methods

Purification of recombinant apolipoproteins A-I and A-IV and efficient affinity tag cleavage by tobacco etch virus protease

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Abstract **The expression of recombinant apolipoproteins provides experimental avenues that are not possible with plasma purified protein. The ability to specifically mutate residues or delete entire regions has proven to be a valuable tool for understanding the structure and function of apolipoproteins. A common feature of many recombinant systems is an affinity tag that allows for straightforward and high-yield purification of the target protein. A specific protease can then cleave the tag and yield the native recombinant protein. However, the application of this strategy to apolipoproteins has proven somewhat problematic because of the tendency for these highly flexible proteins to be nonspecifically cleaved at undesired sites within the native protein. Although systems have been developed using a variety of proteases, many suffer from low yield and, especially, the high cost of the enzyme. We developed a method that utilizes the tobacco etch virus protease to cleave a histidine-tag from apolipoproteins A-I and A-IV expressed in *Escherichia coli*. This protease can be easily and inexpensively expressed within most laboratories. We found that the protease efficiently cleaved the affinity tags from both apolipoproteins without nonspecific cleavage. All structural and functional measurements showed that the proteins were equivalent to native or previously characterized protein preparations. In addition to cost-effectiveness, advantages of the tobacco etch virus protease include a short cleavage time, low reaction temperature, and easy removal using the protease's own histidine-tag.—** Tubb, M. R., L. E. Smith, and W. S. Davidson. **Purification of recombinant apolipoproteins A-I and A-IV and efficient affinity tag cleavage by tobacco etch virus protease. *J. Lipid Res.* 2009.** 50: **1497–1504.**

Supplementary key words bacteria • expression • inexpensive • cost effective • low temperature cleavage • TEV

Apolipoprotein (apo)A-I is the major protein component of HDL, levels of which are inversely correlated with the risk of cardiovascular disease (CVD) (1). Apolipoprotein (apo) A-IV is a component of HDL and is also involved in chylomicron assembly and secretion (2, 3). Anti-inflammatory functions have been attributed to both proteins (4, 5), and apoA-IV may exhibit a satiety function as well (6). ApoA-I and apoA-IV are also involved in reverse cholesterol transport, which is thought to be a major way by which HDL protects against CVD (7). Thus, there is significant interest in the structure and function of these plasma apolipoproteins. Site-directed mutagenesis studies are an excellent tool for narrowing down regions of apolipoproteins important for particular functions, but they require the ability to recombinantly express large quantities of pure protein. Many such expression systems have been developed for apoA-I and, more recently, for apoA-IV. For example, Feng et al. have developed a yeast expression system for apoA-I yielding an impressive 91 mg/L (8). However, this system requires the use of expensive, large-scale fermenters and five days for expression alone. Pyle et al. (9) and Sorci-Thomas et al. (10) have developed baculovirus systems which can be cultured in most laboratories without fermentors and which express large quantities of proapoA-I in insect cells. Still, these systems require days to express adequate quantities of protein and, in some cases, require the need for extensive purification of the secreted proapoA-I. Furthermore, Pyle et al. (11) found that removal of the apoA-I pro segment reduced the yield of this system from 80 to 5 mg/L, significantly decreasing its utility for mature apoA-I.

Such issues have led many groups, including our own, to focus on bacterial expression systems for apolipoproteins. The most popular approaches involve integrated affinity tags

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; apo, apolipoprotein; CD, circular dichroism; CVD, cardiovascular disease; DMPC, dimyristoyl-phospatidylcholine; *E. coli*, *Escherichia coli*; Gly, glycine; HIC, hydrophobic interaction column; His-tag, histidine-tag; igase, IgA protease; IMAC, nickel affinity column; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria broth; MLV, multilamellar vesicles; Pro, proline; STB, standard tris buffer; TEV, tobacco etch virus; Thr, threonine; Trp, tryptophan.

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for efficient product purification. Although some studies have used recombinant apolipoproteins without tag removal (12), it is generally preferred to remove the tag for fear of artifactual effects on protein function. Unfortunately, the tags are often difficult or costly to remove without unintentional alteration of the target protein, a particular problem for dynamic proteins such as apoA-I. Ryan, Forte, and Oda have used an autocleavage technique with a codon-optimized construct that results in high protein yield (100 mg/L) and specific tag cleavage (13), though the protein must be exposed to acidic conditions during cleavage. In the past, we have used IgA protease (14) which can specifically cleave His-tags from both apoA-I and apoA-IV (15, 16). Unfortunately, the protease is expensive from commercial sources and quickly becomes cost prohibitive to use near the recommended ratio of protease to target protein, at least when expressing milligram quantities of multiple mutants for comparison studies.

For these reasons, we investigated alternative protease systems for cleaving affinity tags from apolipoproteins expressed in *Escherichia coli* (*E. coli*). The tobacco etch virus (TEV) protease has been used by numerous investigators to produce recombinant proteins (17–19). One group has worked extensively to produce a stable, highly efficient TEV protease which can be easily expressed in *E. coli* (20) by any laboratory equipped for recombinant protein production. This S219V mutant of TEV protease is resistant to self-cleavage and auto-inactivation. Furthermore, it contains a noncleavable histidine (His)-tag for purification away from the cleaved target protein product.

