methods

Ratio determination of plasma wild-type and L159R apoA-I using mass spectrometry: tools for studying apoA-I$_{Fin}$

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Abstract In this report, methods are described to isolate milligram quantities of a mutant apolipoprotein A-I (apoA-I) protein for use in structure-function studies. Expression of the L159R apoA-I mutation in humans reduces the concentration of plasma wild-type apoA-I, thus displaying a dominant negative phenotype in vivo. Earlier attempts to express and isolate this mutant protein resulted in extensive degradation and protein misfolding. Using an *Escherichia coli* expression system used predominantly for the isolation of soluble apoA-I mutant proteins, we describe the expression and purification of L159R apoA-I (apoA-I$_{Fin}$) from inclusion bodies. In addition, we describe a mass spectrometric method for measuring the L159R-to-wild-type apoA-I ratio in a 1 μl plasma sample. These new methods will facilitate further studies into the mechanism behind the dominant negative phenotype associated with the expression of the L159R apoA-I protein in humans.—Owen, J. S., M. S. Bharadwaj, M. J. Thomas, S. Bhat, M. P. Samuel, and M. G. Sorci-Thomas. **Ratio determination of plasma wild-type and L159R apoA-I using mass spectrometry: tools for studying apoA-I$_{Fin}$. *J. Lipid Res.* 2007.** 48: **226–234.**

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Apolipoprotein A-I (apoA-I) is the major structural protein of HDL particles, and its concentration in plasma is inversely correlated with the risk of coronary heart disease (1–3). HDL apoA-I slows the progression of atherosclerosis through several mechanisms, among which the most well documented relates to a process termed reverse cholesterol transport (4, 5). In this process, the newly secreted and lipidated hepatic apoA-I activates the cholesterol esterification enzyme, LCAT, and represents an important branch point in the determination of plasma HDL concentrations. Numerous studies have demonstrated that a specific domain of apoA-I, consisting of the sixth and seventh α-helical repeats (amino acid residues 143–186 of the mature protein) (6, 7), is essential for activation of the LCAT enzyme.

A single amino acid substitution within the sixth helical repeat, L159R apoA-I, or apoA-I$_{Fin}$, was identified in a kindred in Finland in 1996 (8–10). Affected family members were found to be heterozygous for the mutation and to have only 20% as much HDL cholesterol and 25% as much apoA-I in plasma as unaffected family members. L159R apoA-I thus belongs to a group of apoA-I mutations described as displaying a dominant negative phenotype (11). Individuals carrying a single mutant allele show accelerated clearance of HDL from their plasma, presumably attributable in part to the inability of the mutant apoA-I to activate the enzyme LCAT (9, 10). Reduced LCAT activation could in turn reduce the lipidation and thus the maturation of HDL particles, causing more rapid clearance by the kidney (12). Thus, a defect in HDL particle maturation suggests a possible mechanism for the observed dominant negative phenotype in individuals carrying a single allele for this mutation.

Based on other studies (13), the proteolytic degradation of L159R apoA-I in plasma is believed to account for the dominant negative phenotype and its effect on wild-type apoA-I concentrations. In those studies, the L159R apoA-I protein was expressed in mouse models using recombinant adenovirus-mediated gene transfer. In addition, those authors investigated apoA-I secretion using cultured primary mouse hepatocytes and found evidence for the impaired secretion of L159R apoA-I, apparently attributable to intracellular proteolysis.

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Abbreviations: apoA-I, apolipoprotein A-I; CCB, chitin column buffer; IPTG, isopropylthio-β-D-galactoside; LC-MS, high-performance liquid chromatography-electrospray mass spectrometry; LC-MS/MS, high-performance liquid chromatography-electrospray tandem mass spectrometry; LDLr$^{-/-}$, apoA-I$^{-/-}$, low density lipoprotein receptor-deficient/apolipoprotein A-I-deficient.

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To investigate the metabolic properties of this mutant apolipoprotein, large-scale expression of purified L159R apoA-I was necessary, complete with a method for its quantification in the presence of wild-type apoA-I. In previous studies, the *Escherichia coli* IMPACT™ system from New England Biolabs has been used routinely in our laboratory for the expression and purification of wild-type apoA-I and numerous mutant forms of apoA-I (14, 15). However, our initial attempts to express L159R apoA-I using this system were unsuccessful because of the extensive degradation of the target protein in the bacteria. This problem was overcome by inducing expression at a higher temperature (i.e., 30°C) rather than at the 15°C used previously for apoA-I mutant protein expression (14, 15). Instead of degradation of the L159R apoA-I, expression at 30°C resulted in the formation of inclusion bodies within the bacteria.

The production of recombinant proteins in inclusion body form, rather than soluble form, presents a technical challenge in that the target protein must be solubilized using denaturants and subsequently refolded. The denaturants used should be of the type that can be removed completely before structure-function studies are carried out. In spite of this drawback, however, inclusion body expression is widely used for commercial protein production. An advantage of producing proteins in inclusion bodies is that they enjoy a high degree of protection from cellular proteases. Other advantages include high yield (up to 30% of total cellular protein) and high purity; the target protein is generally the main constituent of inclusion bodies (16).

Based on the isolation of pure L159R apoA-I, we then developed a novel assay capable of quantifying wild-type and L159R apoA-I in plasma containing both proteins. The assay is based on high-performance liquid chromatography-electrospray mass spectrometry (LC-MS) analysis of a trypsin digest of the protein mixture. With the development of this technique, future studies will be able to use this LC-MS assay to probe the mechanism of the dominant negative effect of the helix 6 mutation L159R apoA-I on wild-type apoA-I plasma concentrations.

