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A novel technology for the production of a heterologous lipoprotein immunogen in high yield has implications for the field of vaccine design

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ABSTRACT

We have developed a novel platform technology that can express high levels of recombinant lipoproteins with intrinsic adjuvant properties. In this study, Ag473 (a lipoprotein from *Neisseria meningitidis*) can be produced in high yields using *Escherichia coli* strain C43 (DE3). After testing a non-lipoimmunogen (E3, from dengue virus) fused with different lipid signal peptides from other lipoproteins as well as Ag473 fragments of different lengths, we identified that the fusion sequence has to contain at least the N-terminal 40 residues, D1, of Ag473 to achieve high expression levels of the recombinant lipo-immunogen (rlipo-D1E3). The rlipo-D1E3 was found to elicit stronger anti-E3 and virus neutralizing antibody responses in animal studies than those from rE3 alone or rE3 formulated with alum adjuvant. These results have successfully demonstrated the merit of lipo-immunogens for novel vaccine development.

1. Introduction

Modern vaccines based on recombinant proteins or synthetic peptides are thought to be safer and cause fewer side effects than inactivated and live attenuated vaccines. However, these well-defined vaccine candidates are usually poorly immunogenic and require either appropriate adjuvantation or forming virus-like particles to be efficacious [1]. The outer-membrane lipoprotein, OspA, of *Borrelia burgdorferi* which is a Braun's lipoproteins (BLP) [2], has recently been licensed in US as the vaccine against Lyme disease. Animals immunized with the full-length OspA (lipidated form) were shown to be protected against *B. burgdorferi* challenge [3–6]. In contrast, vaccination with non-lipidated OspA was unable to elicit protective immunity against the pathogen [7,8]. Although these results have clearly demonstrated the superior immunoprotective property of lipoproteins, the function of lipid moiety was not fully appreciated at that time. Now the superior immune-protective property of lipoproteins is shown to trigger both innate and adaptive immunity [9,10]. Therefore, to enhance the potency of recombinant protein subunit vaccines, one can design and engineer immunogens as lipoproteins by covalently linking the antigen proteins with lipid moieties. As the cysteine-linked diacyl lipid moiety of BLP is recognized as a danger signal by the immune system to induce antimicrobial activity and trigger host defense mechanisms through Toll-like receptor stimulation [11,12]. BLPs are characterized by the presence of a lipobox motif which is located in the C-terminal part of their leader peptide and contains a conserved cysteine residue which is the target for *N*-acyl-*S*-diacylglyceryl-cystinyl modification [13] (Fig. 1a). Modification of the precursor protein is mediated by the consecutive activity of three enzymes: the phosphatidylglycerol–pre-prolipoprotein diacylglyceryl transferase (Lgt) responsible for adding a diacylglyerol residue to the thiol group of the lipobox cysteine, the prolipoprotein signal peptidase/signal peptidase II (LspA) which subsequently cleaves the lipidation signal sequence and the phospholipid–apolipoprotein *N*-acyltransferase (Lnt) which completes lipid modification [14].

Recombinant lipoproteins as vaccine candidates have not been fully utilized because of bottle-necks of protein expression. The post-translational modification and expression level of BLPs have been improved by fusing the target gene with selected signal peptide coding sequences [15–18], but the overexpression of lipoproteins in *Escherichia coli* frequently results in incomplete

Abbreviations: E3, envelope-protein domain 3 of dengue virus; rE3, recombinant E3; rlipo-D1E3, recombinant lipidated E3; BLP, Braun's lipoproteins; alum, aluminum phosphate; rAg473, lipidated recombinant Ag473.

