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Both polarity and aromatic ring in the side chain of tryptophan 246 are involved in binding activity of *Vibrio vulnificus* hemolysin to target cells

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ABSTRACT

Vibrio vulnificus secretes a hemolysin/cytolysin (VVH) that induces cytolysis against a variety of mammalian cells by forming pores on the cellular membrane. VVH is known to bind to the cellular membrane as a monomer, and then convert to a pore-forming oligomer. However, the structural basis for binding of this toxin to target cells remains unknown. We show here that the polarity and indole ring on the side chain of Trp 246 (W246) of VVH, which sits on a bottom loop, participates in binding to cellular membrane. To clarify the binding mechanisms of VVH, we generated a series of W246 point mutants that were substituted with Arg (W246R), Ala (W246A), or Tyr (W246Y), and tested their binding and cytotoxicity on Chinese hamster ovary (CHO) cells. At a final concentration of 1 μg/ml of VVH, wild type (Wt), W246A and W246Y could bind and induce cytotoxicity to CHO cells, whereas W246R could not. The cytotoxic activity of W246A was significantly lower than that of Wt. These findings indicate that both the polarity and indole ring on the side chain of W246 were involved in the binding of this toxin to the target cellular membrane. The indole ring plays a particularly important role in toxin binding.

1. Introduction

Many Gram-positive and -negative bacteria produce pore forming toxins [17,23]. Structure-function relationships of pore forming toxins produced by Gram-positive bacteria have been intensively studied using crystal structure analysis techniques for over a decade, whereas only recently have such studies been undertaken in Gram-negative bacteria [15]. *Vibrio vulnificus* is a Gram-negative and opportunistic pathogen that causes severe septicemia with a high mortality rate [9]. *V. vulnificus* produces an extracellular pore-forming toxin called *Vibrio vulnificus* hemolysin/cytolysin (VVH). VVH is a virulence factor that promotes invasion of *V. vulnificus* from intestine to blood stream by causing intestinal tissue damage [5]. VVH binds to cellular membrane as a monomer and then assembles SDS-resistant oligomers [8]. These oligomers form small ion-permeable pores that induce hemolysis in red blood cells [22] and apoptosis in epithelial cells [6].

It was reported that *Vibrio cholerae* cytolysin (VCC), which is considered VVH ortholog, is composed of three domains, namely the cytolysin domain, the β-trefoil lectin domain, and the β-prism lectin domain [16]. Although VVH lacks the β-prism lectin domain that has carbohydrate-binding activity for VCC [7,10,12,18], it shares 47% sequence similarity in amino acid residues with VCC. In addition, regardless of the very low similarity in amino acid sequences between Gram-positive and Gram-negative bacteria producing pore-forming toxins, the three dimensional structure of pore forming domains are similar [15]. The structure and function relationships of Leukocydin F (LukF) and *Staphylococcal* α-hemolysin that are produced by Gram-positive bacteria have structural similarity with VVH, and studies of the membrane binding mechanisms showed that these toxins bind to the cellular membrane via the bottom of the rim domain [4,13,19]. For instance, the tryptophan or tyrosine residue that is found at the bottom of the rim domain of VCC or LukF, respectively, participates in the initial attachment of these toxins to target cellular membranes [19]. Based on our modeling analysis of VVH, a tryptophan residue but not tyrosine residue sits on the tip of the bottom loops in the VVH rim domain.

In this study, we investigated the role of W246, which sits on the tip of loop2 in the VVH rim domain, in the cytotoxic mechanism of VVH, by site directed mutagenesis. We found that both the polarity and indole ring on the side chain of W246 participate in the binding

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of this toxin to cellular membrane. Our analysis of the structure-function relationship of VVH will improve understanding of the evolutionary process of pore-forming toxins as well as of the toxic mechanism of VVH.

2. Materials and methods

2.1. Bacterial strain

V. vulnificus strain K44 was isolated from the blood of a septicemic patient at Kurashiki Central Hospital in Japan. K44 was routinely cultivated in Luria-Bertani (LB) broth at 37 °C.

2.2. Homology modeling and the template toxin

The modeling was performed using the SWISS-MODEL protein comparative modeling server (https://swissmodel.expasy.org) [1]. VCC was used as the template. VCC and VVH share 47% positives in aligned amino acid sequences by BLAST2.

