The rotavirus surface protein VP8 modulates the gate and fence function of tight junctions in epithelial cells

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Summary

Rotaviruses constitute a major cause of diarrhea in young mammals. Rotaviruses utilize different integrins as cell receptors, therefore upon their arrival to the intestinal lumen their integrin receptors will be hidden below the tight junction (TJ), on the basolateral membrane. Here we have studied whether the rotavirus outer capsid proteins are capable of opening the paracellular space sealed by the TJ. From the outermost layer of proteins of the rotavirus, 60 spikes formed of protein VP4 are projected. VP4 is essential for virus-cell interactions and is cleaved by trypsin into peptides VP5 and VP8. Here we found that when these peptides are added to confluent epithelial monolayers (Madin-Darby canine kidney cells), VP8 is capable of diminishing in a dose dependent and reversible manner the transepithelial electrical resistance. VP5 exerted no effect. VP8 can also inhibit the development of newly formed TJs in a Ca-switch assay. Treatment with VP8 augments the paracellular passage of non-ionic tracers, allows the diffusion of a fluorescent lipid probe and the apical surface protein GP135, from the luminal to the lateral membrane, and triggers the movement of the basolateral proteins Na^+-K^+-ATPase, $\alpha_v\beta_3$ integrin and β_1 integrin subunit, to the apical surface. VP8 generates a freeze-fracture pattern of TJs characterized by the appearance of loose end filaments, that correlates with an altered distribution of several TJ proteins. VP8 given orally to diabetic rats allows the enteral administration of insulin, thus indicating that it can be employed to modulate epithelial permeability.

Key words: Rotavirus, VP8, Tight junction, Occludin, Claudin, Drug delivery

Introduction

Rotaviruses are a major cause of diarrhea in young mammals. In developing countries rotavirus infections cause 600,000 childhood deaths per year and constitute an important source of morbidity in industrialized countries (Parashar et al., 2003). These viruses of the Reoviridae family have a genome composed of 11 double-stranded RNA segments, surrounded by three concentric layers of protein. The outermost layer is smooth and formed by a 37 kDa glycoprotein named VP7. From it, 60 spikes formed by an 88 kDa protein named VP4, project outwards (Estes, 2002). VP4 is essential for early virus-cell interactions, because it participates in receptor binding and cell penetration (Lopez and Arias, 2004). In fact, the infectivity of rotaviruses is dependent upon the specific cleavage by trypsin of VP4 into subunits VP5 and VP8 (Almeida et al., 1978; Espejo et al., 1981; Estes et al., 1981).

In vivo rotavirus infects primarily the mature enterocyte of the villi of the small intestine (Kapikian and Chanock, 2002). In vitro rotaviruses bind to a wide variety of cell lines, although only a subset of these, including cells of renal or intestinal origin, and transformed cell lines derived from breast, stomach, bone and lung, are efficiently infected (Ciarlet et al., 2002).

Some rotaviruses bind to a cell-surface receptor containing sialic acid (SA), whereas others (e.g. human rotavirus) apparently do not require SA for infection (Fukudome et al., 1989). Furthermore, association to a secondary SA-independent receptor can overcome the initial interaction of certain rotavirus with SA. SA-dependent rhesus rotaviruses (RRV) initially bind to the cell through VP8 (Fiore et al., 1991; Isa et al., 1997), whereas variants of RRV that no longer depend on the presence of SA (e.g. nar3), interact with the cell through VP5 (Zarate et al., 2000b).

The rotavirus surface proteins contain integrin ligand sequences (Coulson et al., 1997; Guerrero et al., 2000) and several integrins like $\alpha_2\beta_1$ (Zarate et al., 2000a), $\alpha_v\beta_3$ (Guerrero et al., 2000), $\alpha_X\beta_2$ and $\alpha_4\beta_1$ (Coulson et al., 1997; Hewish et al., 2000) are known to function as rotavirus cell receptors (for a review, see Lopez and Arias, 2004).

In epithelial and endothelial cells integrins have a polarized distribution and localize at the basolateral plasma membrane (Gut et al., 1998; Hynes, 1992). Therefore, rotaviruses reaching the intestinal epithelium from the apical surface or being in contact with the lumenal surface of confluent epithelial cell lines would find their integrin receptors hidden beneath the tight junction (TJ) on the basolateral surface. How then might luminal rotaviruses with putative basolateral ligands interact in polarized epithelia? One possibility is that rotavirus outer capsid proteins might be capable of opening the paracellular space sealed by the TJ. In this study we have explored this issue, and have found that the VP8 protein of rotavirus is capable of modulating TJ sealing in epithelia.

Materials and Methods

Cell culture

Madin-Darby canine kidney (MDCK) cultures between 60th and 100th passage were grown as previously described (Perez-Moreno et al., 1998).

Generation of fusion proteins

The RRV *VP8* fragment (*VP4* gene nucleotides 1 to 750) was cloned in pGEX-4T (Pharmacia) and in pET-28 (Novagen) plasmids as previously described (Dowling et al., 2000; Guerrero et al., 2000; Isa et al., 1997). The RRV *VP5* fragment (*VP4* gene nucleotides 749 to 2347) was cloned in pGEX-4T-2 (Pharmacia) as previously described (Zarate et al., 2000b). Expression and purification of fusion proteins was performed following previously described standard procedures (Frangioni and Neel, 1993). For their experimental use, fusion proteins VP5 and VP8 were dissolved in fresh Dulbecco's minimal essential medium (DMEM) and sterilized by passage through a 0.22 μm filtration unit.

Measurements of transepithelial electrical resistance

The degree of sealing of the TJ was evaluated by measuring the transepithelial electrical resistance (TER) across the monolayers as previously described (Gonzalez-Mariscal et al., 1985). TER determinations were done on single monolayers that were then discarded. The number of monolayers employed for each time point is shown in parenthesis. The values of TER shown have been normalized as previously reported (Balda et al., 1991).

Immunofluorescence

The immunofluorescence was performed according to standard procedures previously described (Jaramillo et al., 2004). The following antibodies were employed: rabbit polyclonals against ZO-1 (diluted 1:100 in 1% Ig-free BSA, Zymed 61-7300), claudin-3 (diluted 1:20 in 1% Ig-free BSA, Zymed 34-1700), occludin (diluted 1:100 in 1% Ig-free BSA, Zymed 71-1500) and $\alpha_v\beta_3$ integrin (diluted 1:75 in 1% Ig-free BSA, Chemicon International MAB1976); and mouse monoclonals against the apical cell surface glycoprotein GP135 (a generous gift of George Ojakian, New York State University) and the β Na^+-K^+-ATPase (a generous gift of Marcelino Cereijido, CINVESTAV, Mexico). As secondary antibodies we employed: FITC-conjugated goat anti-rabbit (catalogue no. 62-6111, diluted 1:100, Zymed), CY5-conjugated goat anti-rabbit (catalogue no. 62-6116, diluted 1:100, Zymed) or FITC-conjugated goat anti-mouse (catalogue no. 62-6511, diluted 1:100, Zymed). The fluorescence of the monolayers was examined using a confocal microscope (Leica SP2) with a Krypton-argon laser.

Ca^{2+}-switch assay for evaluating TJ assembly

The ability of VP8 to inhibit the development of TJs was assessed utilizing the Ca^{2+} switch assay (Gonzalez-Mariscal et al., 1985).

Paracellular flux assay

This assay was done as has been described elsewhere (Cereijido et al., 2002). Briefly, 1.5 ml of the tracer solution (10 μg/ml FITC-Dextran of 4 or 70 kDa; Sigma-Aldrich, catalogue no. FD-4, FD-70S) were added to the apical side of confluent monolayers grown on Transwell filters (Costar, catalogue no. 3450). After one hour of incubation at 37°C the media from both the upper and bottom chambers was collected and the FITC Dextran was measured in a fluorometer (excitation 492 nm; emission 520 nm).

Membrane lipid diffusion assay

This assay was performed as has been previously described (Cereijido et al., 2002; Contreras et al., 2002). Briefly, MDCK cells grown on Transwell inserts (Costar, catalogue no. 3470) were labeled on the apical side for 10 minutes on ice with a 5 nmol/ml solution of the Bodipy®FL-C12-sphingomyelin-BSA complex (Molecular Probes, Eugene, OR, USA). Then they were washed thrice with cold P buffer (NaCl 145 mM, HEPES pH 7.4 10 mM, Na-pyruvate 1.0 mM, glucose 10 mM and $CaCl_2$ 3.0 mM) and incubated on ice for 30 minutes. The membranes were immediately observed by confocal microscopy.