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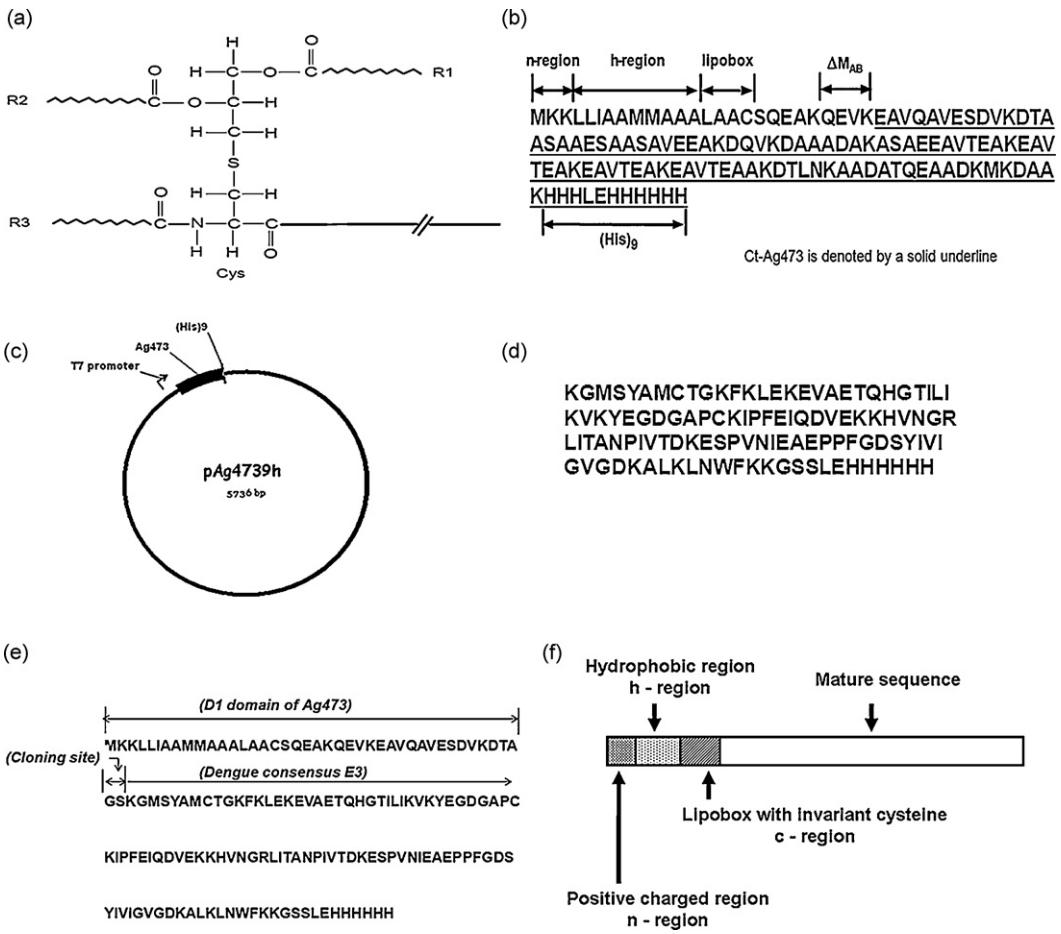

Fig. 1. Sequence and constructs of expressed proteins, and the tripartite nature of the bacterial lipoprotein. (a) The structure of the lipid modification was elucidated by Hantke and Braun. The sulphydryl group of the N-terminal cysteine is modified with a diacylglyceryl group attached through a thioether linkage, and the amino group was acylated with a fatty acid. (b) The amino acid sequence of Ag473 and the three regions of the signal peptide have been labeled, which are n-region, h-region, and lipobox. The ΔMAB represents the sequence difference between the two groups identified by mass spectrometry (see Section 3). A nonahistidine tag was engineered at the C-terminal end ofAg473, symbolized (His)9. Moreover, Ct-Ag473 refers to the C-terminal fragment encompassing amino acid 27–132 of Ag473. (c) The Ag473 gene of *Neisseria Meningitidis* (accession number: AY566590) was engineered to code for a nonahistidine tag (HHLEHHHHHH) at the C-terminus of the antigen and was cloned into the pET-22b vector. The recombinant antigen was expressed in *Escherichia coli* using a phage T7 promoter system. (d) The length of the E3 immunogen is 103 amino acids. We added a histidine tag at the C-terminal end of E3 and two additional amino acids were generated from the cloning site. In addition, there is a methionine was added in the N-terminus of E3. (e) After cloning the construct of D1E3, two amino acids, DP, were introduced between the fusion partner and E3. (f) Tripartite structure of the lipoprotein signal sequence. The n-region has at least two positively charged residues; the h-region, or the hydrophobic region, is made up of 7–22 predominantly hydrophobic and uncharged residues; and the c-region, which has the consensus [LVI][ASTVI][GAS][C] sequence, is known as lipobox.

modification or even in the lack of lipidation [19–21] and thus remains a challenge for large-scale production of lipoproteins. Several types of proteins have been successfully expressed in *E. coli* BL21 (DE3), yet the overexpression of heterologous proteins in the mutant strain C43 (DE3) was generally found to be better than in BL21 (DE3) due to high efficient transformation and lack of toxicity [22]. For the first time, we have overexpressed a potential *Neisseria meningiditis* lipoprotein vaccine candidate [23], Ag473, at high level in C43 (DE3) cells. We further characterized the purified lipidated recombinant Ag473 (rAg473) using mass spectrometry to demonstrate that the rAg473 has correct lipid modification. To explore whether it was possible to engineer potent lipo-immunogens, as a model study we fused the domain III of the dengue virus envelope protein (E3) with the 40 N-terminal residues of Ag473. The resulting lipo-immunogen (rlipo-D1E3) was expressed in high yield with correct lipid modifications. We validated the superior immune-protective property of lipoproteins by showing that this viral lipo-immunogen was able to enhance immune responses in the absence of adjuvant.

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Restriction enzymes and ligase were from New England Biolabs, Inc. (Beverly, MA, USA). Primers used for cloning were from Mission Biotech, Inc. (Taipei, Taiwan). Trypsin was purchased from Promega Co. (Madison, WI, USA) and the matrix used for mass spectrometry analysis was from Promega Co. (Madison, WI, USA).

2.2. Cloning and expression of recombinant proteins

The Ag473 gene (Fig. 1b) was amplified using the forward 5′-GGAATTCCATATGAAAAAATTATTGATTGC-3′ primer contains a Nde I restriction site and the reverse primer, 5′-CCGCTCGAGGTGATGATGTTTGGCGGCATCTTTCATTTTG-3′ is complementary to the coding sequence and contains a Xho I restriction site with three additional histidine codons in front of the Xho I site. As a result,