MATERIALS AND METHODS

Materials

The IMPACT™ protein expression system, including *E. coli* strain ER2566, the pTYB12 plasmid vector, and chitin beads, was purchased from New England Biolabs. Ampicillin was from Fisher. PCR primers were synthesized by International DNA Technologies. Taq DNA polymerase was from Roche. Restriction enzymes and modified sequencing-grade porcine trypsin were from Promega. Isopropylthio-β-D-galactoside (IPTG) and Simply Blue™ protein stain were from Invitrogen. DNase I was from Worthington Biochemical. RapiGest SF was from Waters. Solvents used in MS and LC-MS were "B&J GC^2" grade from Burdick and Jackson. Other materials were from Sigma Chemical.

Plasmid construction, protein expression, and purification

The coding sequence for L159R apoA-I was cloned from the previously described CMV5 vector (17), amplified by PCR as described (14), and inserted into the pTYB12 vector (14). For expression and purification of L159R apoA-I from inclusion bodies, we modified the method reported previously for the soluble expression and purification of apoA-I mutants (14). *E. coli* ER2566 was transformed with 1 μg of plasmid DNA and grown overnight at 37°C in Luria-Bertani broth containing 100 μg/ml ampicillin. The next morning, 1 liter of Luria-Bertani broth was inoculated with 10 ml of overnight culture and grown at 37°C to an optical density at 600 nm of 0.6–0.8. Protein expression was induced with 0.3 mM IPTG at 30°C. After 6 h, cells were pelleted and frozen at −80°C until further use. In some experiments, protein expression was induced with 0.3 mM IPTG overnight at 15°C, and cells were used the next morning without freezing. We found, however, that induction for 6 h at 30°C gave better expression levels of the target protein (see Results; **Fig. 1A**). For protein purifica-

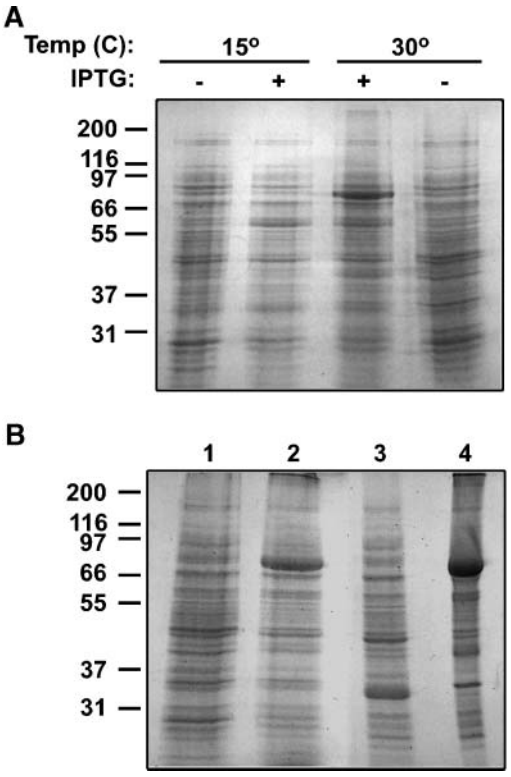

Fig. 1. Full-length L159R apolipoprotein A-I (apoA-I) fusion protein can be expressed at 30°C but not at 15°C. *E. coli* strain ER2566 was transformed with plasmid pTYB12 encoding L159R apoA-I as a fusion protein having the chitin binding domain at the N terminus, as described in Materials and Methods. Aliquots of the cultures were separated by 10% SDS-PAGE, and protein bands were visualized by staining with Simply Blue™. A: Cells were grown at 37°C until reaching an optical density at 600 nm of 0.6 and then incubated in the absence (−) or presence (+) of 0.3 mM isopropylthio-β-D-galactoside (IPTG) at 15°C for 16 h or at 30°C for 6 h. B: The L159R apoA-I fusion protein expressed at 30°C is present in the insoluble pellet fraction. ER2566 cells were transformed and cultured as in A. Upon reaching an optical density at 600 nm of 0.6, cells were incubated at 30°C for 6 h in the absence (lane 1) or the presence (lane 2) of 0.3 mM IPTG. Cells incubated with IPTG at 30°C for 6 h were disrupted as described in Materials and Methods, and the homogenate was centrifuged at 33,000 *g* for 30 min at 4°C. Aliquots of the supernatant (lane 3) and pellet (lane 4) were separated by 10% SDS-PAGE, and protein bands were visualized by staining with Simply Blue™. The band migrating at ~83 kDa corresponds to the chitin binding domain-intein fusion apoA-I protein.

tion, cell pellets from the 1 liter preparation were thawed and resuspended in a total volume of 60 ml of 1× chitin column buffer (CCB) containing 500 mM NaCl, 30 mM HEPES, pH 8.0, 0.1 mM EDTA, and 80 μg/ml PMSF. Cells were disrupted with two passes through an Avestin Emulsiflex-C3 high-pressure emulsifier at a homogenizing pressure of 13,000–14,000 p.s.i. The homogenate was treated with 6 μg/ml DNase I and 1.8 mM $MgCl_2$ for 30 min on ice.

Inclusion bodies were recovered from the homogenate by centrifugation for 30 min at 33,000 *g* at 4°C. The supernatant was discarded. Inclusion bodies were suspended in 80 ml of 8 M urea and again centrifuged for 30 min at 33,000 *g* at 4°C. The supernatant (75 ml) was mixed with 80 ml of 5× CCB (2.5 M NaCl, 150 mM HEPES, pH 8.0, and 0.5 mM EDTA) and then diluted with water to a total volume of 400 ml, resulting in a final composition of 1.5 M urea and 1× CCB, pH 8.0. The diluted protein solution was incubated overnight at 4°C on a rotator in the presence of 80 μg/ml PMSF to refold the protein. Higher yields of correctly refolded protein were obtained when the deionized water used for this dilution step had been gently stirred under vacuum for 30 min to remove dissolved gases.

