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Expression and characterization of an M cell-specific ligand-fused dengue virus tetravalent epitope using *Saccharomyces cerevisiae*

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A fusion construct (Tet-EDIII-Co1) consisting of an M cell-specific peptide ligand (Co1) at the C-terminus of a recombinant tetravalent gene encoding the amino acid sequences of dengue envelope domain III (Tet-EDIII) from four serotypes was expressed and tested for binding activity to the mucosal immune inductive site M cells for the development of an oral vaccine. The yeast episomal expression vector, pYEGPD-TER, which was designed to direct gene expression using the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter, a functional signal peptide of the amylase 1A protein from rice, and the GAL7 terminator, was used to clone the Tet-EDIII-Co1 gene and resultant plasmids were then used to transform *Saccharomyces cerevisiae*. PCR and back-transformation into *Escherichia coli* confirmed the presence of the Tet-EDIII-Co1 gene-containing plasmid in transformants. Northern blot analysis of transformed *S. cerevisiae* identified the presence of the Tet-EDIII-Co1-specific transcript. Western blot analysis indicated that the produced Tet-EDIII-Co1 protein with the expected molecular weight was successfully secreted into the culture medium. Quantitative Western blot analysis and ELISA revealed that the recombinant Tet-EDIII-Co1 protein comprised approximately 0.1–0.2% of cell-free extracts (CFEs). In addition, 0.1–0.2 mg of Tet-EDIII-Co1 protein per liter of culture filtrate was detected on day 1, and this quantity peaked on day 3 after cultivation. *In vivo* binding assays showed that the Tet-EDIII-Co1 protein was delivered specifically to M cells in Peyer's patches (PPs) while the Tet-EDIII protein lacking the Co1 ligand did not, which demonstrated the efficient targeting of this antigenic protein through the mucosal-specific ligand.

[**Key words:** Oral feeding; M cell-specific peptide ligand; Dengue virus; Domain III of the dengue envelope protein; Baker's yeast]

Dengue viruses are enveloped, positive sense RNA viruses that are members of the Flaviviridae family. Dengue infection has been acknowledged as the most important arthropod-borne viral infection in humans. Dengue infections can reach up to 50–100 million cases worldwide annually with approximately 500,000 patients hospitalized for Dengue Hemorrhage Fever (DHF) and Dengue Shock Syndrome (DSS), with up to a 5% fatality rate in some areas (1,2). Recently, there has been a dramatic increase in the annual average number of DHF and dengue fever cases reported to the World Health Organization (WHO) (2). Dengue viruses exist as four distinct serotypes, which makes the development of an effective dengue vaccine a great challenge since the vaccine must be tetravalent (3). The requirement for a tetravalent vaccine is due to the likelihood that a secondary infection with a different viral serotype leads to the most severe forms of dengue virus infection such as DHF and DSS. This increase in disease severity is supposedly due to the presence of sub-neutralizing antibodies generated during the primary infection and their enhancement to viral infection (4,5). Although dengue infections have been considered a global public health priority, the development of a safe and effective dengue vaccine has been hampered by the strict requirements for tetravalency, the complex nature of the disease, and the lack of investment (2). As such, no proven vaccine has been developed to date.

The discovery of dengue envelope domain III (EDIII) as a neutralizing epitope (6–11) has opened up great opportunity for the development of a safe and effective recombinant dengue vaccine. Accordingly, several approaches have been attempted to overcome the tetravalency requirement. These included expression of a single tandem array of each EDIII serotype or a synthetic consensus protein based on the amino acid sequence of all four EDIII serotypes (12–16). Moreover, the immunogenic efficacy of recombinant tetravalent proteins has been confirmed (12,13), and their limitations, including the triggering of a balanced immune response, have been further improved through the expression of the protein in a eukaryotic host (17).

