

Original Articles

# Zonula occludin toxin, a microtubule binding protein

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**Subject headings** microtubules; microtubule proteins; microtubule-associated proteins; intercellular junctions; vibrio cholerae; cholera toxin; zonula occluding toxin

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## Abstract

**AIM** To investigate the interaction of Zot with microtubule.

**METHODS** Zot affinity column was applied to purify Zot-binding protein(s) from crude intestinal cell lysates. After incubation at room temperature, the column was washed and the proteins bound to the Zot affinity column were eluted by step gradient with NaCl ( $0.3 \text{ mol} \cdot \text{L}^{-1}$ – $0.5 \text{ mol} \cdot \text{L}^{-1}$ ). The fractions were subjected to 6.0%–15.0% (w/v) gradient SDS-PAGE and then transferred to PVDF membrane for N-terminal sequencing. Purified Zot and tau protein were blotted by using anti-Zot or anti-tau antibodies. Finally, purified Zot was tested in an *in vitro* tubulin binding assay.

**RESULTS** Fractions from Zot affinity column yielded two protein bands with a  $M_r$  of 60kU and 45kU respectively. The N-terminal sequence of the 60kU band resulted identical to  $\beta$ -tubulin. Zot also cross-reacts with anti-tau antibodies. In the *in vitro* tubulin binding assay, Zot co-precipitate with Mt, further suggesting that Zot possesses tubulin-binding properties.

**CONCLUSION** Taken together, these results suggest that Zot regulates the permeability of intestinal tight junctions by binding to intracellular Mt, with the subsequent activation of the intracellular signaling leading to the permeabilization of intercellular tight junctions.

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## INTRODUCTION

*Vibrio cholerae*, the human intestinal pathogen responsible for the diarrhoeal disease cholera, elaborates a large number of extracellular proteins, including several virulence factors. The severe dehydrating diarrhoea characteristic of cholera is induced by cholera toxin (CT). A number of epidemiological studies<sup>[1-4]</sup> have shown a concurrent occurrence of the CT genes (ctx-A and ctx-B) and the genes for two other virulence factors elaborated by *V. cholerae*, zonula occludens toxin (Zot)<sup>[5]</sup> and accessory cholera toxin (Ace)<sup>[6]</sup>. Zot increases the intestinal permeability by rearranging the intestinal cell cytoskeleton strategically located to modulate intercellular tight junctions<sup>[7]</sup>. However, the first step of Zot signaling after the protein internalization remains to be established.

Microtubules (MT) are intracellular structures functionally and anatomically related to the cell cytoskeleton. MTs are composed of  $\alpha$ -tubulin and  $\beta$ -tubulin. Factors known to regulate microtubule dynamic include microtubule-associated proteins (MAPs). Neuronal MAPs, the most abundant of which are MAP2 and tau, stimulate MT assembly<sup>[8,9]</sup>. The best characterized function of MT network polymers is the bi-directional movement of membrane vesicles driven by the MT-based motor proteins, kinesin and cytoplasmic dynein. Different cargoes can be transported via MT-dependent vesicles, including various types of endocytic and exocytic vesicles<sup>[10,11]</sup>. Connection of actin filament network has been found<sup>[12]</sup>. In eukaryotic organisms, various cell functions, including cell shape and mobility require coordinational interaction between actin and MT cytoskeleton<sup>[13]</sup>.

In our study, we found that when cell lysates from mammalian tissues passed through a Zot affinity column, two proteins bound to Zot, the N-terminal sequence of one of these two proteins revealed that it corresponded to tubulin; Zot cross-reacted with antibody against Tau, another well described MAP isolated from mammalian brain; by using a microtubule binding assay, Zot was found co-precipitated with MT. Taken together, these results suggest that Zot is a new member of MAPs family. This Zot property may be involved in the Zot signaling leading to the regulation of intercellular TJ.