In the current report, we show that the TEV protease can specifically and efficiently remove a His-tag from human apoA-I and apoA-IV, resulting in structurally and functionally identical protein as compared with either plasma apoA-I or our previously used recombinant apoA-IV. Compared with our previous IgA protease system, TEV protease cleavage is less costly, faster, carried out at a lower temperature, removed easily, and results in a recombinant protein with only a single nonnative amino acid, glycine, on the N-terminus.

EXPERIMENTAL PROCEDURES

Materials and equipment

Dimyristoyl-phospatidylcholine (DMPC) was purchased from Avanti Polar Lipids. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Fisher. His bind resin was purchased from Novagen. YM30 centricon concentrators were obtained from Amicon. DMEM was obtained from Invitrogen. [1,2-^{3}H(N)]cholesterol was supplied by Amersham Biosciences. 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-bromo-cAMP) was purchased from Sigma. The RAW264.7 macrophages used in the cholesterol efflux assay were purchased from the American Type Culture Collection. All other reagents were analytical grade.

Circular dichroism (CD) measurements were made on a Jasco J-715 spectropolarimeter. The fluorescence measurements were performed on a Photon Technology International Quantamaster spectrometer. A 1900CA Packard liquid scintillation analyzer was used to count the radioactive cholesterol counts associated with the cholesterol efflux assay. All absorbance measurements were made on an Amersham Biosciences Ultraspec 4000 UV/visible spectrophotometer.

Methods

TEV protease expression and purification. TEV protease vector (pRK793) along with accessory plasmid pRIL (Stratagene), originating from the laboratory of David Waugh, were obtained from addgene.org (Addgene plasmid 8827). The construct has a maltose binding protein fused with the catalytic domain of TEV protease with a self-cleavage site between the two proteins. After self-cleavage, the TEV protease retains an N-terminal His-tag, allowing for easy removal from the sample solution after cleavage. The TEV protease domain contains the mutation S219V (20) which confers resistance to auto-inactivation and is about 2× as active as wild-type TEV protease. The TEV protease was expressed in BL21 (DE3) *E. coli* cells. Briefly, 10 ml Luria broth (LB) cultures with 100 μg/ml ampicillin (pRK792) and 30 μg/ml chloramphenicol (pRIL) were inoculated with a single colony from an LB agar plate and grown overnight at 37°C. In the morning, 100 ml LB cultures were supplemented with the same selection agents and inoculated with 1 ml of the overnight culture. At OD_{600} ~0.5, cultures were induced with 1 mM IPTG, and the temperature was reduced to 30°C for 4 h. The cells were pelleted, resuspended in 10 ml of 20 mM Tris-HCl (pH 7.9) + 500 mM NaCl + 10% glycerol + 5 mM imidizole (lysis buffer) per 1 g of wet cell paste. The cells were lysed by probe sonication for 10 min at 50% duty cycle. 5% polyethyleneimine (pH 7.9) was added to a final concentration of 0.1%, and the sample was mixed by inversion and centrifuged at 15,000 *g* for 30 min. The supernatant containing the TEV protease was applied to a nickel affinity (IMAC) column equilibrated with lysis buffer. After seven washes with lysis buffer and three additional washes with wash buffer (lysis buffer with 50 mM total imidazole), the protease was eluted with 20 mM Tris-HCl (pH 7.9) + 500 mM NaCl + 10% glycerol + 1 M imidizole. EDTA and DTT were added to the eluted sample at a final concentration of 1 mM each, and the sample was concentrated by ultrafiltration using YM30 centricons. The sample was dialyzed against 25 mM K_2HPO_4 (pH 8.0) + 200 mM NaCl + 5% glycerol + 2 mM EDTA + 10 mM DTT. The protease was aliquoted into 1 mg samples, flash frozen in liquid nitrogen and stored at −80°C until use. In a subsequent purification, standard Tris salt buffer (STB: 10 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide) + 5 mM DTT + 10% glycerol was used for storage with no difference in protease activity.

Expression and purification of apoA-I. ApoA-I was expressed in *E. coli* and purified using IMAC columns as described previously (15). In some experiments, apoA-I was then applied to a phenyl sepharose hydrophobic interaction column (HIC). In others, this step was omitted. The His-tag was then cleaved from the protein by TEV protease at a 20:1 protein to protease ratio for 30 min at room temperature (22°C). Cut apoA-I was purified using Superdex 200 (Amersham) size exclusion chromatography. Finally, the protein was dialyzed into STB and stored at 4°C until use.