Cell surface biotinylation

Apical and basolateral plasma membrane proteins of confluent MDCK cells grown in Transwell cell culture inserts (Costar, catalogue no. 3470), were biotinylated by applying 500 μg/ml sulfo-NHS-SS biotin (Pierce, catalogue no. 21331), at either the apical (0.5 ml) or the basolateral (1.5 ml) chambers, as previously described (Gottardi et al., 1995). Filters were then excised and monolayers solubilized. β_1 integrin was immunoprecipitated with a specific antibody (Chemicon International, catalogue no. AB1952) as previously described (Perez-Moreno et al., 1998). The samples (50 μl each) were then separated on a 10% SDS-PAGE, transferred to PVDF membranes and blotted with HRP-streptavidin (Zymed 43-4323; diluted 1:2000). Proteins were then detected with the ECL Plus reagent.

Freeze fracture analysis of TJs

Freeze-fracture replicas of TJs present in confluent monolayers of MDCK cells incubated for 1 hour with or without 4 μg/ml of GST-VP8 were performed according to standard procedures described before (Gonzalez-Mariscal et al., 1985) using a Balzers apparatus (BAF400T). Observations were done in an electron microscope JEOL 200EX.

To assess modifications in the pattern of TJ strands, we counted the number of filaments intercepting lines drawn perpendicular to the main axis of the junction, every 48 nm, as well as the number of loose ends found along the 62 μm and 60 μm of TJ networks analyzed respectively for control and VP8-treated monolayers.

As has been previously reported (Gonzalez-Mariscal et al., 1985), the amount of junction is defined by the following function:

$$\sum_{n=1}^{x} n_i\%_i ,$$

where n_i is the number of strands in a segment of a TJ, and % is the percentage in which that number of strands is present in the sample.

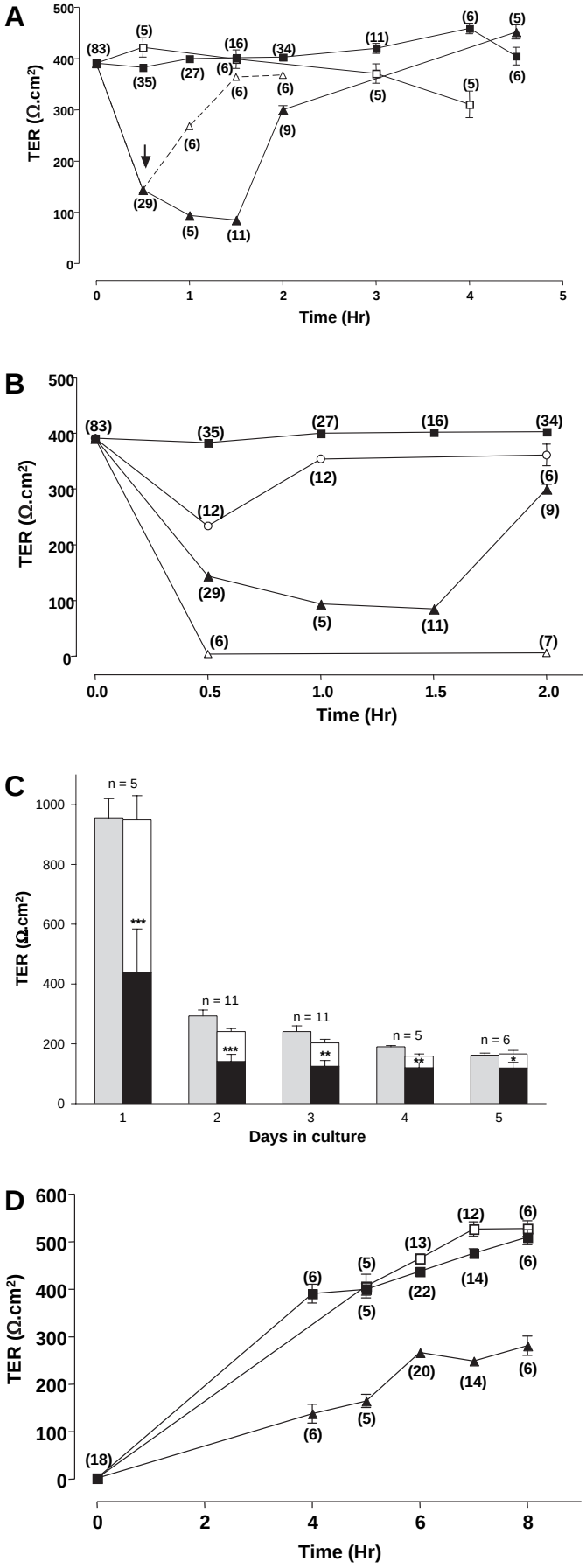
Western blotting

Western blots were made as previously described (Jaramillo et al., 2004) using polyclonal antibodies against ZO-1, claudin-3 and occludin.

Generation of diabetic rats

Male Wistar rats (230-250 g) were maintained on PicoLab® Rodent Diet 20, sterilized by irradiation and water ad libitum, in the animal house (temperature 22-24°C, 50-55% humidity). Care and handling of the animals were in accordance with international recommended procedures.

Diabetes mellitus type I was induced with one intraperitoneal shot of streptozotocin (75 mg/kg weight; Sigma, catalogue no. S-0130), diluted in citrate buffer 0.1 M (Sigma, catalogue no. S-4641) pH 4.5. Streptozotocin solution was prepared immediately before use and protected from light exposure.



Determination of glucose concentration in blood

The animals were anesthetized by ethylic ether inhalation. Using heparinized capillary tubing (Chase Scientific Glass, catalogue no. 2501) a drop of blood was taken from the eye orbital sinus of the rats. Blood glucose concentration was determined using reactive strips (One Touch, LIFESCAN, Johnson & Johnson) and a commercial glucometer (One Touch Basic Plus, LIFESCAN, Johnson & Johnson). Glucose concentration was determined in healthy animals and in those that had received the streptozotocin intraperitoneal shot at least three days before being tested.

Administration of insulin to diabetic rats

In order to study the changes in glucose concentration generated by the administration of insulin, the first blood sample was taken from diabetic animals that had fasted overnight. Immediately after, the animals received their food (Lab Diet 5053), and 30 minutes later they were treated according to the following protocols: (1) Human insulin of intermediate action (3-6 IU/rat; Humulin® N, HI-310, Lilly) was parenterally administered. (2) Human insulin of intermediate action (15-30 IU/rat; Humulin® N, HI-310, Lilly) was given orally through an esophagic rat cannula (Fine Science Tools; catalogue no. 18061-75), in a solution of 400 µl of $NaHCO_3$ (1.5 g/100 ml, pH 8.3-8.4), to neutralize gastric acidity. (3) Employing an esophagic rat cannula, 100 µg of GST-VP8 and human insulin of intermediate action (15-30 IU/rat; Humulin® N, HI-310, Lilly) were administered orally in a solution of 400 µl $NaHCO_3$ (1.5 g/100 ml, pH 8.3-8.4). (4) 100 µg of GST-VP8 was administered orally through an esophagic rat cannula. The level of blood glucose was determined at different times after the start of each of the above described procedures.

Results

Rotaviral protein VP8 induces a reversible and dose dependent decrease in TER

To investigate whether rotaviruses have the ability to open TJs, we proceeded to study the effect of the rotavirus outermost proteins on TER. We started by investigating whether VP5 and VP8 could modulate the TER of MDCK cells. For such aim we employed GST fusion proteins containing the RRV proteins

Fig. 1. Viral protein VP8 alters the transepithelial electrical resistance (TER) of epithelial monolayers (MDCK) in a reversible and dose dependent manner. (A) TER was determined in MDCK monolayers receiving 4 µg/ml of GST-VP5 (open squares) or GST-VP8 (closed triangles). In all the panels of this figure the closed squares correspond to the TER values obtained with MDCK monolayers treated with 4 µg/ml of GST. Monolayers that had been cultured for 30 minutes with 4 µg/ml of GST-VP8 (closed triangles) were washed (arrow) and transferred to media without GST-VP8 (open triangles). (B) MDCK monolayers incubated with 0.4 µg/ml (open circles), 4 µg/ml (closed triangles) or 10 µg/ml (open triangles) of GST-VP8. (C) At different times (1-5 days) after plating at confluence, the epithelial monolayers were treated with control media (gray bars) or 4 µg/ml of GST-VP8 added for 30 minutes (black bars) and 2 hours (white bars). Statistical significance was determined using a one-way ANOVA test. *P<0.05; **P<0.001; ***P<0.0001. (D) Confluent MDCK monolayers cultured for 20 hours in LC (1-5 µM Ca^{2+}) were transferred to NC (1.8 mM Ca^{2+}) (closed squares). Some monolayers were instead transferred to NC media containing either 4 µg/ml of VP5 (open squares) or of VP8 (closed triangles). In all these Figures, the values of TER reported as media±s.e.m. were normalized as explained in the Materials and Methods section. At each experimental point the number of monolayers on which the TER was measured is indicated.

