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Human zonulin, a potential modulator of intestinal tight junctions

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SUMMARY

Intercellular tight junctions are dynamic structures involved in vectorial transport of water and electrolytes across the intestinal epithelium. Zonula occludens toxin derived from *Vibrio cholerae* interacts with a specific intestinal epithelial surface receptor, with subsequent activation of a complex intracellular cascade of events that regulate tight junction permeability. We postulated that this toxin may mimic the effect of a functionally and immunologically related endogenous modulator of intestinal tight junctions. Affinity-purified anti-zonula occludens toxin antibodies and the Ussing chamber assay were used to screen for one or more mammalian zonula occludens toxin analogues in both fetal and adult human intestine. A novel protein, zonulin, was identified that induces tight junction disassembly in non-human primate intestinal epithelia mounted in Ussing chambers. Comparison of amino acids in the active zonula occludens toxin fragment and zonulin permitted the identification of the putative receptor binding domain within the N-terminal region of the two proteins. Zonulin likely plays a pivotal role in tight junction regulation during developmental, physiological, and pathological processes, including tissue morphogenesis, movement of fluid, macromolecules and leukocytes between the intestinal lumen and the interstitium, and inflammatory/autoimmune disorders.

Key words: Zonulin, Tight junction, Zot, Intestine, Human

INTRODUCTION

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 tight junctions (Anderson and Van Itallie, 1995). A century ago, the tight junction was conceptualized as secreted extracellular cement, forming an absolute and inert barrier within the paracellular space (Cereijido, 1992). It is now understood that tight junctions are complex and dynamic structures whose physiological regulation appears to be tightly orchestrated through mechanisms that remain largely undefined (Anderson and Van Itallie, 1995). Furthermore, tight junctions readily adapt to a variety of developmental (Merzdorf et al., 1998), physiological (Madara and Pappenheimer, 1987) and pathological (Milks et al., 1987) circumstances. Data exist that link specific signaling events (Duffey et al., 1981) and actin reorganization (Madara et al., 1986; Gumbiner et al., 1991; Fasano et al., 1995) to the regulation of the tight junction complex.

We have recently demonstrated that zonula occludens toxin (Zot), a protein elaborated by *Vibrio cholerae*, reversibly regulates tight junction permeability (Fasano et al., 1991; Baudry et al., 1992). Zot interacts with a specific surface receptor(s) (Fasano et al., 1997) with subsequent protein kinase C (PKC)α-dependent polymerization of actin microfilaments strategically localized to regulate the paracellular pathway (Fasano et al., 1995). Following this observation, we asked whether Zot might mimic an endogenous modulator(s) of tight junctions. We also postulated that Zot and its putative eukaryotic analogue could be structurally and immunologically related. Accordingly, specific anti-Zot antibodies and the Ussing chamber assay were used in combination to screen for one or more human intestinal Zot analogue(s). Non-primate intestinal tissues were used as an indicator system to identify and purify this analogue.

MATERIALS AND METHODS

Production of affinity-purified anti-Zot antibodies

Purified recombinant His-Zot (Lu et al., 2000), 10 μg in 0.5 ml of PBS mixed with an equal volume of Freund's complete adjuvant, was injected into a rabbit. Two booster doses were administered with Freund's incomplete adjuvant at 4 and 8 weeks. At 12 weeks, the rabbit was bled. The anti-Zot antibodies were then affinity-purified using an His-Zot affinity column prepared by immobilizing 1.0 mg of purified His-Zot (Lu et al., 2000) to a pre-activated gel (Aminolink, Pierce) overnight at room temperature. The column was washed with PBS, and then loaded with 2.0 ml of anti-Zot rabbit antiserum. After 90 minutes incubation at room temperature, the column was washed with 14 ml of PBS and the specific anti-Zot antibodies were eluted with 4.0 ml of a solution comprising 50 mM glycine (pH 2.5), 150 mM NaCl and 0.1% (v/v) Triton X-100. The pH of the eluted 1.0 ml fractions was immediately neutralized with 1.0 N NaOH. To

determine the production of specific antibodies, the affinity-purified anti-Zot antibodies and the affinity column pass-through serum were used to perform both western blotting and neutralization experiments in the Ussing chamber assay. The affinity-purified anti-Zot antibodies (but not the serum pass-through) recognized Zot on western blotting and neutralized its biological effect on rabbit small intestine permeability measured in the Ussing chamber assay.

