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Recombinant VP1 protein expressed in *Pichia pastoris* induces protective immune responses against EV71 in mice

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ABSTRACT

Human enterovirus 71 (EV71) is one of the major causative agents of hand, foot and mouth disease and is also associated with serious neurological diseases in children. Currently, there are no effective antiviral drugs or vaccines against EV71 infection. VP1, one of the major immunogenic capsid proteins of EV71, is widely considered to be the candidate antigen for an EV71 vaccine. In this study, VP1 of EV71 was expressed as a secretory protein with an N-terminal histidine tag in the methylotrophic yeast *Pichia pastoris*, and purified by Ni–NTA affinity chromatography. Immunogenicity and vaccine efficacy of the recombinant VP1 were assessed in mouse models. The results showed that the recombinant VP1 could efficiently induce anti-VP1 antibodies in BALB/c mice, which were able to neutralize EV71 viruses in an *in vitro* neutralization assay. Passive protection of neonatal mice further confirmed the prophylactic efficacy of the antisera from VP1 vaccinated mice. Furthermore, VP1 vaccination induced strong lymphoproliferative and Th1 cytokine responses. Taken together, our study demonstrated that the yeast-expressed VP1 protein retained good immunogenicity and was a potent EV71 vaccine candidate.

1. Introduction

Enterovirus 71 (EV71) is the most frequently detected pathogen in hand, foot and mouth disease (HFMD) patients complicated with the severest forms of neurological disorders [1,2]. Since it was initially identified in 1969 [3], outbreaks and epidemics caused by EV71 have been reported worldwide in the past decades, and hundreds of children have died from severe complications of encephalomyelitis [4–6]. Though there has been a significant increase in EV71 epidemics, effective vaccines or antiviral drugs are not available [7]. There is an urgent need for the development of effective vaccines against EV71.

EV71 belongs to the genus *Enterovirus* in the family *Picornaviridae* [8]. EV71 contains a single-stranded, positive-sense RNA of approximately 7400 nucleotides which is enclosed by capsid proteins VP1, VP2, VP3 and VP4 [9,10]. Because VP1 displays major antigenicity and has been defined as the neutralization determinant [11–13], VP1 is the candidate antigen protein for developing subunit or epitope vaccines [14]. In recent years, several studies have indicated the potential of the VP1 protein to act as an EV71 vaccine candidate. For example, recombinant VP1 protein derived from *E. coli* [15], transgenic tomato [16] or the milk of transgenic mice [17] could induce neutralizing antibodies and protected mice against EV71 infection. However, it was difficult to purify and obtain high-level production of recombinant VP1 proteins in these expression systems.

The methylotrophic yeast *Pichia pastoris* has been widely used in vaccine production with the advantages of easy manipulation, high production levels and low cost [18]. In the present study, the VP1 protein of EV71 was secretory expressed in *P. pastoris* with good antigenicity. The yeast-expressed VP1 induced high levels of neutralizing antibodies, and elicited strong humoral and cellular immune responses in mice, which represents a potential subunit vaccine candidate.

2. Materials and methods

2.1. Cell lines and virus strain

RD cells (rhabdomyosarcoma) were used for the growth of EV71-C4 strain (FY0805, GenBank accession #HQ882182) [19]. EV71 virus stock was collected from the supernatant of infected RD cells 3 days post-infection (dpi) at 37 °C. The virus from the cell culture was purified as described previously [20].

2.2. Codon optimization

The codon-optimized gene was designed based on the protein sequence of VP1 (GenBank Accession #ADC53084) according to the codon bias of *P. pastoris* [21] (http://www.kazusa.or.jp/codon). Codon optimization was performed by using the JCat program [22].

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The entire *VP1* gene with *Eco*R I and *Xba* I restriction sites at each end was designed and was in frame with α-factor of pPICZαA vector (Fig. 1C). The designed *VP1* was synthesized by Takara (Dalian, China).

2.3. Construction of plasmids encoding VP1

The synthesized *VP1* gene was inserted in the pUC57 plasmid (pUC57-VP1). Proper construction was confirmed by DNA sequencing. The pUC57-VP1 was double digested with *Eco*R I and *Xba* I, prior to the insertion into plasmid pPICZαA (Invitrogen, USA). The resulting plasmid was designated pPICZαA-VP1.

2.4. VP1 expression and purification

The recombinant expression vector pPICZαA-VP1 was linearized by *Sac* I (TaKaRa) and electroporated into *P. pastoris* GS115 competent cells. The transformants were selected on YPD plates containing Zeocin™ (Invitrogen) at a final concentration of 100 μg/ml. The positive transformants of *P. pastoris* GS115 were grown in buffered complex glycerol media (BMGY) with vigorous shaking (300 rpm) at 30 °C until the culture reached an OD_{600} = 6. The cells were subsequently centrifuged at 3000×g for 5 min and resuspended to an OD_{600} of 1 in buffered complex methanol media (BMMY). The expression was induced by continuous incubation at 30 °C for 72 h and the addition of methanol every 24 h to a final concentration of 0.5% for transcriptional induction. A negative control containing the empty pPICZαA vector was carried out in parallel. The supernatant of the induced culture was collected by centrifugation and subjected to precipitation at 80% ammonium sulfate saturation, and the 80% precipitate was collected by centrifugation at 10,000×g for 15 min. The precipitated protein was dissolved in distilled water and dialyzed extensively against 50 mM TBS (pH 7.2) by changing the dialysis buffer 3 times at 4 °C overnight. The sample was loaded onto a Ni–NTA affinity column pre-equilibrated with TBS (pH 7.2), and eluted with 0.2 M imidazole in 50 mM TBS (pH 7.2) after extensive washing with TBS. The protein samples were analyzed by 12% SDS–PAGE and western blot with anti-His polyclonal antibody. The enriched VP1 fraction was stored at −20 °C.

