Veterinary Immunology and Immunopathology 167 (2015) 96–103

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Cell-penetrating peptide-mediated subunit vaccine generates a potent immune response and protection against *Streptococcus iniae* in Japanese flounder (*Paralichthys olivaceus*)

Yun Sun[a,b], Yong-hua Hu[a,*]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Fisheries Department, Hainan University, Haikou 570228, China

ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 14 June 2015
Accepted 17 July 2015

Keywords:
Cell-penetrating peptides
TAT
Immune protection
Streptococcus iniae

ABSTRACT

The efficiency of antigen capture, processing, and presentation by antigen-presenting cells is the key to induce an effective immune response. Cell-penetrating peptides (CPPs) are short peptides that facilitate cellular uptake of various molecular cargoes and have an attractive potential for vaccine delivery. In this study, the *Drosophila Antennapedia* homeoprotein (Antp) and the human immunodeficiency virus-1 transactivator of transcription (TAT) peptides were fused to the N- or C-terminus of Sia10, a protective antigen of *Streptococcus iniae*, resulting in four recombinant fusion proteins, i.e., rAntp-Sia10, rSia10-Antp, rTAT-Sia10, and rSia10-TAT. All fusion proteins were expressed and purified, and their ability to penetrate into cells was examined. The results showed that rTAT-Sia10 had the strongest ability to translocate through the cellular membrane into cells. Immunofluorescence microscopy and Western blot assay confirmed that rTAT-Sia10 could penetrate into the head kidney lymphocytes and gill cells of Japanese flounder (*Paralichthys olivaceus*). Immunological analysis showed that rTAT-Sia10 significantly enhanced macrophage activation and peripheral blood leukocyte proliferation, and induced production of specific serum antibodies at 2–8 weeks post-vaccination. Transcriptional analysis showed that vaccination with rTAT-Sia10 up-regulated the expression of the genes encoding IL-1β, IL-8, NKEF, Mx, IgD, IgM, TNFα, MHC I α, MHC IIα, and CD8α. Fish vaccinated with rTAT-Sia10 exhibited significantly higher levels of survival rates (98% at 1 month and 92% at 2 months) compared to fish vaccinated with rSia10 (57% at 1 month and 53% at 2 months). Taken together, these results indicate that TAT-derived peptide has a great potential in the application of bacterial vaccines.

1. Introduction

Vaccines are the most effective means of preventing diseases (Tonheim et al., 2008). In recent years, different types of vaccines for fish have been studied, such as attenuated vaccines, DNA vaccines, and subunit vaccines (Cheng et al., 2010a,b; Sun et al., 2010). However, lack of immunogenicity is one of the biggest barriers to developing effective fish vaccines. The efficiency of antigen capture, processing, and presentation by antigen-presenting cells (APCs) is pivotal for inducing an effective immune response (Mittendorf et al., 2007). Cell-penetrating peptide (CPP)-cargo conjugates have been reported can be transported into the cytoplasm of APCs, through the MHC class I pathway to induce strong CTL responses (Wadia et al., 2004; Foerg and Merkle, 2008), and leads to the generation of potent immune responses (Brooks et al., 2010a,b; Apostolopoulos et al., 2006).

CPPs, which are also called protein transduction domains, usually consist of no more than 30 amino acids with net positive charge (Schwarze and Dowdy, 2000). CPPs are known to transport cargoes such as oligonucleotides (Gait, 2003), plasmids (Rittner et al., 2002), and proteins (Wadia et al., 2004) through cell membranes and into living cells (Kilk, 2004). In 1988, Green and Frankel both reported that the human immunodeficiency virus (HIV)-1 transactivator of transcription (TAT) protein passed efficiently through cell membranes of cultured mammalian cells (Green and Loewenstein, 1988; Frankel and Pabo, 1988). Following this discovery, many other CPPs were discovered, including HSP-VP22 (Cashman et al., 2002), *Drosophila Antennapedia* homeoprotein (Antp) (Derossi et al.,

* Corresponding author at: Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China.
E-mail address: huyonghua@qdio.ac.cn (Y.-h. Hu).

http://dx.doi.org/10.1016/j.vetimm.2015.07.008
0165-2427/

1994), and PEP-1 (Lindgren et al., 2000). To date, several CPPs have been used in protein vaccines (Fawell et al., 1994; Wadia and Dowdy, 2005), DNA vaccines (Michel et al., 2002; Perkins et al., 2005), and tumor vaccines (Hiroshi et al., 2006; Woo et al., 2004).

Streptococcus iniae is a Gram-positive bacterium (Zhang et al., 2014). As one of the most widespread pathogens in aquaculture, *S. iniae* can infect a wide range of fish species (Austin and Austin, 2008; Li et al., 2014; Low et al., 1999). In a previous work, we identified an in vivo-induced antigen, Sia10, from the pathogenic *S. iniae* strain (Sun et al., 2010). In primary structure, Sia10 is closely related to a secretory antigen and is predicted to have subcellular localization in the extracellular membrane. We constructed a Sia10-based subunit vaccine, but it was unsatisfactory in eliciting immunoprotection (Sun et al., 2010).

In the present study, we designed two CPPs (TAT and Antp) to carry the antigen Sia10. We examined the ability of these CPPs to deliver the subunit vaccine into Japanese flounder (*Paralichthys olivaceus*) cells and the effect of the CPPs on the protection induced by Sia10.