Immunoscreening of both fetal and adult human tissues

Human fetal and adult tissues were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Proteins in crude tissue lysates were lyophilized and resuspended in phosphate buffered saline (PBS) (5:1, v/v), resolved by 10% SDS-PAGE, and either silver stained or transferred onto PVDF membrane (Millipore) and immunoblotted using affinity-purified, polyclonal anti-Zot antibodies as primary antibodies and anti-rabbit IgG (peroxidase conjugate, Sigma) as secondary antibodies. Non-specific binding sites were blocked by PBS with 5% milk plus 0.1% Tween-20. Films were exposed with ECL detection reagent (Amersham) for 1 minute.

Immobilization of anti-Zot antibodies to AminoLink Plus column

2 mg of affinity-purified polyclonal anti-Zot antibodies in 4 ml coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) and 40 μl of 5 M 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 M Tris-HCl (pH 7.4) and 40 μl of 5 M 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 M NaCl and stored in PBS containing 0.05% sodium azide.

Purification of human intestinal zonulin

Segments of human small intestines were obtained from the Brain and Tissue Bank for Developmental Disorders of the University of Maryland. It is general policy of this facility to harvest and store human tissues under sterile conditions. The intestinal mucosa was scraped from the muscle and serosal layers using a glass coverslip under sterile conditions. The mucosa was then suspended in standard PBS (Biofluids, Rockville, USA) (5:1, v/v) containing a cocktail of protease inhibitors, disrupted by homogenization, and the suspension centrifuged at 15,000 ***g*** at 4°C for 30 minutes. The supernatant was collected, lyophilized, reconstituted in PBS (10:1, v/v) and filtered through a 0.45 nm pore size filter (Millipore). The filtrates were passed through a 200 ml Sephacryl S200 HR column (Pharmacia), eluted with PBS, and 2.0 ml fractions processed for immunoblotting with affinity-purified polyclonal anti-Zot antibodies. Zot-immunoreactive fractions were pooled, lyophilized, reconstituted in PBS (10:1, v/v), and subjected to ion-exchange chromatography on a 45 ml Q-Sepharose column (Pharmacia). The column was loaded with the Zot immunoreactive fractions, washed with 300 ml of 50 mM Tris-Cl buffer (pH 7.4), and the binding proteins eluted from the column by a step salt gradient (0%-80%, w/v NaCl in 50 mM Tris-Cl buffer, pH 7.4). The fractions so obtained were lyophilized, subjected to western blotting using affinity-purified anti-Zot antibodies, and the immunoreactive fraction (fraction 1 eluted with 20% NaCl) was loaded on an anti-Zot antibody affinity column (see above). The binding protein(s) were finally eluted with a solution containing 50 mM glycine-Cl and 0.15 M NaCl, pH 2.5, and dialyzed against PBS.

Ussing chamber assay

The Ussing chamber assay was carried out as previously described (Fasano et al., 1995; Fasano et al., 1991; Baudry et al., 1992; Fasano et al., 1997) using different tracts of intestine from 5-6 kg adult male Rhesus monkeys. Animal housing, care and euthanasia were performed in the Central Animal Facility strictly in accordance with the University of Maryland School of Medicine Animal Facility guidelines and in line with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985). Eight sheets of mucosa (three jejunum, three ileum and two colon), stripped of their serosal and muscular layers, were mounted in lucite Ussing chambers (1.12 cm^2 opening), connected to a voltage clamp apparatus (EVC4000 WPI, Saratosa, FL, USA), and bathed with freshly prepared Ringer's solution (Fasano et al., 1995). The transepithelial potential difference (PD) was measured every 10 minutes, and the short-circuit current (*I*sc) and tissue resistance (*R*t) were calculated as previously described (Fasano et al., 1995; Fasano et al., 1991; Baudry et al., 1992; Fasano et al., 1997). Given the variability of absolute *R*t among different animals, the results were expressed as Δ*R*t (peak *R*t change – baseline *R*t value).

