Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 41 (2005) 447–453
Protein Expression & Purification
www.elsevier.com/locate/yprep

Bacterial expression and characterization of rat apolipoprotein E

Kevin Pearson, Min Liu, Ling Shen, Patrick Tso, W. Sean Davidson *

Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, OH 45267, USA

Received 6 January 2005, and in revised form 16 February 2005
Available online 10 March 2005

Abstract

Apolipoprotein (apo) E is a protein involved in both lipid metabolism and neuroprotection. Recently, it has been suggested that apoE may play a role in the regulation of food intake and body weight in rodents. However, rodent plasma apoE is difficult to purify in reasonable amounts due to numerous time-consuming steps. To circumvent this, we created a bacterial expression system for the efficient production of large amounts of rat apoE. We inserted rat apoE DNA into the pET30 expression vector and overexpressed the proteins in *Escherichia coli* strain BL21 (DE3). A histidine tag present at the N-terminus allowed for easy purification of the recombinant protein. The tag was removed with an IgA protease (*Igase*) from *Neisseria gonorrhoeae* leaving the mature form of the protein. The use of *Igase* was important as several more common proteases routinely cleave apolipoproteins at undesired sites. The recombinant protein was then compared both structurally and functionally to rat plasma apoE. This expression system will be highly useful for probing the ability of rat apoE to mediate food intake in rats.

Keywords: Apolipoprotein E; Rat; Recombinant; Apolipoprotein; ApoE; Cholesterol efflux; Lipid binding

Apolipoprotein (apo) E[1] is a 34 kDa protein that was discovered in 1973 as a protein constituent of human very low-density lipoprotein [1]. ApoE is synthesized by many tissues, including the liver and brain, and various cell types within these tissues [2]. It plays a critical role in lipid transport and cholesterol homeostasis as it is a ligand for the low-density lipoprotein (LDL) receptor [3,4]. ApoE also has an important role in neuroprotection [5–7] and the human apolipoprotein E4 isoform is linked to an increased risk for Alzheimer's disease [8–10] in humans. Many studies have focused both on the structure and function of apoE, making it one of the most well-studied apolipoproteins.

Several groups have shown that apoE production is increased in response to certain dietary conditions [11,12]. Recently, our preliminary data showed that centrally administered apoE is involved in the regulation of food intake (Min Liu, personal communication). Large amounts of apoE will be required to determine whether peripheral administration will also exhibit an inhibitory effect on food intake. However, purifying native rat apoE is difficult, requiring several time-consuming steps that result in low protein yields. Although several bacterial expression systems have been developed for human and mouse apoE [13–16], there is still a need for recombinant systems that produce large amounts of pure rat apoE for in vivo experiments in the rat model.

In this work, we used PCR techniques to isolate and amplify apoE cDNA from a rat liver cDNA library and insert it into a bacterial expression vector with an N-terminal histidine tag. A key feature of our design was the

* Corresponding author. Fax: +1 513 558 2289.
E-mail address: Sean.Davidson@uc.edu (W.S. Davidson).

[1] *Abbreviations used:* ABCA1, ATP-binding cassette transporter A1; apo, apolipoprotein; BSA, bovine serum albumin; CD, circular dichroism; DMEM, Dulbecco's modified Eagle's medium; DMPC, dimyristoylphosphotidylcholine; HDL, high-density lipoprotein; His, histidine; IPTG, isopropyl-β-D-thiogalactoside; LDL, low-density lipoprotein; PCR, polymerase chain reaction; Rec., recombinant; STB, standard Tris buffer.

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.02.011

use of the *Igase* protease which we demonstrate does not cleave the apoE at unwanted sites in the sequence. We found that the expression system was able to produce at least 20 mg of highly pure rat protein per liter of culture media. Importantly, the recombinant rat apoE produced in this expression system was nearly identical to native apoE in terms of secondary structure and ability to promote cholesterol efflux from cells. This rodent apoE expression system will be useful for in vivo studies of the effects of apoE on satiety and lipid metabolism.