## MATERIAL AND METHODS

### *Purification of 6-his Zot*

Plasmid pSU111, containing the clone Zot gene in a pQE-30 vector with a 6-histidine tag at its N-terminal, was grown in LB medium with 20 g/L glucose, 25 µg/L kanamycin and 200 µg/L ampicillin at 37°C with vigorous mixing until the  $A_{600}$  reached 0.7–0.9. Cultures were then induced with 2 mmol·L<sup>-1</sup> isopropylthio-β-D-galactoside (IPTG, Fisher), followed by an additional 2 h culture period at 37°C with vigorous shaking. The cells were harvested by centrifugation at 4 000×g for 20 min and resuspended in buffer A (6 mol·L<sup>-1</sup> Guanidine-HCl, 0.1 mol·L<sup>-1</sup> Na-phosphate, 0.01 mol·L<sup>-1</sup> Tris-HCl, pH 8.0; 5 mL/g wet cell weight). After stirring for 1 h at room temperature, the mixture was centrifuged at 10 000×g for 30 min at 4°C. A 50% slurry of Superflow (QIAGEN, 1 mL/g wet cell) was added to the supernatant and stirred for 1 h at room temperature. The mixture was loaded onto a 5 cm×1.5 cm column and washed sequentially with buffer A, buffer B (8 mol·L<sup>-1</sup> urea, 0.1 mol·L<sup>-1</sup> Na-phosphate, 0.01 mol·L<sup>-1</sup> Tris-HCl, pH 8.0) and buffer C (8 mol·L<sup>-1</sup> urea, 0.1 mol·L<sup>-1</sup> Na-phosphate, 0.01 mol·L<sup>-1</sup> Tris-HCl, pH 6.3). Each wash step was continued until the  $A_{280}$  of the flow-through was less than 0.01. His-Zot was eluted by addition of 250 mmol·L<sup>-1</sup> imidazole (1,3-diaza-2,4-cyclopentadiene) to buffer C. After dialysis against urea, the eluate was diluted 200–500 times in PBS, stirred with 50% slurry Superflow (1 mL/g wet cell weight) for 2 h at room temperature, loaded onto another 5 cm×1.5 cm column, washed with phosphate-buffered saline (PBS) and eluted with 250 mmol·L<sup>-1</sup> imidazole in PBS. Purity of the His-Zot protein was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blot using polyclonal anti-Zot antibodies.

### *Analytical procedures*

**SDS-PAGE** It was carried out on a 50 g·L<sup>-1</sup>–150 g·L<sup>-1</sup> gradient gel, stained with Coomassie brilliant blue dye, destained by 75 mL·L<sup>-1</sup> acetic acid with 100 mL·L<sup>-1</sup> methanol and dried with Gel Drying Film (Promega).

**Western blot analysis** Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (MILLIPORE). Non-specific binding sites were blocked by PBS with 50 mL·L<sup>-1</sup> milk plus 1 g·L<sup>-1</sup> Tween-20. Primary and secondary antibodies were rabbit polyclonal anti-Zot antibody and anti-rabbit IgG (peroxidase conjugate, Sigma), respectively. Films were exposed with ECL detection reagent (Amersham) for 1 min, and developed by Konica SRX-101 developer.

### *Immobilization of his-Zot to AminoLink plus column*

One mg of his-Zot in 4 mL coupling buffer (0.1 mol·L<sup>-1</sup> sodium phosphate, 0.15 mol·L<sup>-1</sup> NaCl, pH 7.2) and 40 µL of 5 mol·L<sup>-1</sup> sodium cyanoborohydride, were added to an equilibrated AminoLink plus column (Pierce) and gently mixed overnight at 4°C. After washing with coupling buffer, 4 mL of 1 mol·L<sup>-1</sup> Tris-HCl (pH 7.4) and 40 µL of 5 mol·L<sup>-1</sup> sodium cyanoborohydride were added to the column followed by gently mixing for 30 minutes at room temperature to block the remaining active sites. The column was washed with 1 mol·L<sup>-1</sup> NaCl and stored in PBS containing 0.5 g·L<sup>-1</sup> sodium azide.