Zonulin N-terminal sequencing

Purified zonulin obtained from both fetal and adult human intestine was resolved by 8.0% (w/v) SDS-PAGE and transferred onto PVDF membranes using CAPS buffer containing 100 ml of 10×(3-[cyclohexylamino]-1 propanesulfonic acid), 100 ml of methanol and 800 ml of distilled water. The filters were then stained with Coomassie Blue, and the visualized protein was excised and subjected to N-terminal sequencing (Hunkapiller et al., 1984) using a Perkin-Elmer Applied Biosystems Apparatus Model 494.

Partial digestion of zonulin

A 1.0 mm strip of PVDF filter containing zonulin purified from human adult intestine was placed in a plastic tube previously washed with 0.1% (w/v) trifluoracetic acid (TFA), and rinsed with methanol. The filter was incubated in 100 mM Tris, pH 8.2, 10% (v/v) CH_3CN and 1.0% (v/v) reduced Triton X-100 at 37°C for 60 minutes. The filter was then incubated with 150 ng of trypsin for an additional 24 hours. The solution with filter was sonicated on ice for 10 minutes and the supernatant decanted and saved. The filter was then incubated with 0.1% (w/v) TFA and the solution sonicated for additional 10 minutes, and the supernatant decanted. The two samples containing digested zonulin were loaded on a 0.5 mm × 250 mm C_{18} column, 5.0 μm particle size, 300 Å pore size. A gradient from 0.1% (w/v) TFA to 45% CH_3CN water + 0.1% (w/v) TFA was developed for 2.25 hours. The peaks were collected and sequenced.

Statistical analysis

Results are presented as means ± s.d. Data were analyzed using one-way analysis of variance (ANOVA), and $P<0.05$ was considered statistically significant.

RESULTS

Immunoscreening of adult human intestine

Initially, increasing amounts of proteins from crude intestinal mucosa lysates were resolved by SDS-PAGE, and either silver stained or transferred to PVDF filters and immunoblotted using affinity-purified, polyclonal anti-Zot antibodies. The silver staining gel revealed the presence of multiple bands (Fig. 1A), while a single immunoreactive band with an apparent molecular mass of 47 kDa was detected by western immunoblotting. (Fig. 1B). The intensity of this band increased by increasing the amount of proteins loaded on the gel (Fig. 1B).

Zonulin purification from human intestine

To purify the human intestinal Zot analogue (that we named zonulin), the human small intestinal mucosa was subjected to

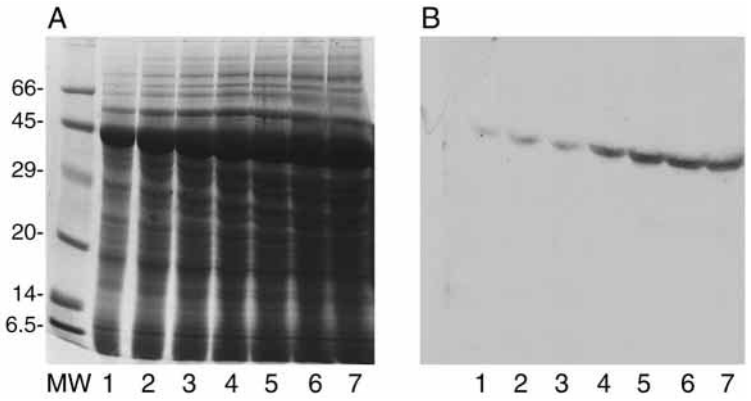

Fig. 1. Immunoscreening of human adult intestinal mucosa with affinity-purified polyclonal anti-Zot antibodies. Increasing amounts of mucosal lysate suspensions (20-140 µl, lanes 1 through 7) of human adult intestine were resolved by SDS-PAGE, and either silver stained (A) or transferred and immunoblotted using affinity-purified polyclonal anti-Zot antibodies (B). Western immunoblotting showed a single immunoreactive band of approx. 47 kDa whose intensity increased with the increasing amount of proteins loaded. The positions of marker proteins (kDa) are shown.

size exclusion chromatography followed by salt gradient chromatography and immuno-affinity purification. After each step of the purification protocol, the protein preparation was resolved by SDS-PAGE, and both silver stained and immunoblotted. The stained eluate of the anti-Zot antibody affinity column revealed a single protein band that migrated with an apparent molecular mass of 47 kDa (Fig. 2A) and was recognized by the anti-Zot antibodies (Fig. 2B). Western immunoblotting demonstrated a progressive increase in zonulin protein (Fig. 2B) that corresponded to a progressive increase in specific permeabilizing activity (Table 1) after each sequential purification step. Laser densitometry analysis of the immunoreactive zonulin band present in the different purification step samples revealed an approximately 30-fold increase from the starting material to the affinity-purified fraction (data not shown). No immunoreactive bands were detected in either *V. cholerae* or *E. coli* cell lysates (Fig. 2A, lanes 1 and 2, respectively), ruling out the possibility of a cross reaction of the intestinal tissue preparations with unrelated, bacterial-associated proteins eventually contaminating the intestinal specimens. The final yield of purified zonulin was 465 µg/100 g of intestinal mucosa.