Materials and methods

Materials

Primer synthesis and DNA sequencing were performed by the University of Cincinnati DNA Core (Cincinnati, OH). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Rat Liver QUICK-Clone cDNA was purchased from BD Biosciences Clontech (Palo Alto, CA). Cloned *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). SDS–PAGE gels were obtained from Bio-Rad (Hercules, CA) or Amersham-Pharmacia (Piscataway, NJ). The low molecular weight marker was from Amersham-Pharmacia (Piscataway, NJ). Pre-stained protein ladder was from Invitrogen Life Technologies (Carlsbad, CA). Immun-Blot PVDF membrane was from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) system was from Amersham Life Science (Princeton, NJ). IgA protease (*Igase*) was purchased from MoBiTec (Germany). BL-21 (DE3) *Escherichia coli* and the pET30 vector were from Novagen (Madison, WI). Kanamycin was purchased from Calbiochem (San Diego, CA). Isopropyl-β-D-thiogalactoside (IPTG) was from Fisher Scientific (Pittsburgh, PA). 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was acquired form Avanti Polar Lipids (Birmingham, AL). HisBind Resin was from Novagen (Madison, WI). Centriprep centrifugal concentrators were from Millipore/Amicon Biosepara-tions (Bedford, MA). Fatty acid-free bovine serum albumin (BSA) was from Calbiochem (San Diego, CA). 8-Bromoadenosine 3′:5′-cAMP was from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum, Dulbecco's modified Eagle's medium, and phosphate-buffered saline were from Invitrogen Life Technologies (Carlsbad, CA). RAW 264.7 mouse macrophage cells were from American Type Culture Collection (Manassas, VA). All chemical reagents were of the highest quality available.

Methods

Cloning and mutagenesis of rat apoE

A rat liver cDNA library was used to clone rat apoE. PCR primers were first designed to amplify the coding region of rat apoE (forward 5′-GCCCTGCTGTTGG TCCC-3′ and reverse 5′-GCGGCAGGGCGTAGGTG AGGG-3′) and PCR amplification was carried out using a Perkin–Elmer thermocycler: 1 min hold at 94 °C; 30 rounds of 94 °C for 1.5 min, 55 °C for 1.5 min, and 72 °C for 1.5 min; followed by a hold for 10 min at 72 °C to ensure full extension. Cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used for this and the subsequent reaction. Following amplification, forward and reverse flap primers were designed to contain restriction enzyme sites. The forward primer also contained the sequence encoding a proteolytic cleavage site specific for *Igase* that is required for removing the histidine tag from the purified protein [17]. The forward primer sequence is shown in Fig. 1 with the flap region containing the *Nco*I restriction site in italics and the *Igase* site in underlined bold lettering. The clamp region is shown in capital letters and matches the forward sequence for apoE. The reverse primer contained a clamp region and a flap region containing a *Hin*dIII restriction enzyme site. The amplified DNA from the first PCR was used as a template for these flap primers. The apoE DNA and empty pET30 vector were cut with *Nco*I and *Hin*dIII, ligated together and the entire construct was sequenced. Only the DNA for the mature sequence of rat apoE was included in the expression vector.

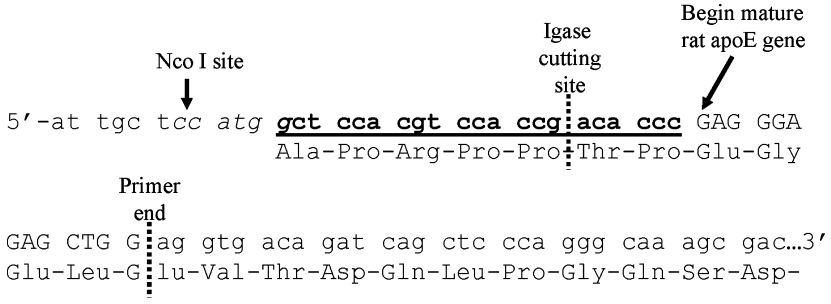

Fig. 1. Forward oligonucleotide primer sequence. The flap primer was designed so that its 3′ end clamps onto the rat apoE mature DNA sequence at its 5′ end. The clamp region is shown in capital letters. The primer also contained an *Igase* cleavage site for cleavage of the histidine tag located at the N-terminus of the protein. This sequence is shown in underlined bold lettering. An *Nco*I restriction site was located near the 5′ end of the primer and was used to ligate the resulting PCR product into the pET30 vector. The *Nco*I site is shown in italics. The reverse primer was similar but contained a clamp region and a *Hin*dIII site for ligation into the pET30 vector.