### *Preparation of human tissue plasma membranes*

Adult human brain tissues were obtained from the Brain and Tissue Banks for Developmental Disorders at the University of Maryland and used under the approval of the University's Institutional Review Board. Adult human heart and intestinal tissues were utilized for comparative analysis. Tissues were washed with buffer D (20 mmol·L<sup>-1</sup> Tris-HCl, 20 mmol·L<sup>-1</sup> EDTA, 250 mmol·L<sup>-1</sup> sucrose, pH 7.5), homogenized in buffer E (buffer D containing 5 mg·L<sup>-1</sup> leupeptin, 2 mg·L<sup>-1</sup> aprotine, 1 mg·L<sup>-1</sup> pepstatin, 10 mg·L<sup>-1</sup> phenylmethanesulfonyl fluoride (PMSF), and centrifuged at 5 000×g, 4°C for 10 min. Supernatants were centrifuged at 12 000×g, 4°C for 45 min. Precipitates were discarded and supernatants were centrifuged at 30 000×g, 4°C for an additional 90 min. Precipitates were dissolved in buffer E with 5 g·L<sup>-1</sup> 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sitting on ice for 60 min with gentle mixing every five minutes.

### *Affinity purification of his-Zot binding proteins*

Membrane preparations obtained from human brain were loaded on an AminoLink plus -Zot affinity column, washed, and equilibrated with PBS at room temperature containing 1 g·L<sup>-1</sup> Triton X-100. The columns were incubated for 90 min at room temperature, washed with 8 volumes of PBS containing 1 g·L<sup>-1</sup> Triton X-100, and eluted with PBS containing 1 g·L<sup>-1</sup> Triton X-100 with 0.1 mol·L<sup>-1</sup> NaCl, 0.3 mol·L<sup>-1</sup> NaCl, 0.5 mol·L<sup>-1</sup> NaCl, 0.8 mol·L<sup>-1</sup> NaCl and 1.0 mol·L<sup>-1</sup> NaCl, respectively. Fractions were collected and subjected to SDS-PAGE. N-terminal amino acid sequence analysis.

### *N-terminal amino acid sequence analysis*

The fractions of human tissue lysates containing Zot binding proteins were resolved by 50 g·L<sup>-1</sup>

150 g·L<sup>-1</sup> gradient SDS-PAGE and transferred onto PVDF membranes using CAPS buffer [10mmol·L<sup>-1</sup> 3-(cyclohexylamino)-1 propanesulfonic acid and 100 mL·L<sup>-1</sup> methanol]. The protein bands were excised and subjected to N-terminal sequencing using a Perkin-Elmer Applied Biosystems Apparatus Model 494.

#### Microtubule binding assay

Spin-Down assay kit (Cytoskeleton) was used in the experiments according to the *manufacturer's* recommendations. MT (20 nmol·L<sup>-1</sup> final concentration) were obtained by mixing an aliquate of tubulin (20 µL, 5 g/L) and 2 µL, 200 mmol·L<sup>-1</sup> taxol in G-PEM buffer (80mmol·L<sup>-1</sup> Pipes pH 6.8, 1mmol·L<sup>-1</sup> MgCl<sub>2</sub>, 1mmol·L<sup>-1</sup> EGTA and 1mmol·L<sup>-1</sup> GTP). One µg of Zot was incubated with 20 nmol·L<sup>-1</sup> microtubule at room temperature for 20 min, while MT-associated protein MAP2 and bovine serum albumin (BSA) were used as positive and negative control, respectively. Proteins attached to MT and unbound proteins were separated by centrifugation. Each reaction product was carefully placed on the top of a cushion (G-PEM buffer plus 400 g·L<sup>-1</sup> glycerol) in Ultraclear TM ultracentrifuge tube. Following centrifugation, supernants and pellets were carefully removed and supernants were mixed with 1/20<sup>th</sup> volum of 500 g·L<sup>-1</sup> TCA solution for precipitation protein using centrifuge. Both supernants and pellets of the preparations containing the tested proteins were analyzed by either SDS-PAGE (MAP2 and BSA) or western blotting (Zot).

## RESULTS

#### Isolation of Zot binding proteins from human brain

His-Zot was successfully immobilized to AminoLink Plus gel with immobilization yields of 89%-95%, as established by the protein assay (Bio-Rad detergent-compatible protein assay). Plasma membrane preparations from human brain loaded on the Zot affinity column contained two major Zot-binding proteins with apparent molecular masses of approximately 45kU and 55kU, respectively (Figure 1, lane 2).