Four 3 × 30 cm columns were prepared with 25 ml of chitin slurry (~15 ml of chitin beads) in each column. The chitin matrix was washed thoroughly with water to remove ethanol. Each column was then washed with 100 ml of 1× CCB, pH 8.0, containing 1.5 M urea, and the protein solution was added to the columns (100 ml per column) and mixed on a rotator for 1 h at 4°C. The matrix was pooled into two columns, and each column was washed with 20 bed volumes (600 ml) of 1× CCB containing 1.5 M urea, followed by 20 bed volumes of 1× CCB without urea. The matrix was then incubated at 4°C for 2 h to allow protein refolding. The intein cleavage reaction was initiated by quickly (gravity flow rate) washing each column with 1 bed volume (30 ml) of a cleavage buffer composed of 1× CCB with 100 mM DTT and 80 μg/ml PMSF. We found it helpful to readjust the pH to 8.0 after adding the DTT. Each column was then mixed on a rotator with 70 ml of cleavage buffer overnight at 16°C. The cleavage buffer containing the liberated L159R apoA-I was drained from the column, and a second cleavage reaction was carried out for 4 h at room temperature using freshly prepared cleavage buffer. The products from both cleavage reactions were thoroughly dialyzed against 10 mM ammonium bicarbonate, pH 7.4, containing 3 μM EDTA and 15 μM sodium azide. The entire cleavage reaction (~500–600 ml) was lyophilized to dryness, dissolved in 8–10 ml of 6 M guanidine hydrochloride (final protein concentration, 0.2–0.5 mg/ml), and then dialyzed exhaustively against 10 mM ammonium bicarbonate, pH 7.4. This relatively low protein concentration is necessary to minimize precipitation of the L159R apoA-I during refolding; however, once refolding is complete, L159R apoA-I can be concentrated without precipitation, and the refolded mutant protein appears as soluble as the wild-type protein. The refolding step was essential for the complete removal of a large amount of HEPES that was associated with the apoA-I and could not be removed by direct dialysis. This step is recommended because HEPES interferes with the Lowry assay for quantification of total protein yield (18).

Mass spectrometric quantitation of the L159R-to-wild-type apoA-I ratio

To compare and quantify unknown samples composed of wild-type and L159R apoA-I mixtures, pure stocks of these apolipoproteins were mixed at various ratios, and a standard curve was created. The stock solutions for L159R and wild-type apoA-I were diluted to a working solution of 100 ng/μl with 10 mM ammonium bicarbonate, pH 7.4, and then mixed in equal volumes (10 μl of each), and a 10 μl aliquot was withdrawn that represented standard 1 [50% L159R + 50% wild-type apoA-I (mutant-to-wild-type ratio = 1)]. Another 10 μl from the wild-type apoA-I was added to standard 1 and mixed, and a 10 μl aliquot was again withdrawn, which represented standard 2 [25% L159R + 75% wild-type apoA-I (mutant-to-wild-type ratio = 0.33)]. Two-fold serial dilutions were continued in this manner to generate five standards, each containing 1 μg of total protein. All standards were loaded in their entirety onto individual lanes on a 12% SDS-PAGE apparatus and subjected to trypsin digestion and LC-MS (19) in parallel with mouse plasma samples.

The peptide mixture from each trypsin digestion was analyzed by LC-MS on a Waters Q-TOF API-US mass spectrometer running MassLynx™ 4.0 software and connected to a Waters CapLC. The HPLC column was a 100 μm × 100 mm Waters Symmetry C_{18} column with 3.5 μm particle size. Samples were injected in water containing 0.02% heptafluorobutyric acid at 0.5 μl/min. The column was eluted with solvent A for 12 min, followed by a linear gradient to 80% solvent B over 8 min, then holding at 80% solvent B for 10 min, before returning to the initial conditions. Solvent A was water-acetonitrile (99:1) with 0.2% formic acid, and solvent B was water-acetonitrile (3:97) with 0.2% formic acid. A gradient flow rate of 0.4 μl/min was achieved by operating the CapLC pump at 4 μl/min and splitting the solvent stream before the column. Positive ion electrospray spectra from *m/z* 300–1,600 were recorded in continuum mode with a 2.4 s accumulation time.

Tryptic fragment T25, which contains the mutated residue 159, provided the basis of the quantitative assay. The ratio of chromatographic peak areas observed for the $[M+2H]^{2+}$ charge states of the wild-type and L159R versions of the T25 peptide was plotted against the ratios of protein amounts to generate a calibration curve. Identities of the T25 peptides from wild-type and L159R apoA-I were confirmed by sequencing by high-performance liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) as described (19).

ELISA and the analysis of transgenic and knockout mouse plasma

ELISA conditions for the quantification of human wild-type or human L159R apoA-I in plasma samples were the same as those used previously for human wild-type apoA-I (6). When recombinant purified L159R apoA-I was used in place of wild-type apoA-I for the ELISA standard curve, no difference in slope was observed. Thus, to quantify the total human apoA-I concentration from plasma containing a mixture of the two apolipoproteins, ELISA should be conducted followed by determination of the mass ratio of wild-type to L159R apoA-I using the mass spectrometry method described here.