Protein expression and purification

To generate recombinant apoE, we modified our highly efficient *E. coli* expression system used previously for human apoA-I [17]. The expression construct was transfected into *E. coli* BL-21 cells and grown on Luria–Bertani (LB) agar plates that contained 30 μg/ml kanamycin for 16 h at 37 °C. A single colony from the plate was inoculated into 10 ml cultures that included 30 μg/ml kanamycin and grown overnight in a 37 °C shaking incubator (225 rpm). Next, 100 ml culture flasks containing kanamycin had 1 ml of the confluent 10 ml culture tubes added and were grown in the shaking incubator for approximately 3 h. The cells were grown to an A_{600} between 0.6 and 0.7. Protein expression was driven by the T7 promoter upon exposure to 50 μl of 1 M IPTG for 2 h. After expression, the bacterial cells were pelleted at 9700*g* in a Sorvall SLA1500 for 10 min, the supernatant was removed and the cells were frozen at −20 °C overnight.

The cells were resuspended in 4 ml of 1× histidine (His)-bind buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl) per 100 ml of original culture with a final concentration of protease inhibitors; 100 μM PMSF, 20 μM leupeptin, and 1 μM pepstatin A. The cells were lysed with a Model 550 Sonic Dismembrator at level 5 (Fisher, Pittsburgh, PA) three times for 1 min with a 1 min incubation on ice in between. The lysed cells were then spun at 28,384*g* for 20 min in a Sorvall SLA600, leaving the recombinant apoE in the supernatant. The presence of the histidine tag allowed for purification by using a His-bind column (Ni^{2+}-affinity column). The supernatant was passed through a 0.45 μM filter and added to a prepared His-bind column (Novagen: #69673-3) poured with HisBind Resin. The columns were prepared by packing with 4 ml resin by gravity flow. Using a vacuum manifold, the columns were washed with 15 ml H_2O, followed by 25 ml of 1× His-charge buffer (0.05 M nickel sulfate) and finally 15 ml of 1× His-bind buffer. At this step, the filtered supernatant was added and allowed to flowthrough. The column was then washed with 30 ml of 1× His-bind buffer and 30 ml of 1× His-wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl). Next, the protein was collected with 9 ml of 1× His-elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl). The column was then regenerated by adding 20 ml of 1× Strip buffer (100 mM EDTA, 0.5 M NaCl, and 20 mM Tris–HCl). The protein sample was concentrated using Centriprep centrifugal concentrators (30 kDa cutoff) to a concentration of approximately 5 mg/ml and then dialyzed into standard Tris buffer (STB, 10 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, and 0.2% NaN_3).

The protein concentration was determined and the protease, *Igase*, was added at a mass ratio of 1:5000 (*Igase*/uncut apoE) for 24 h at 37 °C in a shaking incubator. *Igase* is an IgA protease from *Neisseria gonorrhoeae* that recognizes and cleaves the sequence Ala-Pro-Arg-Pro-Pro-Thr-Pro. It cleaves the leader portion along with the histidine tag of the protein, leaving the mature form of apoE with an additional Thr-Pro on the N-terminus. A denaturing gel was run to ensure complete cleavage of the protein which occurs at 24 h. In a time-course experiment, we found that 24 h was best for removal of the His-tag from the recombinant apoE (data not shown). However, for recombinant human apoA-I, full cleavage occurs at 16 h. The time requirement difference is probably due to the availability of the cleavage sequence to the *Igase* because of the protein conformation differences.

Next, 10× His-bind buffer was added to the sample to make it 1× and the sample was applied to a prepared His-bind column a second time to separate the mature protein from the cleaved tag. The sample was added to the column and the flowthrough was collected, then the column was washed with 9 ml of 1× His-bind buffer, and finally with 9 ml of 1× His-wash buffer (both washes were collected). The mature cut protein was located in the flowthrough, His-bind, and His-wash buffer fractions. Any uncut protein could be collected by washing the column with His-elute buffer. The fractions were combined, concentrated, and dialyzed into STB or PBS for storage.

Native protein purification

Rat plasma was obtained from abdominal aorta blood of Long–Evans rats. The samples were centrifuged at a density of >1.065 g/ml to isolate any apoB containing lipoproteins. After dialysis to remove KBr that was used to set the density, the rat apolipoproteins present in the HDL density and greater were then separated by SDS–PAGE and purified by gel extraction as described previously [18].

Western blot analysis

Purified proteins (100 ng) were loaded on an 18% denaturing polyacrylamide gel and separated by electrophoresis and transferred to Immun-Blot PVDF membrane. Non-specific sites were blocked overnight (20 mM Tris-base, 0.137 M NaCl, 0.1% v/v Tween 20, and 5% w/v powdered milk, pH 7.6). The membrane was then incubated for 2 h with the primary polyclonal goat against native rat apoE at a dilution of 1:3000. The membrane was washed and incubated with rabbit anti-goat IgG coupled to horseradish peroxidase for 45 min (1:2000) and subsequently washed. An enhanced chemiluminescence (ECL) system was used to visualize the bound antibodies following manufacturer's protocol.