Expression and purification of apoA-IV. ApoA-IV was also expressed in *E. coli* and purified using IMAC columns as described previously (21). An appropriate protein:protease ratio was determined using TEV G apoA-IV and TEV protease at ratios from 2:1 to 1000:1, and it was determined that cleavage at room temperature overnight (16 h) with a 20:1 ratio was sufficient to achieve complete cleavage of the His-tag (data not shown). After dialysis into STB, TEV cut apoA-IV was reapplied to the IMAC column in 1× Bind buffer to capture the cleaved tag, the pro-

tease, and any uncut protein. Purified protein was stored in STB at 4°C until use.

Circular dichroism. For CD studies, proteins were freshly dialyzed against 20 mM phosphate buffer (pH 7.4), and relative concentration was determined by the Markwell-Lowry method (22). Proteins were then diluted to 100 μg/ml, and spectra were collected in a 1 mm cell as an average of three scans. The scans were from 260 to 190 nm at 100 nm/min with a 0.5 nm step size and 0.5 s response. Bandwidth was set to 1 mm, and slit width was 500 μm. To confirm the accuracy of dilution, protein concentration was verified by A_{280}. Mean residual ellipticity was calculated based on this value as described by Woody (23) using 115.3 as the mean residual weight for apoA-I and apoA-IV. Fractional helical content was calculated using the formula of Chen et al. (24) and the mean residual ellipticity at 222 nm. Each experiment was repeated on two independent protein preparations.

Tryptophan fluorescence. Protein samples from the above CD experiments were used to monitor tryptophan (Trp) fluorescence. Fluorescence emission spectra of plasma apoA-I, TEV G-apoA-I, IgA protease apoA-IV, and TEV G-apoA-IV were recorded at room temperature. The emission spectra were collected from 305 to 380 nm using the Trp excitation wavelength of 295 nm chosen to minimize the tyrosine fluorescence. The appropriate buffer controls were also included.

DMPC liposome solubilization assay. The rate of lipid association and reorganization was determined using the DMPC liposome clearance assay (25, 26). Briefly, DMPC multilamellar vesicles (MLVs) at 5 mg/ml were prepared in STB by brief probe sonication. MLVs and STB were mixed, so that once protein was added, the final DMPC concentration was 0.425 mg/ml. Protein was quickly added to the above mixture to a final concentration of 0.17 mg/ml (2.5:1 DMPC/protein, w/w). Proteins for all experiments were in STB. Absorbance at 325 nm was measured at 24.5°C at 30 s intervals for 20 min. Samples were run in triplicate, and plots were normalized to the initial absorbance of the sample (OD_0). The clearance of the reaction is due to apolipoprotein binding to, and solubilizing the MLVs to small discoidal lipoproteins that do not scatter light at 325 nm. The assay was carried out at 24.5°C, the gel/liquid crystal transition temperature of DMPC, where an optimal number of lattice defects exist on the MLV surface. Each result was verified by an additional DMPC experiment on a separate day from an independent protein expression. The DMPC liposome clearance assay is a multistep reaction that involves binding to the lipid surface followed by an unknown reorganization step. Thus, this assay is not strictly a measure of lipid binding. However, it has been proposed that the initial binding steps are rate-limiting and that once a critical number of apolipoproteins bind to the lattice defects on the MLV surface, the rate of reorganization is similar for all proteins (26).

Cholesterol efflux. RAW264.7 macrophages were maintained in DMEM with 10% FBS and 50 μg/ml gentamycin. Cells were grown to 70% confluency, then washed twice with a minimal media (DMEM and 0.22% BSA). Next 0.5 ml labeling media (DMEM, 0.22% BSA, +/− 0.3 mM 8-bromo-cAMP, and 1.0 μCi/ml [1,2-^{3}H(N)]cholesterol was added to the cells for 18 h. Cells were washed twice with minimal media, then 0.5 ml of efflux media (DMEM, 0.22% BSA, +/− 0.3 mM 8-bromo-cAMP, and 10 μg/ml of acceptor protein) was incubated with cells for 8 h. Percent efflux was calculated by dividing the efflux media counts by the total counts (calculated as the efflux media counts plus the intracellular counts after cells were solubilized in isopropanol). Efflux media was 0.45 μm filtered before counting to remove any floating cells.

RESULTS

TEV protease purification

We obtained the TEV protease expression construct from Dr. David S. Waugh via Addgene.org, a nonprofit site dedicated to the dispersal of plasmids to the scientific community. The expression and purification of the protease was performed as described in Methods. After purification, we obtained a single band on SDS-PAGE at a molecular mass of 30 kDa along with some minor bands that may represent autoproteolysis (data not shown).

We first tested the protease to make sure that it lacked the ability to nonspecifically cleave lipid-free recombinant apoA-I or apoA-IV [produced by our IgA protease method (15, 16)]. SDS-PAGGE analysis showed no evidence of nonspecific cleavage of either apolipoprotein even at high ratios of TEV protease to apolipoprotein (1:2 mol:mol A-IV) for 16 h at room temperature (data not shown). This indicates that TEV protease should be a suitable protease for specific cleavage of His-tags from apoA-I and apoA-IV, provided they contain an optimal recognition sequence.