VP5 and VP8. Fig. 1A shows how the addition of 4 μg/ml of GST-VP8 but not of GST-VP5 or of GST alone, significantly reduces the TER of epithelial monolayers after 30 minutes of treatment. Observe how the effect of VP8 upon the electrical resistance of the monolayer becomes reversible with time. To further illustrate the latter point we developed another assay in which GST-VP8 was withdrawn from the culture media (arrow). Observe how in these monolayers (open triangles) the TER recovers in a shorter time period. Fig. 1B shows how when 0.4 μg of GST-VP8 protein were used, the decline in TER obtained was smaller and lasted for a shorter period. In accordance, when a larger amount of GST-VP8 was employed, the TER was completely debased and the monolayers showed no recovery for the next two hours (Fig. 1B). We have also employed VP8 His-fusion protein and obtained similar results (data not shown). To analyze whether the effect of VP8 upon TER is dependent on the age of the monolayer, we proceeded to plate confluent monolayers and to determine their TER at different times of culture with and without VP8. As can be observed in Fig. 1C, MDCK monolayers exhibit an initial rapid increase in TER followed by a decrease to a stable level (gray bars). This variation may be associated, as previously reported (Cereijido et al., 1983; Rabito, 1986), with the number of cells in the monolayer. Thus, as the density increases, the length of the intercellular space per unit area of monolayer also augments. The amount of current that traverses the paracellular pathway depends not only on the resistance of this route, but also on how much pathway is available per epithelial surface [for a review on the structure-function relationship of TJs, see the following (Anderson and Cereijido, 2001; Gonzalez-Mariscal et al., 2001)]. Therefore the increased cell density of the monolayers might be responsible for the reduction in the value of TER. Treating the cells with 4 μg/ml of GST-VP8 for 30 minutes diminishes the TER values of all the monolayers (Fig. 1C, black bars). The white bars of Fig. 1C reveal how when a group of these monolayers is maintained for 2 hours with 4 μg/ml of GST-VP8 the effect disappears and the TER recovers.

Rotaviral protein VP8 delays the development of the epithelial paracellular barrier

Once we had demonstrated that VP8, but not VP5, could decrease the TER of confluent epithelial monolayers with well-formed TJs, we proceeded to study whether it could also alter the development of resistance in monolayers that are in the process of establishing cell-cell contacts. Such experiments named Ca-switch assays are based on our previous reports demonstrating that monolayers incubated for 20 hours in Ca-free media (LC) are devoid of TJs, and develop them with a fast kinetics once they are transferred to media containing 1.8 mM Ca^{2+} (NC) (Gonzalez-Mariscal et al., 1985; Gonzalez-Mariscal et al., 1990). Fig. 1D shows how the addition of 4 μg/ml of GST-VP8 but not of GST-VP5, to monolayers transferred from LC to NC media, delays the development of TER.

VP8 disrupts the TJ gate function for non-ionic molecules

TJs also function as selective barriers to the diffusion of non-ionic molecules through the paracellular route. Therefore we examined whether GST-VP8 could affect the passage of two non-ionic tracers: 4 kDa and 70 kDa FITC-Dextrans (Fig. 2). In confluent MDCK monolayers a small amount of these tracers diffuses across the monolayers. Treatment with 4 μg/ml of GST-VP5 induces a slight increase in the passage of the 4 kDa dextran. In contrast, a one-hour treatment with 4 μg/ml GST-VP8 induces a 25-fold increase in the paracellular permeability of 4 kDa FITC-Dextran and a 6-fold increase in the diffusion of the 70 kDa FITC-Dextran. We therefore conclude that the treatment with VP8 induces a leakage of small non-ionic tracers from the apical domain.

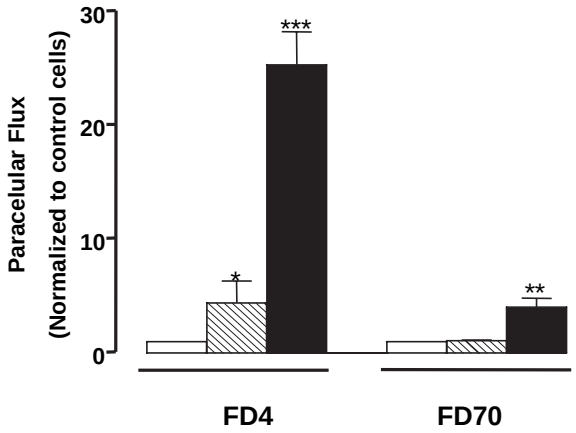


Fig. 2. VP8 disturbs the gate function of the tight junction (TJ) as determined by the paracellular passage of non-ionic tracers. MDCK cells were plated at confluency on Transwells and grown for 3 days. Paracellular flux of 4 kDa FITC-Dextran (FD4), and 70 kDa FITC-Dextran (FD70) was measured in the apical to basolateral direction. The amount of tracer diffusion was normalized to control MDCK cells. The data of six monolayers are reported as the mean±s.e.m. Open columns=control monolayers, closed columns=monolayers treated for 1 hour with 4 μg/ml GST-VP8, broken columns=monolayers treated for 1 hour with 4 μg/ml GST-VP5. Statistical significance was determined using the ANOVA test. **P*<0.05; ***P*<0.001; ****P*<0.0001.

Involvement of VP8 in the TJ fence function

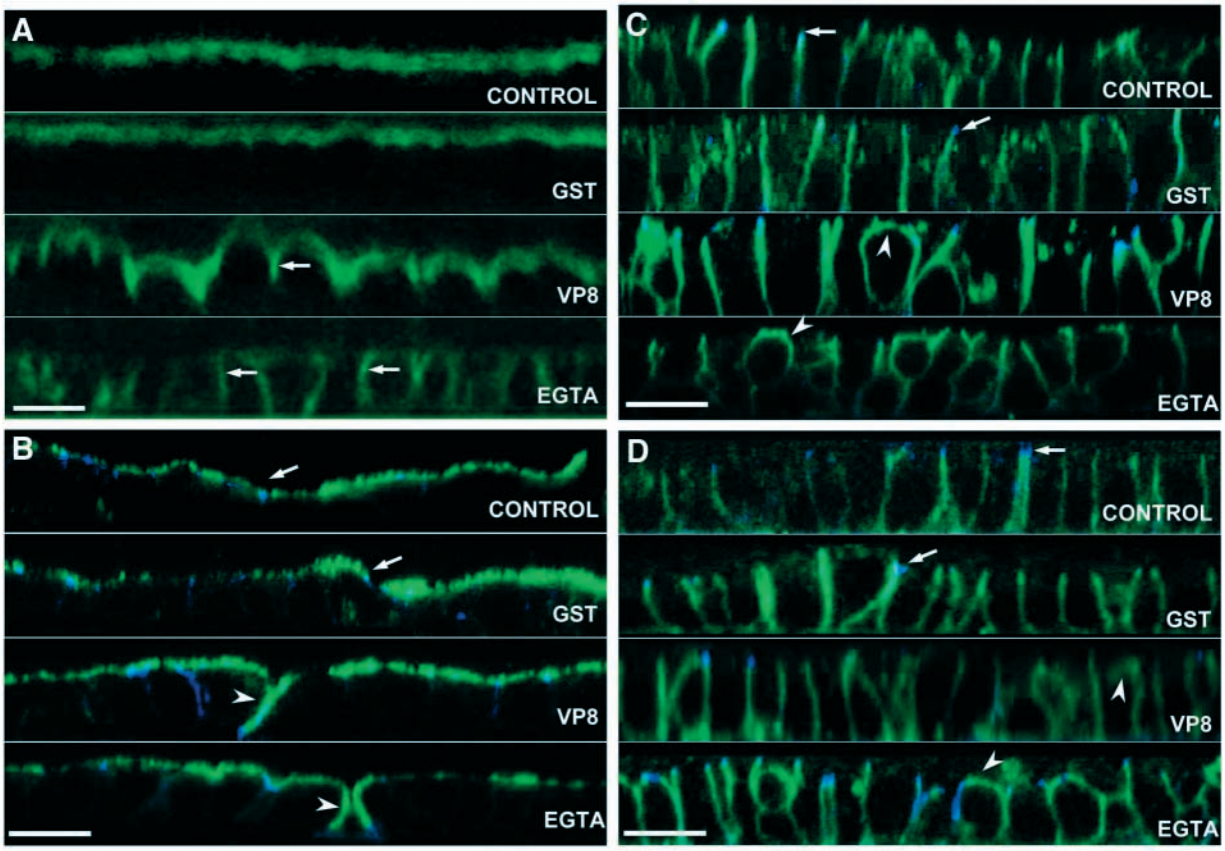
To examine whether VP8 could modify the fence function of the TJ, we analyzed the distribution of a lipid (Bodipy®FL-C12-sphingomyelin/BSA) and apical and basolateral membrane proteins in monolayers treated with VP8. For this set of experiments, confluent MDCK monolayers grown on Transwell® filters, remained untreated (control) or were incubated for 1 hour with 4 μg/ml of GST or GST-VP8. Another set of monolayers was incubated for 10 minutes with 1.8 mM EGTA, because previous reports demonstrated that incubation of epithelial cells with EGTA opens TJs and generates epithelial depolarization (Mandel et al., 1993; Martinez-Palomo et al., 1980).

