in Materials and Methods. Each lane contains the equivalent of 6 μg of protein. Dalibrating high molecular weight standards and their corresponding Stokes' diameter (nm): thyroglobin, 17.0; ferritin, 12.4; catalase, 9.8; lactate dehydrogenase, 8.6; and albumin, 7.0.

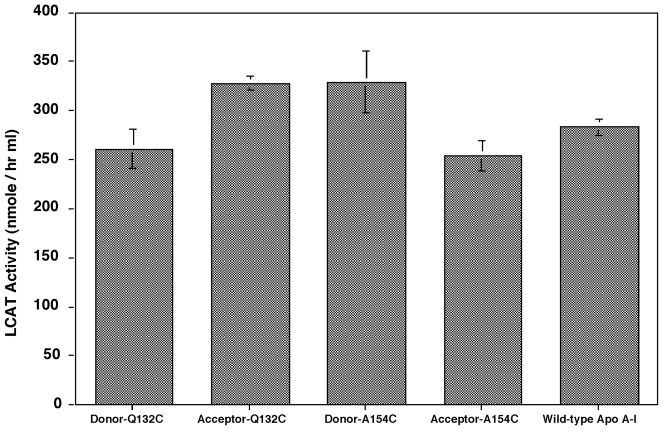

Fig. 6. LCAT reactivity assays on purified DPPC rHDL containing either pure acceptor or donor probe-labeled Q132C apoA-I, pure acceptor- or donor-labeled A154C apoA-I, or recombinant wild-type apoA-I. The rHDL assayed for LCAT reactivity was prepared and purified as described in Materials and Methods at a starting molar ratio of 100:5:1 DPPC:cholesterol:apoA-I. The complexes were purified by FPLC and then used in the LCAT assays. Each assay contained 20 ng of purified human LCAT. All points represent the average of three individual experiments of duplicate determinations for each cholesterol concentration.

DISCUSSION

We describe here a novel protein expression system for production and purification of mature apoA-I to be used in FRET studies. The New England Biosystems "Impact" system (32, 33, 36) offers several advantages over other protein expression systems. First, it uses *E.coli* ER2566 cells that produce high levels of apoA-I-fusion protein when induced with IPTG and grown for 20 h at 16°C. Shorter incubation times at higher temperature (4–6 h at 37°C in the presence of IPTG) yielded higher amounts of insoluble fusion protein, likely resulting from inaccurate protein folding at the higher temperature (data not shown). Second, release of apoA-I from the fusion protein via the intein self-cleavage reaction allows apoA-I to be selectively released from the chitin affinity column after DTT treatment. Optimal DTT-initiated on-column cleavage conditions were identified and consistently yielded 10–15 mg of apoA-I per liter of bacterial culture with a purity of ~85–90%. Finally, construction of the apoA-I fusion protein using our method does not require the addition of his tags or flag sequences leading to additional purification steps after their cleavage from the protein. In our studies, alanine was added to the NH_2 terminus of the mature apoA-I sequence in order

to optimize the intein self-cleavage reaction, which has been reported to favor methionine, alanine or glutamine at this position (32, 33, 37). Thus, for these three reasons, this expression system represents a major advance over previous systems currently in use.

The characterization and optimization of the NEB "Impact" system was carried out in order to obtain rapid high-yield apoA-I expression for use in FRET experiments. In our experience, we have found that FRET studies of rHDL require apoA-I purity >99% because slight alterations in the acceptor-to-donor probe ratio within the rHDL can have confounding effects on data interpretation. Therefore, apoA-I released from the chitin affinity column was further purified by size exclusion and Q-sepharose chromatography. These additional protein purification steps were necessary because even low levels of impurities dramatically reduced the yield and homogeneity of probe-containing rHDL.

In this report we also show that the covalent attachment of either 5-IAF or 5-TMRIA fluorescent probes within apoA-I repeats 5 or 6 had little or no effect on the size, composition, or ability of rHDL to activate LCAT. This is not surprising because these probes are relatively small in size and located on the hydrophilic face of each repeat, thereby minimizing any potential disruption between LCAT and apoA-I. In our studies (16) as well as in studies using acrylodan (6-acryloyl-2-dimethylaminonaphthalene) (38) or ALEXA-546 (39), conformational alterations to protein structure resulting from probe attachment are evident only in the lipid-free state of the modified apoA-I.

The ALEXA series has been recently used to study apoA-I conformation bound to rHDL (39). These probes are similar to the basic fluorescein (5-IAF) and rhodamine (5-TMRIA) probes used in our study but are much larger and more water soluble than 5-IAF and 5-TMRIA. In our hands, large amounts of the ALEXA-546 bound to apoA-I after the probe coupling procedure and thus co-purified with the probe-labeled apoA-I (H-h. Li, unpublished observations) under native conditions. This is a serious problem when interpreting FRET data because noncovalently bound or free probe can associate with the rHDL under evaluation. The contaminating noncovalently bound probe can efficiently quench the signal and lead to erroneous conclusions regarding the protein conformation.

In conclusion, we described herein an expression system and probe-labeling protocol that represents a dramatic improvement over previously described systems. Our method allows rapid acquisition of purified tag-free recombinant wild-type and/or mutant human apoA-I that can then be labeled with fluorescent probes and used in FRET studies of apoA-I bound to rHDL.

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