Circular dichroism spectroscopy

The average secondary structural content of recombinant and native rat apoE was determined by obtaining circular dichroism (CD) spectra at room temperature

using a Jasco S-720 spectropolarimeter. The samples were analyzed at 0.1 mg/ml in 20 mM phosphate buffer. The α-helix content was calculated from the mean residual ellipticity at 222 nm, as described [19].

Dimyristoylphosphatidylcholine liposome solubilization

Dimyristoylphosphatidylcholine (DMPC) was dried in a glass tube and STB was added to a final concentration of 5 mg/ml. Multilamellar liposomes were formed by a brief bath sonication. The turbidity clearance experiments were performed in an Amersham Biosciences Ultraspec 4000 at a constant temperature of 24.5 °C maintained by a circulating water bath. The absorbance was recorded over 30 min at 325 nm after protein samples were added (5:1 mass ratio of DMPC/protein). The *Y*-axis of the graph is the ratio of the absorbance at any given time point (OD) to the initial absorbance at time zero (OD_0) [20]. For a more detailed description of the assay, please refer to [21].

Cholesterol efflux studies

The transformed mouse macrophage cell line RAW264.7 was maintained in growth medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 50 μg/ml gentamicin). The cells were grown to 70–80% confluency in 48-well plates. The cells were then labeled with 1.0 μCi/ml [^{3}H]cholesterol in efflux medium (DMEM + 0.2% BSA) with or without 0.3 mM 8-bromo-cAMP for 24 h. After 24 h, the cells were washed and efflux medium containing differing concentrations of protein acceptors were added to the wells, maintaining the cAMP concentration used in the labeling media. After 8 h, the samples were filtered through a 0.45-μm filter to remove any floating cells and analyzed by liquid scintillation counting. The percent cholesterol efflux was calculated by dividing the counts in the medium at 8 h by total cell counts at time zero [22].

Results and discussion

Fig. 2A shows an 18% SDS–PAGE gel stained with Coomassie blue that summarizes the purification of rat apoE at various stages of the expression/purification. The cell lysate from bacteria overexpressing rat apoE is shown in lane 2. Recombinant rat apoE exhibited a high degree of induction with approximately 25 mg of recombinant apoE per liter of culture media from roughly 6 g of wet weight cells per liter at harvest. Following sonication, the cellular proteins were purified on a His-bind column and the resulting His-tagged rat apoE is shown in lane 3. It is clear that the recombinant protein was cleanly isolated from the cellular proteins resulting in high purity samples. The protein was then cleaved with *Igase*, removing the N-terminal His-tag and leaving the mature protein with an additional Thr-Pro on the N-terminus. The mature cut protein was then purified away from the His-tag by applying the sample to the His-bind column a second time (lane 4). The total protein, approximate purity, estimated protein product, and approximate yield are given in Table 1. Fig. 2B shows a Western blot analysis of recombinant rat and native rat apoE in lanes 2 and 3, respectively. Both proteins were recognized by goat polyclonal native rat apoE anti-serum and generated a single band present at 34 kDa. The predicted size for the recombinant rat apoE was 34,053 Da. This was verified by MALDI mass spectrometry at the Genome Research Institute, University of Cincinnati Proteomics Core which generated essentially a single peak with a mass of 34,077 Da. The discrepancy between the theoretical value and the experimentally derived value was well within the expected error of the machine.

