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Pathogenic bacteria induce colonic PepT1 expression: an implication in host defense response

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Abstract

Background & Aims—Expression of the di/tripeptide transporter PepT1 has been observed in the colon under inflammatory conditions, however, the inducing factors and underlying mechanisms remain unknown. Here, we address the effects of pathogenic bacteria on colonic PepT1 expression together with its functional consequences.

Methods—Human colonic HT29-Cl.19A cells were infected with the attaching and effacing (A/E) enteropathogenic *E. coli* (EPEC). Wild-type and PepT1 transgenic mice or cultured colonic tissues derived from these mice were infected with *Citrobacter rodentium*, a murine A/E pathogen related to EPEC.

Results—EPEC induced PepT1 expression and activity in HT29-Cl.19A cells by intimately attaching to host cells through lipid rafts. Induction of PepT1 expression by EPEC required the transcription factor Cdx2. PepT1 expression reduced binding of EPEC to lipid rafts, as well as activation of NF-κB and MAP kinase and production of IL-8. Accordingly, *ex vivo* and *in vivo* experiments revealed that *C. rodentium* induced colonic PepT1 expression and that, compared to their wild-type counterparts, PepT1 transgenic mice infected with *C. rodentium* exhibited decreased bacterial colonization, production of pro-inflammatory cytokines, and neutrophil infiltration into the colon.

Conclusions—Our findings demonstrate a molecular mechanism underlying the regulation of colonic PepT1 expression under pathological conditions and reveal a novel role for PepT1 in host defense via its capacity to modulate bacterial-epithelial interactions and intestinal inflammation.

Keywords

PepT1; EPEC; *Citrobacter rodentium*; Cdx2; HT29-Cl.19A; Lipid rafts

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Introduction

The di/tripeptide transporter PepT1 is normally expressed in the brush border membranes of enterocytes in the small intestine, the proximal tubular cells of the kidney, bile duct epithelial cells and immune cells¹. It has been reported that PepT1 expression is induced in colonic epithelial cells under inflammatory conditions^{2, 3}, and that PepT1 can mediate the transport of bacterial pro-inflammatory peptides into colonic epithelial cells¹. Although the mechanism of PepT1 expression under pathological conditions remains unknown, it has been suggested that PepT1 expression is likely induced at a transcriptional level, and that specific transcriptional regulation by signaling pathway(s) may be activated^{2–5}. We previously showed that PepT1 is localized in lipid rafts (LRs) of intestinal epithelial cells (IECs) and immune cells⁶. LR has been proposed to compartmentalize proteins and lipids to regulate many cellular functions, such as sorting and trafficking of proteins and signal transduction⁷. Recently, several lines of evidence have suggested a role for LR as docking sites for pathogens to attack host cells^{8, 9}.

Enteropathogenic *Escherichia coli* (EPEC) is a food-borne pathogen that is implicated in the pathophysiology of infantile diarrhea¹⁰. EPEC intimately attaches to host IECs through the binding of the bacterial outer membrane protein intimin to Tir, a bacterium-encoded factor translocated into the host plasma membrane upon infection. Attachment of EPEC causes attaching and effacing (A/E) lesions, characterized by formation of actin-filled membranous protrusions (pedestal) and destruction of microvilli¹¹. EPEC also induces inflammatory responses in IECs, characterized by activation of NF- κ B¹² and MAP kinases^{13, 14} and production of pro-inflammatory cytokines, such as IL-8^{13, 14}. EPEC has been reported to affect epithelial membrane transport activities, including butyrate uptake¹⁵ and Na⁺/H⁺¹⁶ and Cl⁻/OH⁻¹⁷ exchange. These activity changes are suggested to be consequences of the redistribution of surface proteins from the apical membranes into the intracellular compartments^{15, 17}. However, the transcriptional mechanisms underlying regulation of membrane transport by EPEC remain unknown.

Here, we address i) the role of EPEC in the induction of PepT1 expression and function in colonic epithelial cells, ii) the molecular mechanisms underlying EPEC-induced PepT1 expression, and iii) the role of colonic PepT1 in bacterial-epithelial interactions and bacteria-induced intestinal inflammation. *In vitro* studies were validated by *ex vivo* and *in vivo* experiments using a PepT1 transgenic mouse model and *Citrobacter rodentium*, a murine A/E pathogen closely related to EPEC, which has been shown to produce comparable ultra-structural changes in mouse distal colons^{18, 19}.

Materials and Methods

Cell culture and *in vitro* infection

HT29-Cl.19A cells were grown in DMEM supplemented with 10% FBS and 1.5 μ g/ml plasmocin. Wild-type (WT) EPEC E2348/69 and EPEC mutant Δeae and Δtir strains were grown in Luria-Bertani (LB) broth. For infection, EPEC suspension in DMEM was added to confluent cell monolayers with a multiplicity of infection of 10. For adhesion assay, infected cells were washed, lysed and plated on LB plates.

Ex vivo and *in vivo* infection

8-week-old FVB WT and PepT1 transgenic mice²⁰ were used (Supplementary Material). *C. rodentium* (ATCC, S1116) were cultured overnight at 37°C in LB.

For *ex vivo* infection, mouse colonic tissues cultured as described previously²¹ were infected with *C. rodentium*. 6 h after infection, supernatants were collected and specimens were washed with PBS.

For infection of mice, food was withdrawn and drinking water was replaced with *C. rodentium* suspension in 20% sucrose distilled water overnight (4×10^8 CFU/mouse) as previously described²². Seven days after infection, serum was collected and mice were euthanized.