The production of human L159R apoA-I transgenic mice was carried out by DNA microinjection into C57BL/6J×D2F1 zygotes. The coding sequence for human L159R apoA-I was cloned as described previously (17), amplified by PCR, and inserted into the liver-specific apoE promoter LE6 vector (20). After weaning of founder mice, a mouse tail biopsy of ~1 cm in length was used for DNA analysis. Tail DNA was analyzed by PCR for the presence of the L159R apoA-I transgene. All transgenic founders were further genotyped by Southern blot, Western blot, and ELISA specific for human apoA-I. Founder animals carrying the L159R apoA-I transgene were fully crossed into low density lipoprotein receptor-deficient/apolipoprotein A-I-deficient ($LDLr^{-/-}$, $apoA\text{-}I^{-/-}$) double knockout mice to obtain transgenic L159R apoA-I, $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ over five generations (21). Transgenic mice expressing the human wild-type apoA-I gene were purchased from Charles River Laboratories and were also fully crossed into the $LDLr^{-/-}/apoA\text{-}I^{-/-}$ double knockout mouse

genotype to obtain transgenic wild-type apoA-I, LDLr$^{-/-}$, apoA-I$^{-/-}$. Crosses between transgenic L159R apoA-I, LDLr$^{-/-}$, apoA-I$^{-/-}$ and transgenic wild-type apoA-I, LDLr$^{-/-}$, apoA-I$^{-/-}$ were also made to obtain the double transgenic L159R×wild-type apoA-I in the double knockout background (LDLr$^{-/-}$, apoA-I$^{-/-}$). All mice were maintained in specific pathogen-free barrier facilities in Microisolater™ caging (Lab Products, Maywood, NJ). All mice were housed at the Wake Forest University Baptist Medical Center, where procedures were approved by the Animal Care and Use Committee of the Wake Forest University Health Sciences in accordance with all Public Health Service guidelines.

Experimental mice were weaned at 21 days and fed a chow diet (Purina). Blood was obtained by orbital sinus bleeding after a 4 h fast for evaluation. The animals were anesthetized using a 1:1 mixture of ketamine (50 mg/ml) and xylazine (10 mg/ml) in which 1 μl per gram of body weight was injected intramuscularly behind the knee. Orbital sinus bleeding was performed by inserting a micro hematocrit capillary tube into the retro-orbital plexus. Up to 100 μl of whole blood was drawn. The animal's eye was treated with an ophthalmic ointment (Lacri-Lube) to keep the eyes lubricated, and the mouse was then placed on a 37°C heating pad.

RESULTS

In previous studies, we have detailed the expression and purification of wild-type and mutant forms of apoA-I using the *E. coli*-based, commercially available IMPACT™ system (14, 15). This system expresses the target protein fused to an intein and a chitin affinity tag sequence. The intein is engineered so that the addition of DTT causes self-cleavage of the fusion protein, releasing free target apolipoprotein into solution while the chitin binding domain-intein fusion protein remains attached to the chitin affinity matrix. One important advantage of this expression system is that the native apolipoprotein can be obtained without the usual addition of numerous amino acid sequences at the N or C terminus of the target protein for the addition of histidine tags and protease cleavage sites necessary for their partial removal after affinity purification.

Our initial attempts to express L159R apoA-I using the IMPACT™ system failed, apparently because of the extremely rapid degradation of the target protein within the bacteria. Figure 1A shows the whole cell extracts from *E. coli* harboring the pTYB12 plasmid for the expression of L159R apoA-I grown at two different temperatures. Induction of the cells with or without IPTG at 15°C overnight (Fig. 1A, left two lanes) led to the accumulation of several bands of ~60 kDa, smaller than the expected 83 kDa chitin binding domain-intein-apoA-I fusion protein. In contrast, when induction was carried out for 6 h at 30°C, IPTG induced a strong 83 kDa band (Fig. 1, far right lane).

Furthermore, induction at 30°C caused the target protein to be insoluble, presumably in the form of inclusion bodies, as shown by its presence in the pellet fraction after centrifugation of the cell homogenate (Fig. 1B). The 83 kDa band induced by IPTG at 30°C (Fig. 1B, lane 2) was not present in the absence of IPTG (Fig. 1B, lane 1). When the IPTG-induced 30°C cell homogenate was centrifuged, the 83 kDa product was not found in the soluble fraction of disrupted cells (Fig. 1B, lane 3) but was present in the pellet fraction (Fig. 1B, lane 4). Thus, to purify the target protein from the insoluble fraction or inclusion bodies, we outlined an optimized procedure in Materials and Methods for the solubilization and refolding of the chitin binding domain-intein-L159R apoA-I fusion protein and its subsequent cleavage to release pure native human apoA-I protein. A critical feature of this procedure is that the apoA-I fusion protein is bound to the chitin affinity matrix under mild denaturing conditions (1.5 M urea), but intein cleavage is carried out in the total absence of urea. In contrast to the manufacturer's recommendations (http://www.neb.com/nebecomm/products/productE6900.asp), we found that in our hands, even low concentrations of urea in the cleavage buffer (~0.5 M) completely prevented the intein cleavage reaction (data not shown). Using this protocol, typically, 1 liter of bacterial culture yielded >3 mg of pure L159R apoA-I.

After the isolation and purification of L159R apoA-I, our next goal was to develop a method to distinguish and quantify L159R from wild-type apoA-I in mixtures of the two proteins. To do this, a standard curve containing mixtures of pure human wild-type and L159R apoA-I in known proportions was created, run on 12% SDS-PAGE gels, and then subjected to in-gel trypsin digestion followed by LC-MS analysis. As would be expected, the standard curve using both purified proteins allows for the mass spectrometry response ratio to be calculated and used for the quantification of unknown plasma samples, as shown in **Fig. 2**.