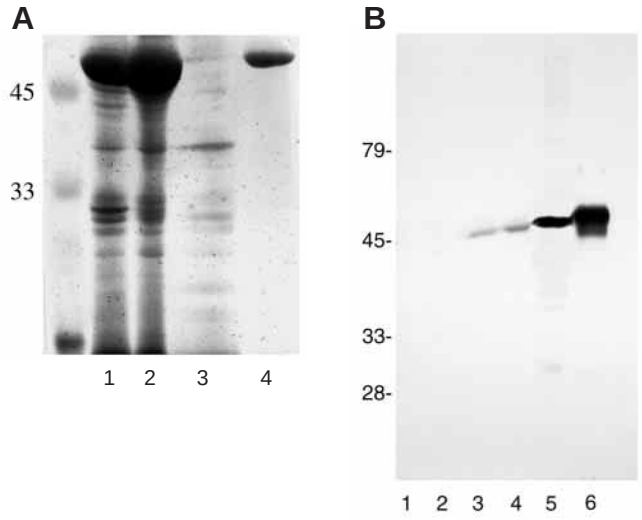

Fig. 2. Purification of human intestinal zonulin. (A) Total lysates of human intestinal mucosa (lane 1), pooled Zot-immunoreactive fractions harvested by size exclusion chromatography (lane 2) followed by salt gradient chromatography (lane 3) and the eluate from the subsequent anti-Zot antibody affinity column chromatography (lane 4) were each normalized by protein concentration, resolved by SDS-PAGE, and silver stained. The sequential purification steps yielded a single band of approx. 47 kDa. (B) *V. cholerae* cell lysate (lane 1), *E. coli* cell lysate (lane 2), human intestinal mucosa lysates (lane 3), pooled Zot-immunoreactive fractions harvested by size exclusion chromatography (lane 4) followed by salt gradient chromatography (lane 5) and the eluate from the subsequent anti-Zot antibody affinity column chromatography (lane 6) were each normalized by protein concentration, resolved by SDS-PAGE, and transferred and immunoblotted using affinity-purified anti-Zot antibodies. No immunoreactive bands were detected in either *V.cholerae* wild-type strain 395 (lane 1) or *E.coli* strain DH5α (lane 2). The western analysis of the different fractions of human intestinal mucosa after each purification step revealed an increasing amount of immunoreactive material during the process.

Zonulin bioactivity in non-human primate intestinal system

To establish whether the zonulin preparations were biologically active, they were tested on Rhesus monkey intestine mounted in Ussing chambers (Fasano et al., 1995; Fasano et al., 1991; Baudry et al., 1992; Fasano et al., 1997). Intestinal tissues from the same animal with comparable baseline *R*t (average stripped jejunal *R*t: 37 Ωcm^2 (range 35-41); average stripped ileal *R*t: 30 Ωcm^2 (range 26-33)) were simultaneously exposed to either zonulin or medium alone. Affinity-purified zonulin reduced *R*t compared to the medium control in both monkey jejunum (35.3% decrement) and ileum (25.6% decrement), but not in the colon (see Table 2). No significant changes in either PD or *I*sc were observed (data not shown). The biological effect of zonulin on *R*t normalized by protein concentration increased approximately 20-fold from the tissue lysate preparation (Fig. 2A, lane 3) to the affinity-purified preparation (Fig. 2A, lane 6) (Table 1). *R*t changes were evident only when zonulin was added

Table 1. Specific activity, protein concentration and activity increase of zonulin during the purification process

Purification steps	Activity ΔRt (Ωcm^2)	Protein* (mg/ml)	Specific activity (ΔRt/mg protein)	Fold activity
Tissue lysate	–5.3±2.5	7.4	0.72	1
Sephacryl-S300	–7.3±3.2	6.8	1.07	1.5
Q-sepharose	–8.0±2.8	1	8	11.1
Affinity column	–10.0±5.0	0.7	14.3	19.9

*Zot-immunoreactive fractions were pooled, lyophilized, reconstituted in PBS (10:1, v/v), and subjected to the next purification step.

