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Zonula Occludens Toxin Structure-Function Analysis

IDENTIFICATION OF THE FRAGMENT BIOLOGICALLY ACTIVE ON TIGHT JUNCTIONS AND OF THE ZONULIN RECEPTOR BINDING DOMAIN*

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Zonula occludens toxin (Zot) is an enterotoxin elaborated by *Vibrio cholerae* that increases intestinal permeability by interacting with a mammalian cell receptor with subsequent activation of intracellular signaling leading to the disassembly of the intercellular tight junctions. Zot localizes in the bacterial outer membrane of *V. cholerae* with subsequent cleavage and secretion of a carboxyl-terminal fragment in the host intestinal milieu. To identify the Zot domain(s) directly involved in the protein permeating effect, several *zot* gene deletion mutants were constructed and tested for their biological activity in the Ussing chamber assay and their ability to bind to the target receptor on intestinal epithelial cell cultures. The Zot biologically active domain was localized toward the carboxyl terminus of the protein and coincided with the predicted cleavage product generated by *V. cholerae*. This domain shared a putative receptor-binding motif with zonulin, the Zot mammalian analogue involved in tight junction modulation. Amino acid comparison between the Zot active fragment and zonulin, combined with site-directed mutagenesis experiments, confirmed the presence of an octapeptide receptor-binding domain toward the amino terminus of the processed Zot.

Vibrio cholerae produces a variety of extracellular products including zonula occludens toxin (Zot)[1] (1). The *zot* gene, along with other genes encoding virulence factors such as *ctxA*, *ctxB* (2, 3), and *ace* (4), is part of the chromosomally integrated genome of a filamentous phage designated CTXΦ (5–10). The *zot* product seems to be involved in the CTXΦ morphogenesis because Zot mutagenesis studies demonstrated the inability of CTX elements to be self-transmissible under appropriate conditions (5). The high concurrence among *V. cholerae* strains of the *zot* gene and the *ctx* genes (11, 12) also suggests a possible synergistic role of Zot in the causation of acute dehydrating diarrhea typical of cholera. The recently completed genomic sequence of *V. choleare* El Tor N16961 revealed that the CTXΦ filamentous phage is integrated in one of the two circular chromosomes of the bacterium (13).

Beside its role in phage morphogenesis, Zot also increases the permeability of the small intestine by affecting the structure of the intercellular tight junctions (tj) (1). This effect was initially described on rabbit ileal tissues mounted in Ussing chambers by using filtered supernatants from *V. cholerae O1* strains, suggesting that Zot is secreted (1, 14). Zot also possesses a cell specificity related to the toxin interaction with a specific receptor whose surface expression differs on various cells (15–17). Zot induces modifications of cytoskeletal organization that lead to the opening of tj secondary to the transmembrane phospholipase C and subsequent protein kinase Cα-dependent polymerization of actin filaments strategically localized to regulate the paracellular pathway (15). Furthermore, *in vivo* experiments suggested that the effect of Zot on tj might lead to intestinal secretion after the permeation of the intercellular space (16). This modulation is reversible, time- and dose-dependent, and confined to the small intestine because Zot does not affect colon permeability (1, 16). Furthermore, the number of Zot receptors seems to decrease along the intestinal villous axis (16).

To clarify the Zot bifunctional activity, we analyzed the structure-function properties of the toxin by constructing a series of deletion mutants that were tested for their ability to both modulate tj and bind to the Zot/zonulin receptor (18). The results provide evidence that the active domain responsible for Zot enterotoxic activity resides toward the carboxyl-terminal region of the toxin.

EXPERIMENTAL PROCEDURES

Construction of His-Zot In-frame Deletion Mutants—In-frame deletion derivatives of the *zot* gene were obtained by using polymerase chain reaction techniques (Table I). To develop deletions of the Zot carboxyl or amino terminus, several oligonucleotides were designed to amplify various portions of the 5′ and 3′ ends of the *zot* gene (Table II). The *zot* gene was cloned in plasmid vector pQE30 (Qiagen, Inc., Valencia, CA), which provides high-level expression in *Escherichia coli* of proteins containing a 6-histidine (6xHis) affinity tag at their amino terminus. The His tag allows a one-step method for protein purification using the Nickel-nitrilotriacetic acid resin capture column. To obtain Zot internal in-frame deletions (ΔE and ΔC clones), pSU113 (19) was subjected to enzymatic digestion (*Stu*I and *Hin*dIII). The right end of Zot was replaced with polymerase chain reaction products obtained by using F7/F2 and F11/F2 primers pairs, respectively, that were subsequently re-ligated. Oligonucleotides were designed to introduce restriction sites needed for cloning procedures (Table II). Amplification prod-

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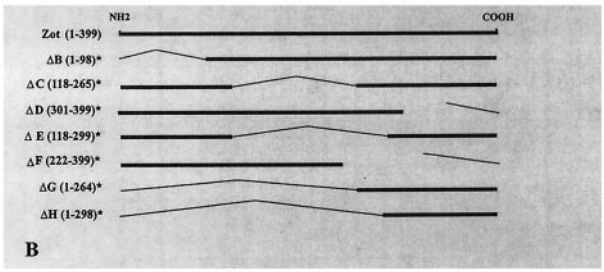
[1] The abbreviations used are: Zot, zonula occludens toxin; tj, tight junction(s); PBS, phosphate-buffered saline; Rt, tissue resistance; AA, amino acid.