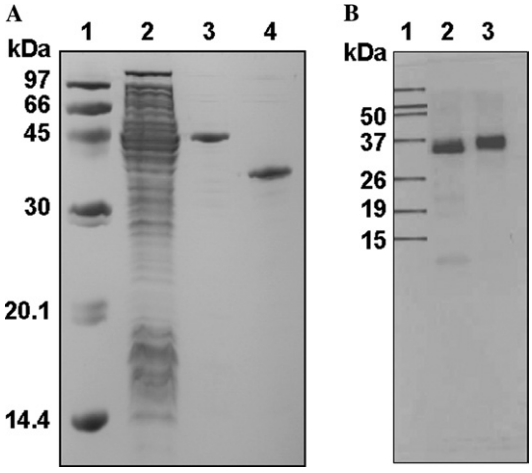

Fig. 2. SDS–PAGE analysis of recombinant rat apoE at different stages of purification and Western blot analysis following purification. (A) Samples of apoE were electrophoresed on an 18% denaturing polyacrylamide gel and stained with Coomassie blue. Lane 1: Amersham-Pharmacia low molecular weight marker. Lane 2: whole bacterial cell lysate expressing rat apoE. The cells were solubilized in SDS containing loading buffer and boiled for 3 min. Lane 3: uncut His-tagged recombinant rat apoE after purification on His-bind column. Lane 4: recombinant rat apoE with His-tag removed. (B) Western blot analysis of recombinant rat apoE (lane 2) along with native rat apoE (lane 3). The proteins were purified and 100 ng of each was loaded on an 18% denaturing polyacrylamide gel along with a protein ladder (lane 1, Invitrogen BenchMark Pre-stained protein ladder). The proteins were transferred to PVDF membrane and probed with goat polyclonal native rat apoE serum as described under Materials and methods.

To determine the stability of the purified recombinant rat apoE, samples of the protein were stored in STB at both 4 and −20 °C for three weeks each. These were compared to a sample that had been mixed with SDS loading buffer and stored at −20 °C from the beginning of the experiment. Fig. 3 is an SDS gel showing the proteins under these various storage conditions. There was no difference between the three different storage conditions over the 3-week period. This demonstrates that the protein is stable in buffer and does not precipitate over time. Furthermore, this indicates that the purity is such

Table 1
Purification of recombinant rat apolipoprotein E

Purification stage	Total protein (mg)[a]	Approximate purity of apoE (%)[b]	Estimated protein product (mg)[c]	Approximate yield (%)[d]
Total cell extract[e]	180	14	25	—
Uncut apoE (His-tag present)	25	93	23	96
Cut apoE (His-tag removed)	22	92	20	80

[a] Total protein was determined by Markwell–Lowry protein assay [26].
[b] Approximate purity was determined by densitometric scanning.
[c] Estimated protein product was calculated by multiplying the total protein by the approximate purity.
[d] Approximate yield was calculated by dividing the estimated protein product of both the uncut and cut apoE by the estimated protein product in the total cell extract.
[e] One liter of culture was used for this preparation and the total cell extract was collected from approximately 6 g of wet weight cells.

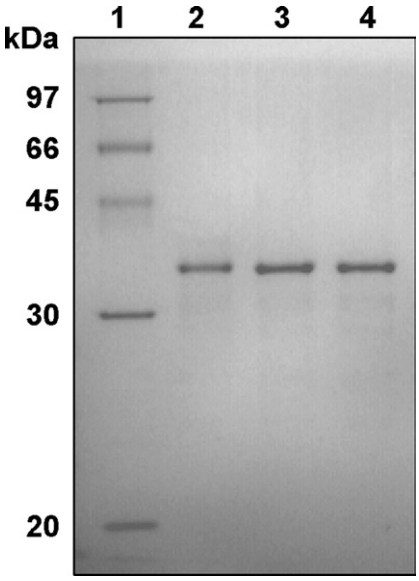

Fig. 3. SDS–PAGE analysis of rat apoE under different storage conditions. Samples of apoE were electrophoresed on an 18% denaturing polyacrylamide gel and stained with Coomassie blue following storage under different conditions. Lane 1: Amersham-Pharmacia low molecular weight marker. Lane 2: recombinant rat apoE stored in SDS loading buffering under denaturing conditions at −20 °C for three weeks. Lane 3: recombinant rat apoE stored at 4 °C in STB for three weeks. Lane 4: recombinant rat apoE stored at −20 °C in STB for three weeks.

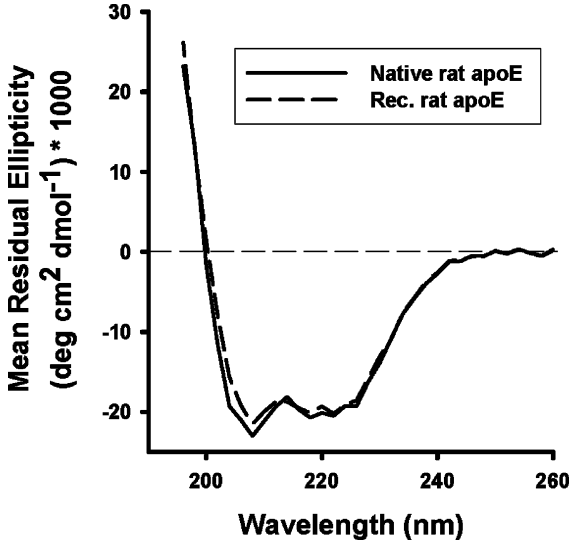

Fig. 4. Far-UV circular dichroism spectra of apoE. The spectra of native rat apoE (solid line) and recombinant rat apoE (broken line) were recorded on a Jasco J-720 spectropolarimeter from 195 to 260 nm. All samples were analyzed at 0.1 mg/ml at room temperature.

that contaminating proteases are not present in high enough amounts to degrade the protein significantly over three weeks.