Table 2. Effect of zonulin on transepithelial intestinal tissue resistance (*R*t)

Treatment (*n*)	ΔRt (Ωcm²) Jejunum	Ileum	Colon
Medium control (4)	−1.0±0.58	−1.3±0.33	−1.1±0.48
Zonulin* (4)	−13.0±0.51	−7.7±1.45	−1.3±0.59
P value (4)	<0.00001	0.013	NS

*Estimated final concentration: 10^{-13} M.
NS, not significant.

to the mucosal and not to the serosal aspect of the mucosa and were completely reversible within 20 minutes of zonulin withdrawal from the chamber reservoir (data not shown). The zonulin-induced decrement in ileal *R*t was blocked by pre-incubation of zonulin with anti-Zot antibodies (100% inhibition) but not with normal rabbit serum (3% inhibition), by coadministration with the PKC inhibitor CGP41251 (Fasano et al., 1995) (87.5% inhibition) and by pretreatment of the intestinal tissue with maximal concentrations of Zot (100% inhibition). These combined data suggest that zonulin and Zot share one or more domains recognized by the same affinity-purified antibodies and act through the same intracellular signaling pathway(s) (Fasano et al., 1995).

Screening of extraintestinal tissues for the presence of zonulin

We have recently reported the purification of a putative zonulin receptor from both human intestinal and extraintestinal tissues (Lu et al., 2000), suggesting that the zonulin system is not confined to the gastrintestinal tract. To establish whether zonulin could also be detected in extraintestinal sites, both fetal and adult human tissues, including intestine, heart and brain, were immunoscreened. The zonulin band of approx. 47 kDa was detected in all human tissues screened (Fig. 3).

Fetal and adult human intestinal zonulin N-terminal sequences

Zonulin from both adult and fetal human intestinal tissues was subjected to N-terminal microsequencing (Table 3). The N-terminal sequence of fetal zonulin was found to be 60% identical to the N terminus of adult zonulin (Table 3). The structural differences between fetal and adult zonulin may be operative during fetal development. Internal sequences of the adult zonulin failed to reveal any similarities to proteins in the database using the Blast search (data not shown).

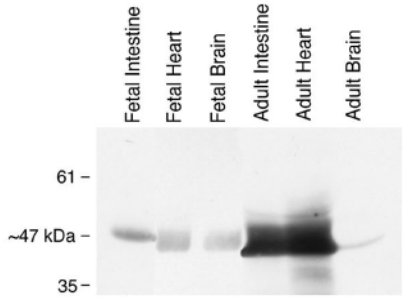

Fig. 3. Immunoscreening of both fetal and adult human tissues with affinity-purified polyclonal anti-Zot antibodies. Tissue lysates of human fetal and adult brain, heart and intestine were resolved by SDS-PAGE, transferred on PVDF membranes, and immunoblotted using affinity-purified polyclonal anti-Zot antibodies. Western immunoblotting showed the single zonulin band in all tissues screened, suggesting that the protein is also present in extraintestinal tissues both during fetal development and in mature organs.

Structural requirements for receptor binding and permeabilizing activity

That *V. cholerae*-derived Zot and human zonulin both act on intestinal tight junctions (Fasano et al., 1995; Fasano et al., 1991; Baudry et al., 1992; Fasano et al., 1997) (see above) and display the same regional activity (Fasano et al., 1997) (see above) coincident with Zot receptor distribution within the intestine (Fasano et al., 1995; Fasano et al., 1997), suggesting that these two molecules interact with the same receptor. We compared the primary amino acid structures of Zot and zonulin to provide insights into the structural requirements of the ligand for engagement with the receptor coupled to intestinal tight junction regulation. The N termini of zonulin and the Zot active fragment (Uzzau et al., 1999) shared a common motif (see shaded area in Table 3). G in position 8, V in position 12, Q in position 13, and G in position 15 all were highly conserved in Zot and zonulin (see Table 3) and therefore, may be critical for intestinal receptor binding. To address this hypothesis, a synthetic octapeptide corresponding to amino acid residues 8-15 of human fetal intestinal zonulin was synthesized and purified by reverse-phase HPLC. Ileal tissues were exposed to 10 μg/ml of the synthetic peptide, either alone or in combination with 0.1 μg/ml of Zot or zonulin. Both Zot and zonulin reduced tissue *R*t, whereas the synthetic peptide did not (Fig. 4). Treatment of the ileal tissue preparation with the synthetic peptide for 20 minutes prior to and throughout the study period protected against the reduction of *R*t in