The lipid complex was applied to the apical surface of confluent MDCK monolayers. After washing, the diffusion of the Bodipy®FL-C12-sphingomyelin-BSA complex was immediately analyzed. The confocal *z*-sections shown in Fig. 3A illustrate how in control and GST-treated cells, the fluorescent lipid is confined to the apical domain and no lateral staining is detected. However, in MDCK cells treated with

EGTA the Bodipy®FL-C12-sphingomyelin complex diffuses across the TJ and stains the lateral membranes, leaving almost no detectable stain on the apical surface. The cultures incubated with GST-VP8 display a pattern, in which lateral membrane staining starts to be detected, thus indicating alterations in the TJ fence function.

Next we proceeded to detect by immunofluorescence the distribution of polarized membrane proteins. In these assays occludin was simultaneously employed, because it allowed us to distinguish with precision the limit between the apical and the basolateral domains of the plasma membrane in control monolayers (arrows). Fig. 3B illustrates how in control and GST-treated monolayers, the apical cell surface glycoprotein GP135 (Ojakian and Schwimmer, 1988) is confined to the luminal surface, whereas in the cultures treated with GST-VP8 or EGTA the staining moves from the apical to the lateral membrane. In Fig. 3C we illustrate the effect of GST-VP8 on the distribution of the Na^+K^+-ATPase, a basolateral membrane marker. Observe how the clear basolateral distribution of the Na^+K^+-ATPase in control and GST-treated monolayers is altered when the monolayers are incubated with GST-VP8. This situation is more intensively observed in monolayers treated with EGTA.

As stated in the Introduction, because rotaviruses employ integrins as cell surface receptors, we proceeded to analyze whether VP8 altered their polarized distribution in epithelia. Fig. 3D reveals how in control and GST-treated monolayers, the rotavirus receptor, integrin $\alpha_v\beta_3$, is detected along the basolateral membrane. In contrast, treatment with VP8 or EGTA induces the appearance of the integrin on the apical surface. This effect was further confirmed in an assay in which either the apical or the basolateral membrane surfaces of



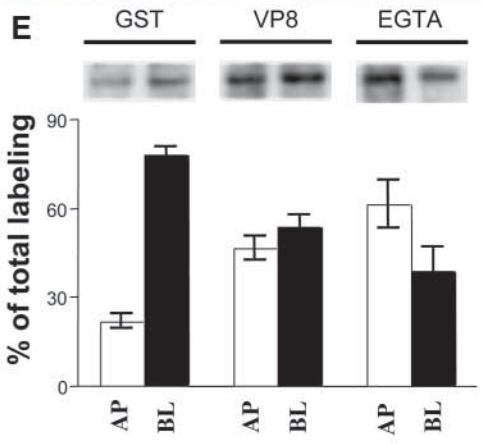


Fig. 3. VP8 perturbs the fence function of the tight junction (TJ). In all the experiments presented here, cells were plated at confluency on Transwell inserts for 3 days. Some monolayers remained in control media, whereas others were treated for 1 hour with 4 μg/ml of GST or GST-VP8, or for 10 minutes with 1.8 mM EGTA. The membrane distribution of the fluorescent markers was determined by *z*-sectioning on a confocal microscope. Bar, 8 μm. (A) Bodipy®-sphingomyelin-BSA complex diffusion assay. Fluorescent lipid/BSA was loaded to the apical surface. Arrows indicate lateral membrane staining. (B) Movement of the apical membrane protein GP135 (green) to the lateral membrane. In this and the following images arrows indicate occludin (blue) staining in the TJ region of control and GST-treated monolayers. The arrowheads in GST-VP8 and EGTA-treated monolayers denote the protein that has moved to the opposite plasma membrane. (C) Movement of the basolateral membrane protein Na^+K^+-ATPase (green) to the apical surface. (D) Redistribution of $\alpha_v\beta_3$ integrin (green) from the basolateral to the apical surface. (E) Another set of monolayers treated in the same manner was employed for selective surface membrane labeling (AP, apical; BL, basolateral) with activated biotin. Densitometric values of β_1 integrin labeling are expressed as mean percentages of the total (apical plus basolateral)±s.e.m. Immunoblots are representative of those from three separate experiments.

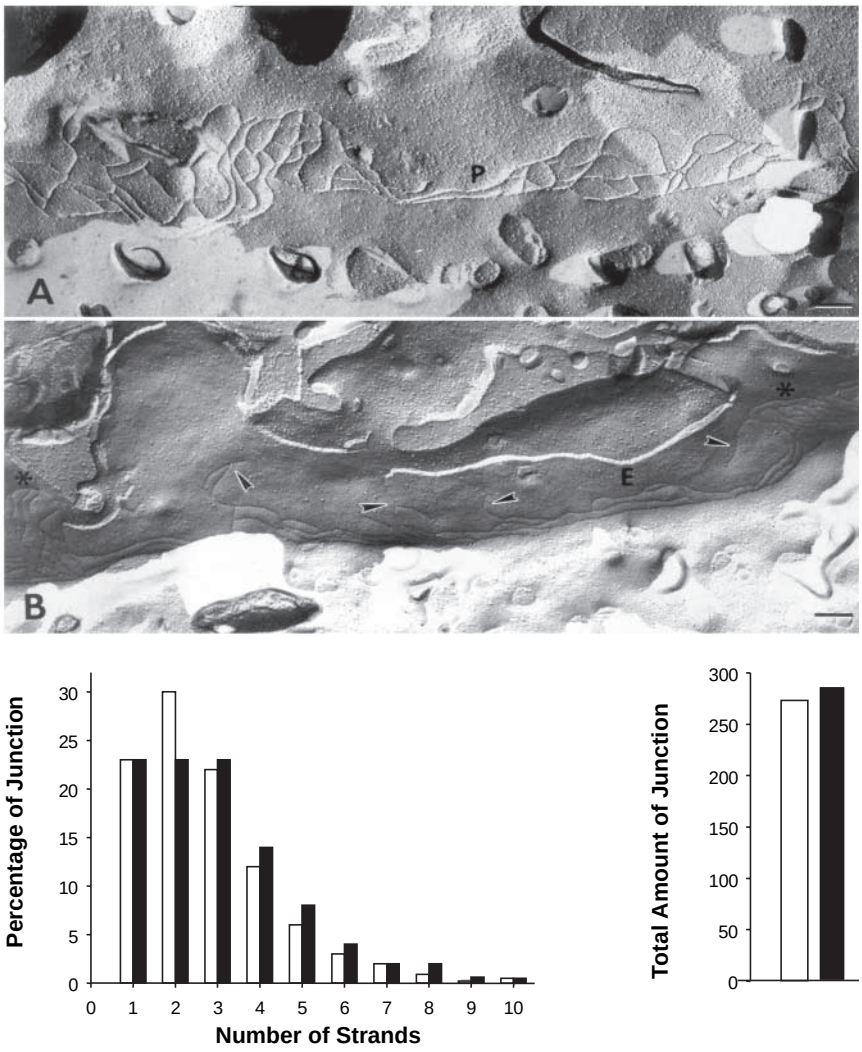


Fig. 4. VP8 induces changes in the tight junction (TJ) strand organization observed by freeze-fracture. The upper panel shows representative freeze-fracture images of control (A) and VP8 (4 μg/ml) treated monolayers (B). In the latter several loose ends (arrowheads) are found between regions where the network is profound and complex (asterisks). P=protoplasmic face; E=exoplasmic face; Bar, 200 nm. The lower panel corresponds to the morphometric results obtained from 1239 and 1124 TJ sites analyzed for the control (white bars) and VP8-treated monolayers (black bars), respectively. The distribution of the number of TJ strands and the total amount of junction were studied as described in the Materials and Methods section.

monolayers were labeled with sulfo-NHS-SS-biotin. The results in Fig. 3E reveal a redistribution of β1 integrin from the basolateral to the apical surface. These observations thus demonstrate that VP8 has the capacity to alter the distribution of integrins in epithelial cells. This effect might be of importance for rotaviruses infectivity because it would allow enhanced exposure of integrins to the luminal environment containing the viral particles.