The peptides containing residue 159 (the 25th tryptic peptide from the N terminus, designated T25) showed different HPLC retention times and *m/z* values characteristic of the wild-type and L159R apoA-I proteins. For both wild-type and L159R apoA-I T25 peptides, the $[M+2H]^{2+}$ charge state was by far the most abundant (data not shown). Mixtures of purified wild-type and L159R apoA-I proteins, containing from 50% L159R apoA-I (ratio of L159R to wild type = 1) to 3.1% L159R apoA-I (ratio = 0.0323), gave reproducible chromatographic peak areas for the $[M+2H]^{2+}$ charge states of the two T25 peptides (Fig. 2A, B). In the mixture containing 3.1% L159R apoA-I, the mutant T25 peptide was detected with a signal-to-noise ratio of 5 (Fig. 2B). Thus, the peak area of the L159R apoA-I T25 peptide reflected the known starting percentage of L159R apoA-I mass in a linear manner, as seen in Fig. 2C.

Additionally, we also conducted spiking experiments in which a known mass of pure L159R apoA-I was added to plasma from a transgenic wild-type apoA-I, LDLr$^{-/-}$, apoA-I$^{-/-}$ mouse and pure wild-type apoA-I was added to the plasma from a transgenic L159R apoA-I, LDLr$^{-/-}$, apoA-I$^{-/-}$ mouse. From these experiments, spiking various masses of pure apolipoprotein into mouse plasma, the results were essentially the same as with mixing purified wild-type and mutant protein (Fig. 2).

We next investigated whether this method would provide an assay allowing the mass determination of L159R and wild-type apoA-I from plasma mixtures of the two apolipoproteins. This assay would be used in conjunction with the total human apoA-I values derived from ELISA

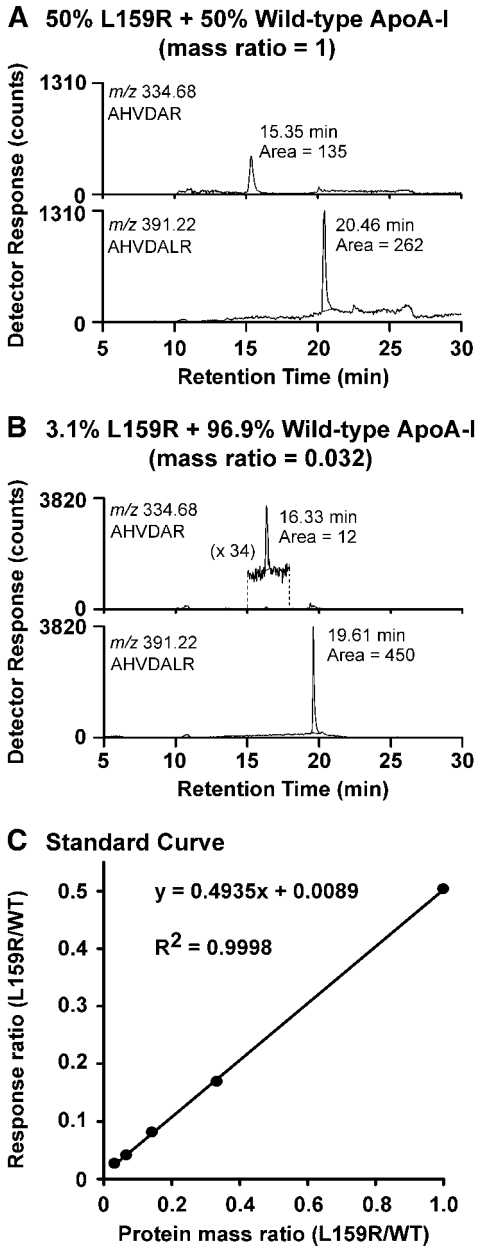

Fig. 2. Generation of a wild-type-L159R apoA-I standard curve. Purified human wild-type apoA-I and L159R apoA-I were mixed so that the mutant protein represented 50, 25, 12.5, 6.25, or 3.125% of the total protein mass. Expressed as the ratio of mutant to wild-type protein, these points are 1, 0.33, 0.14, 0.067, and 0.032, respectively. One microgram of the mixture was run on 12% SDS-PAGE, visualized using Simply Blue™ stain, excised from the gel, and subjected to in-gel trypsin digestion, and the resulting peptide mixtures were analyzed by high-performance liquid chromatography-electrospray mass spectrometry (LC-MS) as described in Materials and Methods. The amino acid sequence of the wild-type T25 peptide is AHVDALR (monoisotopic mass, 780.42 Da; $[M+2H]^{2+}$ *m/z* 391.22); the L159R T25 peptide sequence is AHVDAR (monoisotopic mass, 667.34 Da; $[M+2H]^{2+}$ *m/z* 334.68). A: Chromatogram of a trypsin digest of 50% L159R apoA-I and 50% wild-type apoA-I. B: Chromatogram of a trypsin digest of 3.125% L159R apoA-I and 96.875% wild-type apoA-I. In the inset, the indicated portion of the upper trace is shown magnified 34-fold on the vertical axis. C: Linear relationship between the peak area of L159R apoA-I and the percentage of L159R apoA-I, as analyzed by least-squares linear regression. WT, wild-type.

and would represent a valuable tool in delineating the proportion of mutant to wild-type protein from biological samples. To do this, plasma was obtained from transgenic mice expressing human wild-type apoA-I, L159R apoA-I, or both in a null mouse apoA-I and LDLr background. **Figure 3** shows the results of a 12% SDS-PAGE comparing the migration of pure apoA-I with that of 1 μl of plasma from transgenic and knockout mice. Plasma from transgenic wild-type apoA-I, $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ (Fig. 3, lane 2) contained a band that comigrated with the purified wild-type apoA-I (Fig. 3, lane 1). Likewise, the 28,000 Da band found in plasma from transgenic L159R apoA-I, $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ mice (Fig. 3, lane 4) comigrated with the purified L159R apoA-I protein (Fig. 3, lane 3).