Table 3. N-terminal sequences of fetal and adult human intestinal zonulin

Sample	Source	N-terminal residues 1	5	10	15	20	Intestinal permeability (Ussing chambers)
Zot*	*V. cholerae*		F C I	G R L C V Q D G		F V T	+
Zonulin	Human adult intestine	E V Q L V E S		G G X L			+
Zonulin	Human fetal intestine	M L Q K A E S		G G V L V Q P G		X S N R L	ND
Synthetic peptide	Synthetic			G G V L V Q P G			–
Scrambled peptide	Synthetic			V G V L G R P V			–

The shaded amino acids (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).
*N terminus of the biologically active fragment (amino acid residues 288-399) produced by *Vibrio cholerae* after processing (Uzzau et al., 1999).
The first glycine corresponds to residue #291 of the native Zot molecule (Baudry et al., 1992).

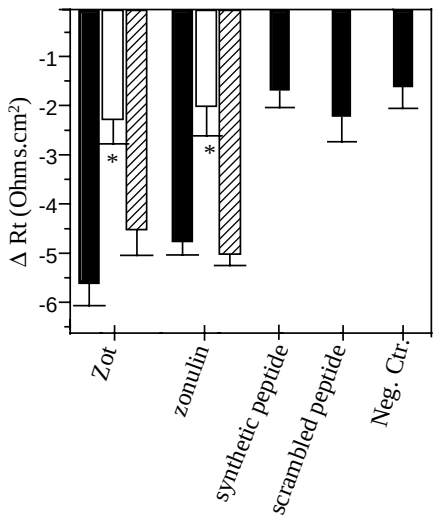

Fig. 4. Zonulin/Zot N termini are involved in receptor binding. The ability of either the synthetic peptide GGVLVQPG (corresponding to residues 8-15 of the zonulin N terminus) or a scrambled control peptide (VGVLGRPV), in which amino acids in position 8, 12, 13 and 15 were substituted, to block Zot/zonulin-induced reduction of ileal *R*t was tested. As anticipated, 0.1 μg/ml of either Zot (final concentration 10^{-12} M) or zonulin (final concentration 1.2×10^{-12} M) decreased peak *R*t compared to the negative control, whereas 1.0 μg/ml of either synthetic peptide did not (closed bars). Pretreatment with the zonulin N terminus-based synthetic peptide (1.0 μg/ml) for 20 minutes prior to and throughout exposure to Zot or zonulin (open bars) prevented *R*t reduction, whereas pretreatment with the scrambled peptide (dashed bars) did not. *n*=3-5 observations. *Significantly diminished Δ *R*t compared to the Zot/zonulin agonist alone at $P<0.008$.

response to Zot or zonulin (Fig. 4). In contrast, a scrambled octapeptide in which amino acids at positions 8, 12, 13 and 15 were substituted had no such inhibitory effect (Fig. 4). Therefore, an N-terminal region within Zot and zonulin (shaded in Table 3) appears to be a key determinant for receptor binding.

DISCUSSION

To meet the many diverse physiological challenges to which the intestinal epithelium is subjected, tight junctions must be capable of rapid and coordinated responses. This requires the presence of a complex regulatory system that orchestrates the state of assembly of the tight junction multiprotein network. While it is well accepted that tight junctions are dynamic structures, surprisingly little is known about their regulation. The discovery of Zot has shed some light on the intricate mechanisms involved in the modulation of the intestinal paracellular pathway and has allowed us to identify an intestinal mammalian analogue that participates in tight junction regulation. This analogue, which we have named zonulin, represents a novel eukaryotic protein that reversibly opens intestinal tight junctions. Our experimental approach did not allow us to establish whether the 47 kDa zonulin is the mature product or whether the protein is processed before being secreted. However, we have preliminary evidence suggesting that, following appropriate stimulation, the intestinal mucosa secretes the intact 47 kDa protein into the luminal but not the basolateral aspect of the small gut mucosa (A. Fasano, unpublished).