VP8 treatment induces changes in TJ strand organization

Next, we asked whether VP8 could affect the TJ structure. For such a purpose we examined by freeze-fracture EM the TJ strand organization (Fig. 4, upper panel). In control monolayers (A) fractured faces of the apical plasma membrane display the characteristic organization of TJs: a network of interconnecting fibrils in the P face of the plasma membrane complementary to grooves on the E face. Treatment with VP8 (B) induces the appearance of loose ends that depart from the main network of TJ fibrils (arrowheads) together with discontinuous series of stranded clusters (asterisks). The arrangement of the main TJ axis is also simpler, as filaments have lost their interconnected appearance.

The morphometric analysis (Fig. 4, lower panel) reveals no changes neither in the number of TJ strands nor in the total amount of junctions between control and VP8-treated monolayers. In contrast, in VP8-treated monolayers, the number of loose ends that run quasi perpendicular to the main junctional axis is increased (Table 1). This change in the junctional pattern could explain the altered fence and gate properties of the TJs observed in VP8-treated monolayers.

VP8 affects the subcellular localization of TJ proteins ZO-1, claudin-3 and occludin

We then examined whether the observed TJ strand disorganization induced by VP8, correlated with changes in the localization of TJ proteins. For this purpose, confluent MDCK monolayers receiving no treatment or incubated for 1 hour with 4 μg/ml of GST or GST-VP8 were examined by confocal immunofluorescence. The distribution of ZO-1, a submembranous protein known for its scaffolding characteristics, as well as of two transmembrane proteins, claudin-3 and occludin [for a review on TJ proteins, see Gonzalez-Mariscal et al. (Gonzalez-Mariscal et al., 2002)], was analyzed in *xy* and *xz* sections. Fig. 5A shows conspicuous sharp ring-like structures of ZO-1, claudin-3 and occludin on

the lateral membranes between neighboring cells in control monolayers and in those treated with GST. The *xz* sections of control and GST-treated cells show the typical TJ dotted staining of ZO-1, claudin-3 and occludin. In GST-VP8-treated monolayers, staining for these proteins is greatly diminished, thus indicating that VP8 alters the molecular architecture of TJs.

Next, we proceeded to study by western blot the presence of ZO-1, claudin-3 and occludin in monolayers treated with GST-VP8. We analyzed Triton X-100 soluble and insoluble fractions, because certain TJ altering treatments displace TJ proteins from the insoluble to the soluble fraction (Chen et al., 2000). After VP8 treatment, no significant change was detected in the amount or in the distribution of these TJ proteins in the soluble and insoluble fractions (Fig. 5B). Therefore the discontinuous staining of the TJ proteins observed by immunofluorescence suggests that the TJs can be disorganized by VP8 without decreasing the cellular content of these TJ proteins, as has been previously observed in epithelial cells treated with tumor necrosis factor-alpha (Poritz et al., 2004) and peptides from the extracellular loops of occludin (Tavelin et al., 2003).

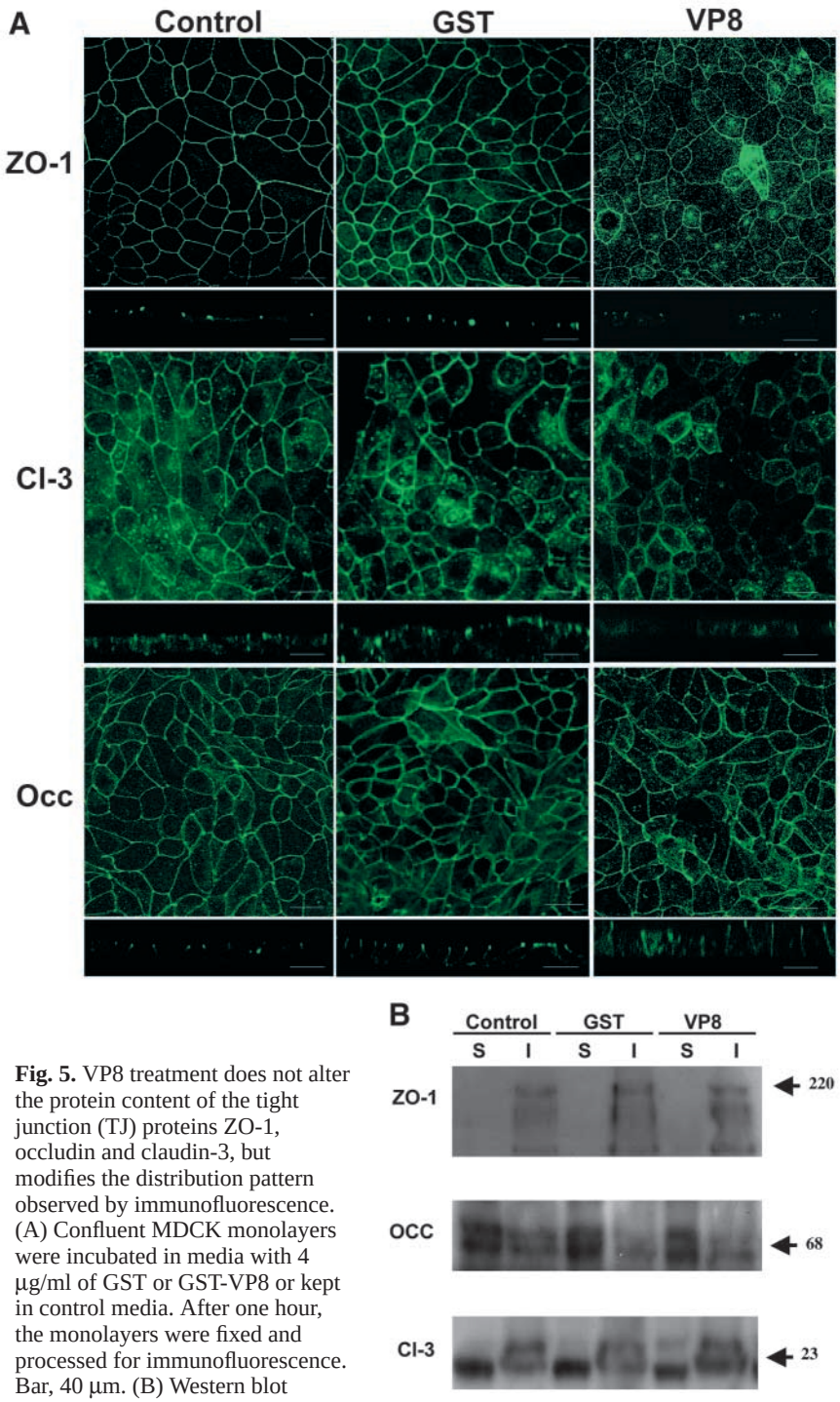


Fig. 5. VP8 treatment does not alter the protein content of the tight junction (TJ) proteins ZO-1, occludin and claudin-3, but modifies the distribution pattern observed by immunofluorescence. (A) Confluent MDCK monolayers were incubated in media with 4 μg/ml of GST or GST-VP8 or kept in control media. After one hour, the monolayers were fixed and processed for immunofluorescence. Bar, 40 μm. (B) Western blot detection of ZO-1, claudin-3 and occludin in the Triton X-100 soluble and insoluble fractions, obtained from monolayers cultured in control media, treated for 1 hour with 4 μg/ml of GST or GST-VP8.