#### N-terminal sequencing of the Zot binding proteins from human brain

The N-terminal sequences of the two Zot/zonulin binding proteins are shown in Table 1. The two proteins were also compared to other protein sequences by Blast search analysis. The N-terminal sequence of the 55kU protein was 100% identical to the N-terminal sequence of tubulin (Table 1) whereas the -45kU protein band resulted 72% identical to the N-terminus of calprotectin, a

calcium binding protein associated to chronic inflammatory processes<sup>[14]</sup> and the cystic fibrosis antigen (CFA)<sup>[15]</sup>. This second protein resulted to be the Zot/zonulin brain receptor<sup>[16]</sup>.

#### Western immunoblotting experiments

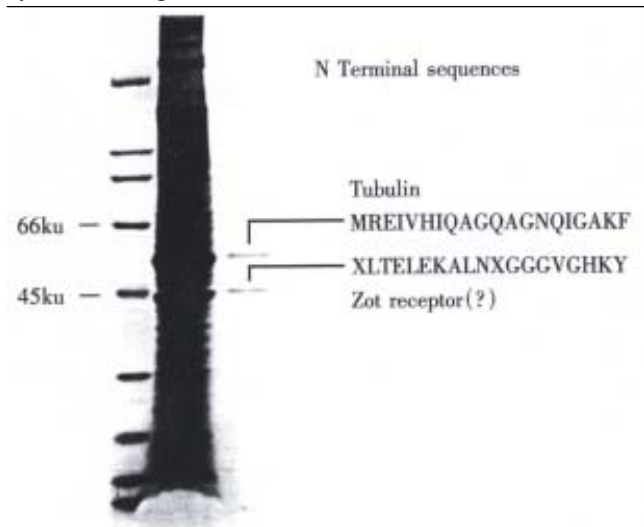
To investigate whether Zot and tau (a well characterized MAP) are immunologically related, cross immunoscreening experiments were performed. As shown in Figure 2, both proteins were recognized by either anti-Zot antibodies (left panel) or anti-tau antibodies (right panel). These results suggest that Zot and tau are immunologically related.

#### Microtubule binding assay

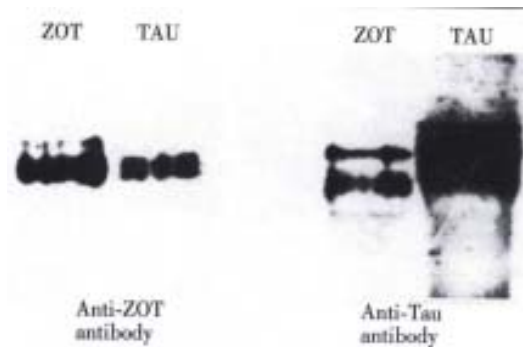
To confirm that Zot possesses MT binding properties, an *in vitro* binding assay was performed. As shown in Figure 3, we observed that Zot co-precipitated with MT as shown by Western immunoblotting analysis, while no Zot was found unbound. BSA lacking MT-binding properties was present only in the supernant. MAP2, a well defined MT associated protein, co-precipitated with MT and was not present in the supernant. These results confirmed the Zot ability to bind to MT.

**Table 1** N-terminal amino acid sequences of Zot binding protein (55kU),  $\beta$ -tubulin, Zot binding protein (45kU), calprotectin (MRP-8), and cystic fibrosis antigen

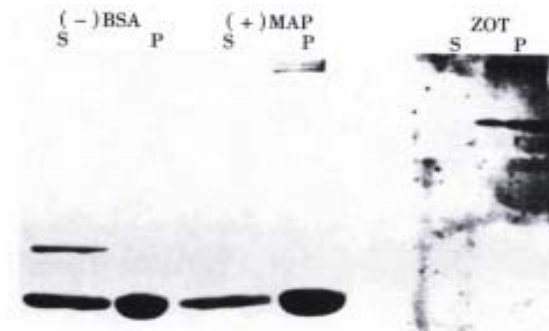
Sample	N-terminus	Identity(%)
Zot binding protein-55kU	MREIVHIQAGQAGNQIGAKF	100
$\beta$ -tubulin	MREIVHIQAGQAGNQIGAKF	
Zot binding protein-45kU	LTELEKALNXGGGVGHKY	77
Calprotectin (MRP-8)	LTELEKALNSIIDVYHKY	
Cystic fibrosis antigen	LTELEKALNSIIDVYHKY	77



**Figure 1** SDS-PAGE of Zot binding proteins isolated by affinity column chromatography from human brain cortex plasma membrane preparations. Lane 1, molecular mass standards; Lane 2, whole-plasma membrane lysate; lane 3, eluate with 0.5 mol·L<sup>-1</sup> NaCl in PBS containing 1 g·L<sup>-1</sup> Triton X-100.