It was interesting that the L159R apoA-I from either the pure source or from plasma always appeared on SDS-PAGE to migrate slightly slower than the wild-type form of the protein. Finally, as a control for unrelated plasma proteins, 1 μl of plasma from nontransgenic $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ mouse plasma (Fig. 3, lane 6) did not contain any apparent staining proteins in that region of the gel.

All gel bands corresponding to apoA-I were excised, digested with trypsin, and analyzed by LC-MS; chromatograms are shown in **Fig. 4**. Wild-type human apoA-I contained a $[M+2H]^{2+}$ peptide with *m/z* = 391.22, the predicted *m/z* of the wild-type T25 peptide, as shown in Fig. 4A, lower trace. LC-MS/MS sequencing of this peptide gave the expected wild-type T25 sequence of AHVDALR (data not shown). Pure wild-type apoA-I also gave a small peak at a slightly earlier retention time with *m/z* 334.68, the predicted *m/z* of the $[M+2H]^{2+}$ state of the mutant T25 (Fig. 4A, upper trace; also visible in Fig. 2B, upper trace). This unknown peak did not interfere with the de-

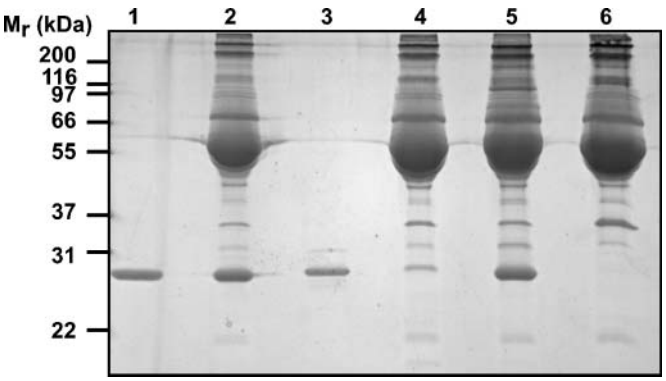

Fig. 3. SDS-PAGE analysis of plasma containing L159R-wild-type apoA-I. One microliter aliquots of mouse plasma were run on 12% SDS-PAGE and then stained with Simply Blue™. Lane 1, 1 μg of pure wild-type apoA-I. Lane 2, plasma from a transgenic mouse expressing wild-type human apoA-I in the low density lipoprotein receptor-deficient/apolipoprotein A-I-deficient ($LDLr^{-/-}$, $apoA\text{-}I^{-/-}$) double knockout background. Lane 3, 1 μg of pure L159R apoA-I. Lane 4, plasma from a transgenic mouse expressing human L159R apoA-I in the $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ double knockout background. Lane 5, plasma from a transgenic mouse expressing both human L159R and wild-type apoA-I in the $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ double knockout background. Lane 6, plasma from an $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ double knockout mouse.

