The Vibrio Cholera-generated Zonulin occludens Toxin (Zot) N-terminal Cleavage Site Contains a Protease-activated Receptor Activating Peptide (PAR-AP) That Retains Biological Activity on Intestinal Tight Junctions

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ABSTRACT

Background: We have previously demonstrated that Zonulin occludens toxin (Zot) elaborated by Vibrio Cholera anchors to the bacterial outer membrane through its single spanning domain and undergoes to a Vibrio-specific cleavage at amino acid residue 288. The resulting 12 Kda c-terminal fragment is then released in the intestinal micromilieu were exerts its permeating effect on intercellular tight junctions (tj). The N-terminus of th cleaved Zot fragment contains a conserved 6-mer protease activated receptor (PAR)-activating peptide (AP) motif.

Aim: We have previously reported that Zot/Zonulin receptor is similar to PAR2, we elected to establish whether the six-mer motif (called AT1002) retains the Zot biological activity on tj.

Methods: Rat small intestine was mounted in ussing chambers and AT1002-induced changes in transepithelial electrical resistance TEER) monitored. Rat epithelial cells (IEC6) were used to study the intracellular signaling, including Ca2+ release, changes in tj protein-protein interactions, and phosphorylation of tj proteins.

Results: Rat small intestine exposed to AT1002 showed a significant reduction in TEER as compared to the negative control starting at 30 minutes and reaching a plateau after 120 minutes. At a time point coincident with the effect con TEER (30 min) AT1002 induced an increment in ZO-1 phosphorylation that was associated to a decrease in ZO1-occludin interaction and an increase in ZO1-ZO2 interaction. Addition of AT1002 to IEC6 cells did not cause any increase in intracellular Ca2+, while the PAR-AP caused a dose-dependent increased in intracellular Ca2+ that reached a plateau at 50 fym.

Conclusions: The 6-mer synthetic peptide At1002 retained the Zot biological activity an intercellular tj and caused a decrease in TEER. This effect was not associated to Ca2+ release, however was related to changes in protein-protein interaction of tj elements following Z)1 phosphorylation.
We have previously demonstrated that Zonula occludens toxin (Zot) elaborated by *Vibrio cholerae* anchors to the bacterial outer membrane through its single spanning domain.
Background 2

**V. cholerae cleavage site**

1  msifihhgap gsyktsgalw lrlpaiksg rhiitnvrgl nlermakylk mdvsdisief
61  idtdhpdrgrl tmarfwhwar kdaflfidec griwpprlta tnlkaldtop dlvaedrpes
121  fevafdmhrh hgwdiicttp niakvhnmir eaaeigyrhf nratvglgak fttthdaan
181  sgqmdshalt rqvkkipsi fkmyastttg kardtmagta lwkdrkilfl fgmvflmfsy
241  sfyglhdnpi f tggndatie seq sepqska tagnavska vapasfg fci grl cvqdgvf
301  tvgderyrlv dnlidpyrpl watghhiykd kltvffetes gsvptelfas syrykvlplp
361  dfnhfvvfdd faaqalwvev krgrlpktek dkkglnsif

Following to this anchorage, Zot undergoes to a vibrio-specific cleavage at amino acid residue 288, with subsequent release of its C-terminal fragment in the intestinal micromilieau.
The N-terminus of the cleaved Zot fragment contains a conserved 6-mer protease activated receptor (PAR)-activating peptide (AP) motif. We have previously reported that zonulin, the Zot eukaryotic counterpart, is a protease that activates a receptor similar to PAR2.
Aims

1. To establish whether the six-mer motif (called AT1002) retains the Zot biological activity on tight junctions.
2. To verify whether AT1002 induces immediate intracellular Ca\(^{+2}\) increase as caused by the PAR-2 peptide activator SLIGRL.
3. To establish whether AT1002 signaling affect ZO1 phosphorylation and its interaction with partner proteins.
Methods

1. Transepithelial electrical resistance (TEER) was monitored either in presence or absence of AT1002 added to the mucosal aspect of rat small intestine mounted in Ussing chambers.

2. The effect of AT1002 on Ca\(^{+2}\) release was studied on the IEC6 rat epithelial cell line.

3. Phosphorylation of ZO-1 induced by AT1002 was analyzed by Western immunoblotting.

4. The effect of AT1002 on protein-protein interaction of ZO-1 with partner proteins was investigated by co-immunoprecipitation analysis.
AT1002 50 μM added to the mucosal aspect of rat small intestine induced a significant decrease in TEER. This effect was reversible upon AT1002 removal (data not shown).
**Results 2**

**Effect of AT1002 and SLIGRL on Ca2+ Signaling**

Figure 1

PAR-2 AP on IEC-6 Cells

Δ[Ca²⁺] (μM)

[SLIGRL-NH₂] (μM)

Dose-Response

$B_{max} = 0.66 ± 0.05$

$K = 20 ± 3$

$\chi^2 = 0.00033$

$R^2 = 0.98726$

SLIGRL induced a dose-dependent intracellular Ca release in IEC6 cells (Fig. 1 and top panel Fig. 2), while no Ca release was detected in either human intestinal Caco2 or T84 cell lines. AT1002 failed to induce Ca release in any of the cell lines tested.
Results 3

AT1002-induced ZO-1 serine phosphorylation: Dose-response curve

The total protein from IEC6 cell lines was subjected to ZO-1 immunoprecipitation after exposure to increasing AT1002 concentrations and then immunoblotted using anti-phosphoserine antibodies. The filter was stripped and re-probed with anti-ZO-1 antibodies to control for equal loading. The phosphoserine western blot shows increase in ZO-1 phosphorylation with increasing dose of AT1002.
AT1002 was used at a concentration of 10 μM. Immunoprecipitation was performed on IEC6 total protein using anti ZO-1 antibodies. The filter was stripped and re-probed with anti-ZO-1 antibodies to control for equal loading. The phosphoserine Western immunoblot shows an increase in phosphorylation of ZO-1 at 30 minutes compared to the control. No change in the phosphorylation was seen at 90 minutes time point.
IEC6 cells were treated with AT1002 10 \( \mu \)M for either 30 or 90 minutes. IEC6 cells were immunoprecipitated with anti-ZO-1 antibodies and immunoblotted with anti-ZO2 antibodies. The filter was stripped and re-probed with anti-ZO-1 antibodies to control for equal loading. The ZO-2 Western immunoblot shows an increased signal both at 30 and 90 minutes, suggesting an increase in ZO-1/ZO2 protein protein interaction following AT1002 exposure.
Conclusions

1. AT1002 caused the tight junctions disassembly as shown by decrease in TEER in rat tissues mounted in Ussing chambers.

2. Contrary to PAR-2 peptide activator SLIGRL, AT1002 did not induce early Ca$^{+2}$ signaling.

3. AT1002 induced ZO-1 phosphorylation that temporarily preceded tight junction disassembly.

4. AT1002 induced increase in ZO-1/ZO2 protein-protein interaction.
Working Model 1

Zonulin

PAR-like

AT1002

PAR-like

AT1002
References