Modulation of TJ permeability by VP8 allows the enteral administration of insulin in an animal model

Once we had demonstrated that the modulation of TJs by VP8 is reversible, time and dose dependent, we proceeded to study whether it could be used in an animal model to facilitate the passage of molecules through epithelia. We selected insulin as a prototype molecule to be tested for oral delivery, based on its relative small size, structure, biological activity and therapeutic relevance. We worked with streptozotocin-induced diabetic male rats in which we evaluated the bioactivity of insulin after enteral co-administration with 100 μg VP8. Fig. 6A illustrates how three days after a single streptozotocin intraperitoneal shot (75 mg/kg body weight) the rats display a much higher level of blood glucose (broken lines) after an overnight fasting period (time zero) and at different times after food is allowed, as compared with animals receiving a mock shot (continuous lines). When the streptozotocin-diabetic rats receive, via an esophagic cannula, oral insulin (30 IU), the blood glucose level does not decrease (Fig. 6B, broken lines), whereas as expected those animals parenterally receiving insulin (6 IU) display a sharp drop in their blood glucose concentration that is maintained for at least 3 hours after the animals are allowed to eat (Fig. 6B, continuous lines). When the streptozotocin-diabetic rats receive an oral administration of GST-VP8 (100 μg) no change in the animals' glucose level is detected (Fig. 6C, broken lines). Instead, when the diabetic rats receive a co-administration of GST-VP8 (100 μg) and insulin (30 IU), the decrease in their blood glucose level resembles that observed in rats receiving parenterally administered insulin

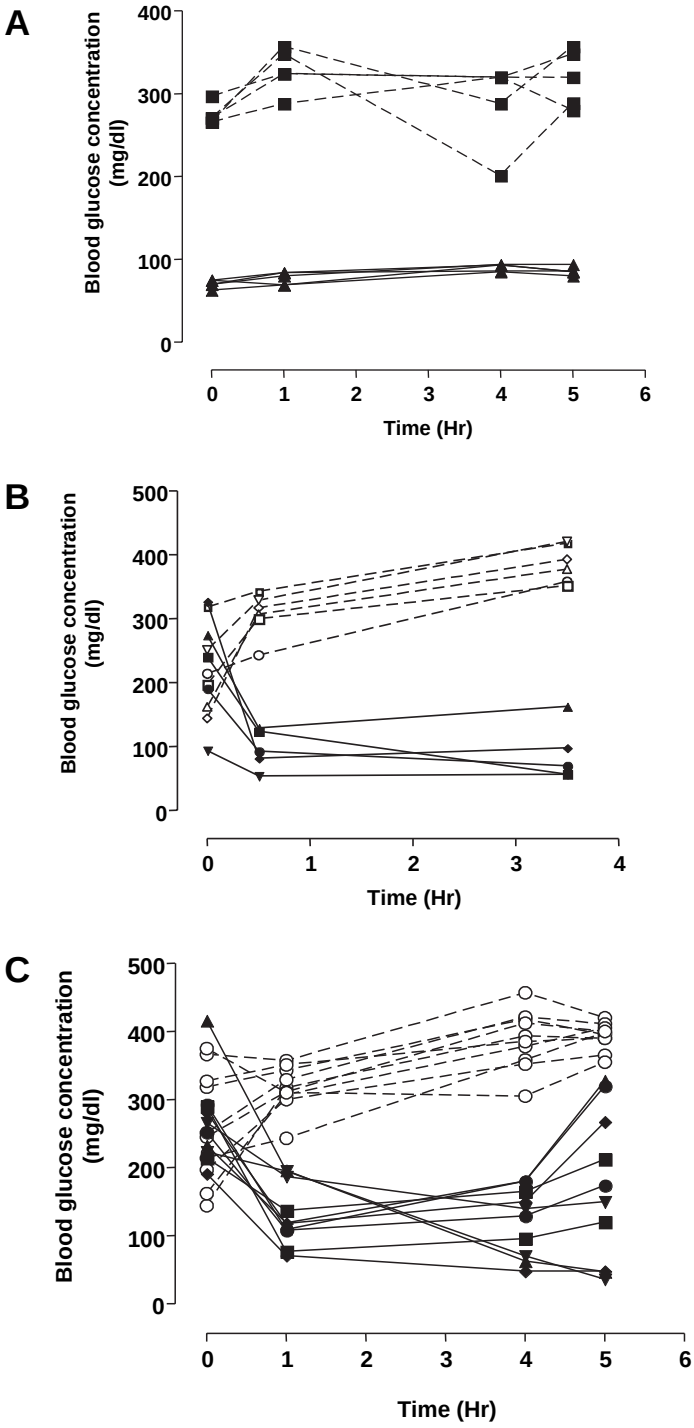


Fig. 6. VP8 facilitates the passage of molecules through the intestinal epithelium in an animal model. (A) Experiments were performed either in rats that had previously received an intraperitoneal injection of streptozotocin (75 mg/kg of body weight) to induce them to become diabetic (closed squares) or a mock injection (closed triangles). (B) Rats with streptozotocin-induced diabetes received insulin (Humulin, intermediate action) parenterally (6 IU; continuous lines) or orally (30 IU; broken lines). (C) Rats with streptozotocin-induced diabetes orally received VP8 (100 μg) (broken lines) or VP8 (100 μg) and insulin (30 IU) (continuous lines). Blood samples were taken after overnight fasting (time=0) or at different times after the animals received their diet. Each line corresponds to the results obtained with one animal.

Table 1. Freeze-fracture analysis of TJs in VP8-treated monolayers

	Loose Ends/10 μm
Control	2.74
VP8	7.33

The results reveal a higher amount of loose ends per strand length compared with control monolayers. 62 and 60 μm of junctional length of control and VP8 treated monolayers, respectively, were analyzed.

(Fig. 6C, continuous lines). The same results were obtained when instead of GST-VP8, His-VP8 was used (data not shown). None of the animals treated with GST-VP8 experienced diarrhea, fever or other systemic symptoms. These results therefore suggest that VP8 could provide a new strategy for the oral delivery of drugs and proteins not normally absorbed through the intestine.

Discussion

Our results demonstrate that the rotavirus protein VP8 is capable of disrupting the TJs of epithelial cells in a reversible, time and dose dependent manner. The effect of VP8 alters both the gate and fence function of intercellular TJs. The former is evidenced by the VP8-generated decrease in TER and by the observed increase in paracellular permeability to non-ionic tracers, thus indicating that the VP8-induced permeability changes apply to both charged and uncharged compounds. The structural organization of the TJ responsible for the intramembrane fence is impaired by VP8 treatment, as determined by the lateral diffusion of the apically administered lipid probe Bodipy®FL-C12-sphingomyelin-BSA complex and the apical marker GP135, and by the movement to the apical surface of the basolateral proteins Na^+K^+-ATPase and integrins $\alpha_v\beta_3$ and β_1 subunit.

In agreement with these results, we were able to detect an altered pattern of TJ strands in freeze-fracture replicas of VP8-treated monolayers, as well as a displaced distribution of the TJ constituents occludin, claudin-3 and ZO-1.

On analyzing the predicted amino acid sequence of RRV VP8 we found that several segments of this protein had a high similarity to domains present in the extracellular loops of claudins and occludin (Fig. 7), the transmembrane proteins of the TJ which are presumed to constitute the backbone of TJ strands (Tsukita et al., 2001). This observation poses the interesting possibility that rotavirus protein VP8 might open the TJ by perturbing the extracellular domains of TJ proteins in a manner similar to that exerted by synthetic peptides corresponding to the extracellular domains of occludin (Chung et al., 2001; Lacaz-Vieira et al., 1999; Van Itallie and Anderson, 1997; Wong and Gumbiner, 1997).

Besides humans, rotaviruses infect a wide variety of animal species such as monkeys, calves, pigs, horses, sheep, dogs, cats, mice, chicken, etc (Estes and Cohen, 1989; Kapikian and Chanock, 2002). Comparison of the deduced amino acid sequences of the VP4 proteins of rotavirus strains obtained from different animal species, has shown that although the VP4 sequence correlates with the species of origin of the virus strain, a high interspecies relatedness is present (Taniguchi et al., 1994). In addition, although VP8 is the less conserved region of VP4, a substantial degree of identity is still

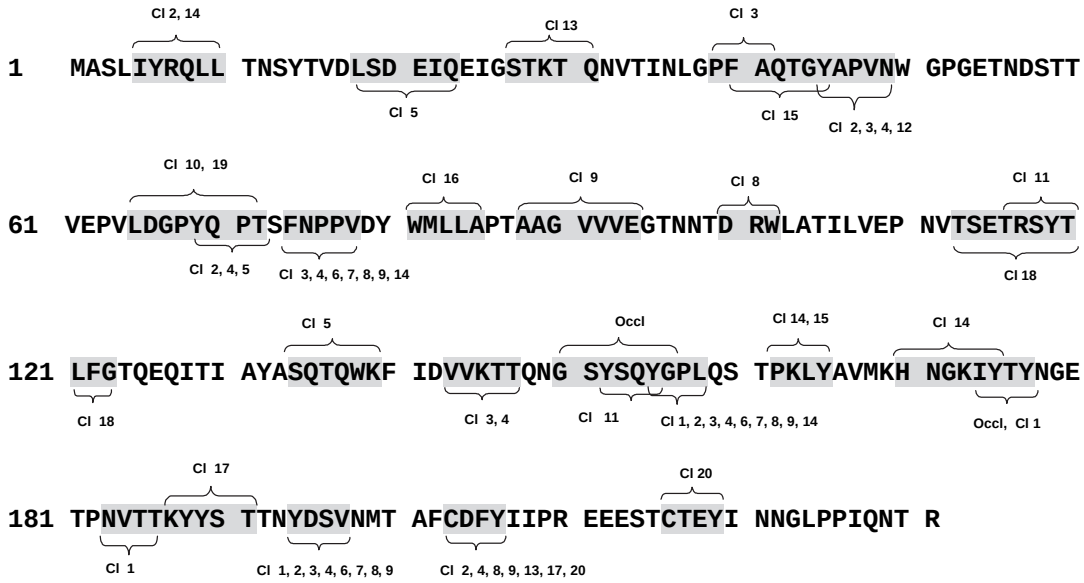


Fig. 7. The sequence of VP8 contains several regions similar to segments present in the external loops of occludin and claudins. The shadowed segments of the VP8 sequence have ⩾50% identity to regions of the extracellular loops of occludin or claudin. The name of the similar protein is indicated next to the brackets (e.g. claudin 2, cl 2; occludin, occl, etc.).

maintained among different rotavirus strains, and, in principle, the VP8 subunits from these strains could generate the same effects observed in this study.