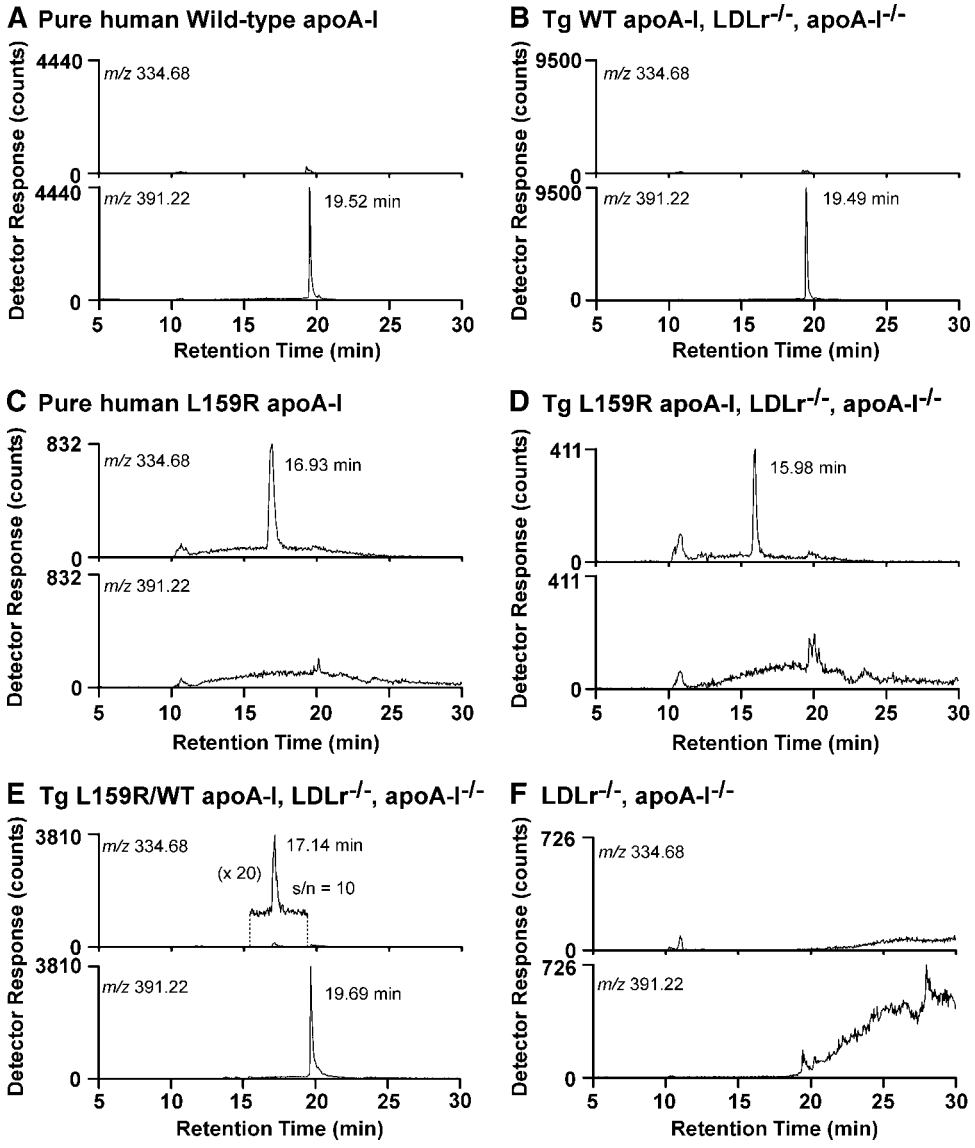

Fig. 4. LC-MS quantification of L159R apoA-I from mouse plasma. Each of the bands corresponding to apoA-I (~28,000 Da) from the gel shown in Fig. 3 was excised and digested with trypsin, and the resulting peptide mixture was analyzed by LC-MS as described in Materials and Methods. A–F show the HPLC elution of ions at the expected *m/z* for the $[M+2H]^{2+}$ states of the T25 fragments of wild-type (lower traces; *m/z* 391.22) and L159R (upper traces; *m/z* 334.68) apoA-I. A: Ions obtained from pure human wild-type apoA-I. B: Ions obtained from apoA-I isolated from the plasma of a transgenic human wild-type apoA-I, $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ mouse. C: Ions obtained from pure human L159R apoA-I. D: Ions obtained from apoA-I isolated from the plasma of a transgenic L159R apoA-I, $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ mouse. E: Ions obtained from apoA-I isolated from the plasma of a double transgenic L159R/wild-type apoA-I, $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ mouse. In the inset, the indicated portion of the upper trace is shown magnified 20-fold on the vertical axis. F: Ions obtained from the region of the gel corresponding to apoA-I from the plasma of an $LDLr^{-/-}$, $apoA\text{-}I^{-/-}$ mouse. Tg, transgenic; WT, wild-type.

velopment of a quantitative assay, however, because its retention time was >2 min later than that of the authentic L159R apoA-I T25 (Fig. 4C, upper trace). Pure L159R apoA-I did not contain this unknown peak (Fig. 4C, upper trace). Sequence analysis by LC-MS/MS showed that this peptide was not related to apoA-I; therefore, it likely represents a contaminant from plasma. The large *m/z* 334.68 peak seen in pure L159R apoA-I (Fig. 4C, upper trace) had the correct $[M+2H]^{2+}$ charge state and was confirmed by LC-MS/MS sequencing to have the mutant T25 sequence of AHVDAR (data not shown). ApoA-I bands excised from the SDS-PAGE lanes from the corresponding transgenic mouse plasma gave chromatograms essentially identical to either pure wild-type or L159R apoA-I, as seen by comparing Fig. 4A, C with Fig. 4B, D. Moreover, plasma from the double transgenic mice expressing both L159R and wild-type apoA-I transgenes in the $apoA\text{-}I^{-/-}$, $LDLr^{-/-}$ null background yielded both wild-type and L159R apoA-I

T25 chromatographic peaks, as shown in Fig. 4E. As a control, plasma from a double null, apoA-I$^{-/-}$, LDLr$^{-/-}$ mouse (Fig. 3, lane 6) contained none of these chromatographic peaks (Fig. 4F).

We conclude that the LC-MS approach described here will provide a means to accurately quantify both wild-type and L159R apoA-I concentrations in mouse plasma when used in conjunction with a standard ELISA method to measure total plasma apoA-I concentrations. This type of approach can, in principle, also be extended to many other situations in which the quantitation of two highly similar isoforms, variants, or modifications of a protein is desirable.

DISCUSSION

Here, we describe a method for the recombinant expression, purification, and quantification of L159R apoA-I. Expression of the mutant protein in *E. coli* proved unexpectedly difficult using the IMPACT™ expression system, which our laboratory has used successfully to express wild-type and numerous apoA-I mutant forms, without major modifications (14, 15). In contrast, L159R apoA-I could not be expressed using these methods except in an insoluble form as inclusion bodies. Attempted soluble expression of the L159R apoA-I at low temperature (15°C) resulted in IPTG-induced products with an apparent molecular mass of lower than expected (<83,000 Da), suggestive of intracellular proteolytic degradation. Proteolysis and inclusion body formation are two branches of the protein quality control pathway in bacteria (22). Thus, it is tempting to speculate that the L159R mutation perturbed the tertiary structure of apoA-I, increasing the probability of misfolding and degradation. These results underscore the importance of using structurally native apoA-I proteins for metabolic and structural studies. The addition of N- or C-terminal amino acids attached for the sake of purification may alter the overall conformation of the protein, as the N- and C-terminal regions of apoA-I appear to be important in its overall tertiary conformation (23).