TABLE I
Bacterial strains and plasmid vectors (top) and schematic description of Zot deletion mutants (bottom)

A

Clones	Amino acid residues deleted or mutated	Plasmid	Oligos	Reference or source
His-Zot	-	p SU113	F28-F2	(19)
His-ΔB	1-98	PSU134	F71-F46	This study
His-ΔC	118-265	PSU133	F141-F2	This study
His-ΔD	301-399	PSU132	F141-F8	This study
His-ΔE	118-299	PSU131	F141-F46	This study
His-ΔF	222-399	PSU130	F141-F92	This study
His-ΔG	1-264	PSU129	S2-F2	This study
His-ΔH	1-298	PSU135	F205-F2	This study
His-ΔG291	GGT→GTT G (291) →V	PSU129*	F183-F184	This study
His-ΔG298	GGT→GTT G (298) →V	PSU129**	F186-F187	This study



*Amino acids deleted in parenthesis

TABLE II
Primer sequences

Primers *	*Sequence (5'→3')* *	*Reference or source*
F2	CCCAAGCTTGGG*TCAAAATATACT*	(19)
F28	CGGGATCCCGT*ATGAGTATCTTT*	(19)
S2	CGGGATCC*GAGCCTCAGTC*	This study
F71	GCGGATCC*ACGGCCACCAATTTAAAGGCG*	This study
F46	GCGGGTACC*TCAAAATATACTATTTAGTCC*	This study
F8	CCCAAGCTTGGG*TCAGACAAAACCATC*	This study
F141	CGGGATCCGGA*ATGAGTATCTTT*	This study
F92	GCGGTACC*TCACAGCGCCGTTCCGGCCGTGTC*	This study
F7	*TCAAAGGCTACTGCTGGG*	This study
F11	*CCTGTTGGTGATGAGCGT*	This study
F205	CGGGATCC*TTTGTCACTGTTGGTG*	This study
F183	*CTTTTGGTTTTTGTATTGTTCGGCTTTGTGTCCAAG*	This study
F184	*CTTGGACACAAAGCCGAACAATACAAAAACCAAAAG*	This study
F186	*CACCAACAGTGACAAAAACATCTTGGACACAAAGCC*	This study
F187	*GGCTTTGTGTCCAAGATGTTTTTGTCACTGTTGGTG*	This study

* Sequences with restriction sites (underlined) of F2, F8 (*Hin*dIII), S2, F71, F28, F205, F141 (*Bam*HI), F46, F92 (*Kpn*I). Sequences corresponding to zot sequences are in *italics*.

ucts were analyzed by agarose gel electrophoresis and purified from salts and free nucleotides (Qiaquick polymerase chain reaction purification kit; Qiagen, Inc.). The fidelity of polymerase chain reaction amplifications was confirmed by DNA sequencing of the plasmid inserts (ABI PRISM 373; Applied Biosystems, Foster City, CA).

Site-directed Mutagenesis—The QuickChange™ Site-directed Mutagenesis Kit (Stratagene, Kingsport, TN) was used to develop point mutations in pSU129 hosted in the DH5α/His-ΔG strain. These mutations resulted in the substitution of either the glycine (G) in position 291 with a valine (V) (DH5α/His-ΔG291) or the glycine in position 298 with a valine (DH5α/His-ΔG298). The oligonucleotides used to obtain the two site-directed derivatives are listed in Table II.

Purification of His-Zot and Its Deletion Mutants—The *zot* gene, its seven deletion mutants, and its point mutated constructs were each inserted into the pQE30 vector to add a 6xHis tag on the amino terminus of each protein. *E. coli* DH5α was then transformed with the plasmids listed in Table I. The clones obtained were grown in Luria Bertani LB medium with 20 g/liter glucose, 25 mg/liter kanamycin, and 200 mg/liter ampicillin at 37 °C with vigorous mixing until A_{600} reached 0.7–0.9. Cultures were then induced with 2 mM isopropyl-1-thio-β-D-galactopyranoside (Fisher), followed by an additional 2-h culture incubation 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 M guanidine-HCl, 0.1 M sodium phosphate, and 0.01 M Tris-HCl, pH 8.0; 5 ml/g wet 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, Inc.; 1 ml/g wet weight) was added to the supernatant and stirred for 1 h at room temperature. The mixture was loaded onto a 5 × 1.5-cm nickel-nitrilotriacetic acid resin column and washed sequentially with buffer A and buffer B (8 M urea, 0.1 M sodium phosphate, and 0.01 M Tris-HCl, pH 8). Each wash step was continued until the A_{280} of the flow-through was less than 0.01. The proteins that bound to the column were eluted by the addition of 250 mM imidazole in buffer C (8 M urea, 0.1 M sodium phosphate, and 0.01 M Tris-HCl, pH 6.3), stirred with a 50% slurry of Superflow (1 ml/g wet weight) for 2 h at room temperature, loaded onto another 5 × 1.5-cm nickel-nitrilotriacetic acid column, washed with phosphate-buffered saline (PBS), and eluted with 250 mM imidazole in PBS. Purity of the His-proteins was established by SDS-polyacrylamide gel electrophoresis analysis followed by Coomassie Blue staining and Western immunoblotting using polyclonal anti-Zot antibodies (19).