Design and cleavage of recombinant apolipoproteins

Site-directed mutagenesis was used to replace the IgA protease recognition (PRPP/TP) site in apoA-I and apoA-IV, both in the pET30 vector (Novagen) (15, 16) (**Fig. 1**), with the cleavage site for TEV protease (ENLYFQ/X). Ideally, position X represents the first amino acid of the mature target protein. However, Kapust et al. (27) have shown that the identity of the amino acid at position X can profoundly affect the efficiency of the TEV cleavage reaction, with G, A, or S found to be the most permissive. Therefore, we generated two different TEV protease recognition constructs for both proteins in this study. The first was a so-called "clean cutting" construct in which position X was the naturally occurring first amino acid of either human apoA-I (D) or human apoA-IV (E), leaving no nonnative amino acids on the N-terminus of either apolipoprotein after cleavage. The second set of constructs placed a G, A, or S at position X, resulting in one additional amino acid on the N-terminus of each apolipoprotein after TEV protease cleavage. ApoA-I constructs were generated with G, A, and S, and apoA-IV was generated with G only. The mutants were sequenced to verify their identity and expressed and purified from *E.coli* as described in Methods. We found no difference in protein expression level for any of the constructs (data not shown).

To determine the efficiency of tag cleavage by the TEV protease, we incubated the various apoA-I and apoA-IV forms with TEV protease at a 20:1 molar ratio (apolipoprotein: protease) for 30 min (apoA-I) or 16 h (apoA-IV) at room temperature. As shown in **Fig. 2A**, the "clean cutting" apoA-I was minimally cleaved after 30 min as indicated by a band of about 34 kDa with no mature apoA-I (28 kDa) visible. By contrast A-apoA-I, G-apoA-I, and S-apoA-I were all

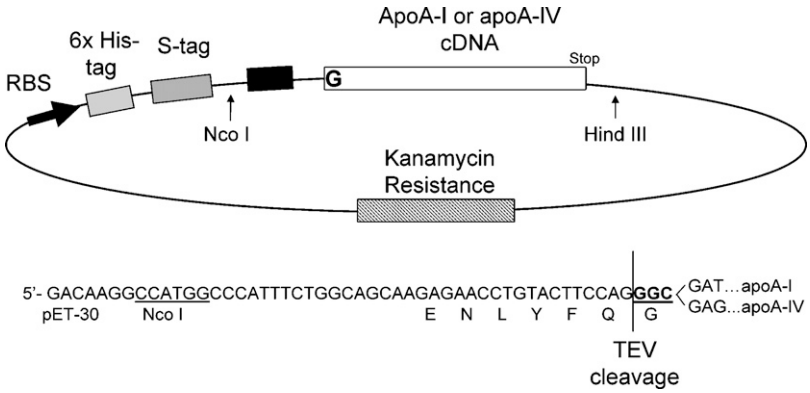

Fig. 1. Vector maps of apolipoproteins A-I and A-IV in pET30. ApoA-I and ApoA-IV coding sequences were previously subcloned into the pET30 bacterial expression vector with an IgA protease recognition site between the histidine tag and the apolipoprotein DNA (15, 32). We used mutagenesis to replace the IgA protease site with the recognition site of the tobacco etch virus (TEV) protease. The arrow represents the transcription start site. The restriction endonucleases used for 5′ and 3′ cloning are shown (*mutagenesis removed the NcoI splice site from the apoA-IV plasmid). The "G" at the beginning of the native apolipoprotein sequence represents the glycine that dramatically improves cleavage of the tag from both apoA-I and apoA-IV (see text). The sequence below shows the exact construction of the TEV cleavage site in relation to the protein coding sequence.

cleaved completely to the 28 kDa band by the TEV protease. Additional bands apparent in the lane represent nonspecific cleavage of recombinant apoA-I that occurs in some preparations, presumably due to proteases released from the bacterial cells during lysis (i.e., the band at about 30 kDa in Fig. 2A, lane 2). Figure 2B shows that "clean-cutting" apoA-IV displayed relatively more cleavage than "clean cutting" apoA-I, exhibiting a band with a molecular mass of the intact fusion protein (47 kDa) as well as a band at 45 kDa corresponding to mature apoA-IV. However, G-apoA-IV was completely cleaved by the protease as indicated by the appearance of the 45 kDa band. Because the cleavage efficiencies of the "clean cutting" constructs were poor, we elected to further study the apolipoproteins containing a G on the N-terminus. We reasoned that the neutral charge and low side-chain volume afforded the least chance of perturbing protein function.

Apolipoprotein purification

G-apoA-I and G-apoA-IV mutants containing the TEV protease site were expressed and purified with regard to amounts of protein at each step to track the yield of mature apolipoprotein after purification. **Figure 3** shows the purification scheme of apoA-I and apoA-IV. As shown in **Fig. 4**, our purification schemes resulted in preparations that were highly pure by densitometry for both apolipoproteins. Protein yield was 26 mg/L (9.6×10^{-7} moles/L) for apoA-I and 84 mg/L (2.0×10^{-6} moles/L) for apoA-IV.