**Figure 2** Western immunoblotting of Zot and tau using either anti-Zot antibodies (left panel) or anti-tau antibodies (right panel). Zot and tau were recognized by both antibodies.



**Figure 3** Microtubule binding assay. BSA (negative control) and MAP2 (positive control) were visualized by Coomassie staining (left panel) while Zot was visualized by immunoblotting (right panel). The bottom protein bands in the left panel are tubulins. As anticipated, BSA remained in the supernatant (S), while MAP2 was entirely precipitated in the pellet (P). Zot also appeared confined in the pellet, confirming that it bound to MT.

## DISCUSSION

Tj is the hallmark of absorptive and secretory epithelia. As a barrier between apical and basolateral compartments, the tj selectively controls the paracellular passage of water, solutes and immune cells between epithelial and endothelial cells. Variations in transepithelial conductance can usually be attributed to changes in the permeability of the paracellular pathway, since the resistances of eukaryotic cells plasma membrane are relatively high<sup>[17]</sup>. Tj represent the major barrier in this paracellular pathway and the electrical resistance of epithelial and endothelial tissues seems to depend on the number of transmembrane protein strands and their complexity as observed by freeze-fracture electron microscopy<sup>[18]</sup>. It has become abundantly clear that, in the presence of  $\text{Ca}^{2+}$ , assembly of the tj is the result of cellular interactions that trigger a complex cascade of biochemical events that ultimately lead to the formation and modulation of an organized network of tj elements, the composition of which has been only partially characterized<sup>[19]</sup>.

Identification and characterization of Zot, a toxin produced by *Vibrio cholerae*, has provided

new information on the regulation of intercellular tj<sup>[5,7,20-22]</sup>. After binding to its surface receptor, Zot is internalized<sup>[23]</sup>, and subsequently triggers a series of intracellular events including phospholipase C and PKC $\alpha$ -dependent actin polymerization which leads to the opening of tj<sup>[7]</sup>. However, the complete cascade of the intracellular events activated by Zot, particularly concerning the early steps, remains undefined. There is now a large body of evidence that protein phosphorylation plays a major role in tj development<sup>[24]</sup> and cytoskeleton rearrangement<sup>[25]</sup>. In eukaryotic cells, junctional complex proteins, actin filaments, microtubules, and intermediate filaments interact to form the cytoskeleton network involved in determination of cell architecture, intracellular transport, modulation of surface receptors, paracellular permeability, mitosis, cell motility, and differentiation<sup>[26]</sup>. We have previously demonstrated that there are two Zot binding proteins in the cell lysates of Zot-sensitive tissues<sup>[16]</sup>. One has been characterized as the Zot/zonulin receptor. With this paper we showed that tubulin is the second Zot-binding protein. Based on these results, it is possible to hypothesize that Zot affects the actin filament network by binding to MT. The association of Zot to MT could be responsible of the effects of Zot on cell uptake and intracellular trafficking of molecules<sup>[10,11]</sup> as well as the changes of tj structure and permeability. Alterations of intestinal tj occur in a variety of clinical conditions affecting the gastrointestinal system, including food allergies, malabsorption syndromes, and inflammatory bowel diseases. The knowledge that can eventually be acquired by studying the regulation of tj may have a tremendous impact on our understanding of the pathogenesis of these disease. It would not be surprising if the modification of tj structure and function by these pathological conditions would be an extension of normal physiologic regulation of tj.

However, several questions remain unanswered: what is the role of Zot-MT interaction on rearrangement of actin filament? Does this interaction affect the permeability of tj? Are MT-dependent cell functions, such as redistribution of organelles and the polarized distribution of membrane proteins, influenced by the MT-Zot binding? Experiments aimed at addressing these questions are presently in progress in our laboratory.

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