In Vitro Ussing Chambers Experiments—Adult male New Zealand White rabbits (2–3 kg) were sacrificed by cervical dislocation. Segments of rabbit small intestine were removed, rinsed free of the intestinal content, opened along the mesenteric border, and stripped of muscular and serosal layers. Eight sheets of mucosa thus prepared were then mounted in Lucite Ussing Chambers (1.12 cm^2 opening) connected to a voltage clamp apparatus (EVC 4000; World Precision Instruments, Sarasota, FL) and bathed with freshly prepared buffer containing 53 mM NaCl, 5 mM KCl, 30.5 mM Na_2SO_4, 30.5 mM mannitol, 1.69 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1.1 mM $MgCl_2$, and 25 mM $NaHCO_3$. The bathing solution was maintained at 37 °C with water jacketed reservoirs connected to a constant temperature circulating pump and gassed with 95% O_2/5% CO_2. Potential difference (PD) was measured, and short-circuit current (I_{SC}) and tissue resistance (Rt) were calculated as described previously (1). Purified Zot (1×10^{-10} M) and the seven purified Zot deletion mutant proteins (1×10^{-10} M) were added to the mucosal side of each chamber. Intestinal tissues exposed to PBS were used as a negative control. Only tissues that, at the end of the experiment, showed an increase in I_{SC} in response to the mucosal addition of glucose (confirming tissue viability) were included in the analysis. In selected experiments, tissues were exposed to the point mutation proteins (1×10^{-10} M) derived from the ΔG clone (Table I), and the assay was conducted as described above.

Cell Cultures—IEC6 cells derived from crypt cells of germ-free rat small intestine (20) were grown in cell culture flasks (Corning Costar Co., Cambridge, MA) at 37 °C in an atmosphere of 95% air and 5% CO_2. The complete medium consisted of Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, containing 5% irradiated fetal bovine serum, 10 μg/ml insulin, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells passage number varied between 15 and 20.

Binding Experiments—IEC6 cells were grown in 8-chamber slides (1×10^5 cells/chamber). The monolayers were exposed to either 1×10^{-10} M Zot or an equimolar amount of each of its deletion mutants and incubated for 30 min at 4 °C. IEC6 cells exposed to PBS were used as a negative control. After washing with PBS (pH 7.4), the cells were fixed in 4% formaldehyde for 10 min at room temperature and then permeabilized with 0.5% Triton X-100 (Sigma) in phosphate buffer (pH 7.4) for 10 min at room temperature. The cells were then washed with PBS and blocked with 0.1% bovine serum albumin for 45 min at room temperature. Primary rabbit polyclonal anti-Zot antibodies (1:500) (19) were then added, and the monolayers were incubated overnight at 4 °C. After washing with PBS, the cells were incubated for 30 min at room temperature with anti-rabbit IgG-fluorescein isothiocyanate-conjugated antibodies (Sigma) (1:100). Finally, the cells were washed with PBS and stained in red using Evans Blue Counterstain (Sigma) in phosphate buffer (1:1000) for 10 min at room temperature. The staining procedure

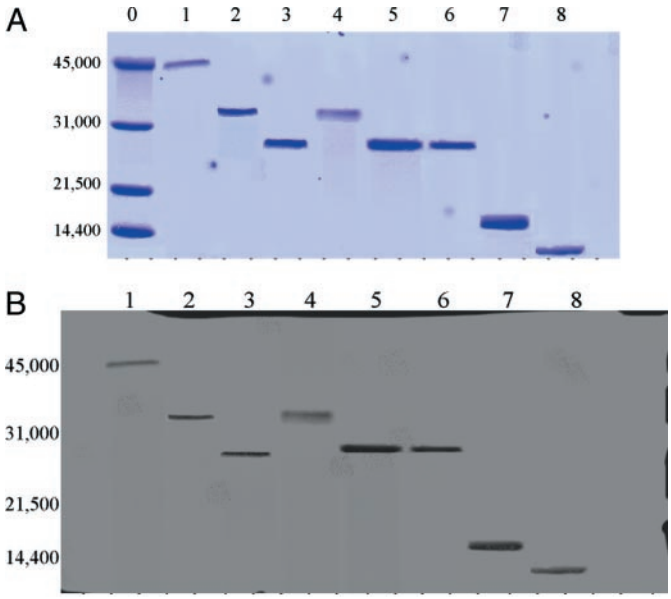


FIG. 1. **SDS-polyacrylamide gel electrophoresis (*A*) and Western immunoblotting (*B*) of Zot and its in-frame deletion mutants.** *Lane 0,* molecular mass standards; *lane 1,* His-Zot; *lane 2,* His-ΔB (1–98); *lane 3,* His-ΔC (118–265); *lane 4,* His-ΔD (301–399); *lane 5,* His-ΔE (118–299); *lane 6,* His-ΔF (222–399); *lane 7,* His-ΔG (1–264); *lane 8,* His-ΔH (1–298) (deleted amino acid residues are shown in parentheses). Conditions were as described under "Experimental Procedures."