Structural and functional characterization of apolipoproteins generated by TEV cleavage

To determine if the presence of the N-terminal glycine perturbed the structure or function of either apoA-I or apoA-IV, we compared G-apoA-I to isolated human plasma apoA-I and G-apoA-IV to our IgA protease-cleaved recom-

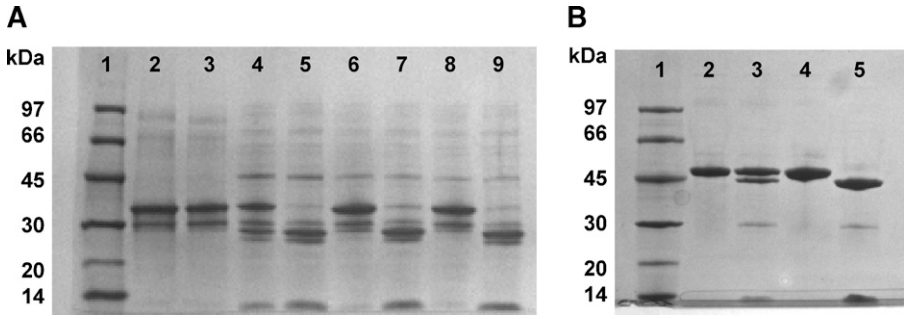

Fig. 2. Histidine tag cleavage from apolipoprotein constructs by TEV protease. (A) Samples of "clean cutting" apoA-I as well as apoA-I containing an N-terminal A, G, or S were incubated at room temperature for 30 min without (lanes 2, 4, 6, and 8, respectively) or with (lanes 3, 5, 7, and 9, respectively) TEV protease. (B) Samples of apoA-IV either clean cutting or containing an N-terminal G were incubated at room temperature for 16 h without (lanes 2 and 4, respectively) and with (lanes 3 and 5, respectively) TEV protease. The apolipoprotein: protease ratio was 20:1 for all samples. 4–15% SDS-PAGGE analysis performed on 6 μg of each reaction mixture and visualized with Coomassie blue. Lane 1 of both gels contains low molecular weight protein standards (Amersham).

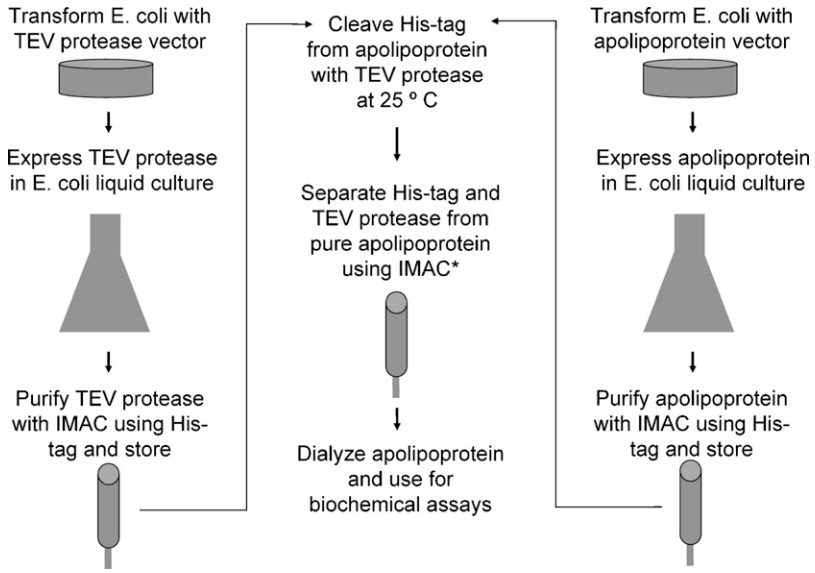

Fig. 3. Expression and purification scheme for TEV protease and apolipoproteins A-I and A-IV. Both the TEV protease and the apolipoproteins were expressed in liquid LB broth after transformation into the BL21 strain of *Escherichia coli*. The TEV protease was captured from cell lysate using its noncleavable histidine tag and immobilized metal affinity chromatography (IMAC). Crude apolipoproteins were captured using IMAC. After dialysis, the apolipoprotein histidine tag was cleaved with TEV protease at a 20:1 protein:protease ratio at room temperature. After cleavage was complete, pure cut apolipoprotein was isolated using IMAC. The protein was dialyzed into STB and stored at 4°C until use. * gel filtration chromatography was used for some experiments with apoA-I.