Having established that the expression system produces significant amounts of stable protein, we next characterized the protein with respect to structure and function. Samples of recombinant and native rat apoE were analyzed using CD to compare average secondary structure content. Fig. 4 shows that native and recombinant rat apoE exhibit nearly identical far-UV CD spectra in both overall shape and the magnitudes of the minima at 208 and 222 nm, which are characteristic of a highly helical protein. The CD values were used to determine α-helical content for each of the proteins [19]. The recombinant and native rat helical contents were very similar at 60 ± 3 and $59 \pm 1\%$, respectively ($n = 6$). These data indicate that the recombinant and native apoE contained a similar overall secondary structure content.

One of the main functions of apolipoproteins is to interact with and bind to lipids. To determine whether recombinant and native rat apoE were similar in their ability to bind and reorganize lipid, a DMPC lipid clearance assay was performed. In this assay, DMPC multilamellar liposomes are generated in buffer, resulting in a turbid solution. Protein samples are then added and the turbidity clears as the protein binds and reorganizes the liposomes into small discoidal structures. Fig. 5 shows that over time, DMPC alone with no protein clears at a very slow rate. However, the addition of apoE caused the solution to clear more rapidly. Both recombinant and native rat apoE were able to clear lipid at a similar rate.

Similar to the related protein apolipoprotein A-I, apoE has been shown to have the ability to promote the efflux of cholesterol via the ABCA1-pathway [23]. Therefore, we tested the ability of the recombinant and native apoE proteins to promote cholesterol efflux from

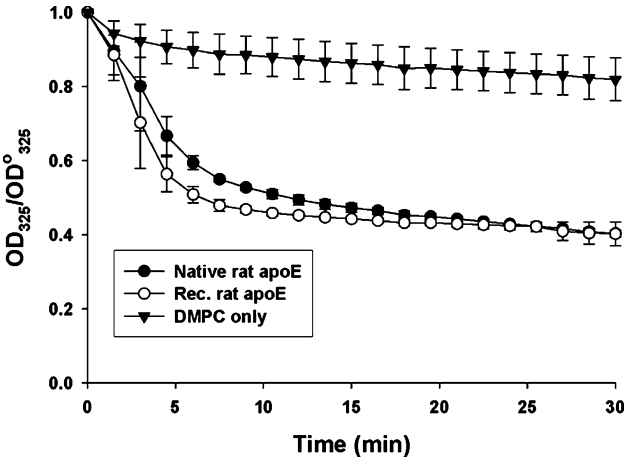

Fig. 5. Dimyristoylphosphatidylcholine (DMPC) liposome solubilization by apoE. DMPC multilamellar liposomes in standard Tris buffer were maintained at 24.5 °C by water bath and monitored at 325 nm for 30 min after 21.3 μg protein (5:1 lipid:protein mass ratio) was added. The filled triangles represent DMPC alone. The filled circles designate native rat apoE and the open circles represent recombinant rat apoE. The *Y*-axis is the ratio of the absorbance at 325 nm at any given time point (OD) to the initial absorbance (OD_0).

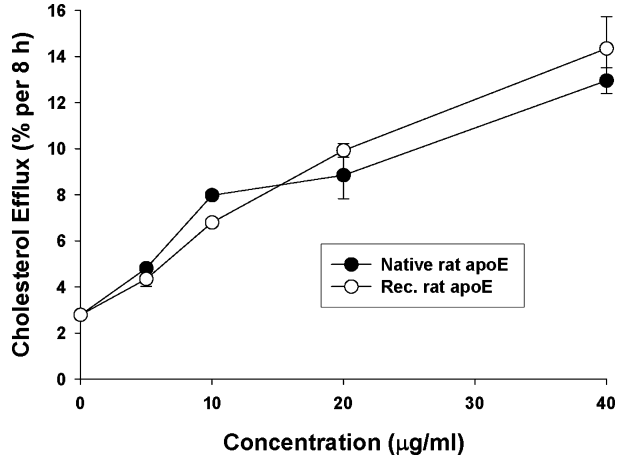

Fig. 6. ABCA1-mediated cholesterol efflux from RAW264 macrophages. Cells were grown to 70–80% confluence in 48-well plates and labeled for 24 h with [^{3}H]cholesterol along with 0.3 mM 8-bromo-cAMP to up-regulate ABCA1. Protein acceptors at different concentrations were added in DMEM with 0.2% BSA and 0.3 mM cAMP. After 8 h, samples were counted in a scintillation counter and divided by total cell labeling to give percentage cholesterol efflux. Filled circles represent native rat apoE and open circles designate recombinant rat apoE.