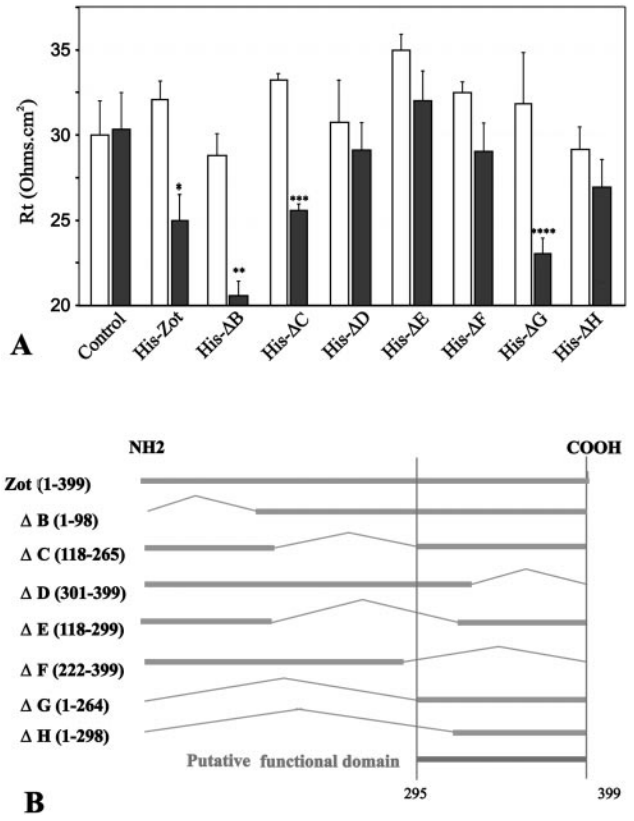


FIG. 2. *A,* effect of Zot and its deletion mutant derivatives on rabbit ileal Rt in Ussing chambers. Tissues were exposed to either 1×10^{-10} M of each protein or to a negative control (PBS), and variation in Rt between the baseline (□) and 90 min postincubation (■) was monitored. All values were expressed as the means ± S.E. *, p between 0.03 and 0.0003 compared with either control, ΔD, ΔE, ΔF, or ΔH. **, $p < 0.01$ compared with control. ***, $p < 0.000006$ compared with control. ****, $p < 0.0001$ compared with control. *B,* schematic description of Zot deletion mutants. The deletion for each protein is shown as a *broken filled line*. The putative Zot functional domain responsible for tj disassembly (delimited by the *two vertical lines*) maps in the toxin's carboxyl-terminal region (AA 295–399). Deleted amino acid residues are shown in *parentheses*.

was used to better visualize the binding particles (stained in green by the IgG-fluorescein isothiocyanate secondary antibodies) that appeared on a red background as fine yellow granules. The wells were washed with PBS, the coverslips were mounted with glycerol-PBS (1:1), pH 8, and then the cells were blindly analyzed by two independent observers with a fluorescence microscope (Optiplot; Nikon Inc., Melville, NY). In selected experiments, the IEC6 cells were incubated with the point mutation proteins (1×10^{-10} M) derived from the ΔG clone listed in Table I, and the binding assay was conducted as described above.

Statistical Analysis—All values are the means ± S.E. The analysis of differences was performed by t test for either paired or unpaired varieties. $p < 0.05$ was considered statistically significant.

RESULTS

Construction of Zot Deletion Mutants—To identify the Zot region(s) involved in both the toxin's permeating effect and its engagement to the target receptor, seven deletion mutants were generated. Each mutant showed the predicted M_r as established by both SDS-polyacrylamide gel electrophoresis analysis (Fig. 1*A*) and Western immunoblotting (Fig. 1*B*). Gene sequencing of these constructs confirmed their correct design (data not shown).

Biological Activity of the Zot Mutants in the ex Vivo Ussing Chamber Assay—To identify the Zot domain(s) responsible for the enterotoxic activity, equimolar amounts of bacterial-expressed, purified His-Zot and its deletion mutants were each tested on rabbit small intestine mounted in Ussing chambers. The ability of the seven Zot mutants to affect the tj competency was analyzed by measuring the changes in Rt induced by a 90-min incubation with each deleted construct as compared with the effect obtained with wild-type His-Zot (positive control) and PBS (negative control). As shown in Fig. 2*A,* Rt reduction induced by His-ΔB and His-ΔC proteins was almost indistinguishable from that induced by His-Zot and was significantly different compared with the negative control. A significant Rt drop was also observed when tissues were exposed to His-ΔG, whereas no significant changes were detected after tissue incubation with His-ΔD, His-ΔE, His-ΔF, and His-ΔH (Fig. 2*A*). These results suggest that the domain responsible for the Zot permeating effect on tj is located toward the protein's carboxyl-terminal section (Fig. 2*B*).