2.3. Generation of the series of W246 mutant VVH expression *V. vulnificus*

The *V. vulnificus* genome DNA was purified by Qiagen Genomic-tip (Qiagen, Hilden, Germany) as recommended by the manufacturer. VVH encoding gene, *vvhA* was amplified with signal sequence by PCR with the primers vvhA5' (5′-GTGGGATCCATGAAAAAAATGACTCTGTTTACC-3'; the underline indicates an *Bam*HI site) and the vvhA3′(5′-GTGGCATGCCTAGAGTTTGACTTGTTGTAATGT -3'; the underline indicates an *Sph*I site), from *V. vulnificus* genome as the template. The amplified DNA was ligated to pGEM-T vector (Promega, Madison, WI) and the sequence was confirmed by DNA sequencing. A QuikChange (Agilent technologies, Santa Clara, CA) site-directed mutagenesis kit was used to introduce the desired mutation at the position of W246. Various mutagenic primers for a series of W246 mutants were designed individually according to the desired mutations with specific restriction enzyme recognition sites (*Sac*II for W246R, *Nae*I for W246A, and *Bam*HI for W246Y). The cycling parameters for the mutagenesis reactions were chosen based on the protocol suggested by the manufacturer. After PCR amplification, the reaction mixture was digested with 10 units of the restriction enzyme *Dpn*I for 1 h. Competent cells were transformed by addition of 10 μl of *Dpn*I-treated reaction mixture. Plasmid DNA was purified using the Qiagen Plasmid Purification Kit (Qiagen, Valencia, CA, USA). The desired mutation was confirmed by digestion with the appropriate restriction enzyme and by DNA sequencing. Each mutant *vvhA* was ligated to suicide vector pYAK1 and transformed to *Escherichia coli* S-17λpir. Mutant *VvhA* encoding pYAK1 were transferred to *V. vulnificus* by conjugation followed by selection for homologus recombination using sucrose counter selection to isolate recombinants. Recombination of mutant *vvhA* with *vvhA* Wt in *V. vulnificus* was confirmed by amplification of *vvhA* and digestion by appropriate restriction enzymes (Fig. 2).

2.4. Purification of VVH

Wt and W246 mutants were purified from the culture supernatant of the *V. vulnificus* following the method of Oh et al. [14]. The protein concentration of each fraction was checked by optical density at 280 nm, and fractions with a high concentration of protein were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with staining solution containing 0.5% Coomassie brilliant blue R-250. Purified VVH was observed as a single band. VVH-containing fractions were dialyzed in 10 mM glycine buffer (pH 9.8)−150 mM NaCl at 4 °C for 16 h. The dialyzed fractions were pooled as purified VVH. The specific activity of purified Wt was 70,000 hemolytic units/mg (HU/mg), which was confirmed by examining the hemolytic activity against mouse erythrocytes. The protein concentrations were determined by the methods of Lowry et al. [11] and densitometry on SDS-PAGE with BSA standard.

2.5. Production of antibodies against VVH in rabbit

Fifteen-week-old New Zealand White rabbits were immunized subcutaneously with purified Wt in complete Freund's adjuvant (Difco, Detroit, Mich.) on day 0. On days 14 and 28, the same rabbits were given booster shots of the same antigen in Freund's incomplete adjuvant (Difco). Immune rabbit sera were collected 10 days after the last immunization. Immunoglobulin G (IgG) was purified with an Affi-Gel protein A MAPS II kit (Bio-Rad, Hercules, CA). Reactivity of the purified IgG was confirmed by western blot analysis against Wt and native VVH. The purified IgG was designated as anti-VVH polyclonal antibody.

2.6. Cell culture

Chinese hamster ovary (CHO) cells were grown in Dulbecco's

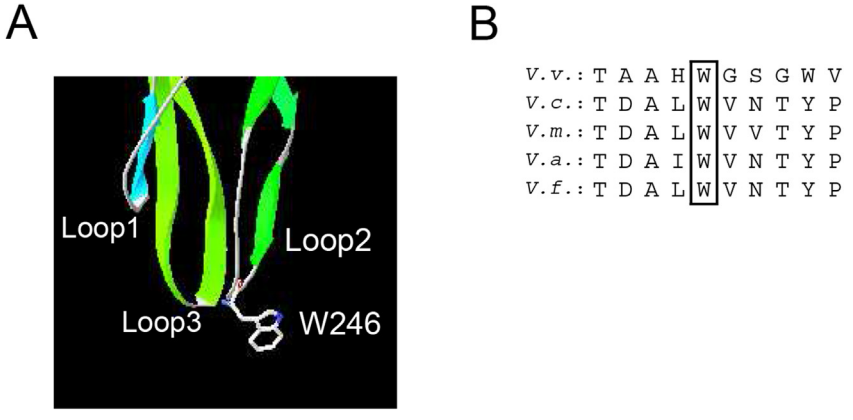

Fig. 1. Conservation and localization of W246. (A) Localization of W246 on predicted 3D-structure of VVH. W246 sits on the tip of loop2 in rim domain. (B) W246 is conserved in other *Vibrionaceae* (*V.c.*; *V. cholerae, V.m.; Vibrio mimicus, V.a.*; *Vibrio anguillarum,* and *V.f.*; *Vibrio fluvialis*) cytolysins. Box corresponds to W246 in VVH.