2. Materials and methods

2.1. Bacterial strain and growth condition

Streptococcus iniae SF1, a fish pathogen, has been reported previously (Cheng et al., 2010a). *Escherichia coli* DH5α and BL21(DE3) were purchased from Takara, Dalian, China. All strains were cultured in Luria-Bertani broth (LB) medium at 37 °C (for *E. coli*) or 28 °C (for SF1). To confirm the authenticity of the cultures, the 16S rRNA genes of the bacteria were amplified using primers 27F and 1492R and the PCR products were purified and sequenced (Zhang and Sun, 2007). SF1 was also verified by PCR analysis using specific primers (Cheng et al., 2010a).

2.2. Fish

Japanese flounder were purchased from a local fish farm (Haiyang, Shandong, China). Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were fed daily with commercial dry pellets and maintained at 20 °C in aerated seawater. Before experiments, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen as reported previously (Li et al., 2015), and no pathogens were detected in these tissues. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA).

2.3. Plasmid construction and preparation

The primers used in this study are listed in Table 1. According to the amino acid sequence of Antp (RQIKIWFQNRRMKWKK) and TAT (GRKKRRQRRRPPQ), and codon preference in *E. coli*, to construct pEtAntp-Sia10, the segments were performed by overlap extension PCR as follows: the first overlap segment was synthesized as 16B, the second overlap PCR was performed with the primer pair 16BF1/BR2, and the fusion PCR was performed with the primer pair 16BF2/BR2. Similarly, pEtSia10-Antp, pEtTAT-Sia10, and pEtSia10-TAT were performed with the primer pair AF2/16AR2, 13BF2/BR2, and AF2/13AR2 respectively. The PCR products were ligated with the T–A cloning vector pEASY-T1 Simple (TransGen, Beijing, China), and the recombinant plasmid was digested with SwaI. The fragments containing Sia10 and CPPs fusion genes were retrieved and inserted into pET259 (Zhou and Sun, 2015a) at the SwaI site, resulting in the recombinant expression plasmids.

Table 1
Primers used in this study.

Primers	Sequence[a] (5′–3′)
16B	CGCCAGATTAAAATCTGGTTTCAGAACCGCCGCATGAAATGGAAG AAAGCAGAATTT
16BF1	ATGAAATGGAAGAAAGCAGAATTTAATCAATCAAATA
16BF2	CCCGGGATGCGCCAGATTAAAATCTGG (SmaI)
BR2	CTCGAGCCCGGGGATATGAATATAATTATATGAGCT (SmaI)
16A	GCGGCGGTTCTGAAACCAGATTTTAATCTGGCGGATATGAAT
16AR1	GATTTTAATCTGGCGGATATGAATATAATTATATGAGCT
16AR2	CCCGGGGCGGCGGTTCTGA (SmaI)
AF2	CCCGGGATGGCAGAATTTAATCAATCAAA (SmaI)
13B	GGCCGCAAGAAGCGCCGTCAGCGCCGTCGCCCGCCGCAGGCAGAATTT
13BF1	CCGCCGCAGGCAGAATTTAATCAATCAAATA
13BF2	CCCGGGATGGGCCGCAAGAAGCG (SmaI)
13A	CTGCGGCGGGCGACGGCGCTGACGGCGCTTCTTGCGGCCGATATGAAT
13AR1	CTTGCGGCCGATATGAATATAATTATATGAGCT
13AR2	CCCGGGCTGCGGCGGGCGA (SmaI)

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

2.4. Purification of recombinant protein

E. coli BL21 (DE3) was transformed with either of the recombinant plasmids. The transformants were cultured in LB medium at 37 °C to OD_{600} 0.5, and the expression of recombinant proteins were induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growth at 20 °C for an additional 6 h, the cells were harvested by centrifugation, and proteins were purified under native conditions using nickel–nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified proteins were dialyzed for 24 h against phosphate-buffered saline (PBS) and treated with Triton X-114 to remove endotoxin as reported previously (Chen et al., 2013). The proteins were concentrated using PEG20000 (Solarbio, Beijing, China). The proteins were analyzed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of purified proteins was determined using the Bradford method with bovine serum albumin as a standard.

2.5. Immunofluorescence microscopy

Japanese flounder (average 451 g) head kidney (HK) lymphocytes were prepared using Percoll gradient as described previously (Hu et al., 2012). The cells were cultured in L-15 medium (Thermo Scientific HyClone, Beijing, China) containing penicillin (100 μg/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) at 22 °C. Japanese flounder gill cells (FG cells) were cultured and maintained as described previously (Tong et al., 1997). The cells were distributed into 6-well tissue culture plates (10^5 cells/well) and cultured in L-15 medium at 22 °C. Immunofluorescence microscopy was performed as reported previously (Qiu et al., 2013). Briefly, 12 μM purified recombinant protein was added to each well of the cells. The cells were incubated at 22 °C for 1 h. After washing with PBS, the cells were fixed for 20 min with 4% paraformaldehyde at RT. After washing with PBS, the cells were incubated with 0.5% Triton X-100 in PBS for 15 min. Then the cells were blocked in the presence of 1% FBS for 1 h after washing. Next, the anti-His tag mouse monoclonal antibody (Bios, Beijing, China) (1/1000 dilution) was applied to the cells for 1 h at RT. Free antibody was washed out, and then fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Bios, Beijing, China) (1/500 dilution) was added to the cells. Finally, a drop of Fluoromount-G (Southern Biotechnology Associates, USA) mounting medium was added to glass cover slips, and Evans blue (Invitrogen, Carlsbad, CA, USA) was used for cells staining according to manufacturer's instructions. The cells were