Binding Activity of Zot Deletion Mutants on IEC6 Cells—To identify the Zot domain(s) that specifically binds to the Zot/zonulin surface cell receptor, IEC6 cell monolayers were exposed to the same purified proteins tested in Ussing chambers. The ability of each deleted Zot derivative to bind to IEC6 cells (Fig. 3, *B–H*) was evaluated using fluorescence microscopy and compared with the binding observed with both wild-type His-Zot (Fig. 3*A*) and the PBS negative control (Fig. 3*I*). Cells incubated with His-ΔG (Fig. 3*G*) showed an amount of binding particles similar to that seen in cells incubated with His-Zot (Fig. 3*A*). The number of fluorescent particles was only minimally decreased in cells incubated with His-ΔB (Fig. 3*B*), His-ΔC (Fig. 3*C*), or His-ΔD (Fig. 3*D*). In contrast, binding capability was completely lost by His-ΔE (Fig. 3*E*), His-ΔF (Fig. 3*F*), and His-ΔH (Fig. 3*H*) mutants. Analysis of these results revealed that the Zot region corresponding to AA residues 265–301 was partially (His-ΔE and His-ΔH) or completely (His-ΔF) deleted in the binding-negative mutants, whereas it was spared in the binding-positive constructs (Fig. 3*J*), suggesting that this region may represent the putative Zot domain involved in receptor binding function.

Characterization of the Zot Binding Domain and the Structure Requirements to Engage to the Zonulin Receptor—We have recently demonstrated that Zot localizes in the *V. cholerae*

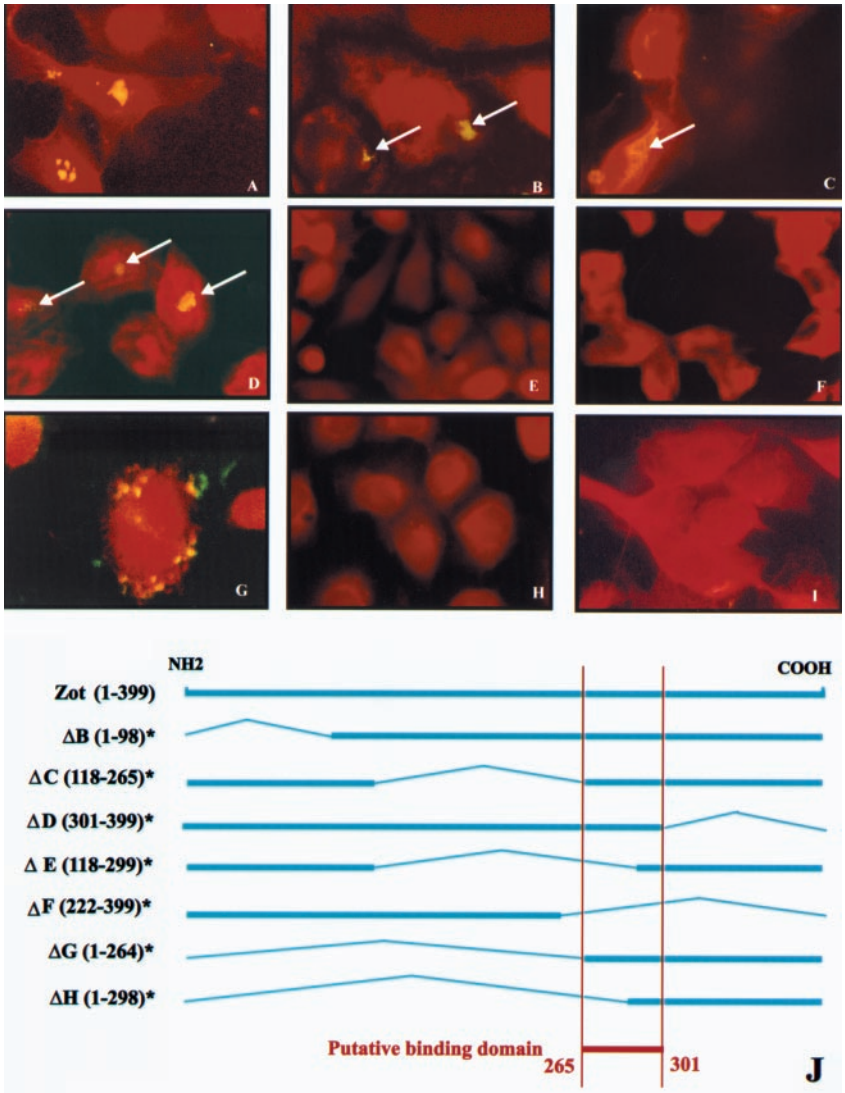


FIG. 3. **Fluorescence microscopy of IEC6 cells.** Cells visualized in *red* by Evans blue counterstain were exposed to either His-Zot (1×10^{-10} M; *A*) or an equimolar amount of Zot deletion mutants (*B–H*) and incubated for 30 min at 4 °C. PBS was used as a negative control (*I*). Green fluorescent binding particles appear as *yellow clusters* on a *red background*. Binding particles were present in cells exposed to His-Zot (*A*) and ΔG (*G*) and, to a lesser extent, in cells exposed to ΔB (*B*), ΔC (*C*), and ΔD (*D*) (see *arrows*). No binding activity was detected in cells incubated with ΔE (*E*), ΔF (*F*), or ΔH (*H*). *J,* identification of the putative binding domain among the seven Zot deletion mutants. The deletion for each protein is shown as a *broken filled line*. The binding of each mutant was compared with His-Zot binding (*A*) and to the PBS negative control (*I*). Comparative binding analysis of the seven deletion mutants tested led to the identification of a region corresponding to Zot amino acid residues 265–301 (delimited by the *two vertical lines*) necessary to display fluorescent signals. *, deleted amino acid residues are shown in *parentheses*.