2.5. Animals and immunization

Three groups, each including twenty 4–6-week old female BALB/c mice, were immunized by intramuscular (i.m.) injection on day 0 with PBS (100 μl), 10 μg VP1 or 10 μg VP1 with Freund's complete adjuvant (50%, v/v) (Sigma, USA). On day 14 and 28, the mice were boosted with PBS (100 μl), 10 μg VP1 or 10 μg VP1 with Freund's incomplete adjuvant (50%, v/v) (Sigma, USA). Blood samples were taken from all the mice on days 0, 14, 28, and 42. The

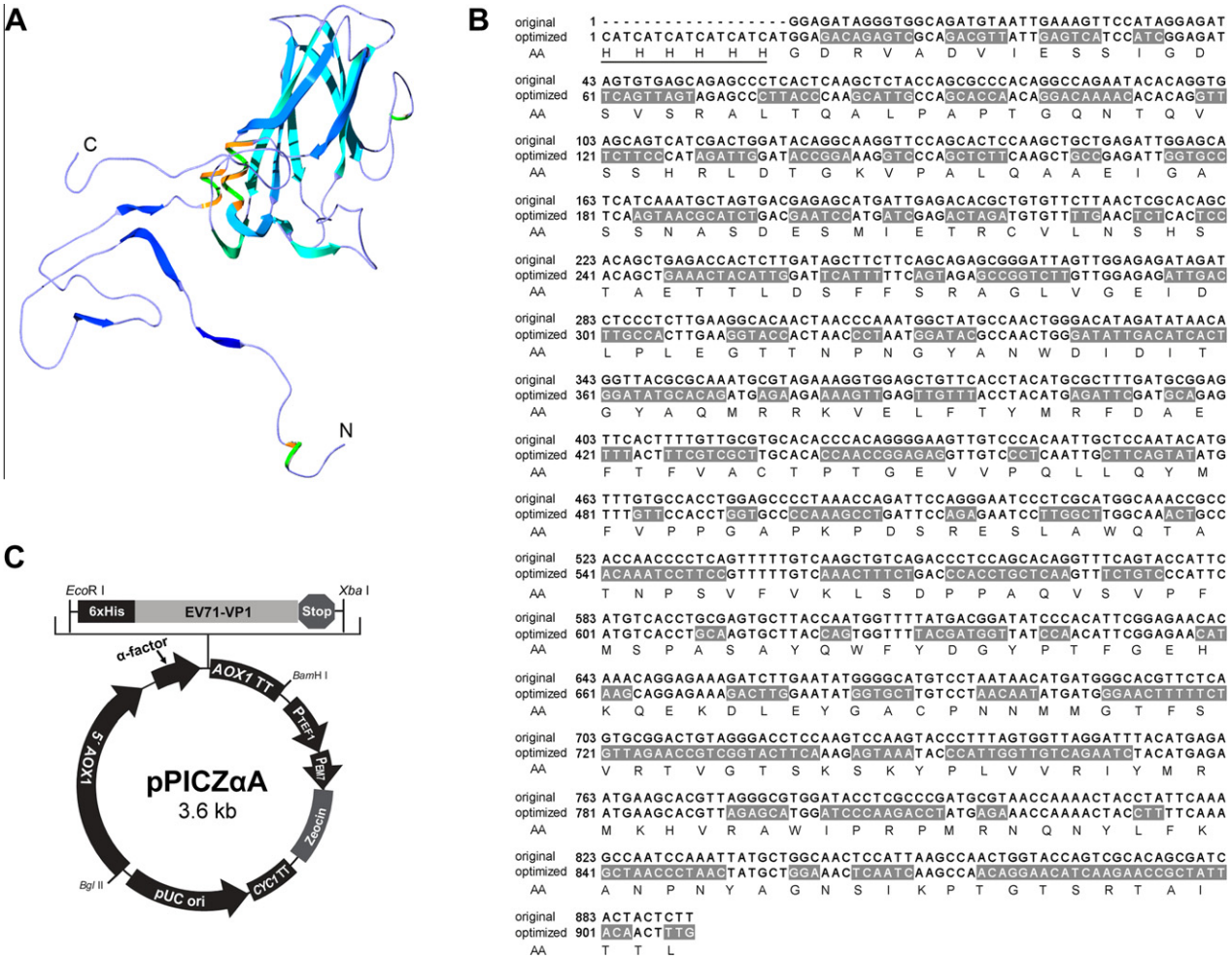

Fig. 1. Construction of the recombinant expression vector pPICZαA-VP1. (A) Crystal structure of VP1. The structure was derived from PDB code 3VBH. (B) Sequence alignment of codon-optimized and original *VP1*. The optimized codons are highlighted in grey; His-tag at the N-terminal is underlined. (C) Schematic diagram of plasmid construct. *VP1* was inserted into pPICZαA for the expression of recombinant protein.