RAW264 mouse macrophages. This system was used because it has been clearly established that the addition of cAMP to the cells causes an increase in ABCA1 at the cell surface [24,25]. Following 24 h treatment of the cells with labeled cholesterol and cAMP, lipid-free protein acceptors were added for 8 h to determine their ability to promote cholesterol efflux by the ABCA1-mediated pathway. Fig. 6 shows ABCA1-mediated cholesterol efflux to recombinant and native rat apoE over a range of concentrations. Again, the recombinant and the native rat apoE were nearly super imposable in terms of their concentration effect on cholesterol efflux.

Conclusion

We have developed a bacterial expression system using the pET30 vector that is highly efficient and effective at producing rat apoE. The recombinant rat apoE compares favorably both structurally and functionally to native rat apoE purified from plasma. This lends confidence that future studies using the recombinant protein will faithfully represent the functionality of the native protein. We should also note that we applied the same strategy to produce recombinant mouse apoE and its characteristics compared favorably to the native rat apoE (data not shown). As stated above, we have determined that centrally administered apoE has inhibitory effects on rat food intake. Large amounts of apoE are required for studying its effects on food intake more thoroughly. Relying solely on the purification of native protein from rat plasma, these types of experiments would not be possible on a large scale. However, this recombinant expression system will allow these tests to be performed in a timely manner. Furthermore, the expression system will allow for the convenient engineering of apoE mutants that can be used to isolate particular regions of apoE that may be involved in food intake regulation.

Acknowledgments

This work was supported by Grants HL67093 and HL62542 (W.S.D.), DK63907 (M.L.), DK56863 and DK56910 (P.T.) from the NIH, and a pre-doctoral fellowship from the Ohio Valley Affiliate of the American Heart Association (K.P.).

References

[1] V.G. Shore, B. Shore, Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components, Biochemistry 12 (1973) 502–507.
[2] N.A. Elshourbagy, W.S. Liao, R.W. Mahley, J.M. Taylor, Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets, Proc. Natl. Acad. Sci. USA 82 (1985) 203–207.
[3] M.S. Brown, J.L. Goldstein, A receptor-mediated pathway for cholesterol homeostasis, Science 232 (1986) 34–47 (Review).
[4] R.W. Mahley, Apolipoprotein E: cholesterol transport protein with expanding role in cell biology, Science 240 (1988) 622–630 (Review).
[5] J.K. Boyles, R.E. Pitas, E. Wilson, R.W. Mahley, J.M. Taylor, Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system, J. Clin. Invest. 76 (1985) 1501–1513.