TABLE III
Comparison of the amino-terminal sequences of human intestinal zonulin and Zot

The shaded amino acids (Zot AA residues 291–298, zonulin AA residues 8–15) represent the putative Zot/zonulins receptor-binding site characterized by the following shared motif: non-polar (G), variable, non-polar, variable, non-polar (V), polar (Q), variable, non-polar (G).

Sample	Source	AA residue*		
		290	295	300
Zot	*V. cholerae*	F C I	G R L C V Q D G	F V T
		5	10 15	20
zonulin	human intestine	M L Q K A E S	G G V L V Q P G	X S N R L
FZI/0	Synthetic peptide		G G V L V Q P G	

outer membrane, to which it is anchored through its single spanning domain (19). The molecule then undergoes to a cleavage that leads to the formation of a ~33-kDa amino-terminal fragment that remains associated to the microorganism and a ≥12-kDa carboxyl-terminal peptide that supposedly is secreted in the host intestinal lumen milieu (19). Based on the Ussing chamber assay results and the binding experiments of the Zot deletion mutants reported above, it is conceivable to hypothesize that this secreted fragment engages to the zonulin receptor and, consequently, causes tj disassembly. Comparison of the amino termini of the secreted Zot fragment (AA residues 288–399) and its eukaryotic analogue zonulin (21) revealed an 8-amino acid shared motif (Table III, shaded area) that encompasses the 265–301-AA region identified by the binding experiments described above as the putative binding domain.

Both zonulin and Zot domains revealed a motif in which 4 of the 8 amino acid residues were identical (G*XXX*VQ*X*G). To confirm that this motif is involved in target receptor engagement, a synthetic octapeptide (GGVLVQPG containing the shared motif) named FZI/0 (see Table III) was engineered and tested on ileal tissue mounted in Ussing chambers either alone or in combination with Zot or zonulin. No changes in Rt in tissues exposed to either FZI/0 or to a scrambled peptide were observed (Fig. 4). Treatment of the ileal tissue preparations with FZI/0 before and throughout the study period prevented Rt changes in response to both Zot and zonulin, whereas the permeating effect of the two proteins was unaffected by pretreatment with the scrambled peptide (Fig. 4). These data suggest that Zot and zonulin bind to the same receptor through a common binding motif (shaded in Table III) localized at the amino termini of both molecules. To establish the structure requirements to engage the target zonulin receptor, two His-ΔG site-directed mutants within the putative binding motif were engineered and tested for both their binding capability on IEC6 cells and their biological activity in Ussing chambers. IEC6 cells incubated with His-ΔG 291 (in which the G in position 291 was substituted with V) showed a reduced number of binding particles (Fig. 5*A, 3*) as compared with cells incu-

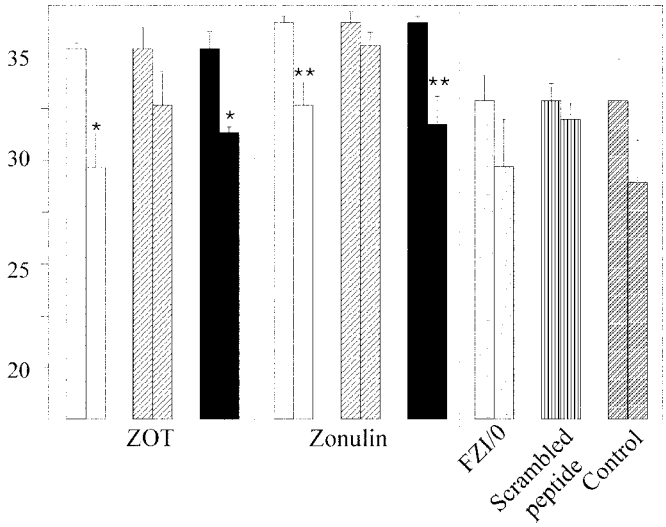
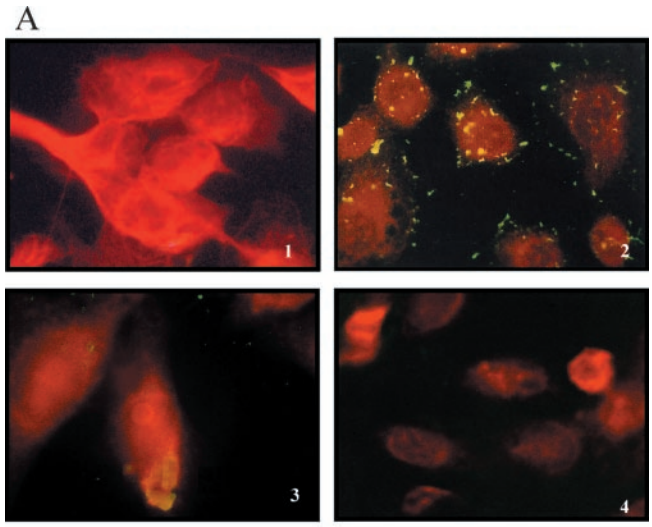