Identification of viral receptors has yielded insight into mechanisms of viral entry. On several occasions these receptors have surprisingly turned out to be cell-cell adhesion proteins. For example, rhinoviruses bind to the intercellular adhesion molecule ICAM-1 (Greve et al., 1989; Staunton et al., 1989), α-herpes viruses to the poliovirus receptor related protein (Prr1) (Geraghty et al., 1998), coxsackie B and adenoviruses 2 and 5 bind to the CAR TJ protein (Bergelson et al., 1997), and reoviruses associate with the JAM protein located at the TJ (Barton et al., 2001). Furthermore, several diarrheogenic bacteria alter the junctional complexes in polarized epithelial cells. For example, infection with *Yersinia pseudotuberculosis* (Tafazoli et al., 2000), *Salmonella enterica*, *Helicobacter pylori*, enteropathogenic and enterohemorrhagic *Escherichia coli* results in alteration of the junctional complexes (for a review, see Sears, 2000). In the case of rotaviruses, entry into the cells appears to be a multi-step process involving the interaction with certain integrins (Lopez and Arias, 2004). Our results suggest that VP8 is capable of generating leaky TJs that allows the exposure of integrins to the luminal surface. After VP8 exposure, the epithelial TJs might still represent a diffusion barrier to large particles such as the rotavirus. Thus, a successful virus infection could alternatively be obtained through the appearance of apically positioned integrins like $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_x\beta_2$ and $\alpha_v\beta_3$, which have been reported to function as rotavirus receptors in epithelial cells. This would allow rotavirus to bind and infect from the luminal domain without having to cross the TJ. Hyper-permeability of the intestine during rotavirus infection has been documented in vivo, with enteral markers such as lactulose and mannitol (Isolauri et al., 1989; Jalonen et al., 1991; Uhnoo et al., 1990), and rotavirus infection of epithelial cell lines has been shown to generate an increased transepithelial macromolecular paracellular permeability, disruption of TJs and loss of TER at late times of infection (Ciarlet et al., 2001; Dickman et al., 2000; Obert et al., 2000).

Finally, the results obtained in this work suggest that VP8 could be employed as a potential tool for the modulation of TJ permeability. In this respect it should be remembered that intestinal absorption of numerous compounds routinely used for the treatment of diseases is limited by their physicochemical characteristics. With the exception of molecules transported by passive diffusion across the cell membrane (lipophilic molecules) or by carrier-mediated transport, absorption of large and hydrophilic molecules is almost exclusively limited by the presence of TJs that control passage through the paracellular pathway. Therefore the design of agents that can effectively and safely increase paracellular permeability via modulation of TJs has become increasingly important (Ward et al., 2000). Here we have shown that VP8 can be used in rats to enhance the intestinal absorption of orally administered insulin through the paracellular pathway. VP8 displays multiple properties that make it a promising tool to promote the transport of drugs, proteins and peptides across epithelia, because: (1) When employed in low doses it partially abolishes the transepithelial resistance, (2) it induces a reversible increase of tissue permeability, (3) in the rat model analyzed, it does not induce diarrhea, and (4) its co-administration in rat with biologically active compounds such as insulin enhances intestinal absorption of the active molecule.

In summary, in this study we have demonstrated that a rotavirus protein of the outer capsid, VP8, is capable of transiently opening TJs. This knowledge can be employed as a tool to enhance enteral administration of macromolecules.

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References

Almeida, J. D., Hall, T., Banatvala, J. E., Totterdell, B. M. and Chrystie, I. L. (1978). The effect of trypsin on the growth of rotavirus. *J. Gen. Virol.* **40**, 213-218.

Anderson, J. M. and Cereijido, M. (ed.) (2001). Introduction, evolution of ideas on the tight junction. In *Tight Junctions* (ed. M. Cereijido and J. Anderson), pp. 1-18. Boca Raton, FL: CRC Press.

Balda, M. S., Gonzalez-Mariscal, L., Contreras, R. G., Macias-Silva, M., Torres-Marquez, M. E., Garcia-Sainz, J. A. and Cereijido, M. (1991). Assembly and sealing of tight junctions, possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J. Membr. Biol.* **122**, 193-202.

Barton, E. S., Forrest, J. C., Connolly, J. L., Chappell, J. D., Liu, Y., Schnell, F. J., Nusrat, A., Parkos, C. A. and Dermody, T. S. (2001). Junction adhesion molecule is a receptor for reovirus. *Cell* **104**, 441-451.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. and Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320-1323.

Cereijido, M., Gonzalez-Mariscal, L. and Borboa, L. (1983). Occluding junctions and paracellular pathways studied in monolayers of MDCK cells. *J. Exp. Biol.* **106**, 205-215.

Cereijido, M., Shoshani, L. and Contreras, R. (2002). Functional analysis of the tight junction. In *Cell-Cell Interactions*, 2nd edn (ed. T. P. Fleming), pp. 71-91. New York, NY: Oxford University Press.

Chen, Y., Lu, Q., Schneeberger, E. E. and Goodenough, D. A. (2000). Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells. *Mol. Biol. Cell.* **11**, 849-862.

Chung, N. P., Mruk, D., Mo, M. Y., Lee, W. M. and Cheng, C. Y. (2001). A 22-amino acid synthetic peptide corresponding to the second extracellular loop of rat occludin perturbs the blood-testis barrier and disrupts spermatogenesis reversibly in vivo. *Biol. Reprod.* **65**, 1340-1351.

Ciarlet, M., Crawford, S. E. and Estes, M. K. (2001). Differential infection of polarized epithelial cell lines by sialic acid-dependent and sialic acid-independent rotavirus strains. *J. Virol.* **75**, 11834-11850.

Ciarlet, M., Crawford, S. E., Cheng, E., Blutt, S. E., Rice, D. A., Bergelson, J. M. and Estes, M. K. (2002). VLA-2 (alpha2beta1) integrin promotes rotavirus entry into cells but is not necessary for rotavirus attachment. *J. Virol.* **76**, 1109-1123.

Contreras, R. G., Shoshani, L., Flores-Maldonado, C., Lazaro, A., Monroy, A. O., Roldan, L., Fiorentino, R. and Cereijido, M. (2002). E-cadherin and tight junctions between epithelial cells of different animal species. *Pflügers Arch.* **444**, 467-475.

Coulson, B. S., Londrigan, S. L. and Lee, D. J. (1997). Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells. *Proc. Natl. Acad. Sci. USA* **94**, 5389-5394.

Dickman, K. G., Hempson, S. J., Anderson, J., Lippe, S., Zhao, L., Burakoff, R. and Shaw, R. D. (2000). Rotavirus alters paracellular permeability and energy metabolism in caco-2 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**, G757-G766.

Dowling, W., Denisova, E., LaMonica, R. and Mackow, E. R. (2000). Selective membrane permeabilization by the rotavirus VP5* protein is abrogated by mutations in an internal hydrophobic domain. *J. Virol.* **74**, 6368-6376.

Espejo, R. T., Lopez, S. and Arias, C. (1981). Structural polypeptides of simian rotavirus SA11 and the effect of trypsin. *J. Virol.* **37**, 156-160.

Estes, M. K. (2002). Rotaviruses and their replication. In *Virology* (ed. B. N. Fields, D. N. Knipe, P. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman and S. E. Straus), pp. 1625-1655. New York, NY: Raven Press.

Estes, M. K. and Cohen, J. (1989). Rotavirus gene structure and function. *Microbiol. Rev.* **53**, 410-449.