Progress in understanding the mucosal immune system has led to the development of various oral vaccines against infectious agents. Mucosal vaccines have several advantages over systemic vaccines: they require less strict regulation since the oral route is more tolerable to endotoxins as well as other impurities than the parenteral route; they do not require special storage (cold-chain free) or properly trained medical staff for delivery; they do not involve complications normally involved with parenteral vaccines that can lead to better compliance; and they provide mucosal as

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well as systematic protection for recipients (18–20). Furthermore, mucosal vaccines have been shown to be advantageous for protection against not only mucosal transmitted infections but also non-mucosal transmitted infections such as malaria, hepatitis B, and Japanese encephalitis caused by a Flaviviridae virus (21).

A key consideration in the development of an oral vaccine is that the introduced antigen must pass through the gut wall where antigen sampling occurs by transcytosis in membranous or microfold cells (M cells). M cell-mediated transcytosis of an internalized antigen incites the initiation of both the antigen-specific mucosal immune response through secretory IgA production and the systemic immune response (22–24). Recent studies have shown effective mucosal immune induction through targeting antigens using M cell-specific antibodies, M cell-specific expression molecules, and M cell-specific ligands (22,25,26).

Baker's yeast, *Saccharomyces cerevisiae*, is an attractive heterologous vaccine expression system because it combines the advantages of simple prokaryotic systems, including high expression level, ease of scale-up, and genetic manipulation and culturing, with the inherent advantage of eukaryotic post-translational modifications and secretion. Moreover, *S. cerevisiae* is a generally recognized as safe (GRAS) organism and is known for its high-quality protein and vitamins levels, which allow for live and oral administration for pharmaceutical, livestock feed, and food applications. Due to the strong adjuvant properties of yeast derivatives, the use of yeast expression systems for recombinant vaccine formulation is desirable. Recently, a well-balanced immune response to dengue viruses was observed when a yeast expression system was used for the production of dengue virus epitope proteins (17).

In this study, *S. cerevisiae* was utilized to express an M cell-specific ligand fused-tetravalent tetrameric antigen created through a fusion of EDIIIs from all four Dengue serotypes in order to create an efficient and balanced mucosal immune response through oral administration. In addition, delivery of the fusion protein to mucosal immune inductive sites was assessed by *in vivo* antigen uptake assays.

MATERIALS AND METHODS

Strains and culture conditions Plasmids were maintained and propagated in *Escherichia coli* HB101 or DH5α according to standard procedures (27). *E. coli* strains were maintained in Luria–Bertani (LB) medium supplemented with appropriate antibiotics.

S. cerevisiae strain 2805 (*MATα pep4::HIS3 prb 1-δ Can1 GAL2 his3 ura3-52*) (28) was selected as the expression host for yeast recombinant proteins. *S. cerevisiae* strains were maintained on YEPD plates (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar). A uracil-deficient selective (ura$^-$) medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acids, 2% dextrose, and 1.5% agar) was employed for the screening of transformants at 30°C.

For expression cultures, a healthy transformant colony was inoculated into 5 ml ura$^-$ medium and incubated for 48 h at 30°C with continuous agitation (200 rpm). Subsequently, 250 μl of the primary inoculum was transferred into 5 ml of YEPD medium and cultured for 16 h at 30°C with continuous agitation (200 rpm). This 5-ml culture was then used as an inoculum for a 40-ml YEPD medium culture in a 300 ml Erlenmeyer flask. Expression cultures were grown at 30°C with continuous agitation (200 rpm), after which the cells and culture filtrates were harvested and examined for the expression of recombinant protein (17).

Construction of yeast expression plasmids and yeast transformation The tetravalent tetrameric EDIII (Tet-EDIII) protein was created by fusing the EDIIIs from all four serotypes in the order of serotype 1, 3, 4 and 2, which were joined using flexible pentaglycine peptide linkers, as described previously (Fig. 1A) (12). The genes encoding EDIII serotypes 1 and 3 were cloned from total RNAs extracted from Vero cells infected with dengue serotype 1 (GenBank no. JF967947) and 3 (GenBank no. JF968056), respectively. The EDIII gene of serotype 4 was obtained from serotype 4 isolate DENV-4/PH/BID-V3361/1956 (GenBank no. GQ868594). The EDIII gene from serotype 2 was obtained from the plasmid pMYV497 in the previous study (29). All EDIII genes were sequenced and subsequently used as templates for constructing the Tet-EDIII sequence by overlapping extension PCR. The resulting Tet-EDIII gene was sequenced and further fused with the M-cell specific peptide ligand (Co1) (22) through PCR using synthetic primers. The fused Tet-EDIII and Co1 gene (Tet-EDIII–Co1) was confirmed by DNA sequencing (Fig. 1B and C).