FIG. 4. **Structural requirements for receptor binding and permeabilizing activity.** To establish whether the Zot/zonulin domain shaded in Table III represents the receptor binding motif involved in the permeabilizing activity of both proteins, the ability of either the synthetic peptide FZI/0 (GGVLVQPG) mimicking the putative binding domain or of a scrambled peptide (VGVLGRPV) to block both Zot and zonulin-induced reduction of ileal Rt was tested. *Left bars* represent baseline Rt values, whereas *right bars* are Rt values after a 90-min incubation. 0.1 μg/ml either Zot or zonulin (□) each induced a significant Rt decrement compared with the negative control, whereas 1 μg/ml either FZI/0 or the scrambled peptide did not. Pretreatment with 1 μg/ml FZI/0 for 20 min before and throughout the exposition to Zot and zonulin (▨) prevented the Rt reduction, whereas pretreatment with the scrambled peptide (■) did not. $n = 3–5$; *, $p < 0.008$ compared with Zot alone. **, $p < 0.005$ compared with zonulin alone.



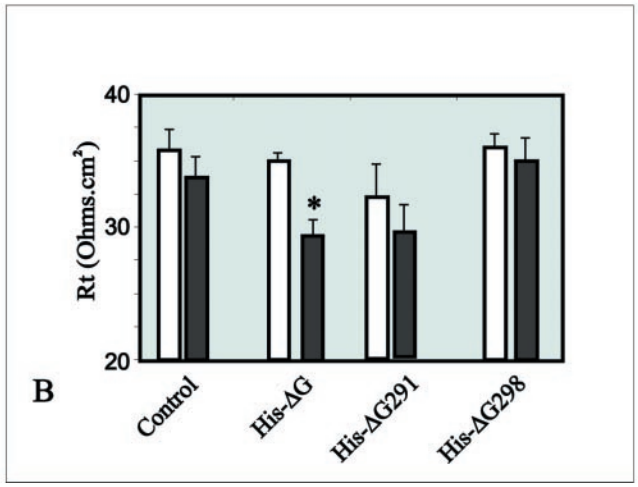


FIG. 5. **Effect of the two His-ΔG site-directed mutants (His-ΔG291 and His-ΔG298) on receptor binding and tj disassembly.** *A,* binding assay. Experiments were performed as described in the Fig. 3 legend. IEC6 cell monolayers incubated with His-ΔG291 showed binding particles (*3*) similar to those detected in His-ΔG-exposed monolayers (*2*), whereas no binding was detected in monolayers exposed to His-ΔG298 (*4*). Negative PBS control (*1*) is shown for comparison. *B,* Ussing chamber assay. The tissue was treated with 1×10^{-10} M of each protein, and Rt change was compared with that in PBS-exposed tissues. His-ΔG291 induced a partial but not a significant reduction in Rt when compared with the changes induced by His-ΔG. The permeating effect was completely ablated when the rabbit ileum was exposed to His-ΔG298. All values were expressed as the means ± S.E. *, $p < 0.009$ compared with His-ΔG298 and $p < 0.0001$ as compared with control.

bated with His-ΔG (Fig. 5*A, 2*), whereas no binding was observed on cells incubated with His-ΔG 298 (G298V) mutant (Fig. 5*A, 4*). Biological assays in Ussing chambers showed that His-ΔG291 had a residual but not significant effect on tj disassembly (Fig. 5*B*). The permeating effect was completely ablated when the intestinal tissues were incubated with His-ΔG 298 (Fig. 5*B*). These results paralleled the effects obtained with these two mutants in the binding assay and confirmed that the G in position 298 plays a crucial role in the capability of His-ΔG to bind to its target receptor, and, consequently, its substitution ameliorated the ligand biological effect on tj.

DISCUSSION

V. cholerae, the human intestinal pathogen responsible for the diarrheal disease cholera, elaborates a large number of extracellular proteins, including several virulence factors. A number of epidemiological studies (22, 23) 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*, Zot (1) and accessory cholera toxin (Ace) (4). This cluster of genes has been recently described as part of a filamentous phage (CTXΦ) that lives in symbiosis with its bacterial host. It has been reported previously that Zot is involved in both CTXΦ morphogenesis (5) and disassembly of the host intestinal intercellular tj (1, 14–16).

