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BacMam production of active recombinant lecithin–cholesterol acyltransferase: Expression, purification and characterization

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ABSTRACT

Lecithin–cholesterol acyltransferase (LCAT) is a key enzyme in the esterification of cholesterol and its subsequent incorporation into the core of high density lipoprotein (HDL) particles. It is also involved in reverse cholesterol transport (RCT), the mechanism by which cholesterol is removed from peripheral cells and transported to the liver for excretion. These processes are involved in the development of atherosclerosis and coronary heart disease (CHD) and may have therapeutic implications. This work describes the use of baculovirus as a transducing vector to express LCAT in mammalian cells, expression of the recombinant protein as a high-mannose glycoform suitable for deglycosylation by Endo H and its purification to homogeneity and characterization. The importance of producing underglycosylated forms of secreted glycoproteins to obtain high-resolution crystal structures is discussed.

1. Introduction

The plasma enzyme lecithin–cholesterol acyltransferase (LCAT) esterifies free cholesterol and plays an important role in the formation and maturation of high density lipoprotein (HDL) particles. Cholesteryl ester (CE) molecules formed by LCAT are more hydrophobic than free or unesterified cholesterol (FC) and partition to the core of the HDL particles resulting in an increase in particle size to a large, spherical shape that are removed from circulation by the liver [1]. LCAT activity is thought to promote cholesterol efflux from peripheral cells along the reverse cholesterol transport (RCT) pathway by creating a gradient of FC between peripheral tissues and the plasma. A therapeutic that increases LCAT activity may promote RCT and prove beneficial for the treatment of dyslipidemia and atherosclerosis [2–5].

LCAT is a member of the α/β hydrolase family [6]. It is produced predominantly in the liver and secreted as a circulating protein into the serum [7]. This protein is highly glycosylated with four potential N-linked glycosylation sites and two putative O-linked sites [8]. Glycan chains can prevent efficient crystal packing making LCAT a challenging candidate for crystallography. The role of glycosylation on LCAT function is unknown but studies of LCAT mutants suggest an association between glycosylation sites and enzyme activity [8–11]. It has also been shown that LCAT produced in different cultured cell lines is made as secreted proteins with different activities [12,13].

The authors have previously expressed a secreted protein for crystallography in insect cells which produce proteins of the paucimannose glycosylation type [14] but LCAT constructs in this host showed little to no expression. We produced recombinant LCAT in human cell lines. Transduction of mammalian cultured cells by recombinant baculovirus (BacMam) has been used for some time; however it is only recently that this technology has been used to express protein targets at a scale useful for crystallization [15]. A laboratory already producing protein using the baculovirus system can cost-effectively incorporate mammalian host cell lines. The development of new culture media and cell lines conducive to suspension growth of mammalian cells can easily allow for the addition to the host mix of bacteria and insect cell lines commonly used to produce recombinant protein for structural studies. The

Abbreviations: LCAT, lecithin–cholesterol acyltransferase; HDL, high density lipoprotein; RCT, reverse cholesterol transport; FC, free cholesterol; CE, cholesteryl ester; CHD, coronary heart disease; HEK, human embryonic kidney; GlcNAc, N-acetyl glucosamine; MANN, mannose; iu/ml, infectious units/milliliter.

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transient production of recombinant protein by BacMam in mammalian cells is also a higher-throughput alternative to making stable cell lines.

The human host cell lines used, HEK293-6E (NRC) [16] and HEK293S GnTI− (ATCC CRL-3022) are derived from the HEK293 cell line which is highly useful as a host for the transient production of recombinant proteins. HEK293 cells produce glycoproteins which are heterogeneous and result in poorly packed crystals of low diffraction but the sugar chain can be removed through the action of glycosidases. HEK293 cells naturally produce hybrid glycoproteins susceptible to the glycosidase PNGase F [17]. Cleavage with PNGase F removes the entire glycan chain which can make proteins prone to aggregation. HEK293-6E cells when grown in the presence of kifunensine express glycoproteins of the high-mannose type (MAN9GlcNAc2) [18,19] while the mutant cell line HEK293S GnTI− is deficient in N-acetylglucosaminyl transferase I [20] and produces MAN5GlcNAc2 glycoproteins. Glycoproteins of the MAN9GlcNAc2 or MAN5GlcNAc2 types are extremely sensitive to activity of the glycosidase Endo H [21] which leaves a single N-acetyl glucosamine (GlcNAc) residue at the glycosylation sites. A single GlcNAc residue at each N-linked glycosylation site can result in protein that is more stable and less prone to aggregation [22].

Underglycosylation may also improve the quality of LCAT crystals [23], which had previously only yielded a low resolution structure [21].

2. Materials and methods

2.1. Cell lines and media

The insect cell line Sf9 was cultivated in ESF921 media (Expression Systems LLC) with no additives. Insect cells were maintained at 27 °C in 2.8 L glass Fernbach flasks shaking at 125 rpm. Sf9 cells were maintained between 1×10^6 and 1×10^7 cells/ml. The human cell line HEK293-6E (NRC) [16] was maintained between densities of 3×10^5 and 3×10^6 cells/ml in Freestyle 293 media (Invitrogen) and cultivated in Fernbach flasks rotating at 125 rpm in 5% CO_2 at 37 °C. HEK293S GnTI− cell line (ATCC CRL-3022) was adapted to Freestyle 293 media from DMEM/F12 in about 2 weeks. Frozen cells were thawed into 30 ml DMEM/F12 (ATCC) in a 15 cm cell culture dish. After approximately 48 h at near confluency, the cell monolayer was removed off the surface of the dish with a pipette. 10 ml of the cell suspension was added to 10 ml of Freestyle 293 in a 125 ml culture flask. The flask was shaken at 125 rpm in 5% CO_2 at 37 °C. HEK293S GnTI− cells were maintained between 3×10^5 and 3×10^6 cells/ml. The cells were scaled up as appropriate in 2.8 L glass Fernbach flasks shaking at 125 rpm. Adaption of HEK293S GnTI− cells from DMEM/F12 to Freestyle 293 or F17 (Invitrogen) media was accomplished in about 2 weeks. The cells adapted to suspension growth and new media without deleterious effects.