These studies will enable the availability of milligram quantities of pure recombinant L159R apoA-I that will facilitate in vitro studies as well as in vivo kinetic turnover studies in mice. This novel mass spectrometric assay will also be indispensable for characterizing mouse models as well as human heterozygous carriers of mutant forms of apoA-I. In humans, L159R apoA-I displays a dominant negative phenotype (i.e., heterozygous carriers of L159R apoA-I have ~10% of the plasma apoA-I concentration of their unaffected relatives). This observation has two implications: *1*) the mutant protein is poorly secreted and/or rapidly cleared from plasma; and *2*) the secretion and/or clearance of the wild-type protein are somehow affected by the presence of the mutant protein. An important question, then, concerns the nature of the interactions of the wild-type and mutant proteins in vivo. Both intracellular and extracellular interactions may contribute to the dominant negative effect of the mutation. COS-1 cells (17) and primary mouse hepatocytes (13) secrete L159R apoA-I poorly, and expression of L159R apoA-I interferes with the secretion of coexpressed wild-type apoA-I (13). Regarding extracellular mechanisms, in human heterozygous carriers of L159R apoA-I, the fractional catabolic rate of apoA-I in plasma is abnormally high (9). Those authors, however, were unable to quantify the ratio of mutant to wild-type apoA-I in plasma, and their kinetic studies used delipidated autologous apoA-I for radiolabeling. Thus, it appears that L159R apoA-I is more rapidly catabolized in plasma than is the wild-type protein, but to date, no direct evidence indicates whether or how the catabolic rate of the wild-type protein may be accelerated by the presence of the mutant protein (11). Thus, the methods we describe here will provide better tools for answering such questions.

The quantification of human L159R apoA-I and human wild-type apoA-I from mouse plasma lacking mouse apoA-I will be used in future studies aimed at defining the mechanism of the dominant negative phenotype. However, these same MS methods would be equally effective in the quantification of mouse and human wild-type apoA-I in mouse plasma. In principle, any tryptic peptide that differs in sequence between the mouse and human proteins could be used to develop such an assay; for example, the mouse sequence for the T25 peptide of apoA-I is ESLAQR, having $[M+H]^+$ at *m/z* 703.37 and $[M+2H]^{2+}$ at *m/z* 352.19, readily distinguishable from both the wild-type and L159R forms of the T25 fragment of human apoA-I. Although ELISA methods currently provide the quantification of mouse and human wild-type apoA-I in mixtures of plasma (6), studies of mutant apoA-I synthesis and catabolism may be carried out in mice not engineered for the lack of endogenous apoA-I expression.

High sensitivity, resolution, and versatility have made mass spectrometry-based techniques the first choice for the identification of protein mutations and posttranslational modifications. The same qualities also make mass spectrometry an ideally suited, underused tool for the differential quantitation of highly similar proteins in biological mixtures. A number of groups have applied mass spectrometry to identify mutations and posttranslational modifications in protein samples (24–29), yet only a few have used mass spectrometry to quantify the relative expression levels of wild-type versus mutant proteins in heterozygous subjects. Nier et al. (30) measured the ratio of mutant to wild-type α-myosin heavy chain in subjects carrying point mutations associated with familial hypertrophic cardiomyopathy. Myosin was extracted from muscle biopsies and digested with protease, and the peptides were separated by HPLC. The HPLC fractions containing the mutant and wild-type peptide fragments were identified by mass spectrometry, but the mutant-to-wild-type ratio was quantified by capillary zone electrophoresis rather than by mass spectrometry. In the realm of posttranslational modifications, Cutillas et al. (31) used one-dimensional gel electrophoresis and in-gel proteolysis to identify and quantify phosphorylation sites in tissue extracts. Those authors quantified changes in protein phosphorylation by comparison with the signal from an internal

standard. Quantitation was based on the integration of chromatographic peaks from an LC-MS analysis of tryptic digests, similar to our method. Different from our method, however, was the proteomics-style approach of those authors, who screened >400 proteins for changes in phosphorylation state.

Our method, in contrast, focuses on a single target protein whose total concentration is measured independently by ELISA. This narrow focus allows us to achieve greater quantitative accuracy and precision by preparing a standard curve from highly purified preparations of the two proteins under study. The importance of this point is apparent in Fig. 2C, where the slope of the calibration curve is markedly different from the theoretical value of 1.0. In other words, to achieve accurate quantitation (as opposed to proteomic screening), it is not valid to assume that two different peptides will be ionized and detected with the same efficiency. Therefore, it should be kept in mind that application of our assay to other proteins has three general requirements: first, the availability of purified materials for making a standard curve; second, a means of measuring absolute concentration, such as ELISA; and third, a means of separating the proteins of interest from other proteins in the sample that may interfere. To meet this third requirement may in some instances require the use of two-dimensional SDS-PAGE, depending on the complexity of the sample.

The results obtained by Nier et al. (30) underscore the biological importance of the differential quantitation of mutant and wild-type proteins. The heterozygous individuals examined have one wild-type and one mutant allele; yet the mutant protein represented, on average, only 12% and 23% of the total for the two point mutations studied. It is increasingly appreciated that the mRNA expression of a gene does not equal protein expression, because a multitude of unknown factors can affect the folding, post-translational modification, trafficking, and catabolism of mutant and wild-type proteins differently, and the resulting phenotype is more a product of protein dose than of gene dose. In familial hypertrophic cardiomyopathy, as in many inherited disorders, the severity of disease varies widely, even within a single family. Nier et al. (30) speculate that such phenotypic variability may be partly attributable to differences in the ratio of wild-type to mutant protein expressed. The same may be true of the familial hypoalphalipoproteinemias and, indeed, of any inherited disorder that manifests in the heterozygous state. Beyond the specific method we describe, then, we anticipate that the general analytical approach we have outlined will find broad applicability in the study of disease-associated genes.

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