The study of the subcellular localization of Zot by affinity-purified anti-Zot antibodies (19) revealed that Zot localizes in the *V. cholerae* outer membrane with a molecular mass of ~45 kDa, which is consistent with the predicted primary translation product from the first methionine of Zot (44.8 kDa) (19). A second immunoreactive molecule, corresponding to the 33-kDa amino-terminal region of Zot, was also detected at the outer membrane site (19). Both molecules are exposed at the bacterial cell surface (19). The 33-kDa Zot processing product is generated by a cleavage site at AA residue 287 (19). This 33-kDa fragment remains associated to the bacterial membrane, whereas the carboxyl-terminal fragment of 12 kDa is excreted in the intestinal host milieu and is probably responsible for the biological effect of the toxin on intestinal tj.

This toxin processing would explain the apparently contrasting results obtained when Zot was originally described (1). Indeed, despite the predicted toxin molecular mass (44.8 kDa) (14), its biological effect on tj was found confined to the <30-kDa *V. cholerae* culture supernatant fraction (1). The importance of the carboxyl-terminal fragment of Zot for its action on tj is further supported by the observation that Tn*pho*A insertions located at the Zot carboxyl-terminal region abolished the enterotoxic activity (14).

Taken together, these data suggest that Zot has a dual function: whereas its ~33-kDa amino-terminal portion is possibly involved in CTXΦ phage assembly (5, 9, 10), the ~12-kDa

carboxyl-terminal fragment of the toxin seems to be responsible for the permeating action on intestinal tj.

Several microorganisms have been shown to exert a cytophatic, pathological effect on epithelial cells that involves the cytoskeletal structure and the tj function in an irreversible manner. These bacteria alter the intestinal permeability either directly (*i.e.* enteropathogenic *E. coli*) or through the elaboration of toxins (*i.e. Clostridium difficile* and *Bacteroides fragilis*) (24). A more physiological mechanism of regulation of tj permeability has been proposed for Zot (1). Zot activates a complex intracellular cascade of events that involve a dose- and time-dependent protein kinase Cα-related polymerization of actin filaments strategically localized to regulate the paracellular pathway (15). These changes are a prerequisite to the opening of tj and are evident at a toxin concentration as low as 1.1×10^{-13} M (16). The toxin exerts its effect by interacting with a specific surface receptor that is present on mature cells of small intestinal villi, but not in the colon (1, 16). The regional distribution of Zot receptor(s) coincides with the different permeating effect of the toxin on the various tracts of intestine tested (16). Our previous results showed that Zot regulates tj in a rapid, reversible, and reproducible fashion and probably activates intracellular signals operative during the physiologic modulation of the paracellular pathway (15, 16). Based on the complexity of the intracellular signaling activated by Zot leading to the tj disassembly (15), we postulated that Zot may mimic the effect of a functionally and immunologically related endogenous modulator of epithelial tj. The combination of affinity-purified anti-Zot antibodies and the Ussing chamber assay allowed us to identify an intestinal Zot analogue that we named zonulin (21). When zonulin was studied in a nonhuman primate model, it reversibly opened intestinal tj after engagement to the same receptor activated by Zot and therefore acts with the same effector mechanism described for the toxin (21). Structure analysis of the zonulin amino terminus and the Zot active fragment identified in this study revealed a shared motif between these two molecules (Table III). Our binding experiments with Zot deletion mutants demonstrated that this motif is crucial for the engagement of the toxin to its target receptor. The importance of this region was confirmed by the ablation of both Zot and zonulin binding and the biological effects on tj competency when intestinal tissues were pretreated with the synthetic octapeptide GGVLVQPG corresponding to the shared motif (Fig. 4). Site-directed mutagenesis revealed the key role of the glycine residue in position 298 (as referred to the Zot entire molecule) for Zot engagement to the target zonulin receptor and thus for the activation of intracellular signaling leading to the opening of intercellular tj.

The paracellular route is the dominant pathway through which passive solutes flux across both the endothelial and epithelial barriers, and its functional status is regulated, in part, at the level of intercellular tj (25). A century ago, the tj was conceptualized as secreted extracellular cement forming an absolute and inert barrier within the paracellular space (26). It is now understood that tj are complex and dynamic structures whose physiological regulation appears to be tightly orchestrated through mechanisms that remain largely undefined (27). Furthermore, tj readily adapt to a variety of developmental, physiological, and pathological circumstances (28, 29). Data exist that support the linkage between the actin cytoskeleton and the tj complex (30–32) and implicate signaling events that regulate paracellular permeability (27). The discovery of Zot shed some light on the intricate mechanisms that govern the permeability of tj and led to the discovery of zonulin, the natural ligand of the Zot target receptor. The partial characterization of this zonulin receptor revealed that it is a 45-kDa glycoprotein containing multiple sialic acid residues with structural similarities to myeloid-related protein, a member of the calcium-binding protein family possibly linked to cytoskeletal rearrangements (18).

With the studies presented here, we have untangled at the molecular level the interplay between the *V. cholerae* Zot toxin and the eukaryotic zonulin pathway used by the microorganism to induce tj disassembly. Our findings on the structural requirements to engage to the zonulin receptor binding pocket and, consequently, to activate the zonulin pathway open new research opportunities to gain more insight on a system possibly involved in developmental, physiological, and pathological processes, including tissue morphogenesis, movement of fluid, macromolecules, and leukocytes between body compartments, and malignant transformation and metastasis.

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