binant apoA-IV (21, 28). CD spectroscopy was used to estimate the overall secondary structural content of the lipid-free proteins. As shown in **Fig. 5A**, the CD spectra of G-apoA-I and human plasma apoA-I revealed the characteristic shape of a predominantly α-helical protein with two minima at 208 and 222 nm. The curves were essentially superimposable. Calculations revealed that the proteins were about 44% helical, which is consistent with previous studies (29). Similarly, as shown in Fig. 5B, the spectra for G-apoA-IV and IgA protease-cleaved apoA-IV were of similar shape to apoA-I and were also superimposable with an estimated helical content of about 77%. We followed up the secondary structure analysis with fluorescence measurements to determine the chemical environment of the four Trp residues in apoA-I or the single residue in apoA-IV. **Figure 6A** and 6B show the tryptophan fluorescence spectra of the apoA-I and apoA-IV proteins, respectively. After excitation at 295 nm, G-apoA-I emitted at a maximal wavelength of 331.3 +/− 0.6 (S.D.) nm, whereas plasma apoA-I emitted at 331.7 +/− 1.5 nm. These values are significantly blue-shifted relative to free tryptophan in solution (352 nm) and indicate that the Trp residues are in

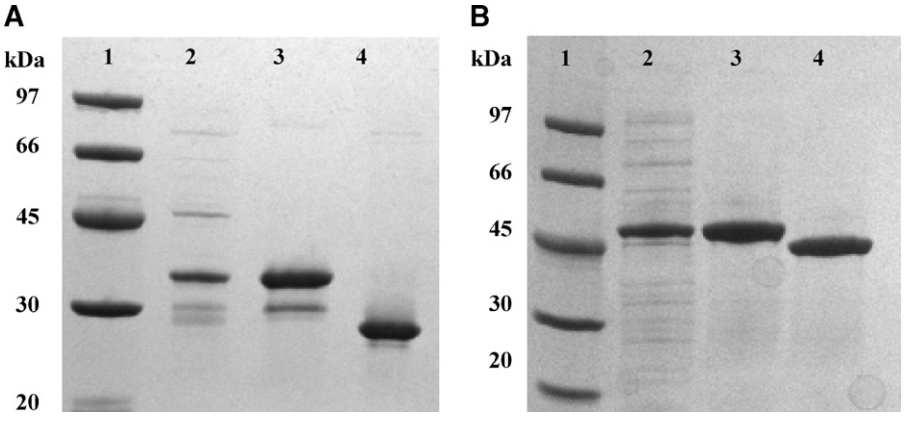

Fig. 4. Purification of recombinant apolipoproteins. 6 μg of each sample (total protein) was analyzed by 4–15% SDS-PAGGE and visualized with Coomassie blue. (A) apoA-I: lane 1, low molecular weight protein standards; lane 2, cleared lysate; lane 3, uncut after IMAC; lane 4, TEV-cut after size exclusion. (B) apoA-IV: lane 1, low molecular weight protein standards; lane 2, cleared lysate, lane 3, uncut after IMAC; lane 4, TEV-cut after IMAC.

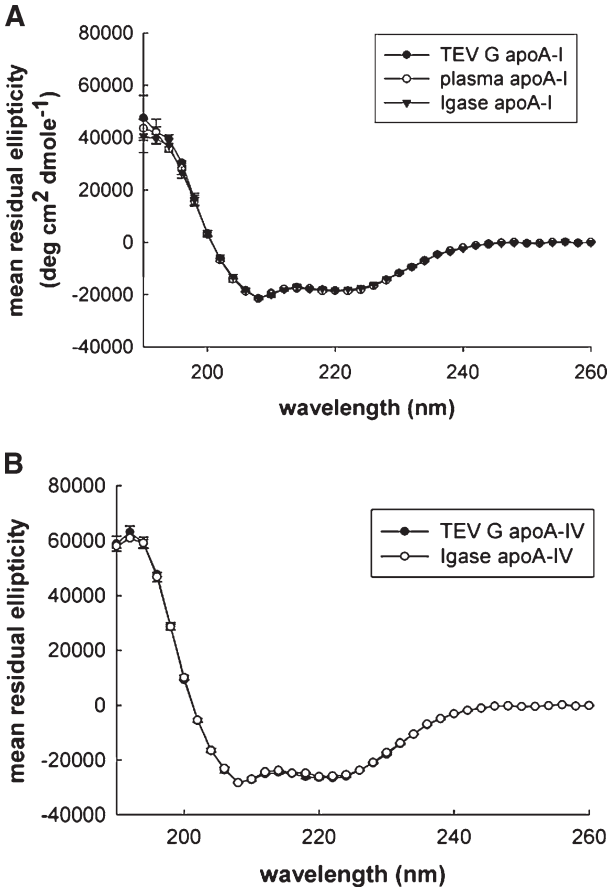

Fig. 5. Far UV circular dichroism spectra of apolipoproteins. (A) apoA-I: recombinant apoA-I cut with IgA protease and TEV protease as well as plasma apoA-I. (B) apoA-IV: recombinant apoA-IV cut with IgA protease (Igase) or TEV protease. Samples were run at room temperature in triplicate with each being the mean of three accumulations. Mean residual ellipticity for all samples was calculated as described in Methods.