[6] M.J. Ignatius, P.J. Gebicke-Harter, J.H. Skene, J.W. Schilling, K.H. Weisgraber, R.W. Mahley, E.M. Shooter, Expression of apolipoprotein E during nerve degeneration and regeneration, Proc. Natl. Acad. Sci. USA 83 (1986) 1125–1129.
[7] H. Sheng, D.T. Laskowitz, E. Bennett, D.E. Schmechel, R.D. Bart, A.M. Saunders, R.D. Pearlstein, A.D. Roses, D.S. Warner, Apolipoprotein E isoform-specific differences in outcome from focal ischemia in transgenic mice, J. Cereb. Blood Flow Metab. 18 (1998) 361–366.
[8] W.J. Strittmatter, A.M. Saunders, D. Schmechel, M. Pericak-Vance, J. Enghild, G.S. Salvesen, A.D. Roses, Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease, Proc. Natl. Acad. Sci. USA 90 (1993) 1977–1981.
[9] E.H. Corder, A.M. Saunders, W.J. Strittmatter, D.E. Schmechel, P.C. Gaskell, G.W. Small, A.D. Roses, J.L. Haines, M.A. Pericak-Vance, Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families, Science 261 (1993) 921–923 (see comments).
[10] A.M. Saunders, W.J. Strittmatter, D. Schmechel, P.H. George-Hyslop, M.A. Pericak-Vance, S.H. Joo, B.L. Rosi, J.F. Gusella, D.R. Crapper-MacLachlan, M.J. Alberts, Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease, Neurology 43 (1993) 1467–1472.
[11] D.S. Knight, D.K. Mahajan, X. Qiao, Dietary fat up-regulates the apolipoprotein E mRNA level in the Zucker lean rat brain, Neuroreport 12 (2001) 3111–3115.
[12] A. Mazur, P. Cardot, C. Felgines, C. Remesy, Y. Rayssiguier, Apolipoprotein E gene expression in the liver of genetically obese Zucker rats: the effect of high-fiber diet, Horm. Metab. Res. 23 (1991) 347–348.
[13] T. Vogel, K.H. Weisgraber, M.I. Zeevi, H. Ben Artzi, A.Z. Levanon, S.C. Rall Jr., T.L. Innerarity, D.Y. Hui, J.M. Taylor, D. Kanner, Human apolipoprotein E expression in *Escherichia coli*: structural and functional identity of the bacterially produced protein with plasma apolipoprotein E, Proc. Natl. Acad. Sci. USA 82 (1985) 8696–8700.
[14] J.A. Morrow, K.S. Arnold, K.H. Weisgraber, Functional characterization of apolipoprotein E isoforms overexpressed in *Escherichia coli*, Protein Expr. Purif. 16 (1999) 224–230.
[15] R.L. Raffai, L.M. Dong, R.V. Farese Jr., K.H. Weisgraber, Introduction of human apolipoprotein E4 "domain interaction" into mouse apolipoprotein E, Proc. Natl. Acad. Sci. USA 98 (2001) 11587–11591.
[16] T. Pillot, A. Barbier, A. Visvikis, K. Lozac'h, M. Rosseneu, J. Vandekerckhove, G. Siest, Single-step purification of two functional human apolipoprotein E variants hyperexpressed in *Escherichia coli*, Protein Expr. Purif. 7 (1996) 407–414.
[17] S.E. Panagotopulos, S.R. Witting, E.M. Horace, M.J. Nicholas, D.W. Sean, Bacterial expression and characterization of mature apolipoprotein A-I, Protein Expr. Purif. 25 (2002) 353–361.
[18] H. Hayashi, D.F. Nutting, K. Fujimoto, J.A. Cardelli, D. Black, P. Tso, Transport of lipid and apolipoproteins A-I and A-IV in intestinal lymph of the rat, J. Lipid Res. 31 (1990) 1613–1625.
[19] Y.H. Chen, J.T. Yang, H.M. Martinez, Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion, Biochemistry 11 (1972) 4120–4131.
[20] R.B. Weinberg, R.A. Anderson, V.R. Cook, F. Emmanuel, P. Denefle, M. Hermann, A. Steinmetz, Structure and interfacial properties of chicken apolipoprotein A-IV, J. Lipid Res. 41 (2000) 1410–1418.
[21] K. Pearson, H. Saito, S.C. Woods, S. Lund-Katz, P. Tso, M.C. Phillips, W.S. Davidson, Structure of human apolipoprotein A-IV: a distinct domain architecture among exchangeable apolipoproteins with potential functional implications, Biochemistry 43 (2004) 10719–10729.
[22] S.E. Panagotopulos, S.R. Witting, E.M. Horace, D.Y. Hui, J.N. Maiorano, W.S. Davidson, The role of apolipoprotein A-I helix 10 in apolipoprotein-mediated cholesterol efflux via the ATP-binding cassette transporter ABCA1, J. Biol. Chem. 277 (2002) 39477–39484.
[23] A.T. Remaley, J.A. Stonik, S.J. Demosky, E.B. Neufeld, A.V. Bocharov, T.G. Vishnyakova, T.L. Eggerman, A.P. Patterson, N.J. Duverger, S. Santamarina-Fojo, H.B. Brewer Jr., Apolipoprotein specificity for lipid efflux by the human ABCAI transporter, Biochem. Biophys. Res. Commun. 280 (2001) 818–823.
[24] J.F. Oram, R.M. Lawn, M.R. Garvin, D.P. Wade, ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages, J. Biol. Chem. 275 (2000) 34508–34511.
[25] J.D. Smith, M. Miyata, M. Ginsberg, C. Grigaux, E. Shmookler, A.S. Plump, Cyclic AMP induces apolipoprotein E binding activity and promotes cholesterol efflux from a macrophage cell line to apolipoprotein acceptors, J. Biol. Chem. 271 (1996) 30647–30655.
[26] M.A. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206–210.