2.2. Construct design

Human LCAT was cloned into the FastBacMam1 vector [24] in which expression is under control of the human cytomegalovirus promoter. A carboxy or amino-terminal hexahistidine tag was fused to LCAT for use in subsequent protein purification. TEV or Factor Xa sites were introduced for tag cleavage and some constructs were truncated for the removal of a proline rich region (residues 402–416) on the carboxy terminus. Equivalent constructs were made with the C31Y mutation, shown to be more stable and active than the native form [25] (Table 1).

Table 1
Constructs of human LCAT in pFastBacMam1.

Construct	Gene specifics	Tag
T282	LCAT(1–416)	C-term TEV::6×His
T283	LCAT(1–416)C31Y	C-term TEV::6×His
T284	LCAT(1–416)	N-term 6×His::FactorXa
T285	LCAT(1–416)C31Y	N-term 6×His::FactorXa
T286	LCAT(1–416)	N-term 6×His::TEV
T287	LCAT(1–416)C31Y	N-term 6×His::TEV
T288	LCAT(1–401)	N-term 6×His::TEV
T289	LCAT(1–401)C31Y	N-term 6×His::TEV
T290	LCAT(1–401)	N-term 6×His::FactorXa
T291	LCAT(1–401)C31Y	N-term 6×His::FactorXa
T292	LCAT(1–401)	C-term TEV::6×His
T293	LCAT(1–401)C31Y	C-term TEV::6×His

2.3. Preparation of LCAT bacmid and transfection of Sf9 cells

Recombinant LCAT BacMam vector was transformed into DH10 Bac cells (Invitrogen) and LCAT bacmid was purified according to the Bac-to-Bac manual. At least 3 bacmid clones were screened after two rounds of streaking. Log phase Sf9 cells were plated in 6 well dishes at 9×10^5 cells/well in 2 ml ESF921 media and transfected with the recombinant LCAT bacmid using Fugene HD (Roche) following the manufacturer's instructions. This transfectant supernatant (P0) was harvested after 3–5 days.

2.4. BacMam virus stock production

Log phase Sf9 cells were plated on 15 cm cell culture plates in 30 ml of ESF921 media at 6×10^5 cells/ml and infected with 100 µl of the P0 supernatant. This first pass virus stock (P1) was harvested after 3–4 days upon exhibition of infection. A second pass virus preparation (P2) was generated in suspension cultures in glass 2.8 L Fernbach flasks shaking at 125 rpm at 27 °C. Sf9 cells at a density of 2×10^6 cells/ml were infected with 3 ml of P1 virus stock. The P2 virus stock was harvested after 3–4 days. Fetal bovine serum was added to all virus stocks at 10% v/v and stored at 4 °C. Virus stocks were titered by Expression Systems LLC by flow cytometric assay of gp64 expression.

The expression of LCAT constructs in log phase HEK293-6E cells was assayed in small scale cultures in a 24 well plate at 37 °C at 5% CO_2. Log phase cells were cultured in the plates at 6×10^5 cells/ml with 20 mM sodium butyrate, 25 µg/ml G418 and 10%v/v P1 LCAT virus stock. 20 µl of the supernatant was analyzed on 4–12% Bis–Tris acrylamide gels (Novex) and blotted using an iBlot transfer device (Invitrogen) following the manufacturer's instructions. The blots were probed using anti-Penta His HRP conjugated antibody (Qiagen) at 1/1000 or an anti-LCAT mouse monoclonal antibody (Amgen) at 1/1000. iBlot (Invitrogen) and Snap ID (Millipore) devices were used to prepare the blots. The blots were visualized using West Pico SuperSignal substrate (Pierce) and exposed to X-ray film for 1 min. The Western blot of Fig. 1 shows testing of several constructs with multiple bacmid clones (e.g. T282 c.1, T282 c.2, etc.).

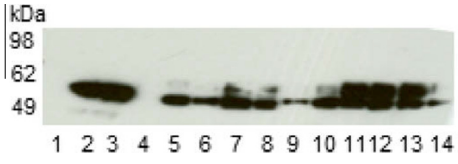

Fig. 1. First pass (P1) virus stock expression testing on 6×10^5 293-6E cells in 1 ml Freestyle medium in a 24 well dish with 20 mM sodium butyrate and G418. 10% v/v virus supe/well. Western blot probed with anti-LCAT 1/1000, anti-mouse HRP 1/5000. Lanes: (1) conditioned media, (2) T286 c.1, (3) T286 c.2, (4) T289 c.1, (5) T290 c.2, (6) T290 c.3, (7) T291 c.1, (8) T291 c.2, (9) T291 c.3, (10) T292 c.1, (11) T292 c.2, (12) T293 c.1, (13) T293 c.2 and (14) T293 c.3.