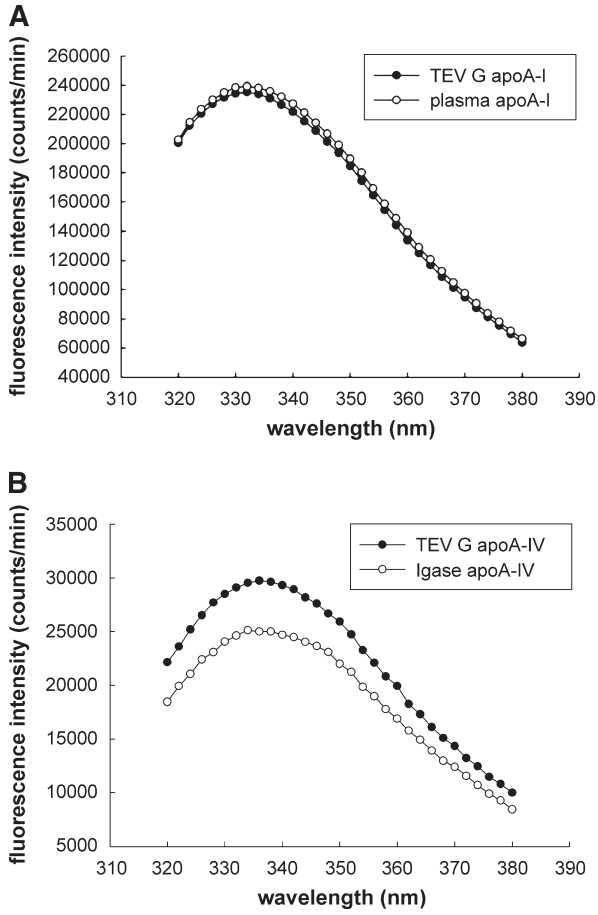

Fig. 6. Tryptophan fluorescence spectra of apolipoproteins. (A) apoA-I: recombinant apoA-I cut with TEV protease (closed circles) as well as plasma apoA-I (open circles). (B) apoA-IV: recombinant apoA-IV cut with TEV protease or IgA protease. Samples were analyzed in triplicate at room temperature, excited with 295 nm light.

a relatively nonpolar environment within the folded protein (30). Similarly, the single tryptophan of TEV G-apoA-IV emitted at 335.8 +/− 1.6 nm whereas IgA protease apoA-IV emitted at 337.2 +/− 2.0 nm (Fig. 6B). We noted a small change in fluorescence intensity between IgA protease–cleaved and TEV-cleaved apoA-IV. The reason for this is unclear; however, the intensity measurement is notoriously dependent on small buffer differences (i.e., pH, salt concentration) and likely does not reflect differences in protein structure. Taken together, these data indicate that the apolipoproteins generated by TEV protease cleavage are structurally similar to either plasma purified protein or that generated by previously validated recombinant methodologies.

To assess the functionality of the TEV protease–cleaved proteins, we performed lipid solubilization and cholesterol efflux comparisons. **Figure 7** shows the results of a DMPC clearance assay, which determines the ability of a given protein to bind and reorganize multilamellar vesicles. Figure 7A shows that G-apoA-I and plasma apoA-I both solubilized DMPC liposomes with similar kinetics, though the plasma protein tended to drive the reaction to a higher degree of completion. Similarly, Fig. 7B shows no difference in lipid binding between IgA protease– and TEV protease–generated apoA-IV. Since a major function of apoA-I is to promote cholesterol efflux via the ATP binding cassette transporter A1 (ABCA1), we incubated the apoA-I proteins with RAW macrophages that had been stimulated to produce ABCA1 with exogenous cAMP (31). At 8 h, it is clear from **Fig. 8** that G-apoA-I and human plasma apoA-I promoted similar degrees of cholesterol efflux in this system at a concentration of the protein at which cholesterol efflux saturation has not been reached (31). Taken together, we found no evidence that apoA-I or apoA-IV cleaved with TEV protease is structurally or functionally different than either plasma purified apoA-I or apoA-IV generated by other strategies.

Stability of TEV protease–generated apolipoproteins

To determine the suitability of these proteins for crystallography studies, we tested the stability of the purified TEV-generated proteins at room temperature for extended periods of time. **Figure 9** shows an SDS-PAGGE analysis of TEV G-apoA-I and TEV G-apoA-IV proteins that had been