A rice amylase signal peptide sequence (RamylA) (30) was added at the 5′ termini of the encoding DNA sequences of Tet-EDIII and Tet-EDIII-Co1 by overlap extension PCR containing *Bam*HI and *Sal*I at their 5′ and 3′ termini, respectively, to direct the secretion of expressed protein. The resulting Ramy1A–Tet-EDIII and Ramy1A–Tet-EDIII-Co1 amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and confirmed by DNA sequencing, yielding the pTet and pTetCo1 plasmids, respectively. The pTet and pTetCo1 plasmids were digested with *Bam*HI and *Sal*I and the excised inserts were directionally cloned into the *Bam*HI/*Sal*I-digested yeast episomal shuttle vector pYEGPD-TER (30), which had the same restriction sites between the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and the galactose-1-P uridyl transferase (*GAL7*) terminator, yielding plasmids pYEGTet and pYEGTetCo1, respectively. The sequence information for each primer is provided in Table 1.

S. cerevisiae 2805 cells were transformed with the constructed recombinant plasmids using the LiAc method (31) and transformants were selected on ura$^-$ selective plates. The stability of the introduced plasmids in yeast was measured as described previously (27).

Analysis of expression Northern blot, Western blot, and ELISA analyses were used to detect the RNA expression and protein production of Tet-EDIII and Tet-EDIII-Co1. For Northern blots, total RNA extraction was carried out as described previously (32). RNA concentration was determined by UV spectrophotometry and 30 μg of total RNA was separated on 1.2% formaldehyde-agarose gels. The RNA was transferred to a nylon membrane (Hybond, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Hybridization was performed with modified Church buffer (250 mM Na_2HPO_4, 1 mM EDTA, 7% SDS, 0.17% H_3PO_4, and 1% hydrolyzed casein) with radioactive probes produced by the random primer method (Promega).

For Western blot analysis, cell-free extracts (CFEs) were prepared as described previously (33). In order to obtain concentrated culture filtrate, the culture media of recombinant *S. cerevisiae* were collected and then concentrated using 30 kDa cutoff Centricon column (Millipore, County cork, Ireland), dialyzed against PBS twice for 4 h at 4°C, and sterilized by passing through a 0.4-μm syringe filter (30). Protein concentration was determined by Bradford assay using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) (33). Sample aliquots of the CFE and culture filtrate were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto Hybond-C Extra nitrocellulose filter membranes

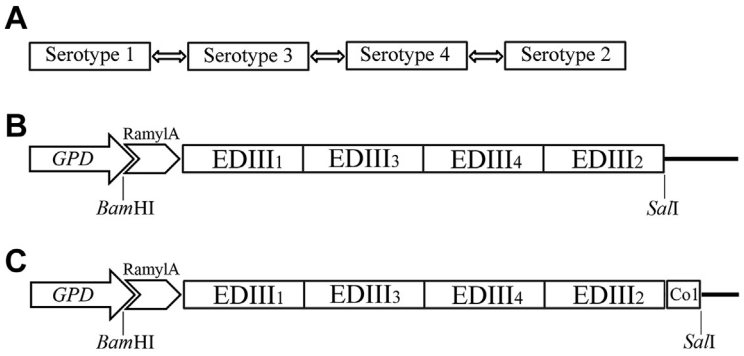

FIG. 1. Schematic diagram of Tet-EDIII protein structure (A) showing the order of each EDIII serotype with pentaglycine linkers (double headed arrows). The Tet-EDIII (B) and Tet-EDIII-Co1 (C) expression cassettes are also shown. The RamylA–Tet-EDIII and RamylA–Tet-EDIII-Co1 were constructed by overlap extension PCR and cloned into the episomal vector pYEGPD-TER using *Bam*HI and *Sal*I. Both constructs were expressed under control of the *GPD* promoter.