The intestinal epithelium represents the largest interface (more than 2,000,000 cm^2) between the external environment and the internal milieu. The appropriate regulation of intestinal tight junctions by modulators such as zonulin is essential to the structural and functional intestinal integrity. The maintenance of tight junction competence prevents movements of potentially harmful environmental factors such as bacteria, viruses, toxins, food allergens and macromolecules across the intestinal barrier. It has been recently reported that celiac disease (CD), an autoimmune disorder (Not et al., 1998) in which tight junctions are opened early in the disease through an as-yet-undefined mechanism (Madara and Trier, 1980; Schulzke et al., 1998) predisposes to autoimmune disorders such as insulin-dependent diabetes mellitus, Hashimoto's thyroiditis, autoimmune hepatitis and connective tissue diseases (Ventura et al., 1999). We have recently demonstrated that zonulin expression is increased during the early stage of the disease (Fasano et al., 2000). One could hypothesize that zonulin-induced opening of intestinal tight junctions, which occurs during the early stage of CD, permits entry of putative allergens into the intestinal submucosa where they can trigger an autoimmune response. In a spontaneous diabetic rat model, β-islet cell destruction and other autoimmune features only develop 3-4 weeks after the increase in gastrointestinal paracellular permeability (Meddings et al., 1999). Interestingly, these permeability changes not only preceded, but were also independent of the autoimmune process (Meddings et al., 1999). Further, the barrier dysfunction was restricted to the small intestine (Meddings et al., 1999), paralleling the regional distribution of the zonulin/Zot receptor within the gut (Fasano et al., 1997). Our recent observation that the increased intestinal permeability in this diabetic rat model is associated to an increase concentration of intraluminal zonulin (Watts et al., 2000) further supports the pathogenic role of this protein on the onset of autoimmune disorders such as diabetes mellitus and CD.

After binding to its surface receptor, Zot is internalized (Fasano, 1998), and subsequently triggers a series of intracellular events including phospholipase C and PKCα activation and actin polymerization (Fasano et al., 1995). That Zot and zonulin are competitively displaced by the same peptide (Fig. 2) and each reversibly opens tight junctions, which are blocked by the same PKC inhibitor (see above) (Fasano et al., 1995; Fasano et al., 1991; Baudry et al., 1992; Fasano et al., 1997), suggests that these two agonists bind to the same receptor and act through the same intracellular effector mechanisms. In eukaryotic cells, actin microfilaments form a cytoskeletal network that regulates cell shape, intracellular trafficking, spatially restricted expression of surface receptors, motility and differentiation (Hurtley, 1998). It is conceivable to hypothesize that, beside its participation in the physiological regulation of intercellular tight junctions of the small intestine, zonulin is involved in one or more of these physiological functions.

At this stage, we do not have a clear understanding of the physiological significance of the structural differences between the fetal and adult zonulin. However, these differences could

further support the hypothesis of possible multiple functions of zonulin, with the adult form mainly in charge of the regulation of the paracellular permeability and its fetal counterpart possibly more involved in the regulation of molecules trafficking between body compartments during embryogenesis. The possibility that zonulin is involved in more than a single function is also supported by our observation that zonulin is present in tissues other than the intestine. Since *Vibrio cholerae* is a pathogen confined to the gastrointestinal tract, we initially anticipated a mammalian Zot analogue whose expression would be restricted to intestinal tissues. Much to our surprise, zonulin was identified in extra-intestinal tissues as well. On the basis of this widespread tissue distribution, it is unclear whether systemically and/or locally produced zonulin(s) are presented to responsive cells. However, the fact that the Zot/zonulin receptor recently isolated in the human heart and brain have distinct N termini (Lu et al., 2000) supports the concept that zonulin might provide regional tissue responsiveness to a given stimulus on the basis of local requirements.

Dysregulation of this conceptual zonulin model may contribute to disease states that involve disordered intercellular communication, including developmental and intestinal disorders leading to autoimmune disease (i.e. CD and diabetes mellitus), tissue inflammation, malignant transformation and metastasis.

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