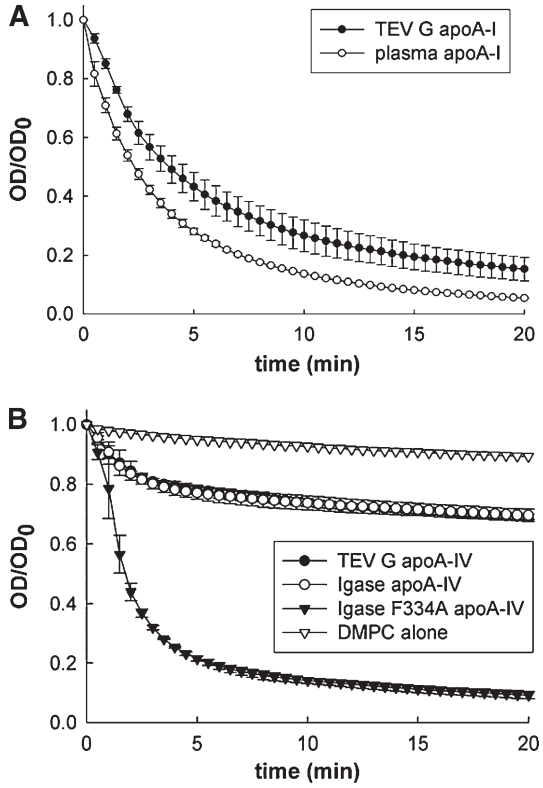

Fig. 7. DMPC clearance assay of apolipoproteins. (A) apoA-I: recombinant apoA-I cut with TEV protease as well as plasma apoA-I. (B) apoA-IV: recombinant apoA-IV cut with TEV protease or IgA protease along with IgA protease-cut, fast-lipid-binding, F334A apoA-IV. All samples were run in triplicate at 24.5°C, the transition temperature of DMPC, for 20 min, readings every 30 sec. Error bars represent one sample standard deviation.

placed at 4°C or room temperature for 2 weeks in STB (see Methods). There was no additional degradation of either protein in the room temperature samples, indicating that the purified samples contained no additional protease activity capable of degrading the protein.

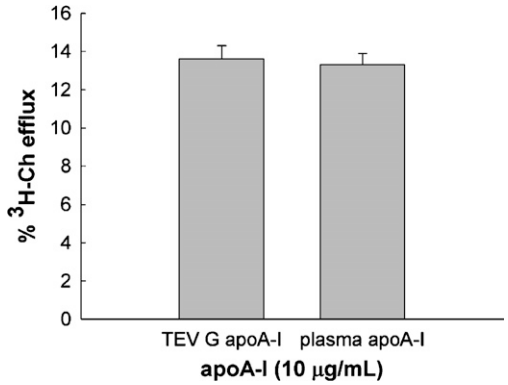

Fig. 8. Cholesterol efflux of apoA-I samples. Plasma-derived and recombinant TEV G-apoA-I samples were used as the cholesterol acceptors from cholesterol-loaded RAW264.7 macrophages. Proteins were added to the media at 10 μg/ml for 8 h. Percent efflux was calculated by dividing the efflux media counts by the total counts (n = 3, error bars represent one sample standard deviation).

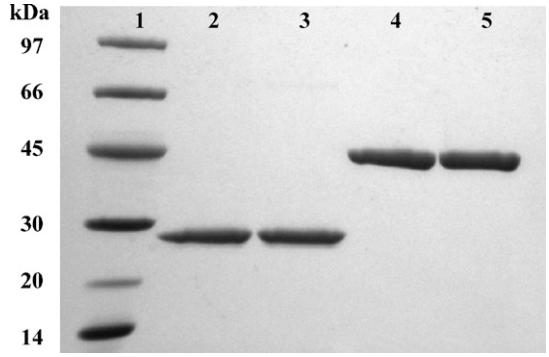

Fig. 9. Long-term stability of recombinant apolipoproteins. ApoA-I and apoA-IV cut with TEV protease were incubated for 2 weeks at 4°C (lanes 2 and 4, respectively) or room temperature (lanes 3 and 5, respectively). 6 μg of each sample was analyzed by 4–15% SDS-PAGGE and visualized with Coomassie blue. Lane 1 contains low molecular weight protein standards.

DISCUSSION

The recombinant *E. coli* expression system incorporating modified TEV protease used in this study has several advantages over other recombinant expression systems that have been utilized for apolipoproteins. Perhaps chief among these is the fact that the protease can be easily expressed in the laboratory, eliminating the need to purchase expensive enzymes commercially. Second, the TEV protease contains an integral His-tag, allowing it to be purified away from the target proteins. This allowed the simultaneous removal of the cleaved tag and the protease from our target products, which typically saves at least one chromatography step that can lower final product yield. Third, TEV protease is highly active at room temperature. Many other proteases require elevated cleavage temperatures that can also activate minor contaminating proteases that can degrade the target protein. Furthermore, shorter, lower temperature incubations minimize oxidation and other protein modifications that can affect target protein function.

Another advantage of the TEV protease is the possibility of generating native proteins that lack extraneous amino acids on the N-terminus. We attempted to produce such so called "clean-cutting" apoA-I and apoA-IV. Unfortunately, the N-terminal residues of apoA-I and apoA-IV did not allow for efficient processing by the TEV protease. The clean-cutting apoA-I, containing an aspartic acid in the X position, was not cut at all in 16 h, and clean-cutting apoA-IV, with a glutamate in the X position, cut with only about 30% efficiency. This was unexpected because the same amino acids, aspartic acid and glutamate, in a NusG-maltose binding protein fusion allowed for around 90% and 60% cleavage, respectively (27). Even though we could not achieve completely native proteins in these cases, the addition of a small and flexible Gly residue would seem to be less obtrusive than the Thr-Pro addition required by the IgA protease or Gly-Pro for the PreScission® protease. Indeed, the structural and functional characterizations performed here bear this out.

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