Estes, M. K., Graham, D. Y. and Mason, B. B. (1981). Proteolytic enhancement of rotavirus infectivity, molecular mechanisms. *J. Virol.* **39**, 879-888.

Fiore, L., Greenberg, H. B. and Mackow, E. R. (1991). The VP8 fragment of VP4 is the rhesus rotavirus hemagglutinin. *Virology* **181**, 553-563.

Frangioni, J. V. and Neel, B. G. (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal. Biochem.* **210**, 179-187.

Fukudome, K., Yoshie, O. and Konno, T. (1989). Comparison of human, simian, and bovine rotaviruses for requirement of sialic acid in hemagglutination and cell adsorption. *Virology* **172**, 196-205.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J. and Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**, 1618-1620.

Gonzalez-Mariscal, L., Chavez de Ramirez, B. and Cereijido, M. (1985). Tight junction formation in cultured epithelial cells (MDCK). *J. Membr. Biol.* **86**, 113-125.

Gonzalez-Mariscal, L., Contreras, R. G., Bolivar, J. J., Ponce, A., Chavez de Ramirez, B. and Cereijido, M. (1990). Role of calcium in tight junction formation between epithelial cells. *Am. J. Physiol.* **259**, C978-C986.

Gonzalez-Mariscal, L., Avila, A. and Betanzos, A. (2001). The relationship between structure and function for tight junction. In *Tight Junctions* (ed. M. Cereijido and J. Anderson), pp. 89-119. Boca Raton, FL: CRC Press.

Gonzalez-Mariscal, L., Betanzos, A., Nava, P. and Jaramillo, B. (2002). Tight junction proteins. *Prog. Biophys. Mol. Biol.* **81**, 1-44.

Gottardi, C. J., Dunbar, L. A. and Caplan, M. J. (1995). Biotinylation and assessment of membrane polarity, caveats and methodological concerns. *Am. J. Physiol.* **268**, F285-F295.

Greve, J. M., Davis, G., Meyer, A. M., Forte, C. P., Yost, S. C., Marlor, C. W., Kamarck, M. E. and McClelland, A. (1989). The major human rhinovirus receptor is ICAM-1. *Cell* **56**, 839-847.

Guerrero, C. A., Mendez, E., Zarate, S., Isa, P., Lopez, S. and Arias, C. F. (2000). Integrin alpha(v)beta(3) mediates rotavirus cell entry. *Proc. Natl. Acad. Sci. USA* **97**, 14644-14649.

Gut, A., Balda, M. S. and Matter, K. (1998). The cytoplasmic domains of a beta1 integrin mediate polarization in Madin-Darby canine kidney cells by selective basolateral stabilization. *J. Biol. Chem.* **273**, 29381-29388.

Hewish, M. J., Takada, Y. and Coulson, B. S. (2000). Integrins alpha2beta1 and alpha4beta1 can mediate SA11 rotavirus attachment and entry into cells. *J. Virol.* **74**, 228-236.

Hynes, R. O. (1992). Integrins, versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Isa, P., Lopez, S., Segovia, L. and Arias, C. F. (1997). Functional and structural analysis of the sialic acid-binding domain of rotaviruses. *J. Virol.* **71**, 6749-6756.

Isolauri, E., Juntunen, M., Wiren, S., Vuorinen, P. and Koivula, T. (1989). Intestinal permeability changes in acute gastroenteritis, effects of clinical factors and nutritional management. *J. Pediatr. Gastroenterol. Nutr.* **8**, 466-473.

Jalonen, T., Isolauri, E., Heyman, M., Crain-Denoyelle, A. M., Sillanaukee, P. and Koivula, T. (1991). Increased beta-lactoglobulin absorption during rotavirus enteritis in infants, relationship to sugar permeability. *Pediatr. Res.* **30**, 290-293.

Jaramillo, B. E., Ponce, A., Moreno, J., Betanzos, A., Huerta, M., Lopez-Bayghen, E. and Gonzalez-Mariscal, L. (2004). Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells. *Exp. Cell Res.* **297**, 247-258.

Kapikian, A. Z. and Chanock, R. M. (2002). Rotaviruses. In *Virology* (ed. B. N. Fields, D. N. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman and S. E. Straus), pp. 1657-1708. New York, NY: Raven Press.

Lacaz-Vieira, F., Jaeger, M. M., Farshori, P. and Kachar, B. (1999). Small synthetic peptides homologous to segments of the first external loop of occludin impair tight junction resealing. *J. Membr. Biol.* **168**, 289-297.

Lopez, S. and Arias, C. F. (2004). Multistep entry of rotavirus into cells, a Versaillesque dance. *Trends Microbiol.* **12**, 271-278.

Mandel, L. J., Bacallao, R. and Zampighi, G. (1993). Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. *Nature* **361**, 552-555.

Martinez-Palomo, A., Meza, I., Beaty, G. and Cereijido, M. (1980). Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* **87**, 736-745.

Obert, G., Peiffer, I. and Servin, A. L. (2000). Rotavirus-induced structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers. *J. Virol.* **74**, 4645-4651.

Ojakian, G. K. and Schwimmer, R. (1988). The polarized distribution of an apical cell surface glycoprotein is maintained by interactions with the cytoskeleton of Madin-Darby canine kidney cells. *J. Cell Biol.* **107**, 2377-2387.

Parashar, U. D., Hummelman, E. G., Bresee, J. S., Miller, M. A. and Glass, R. I. (2003). Global illness and deaths caused by rotavirus disease in children. *Emerg. Infect. Dis.* **9**, 565-572.

Perez-Moreno, M., Avila, A., Islas, S., Sanchez, S. and Gonzalez-Mariscal, L. (1998). Vinculin but not alpha-actinin is a target of PKC phosphorylation during junctional assembly induced by calcium. *J. Cell Sci.* **111**, 3563-3571.

Poritz, L. S., Garver, K. I., Tilberg, A. F. and Koltun, W. A. (2004). Tumor necrosis factor alpha disrupts tight junction assembly. *J. Surg. Res.* **116**, 14-18.

Rabito, C. A. (1986). Reassembly of the occluding junctions in a renal cell line with characteristics of proximal tubular cells. *Am. J. Physiol.* **251**, F978-F987.

Sears, C. L. (2000). Molecular physiology and pathophysiology of tight junctions. V. Assault of the tight junction by enteric pathogens. *Am. J. Physiol Gastrointest. Liver Physiol.* **279**, G1129-G1134.

Staunton, D. E., Merluzzi, V. J., Rothlein, R., Barton, R., Marlin, S. D. and Springer, T. A. (1989). A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell* **56**, 849-853.

Tafazoli, F., Holmstrom, A., Forsberg, A. and Magnusson, K. E. (2000). Apically exposed, tight junction-associated beta1-integrins allow binding and YopE-mediated perturbation of epithelial barriers by wild-type Yersinia bacteria. *Infect. Immun.* **68**, 5335-5343.

Taniguchi, K., Urasawa, T. and Urasawa, S. (1994). Species specificity and interspecies relatedness in VP4 genotypes demonstrated by VP4 sequence analysis of equine, feline, and canine rotavirus strains. *Virology* **200**, 390-400.

Tavelin, S., Hashimoto, K., Malkinson, J., Lazorova, L., Toth, I. and Artursson, P. (2003). A new principle for tight junction modulation based on occludin peptides. *Mol. Pharmacol.* **64**, 1530-1540.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* **2**, 285-293.

Uhnoo, I. S., Freihorst, J., Riepenhoff-Talty, M., Fisher, J. E. and Ogra, P. L. (1990). Effect of rotavirus infection and malnutrition on uptake of a dietary antigen in the intestine. *Pediatr. Res.* **27**, 153-160.

Van Itallie, C. M. and Anderson, J. M. (1997). Occludin confers adhesiveness when expressed in fibroblasts. *J. Cell Sci.* **110**, 1113-1121.

Ward, P. D., Tippin, T. K. and Thakker, D. R. (2000). Enhancing paracellular permeability by modulating epithelial tight junctions. **3**, 346-358.

Wong, V. and Gumbiner, B. M. (1997). A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J. Cell Biol.* **136**, 399-409.

Zarate, S., Espinosa, R., Romero, P., Guerrero, C. A., Arias, C. F. and Lopez, S. (2000a). Integrin alpha2beta1 mediates the cell attachment of the rotavirus neuraminidase-resistant variant nar3. *Virology* **278**, 50-54.

Zarate, S., Espinosa, R., Romero, P., Mendez, E., Arias, C. F. and Lopez, S. (2000b). The VP5 domain of VP4 can mediate attachment of rotaviruses to cells. *J. Virol.* **74**, 593-599.