***C. rodentium* colony counts**

Colonic tissue samples were homogenized in 1 ml PBS and plated on MacConkey agar plates. *C. rodentium* colonies were recognized as pink with a white rim as previously described²³.

Plasmid construction and transfection

cDNA encoding Cdx2 or PepT1 was cloned as previously described^{6, 24}. HT29-Cl.19A cells were transfected with these constructs using lipofectin and stably selected in culture medium containing 1.2 mg/ml geneticin.

Isolation of lipid rafts from HT29-Cl.19A cells

HT29-Cl.19A cells were treated or not with 10 mM methyl- β -cyclodextrin (m β CD) for 30 min and replenished with 2 mM cholesterol for 1 h. Isolation of lipid rafts (LRs) was performed as previously described²⁵. Briefly, cells were lysed with 1% Triton X-100 in TNE buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA). Lysates were adjusted to 40% sucrose in TNE buffer, overlaid with 2 vol of 30% sucrose and 1 vol of 5% sucrose, and then centrifuged at 40,000 rpm for 18 h at 4°C in a Sorval SW 41 Ti rotor (Beckman). Twelve fractions (1-ml each) were collected from the top of the gradient. The floating membrane fraction was defined as LR.

Dual-luciferase reporter assay

HT29-Cl.19A cells were transiently transfected with 5 ng of the construct encoding *Renilla* luciferase (Promega) and 2 μ g of PepT1 promoter construct previously cloned²⁴ using lipofectin. Luciferase activity was detected with a Luminoskan (Thermo) and normalized to *Renilla* luciferase activity.

RT-PCR and Real-time RT-PCR

RT-PCR was performed using GeneJET Fast PCR kit (Fermentas). Real-time RT-PCR was performed using iQ SYBR Green Supermix (Biorad) and an iCycler (Bio-Rad). Primers used are described in Supplementary Material.

Cloning of full-length cDNA encoding PepT1 expressed in EPEC-infected HT29-Cl.19A cells

Total RNA was isolated from EPEC-infected HT29-Cl.19A cells and reverse transcribed using cDNA Synthesis kit (Fermentas). RT-PCR was performed using Platinum Taq DNA Polymerase (Invitrogen) and specific primers (Supplementary Material). The resulting PCR product was cloned into pGEM-T Easy Vector (Promega). Plasmids were grown, purified and the insert was sequenced (Lark Technologies).

Immunofluorescence staining

HT29-Cl.19A cells on cover slips or filter supports were stained with PepT1 antibody and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Molecular Probes) as previously described⁶.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using biotin-labeled double-stranded oligonucleotide encoding the Cdx2⁻⁵⁷⁹-binding site-containing PepT1 promoter (Supplementary Material). Supershift assay was performed using Cdx2 antibody (Zymed Laboratories).

Chromatin immunoprecipitation assay (ChIP)

ChIP was performed using a ChIP kit (Upstate). Briefly, after protein-DNA cross-linking, cell lysates were immunoprecipitated using Cdx2 antibody. The immunoprecipitated chromatin was eluted from the protein A and the cross-linked protein-DNA complexes were reversed. Cdx2⁻⁵⁷⁹-binding sequence in PepT1 promoter was detected by RT-PCR using specific primers (Supplementary Material).

Nuclear run-on assay

See Supplementary Material.

Western blot and dot blot

See Supplementary Material.

Uptake experiments

PepT1-mediated uptake of [³H]Lysine-Proline-Valine (KPV) was performed as previously described²⁶. Briefly, confluent HT29-Cl.19A cells were washed, stabilized in HBSS-10 mM HEPES (pH 7.4) for 15 min at 37°C and incubated with 20 nM [³H]KPV ± 20 mM glycine-leucine for 15 min at room temperature. Cells were washed and radioactivity was counted in a β-counter (Beckman).

Measurement of EPEC attachment to lipid rafts

EPEC attachment was monitored using the electric cell-substrate impedance sensing (ECIS) 1600R device (Applied BioPhysics). EPEC were seeded in ECIS 8W1E electrodes pre-coated with LR fractions at a final total protein concentration of 20 µg/ml and kept at 37°C in 5% CO₂ and 90% humidity. Attachment of cells on the electrode surface changes the impedance in such a way that morphological information of the attached cells can be inferred. Capacitance was measured at 40 kHz and 1 V. The time necessary for EPEC to cover half of the available electrode (*t*_{1/2}) was calculated as previously described²⁷.

ELISA

IL-8 and KC levels were quantified by using the Quantikine IL-8 Immunoassay and KC DuoSet ELISA kits (R&D System).

Histology

Distal colonic sections were fixed in 10% formalin and embedded in paraffin. 5-µm sections were stained with H&E. Photomicrographs were taken using a Nikon Eclipse TS100 microscope.

Manual neutrophil counts

The number of neutrophils per crypt was counted as previously described²².

Myeloperoxidase assay

See Supplementary Material.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. Statistical analysis was performed using unpaired two-tailed Student's *t*-test by InStat v3.06 (GraphPad) software. *P* < 0.05 was considered significant.

Results

EPEC induces PepT1 promoter activity and PepT1 transcription

Although PepT1 is expressed in inflamed colons^{2, 3}, the causation factors have not yet been identified. We hypothesized that pathogenic bacteria could induce colonic PepT1 expression. To test this possibility, human colonic HT29-Cl.19A cells, which do not express detectable level of PepT1², were infected with EPEC. As shown in Figure 1A and B, EPEC induced PepT1 promoter activity and mRNA expression in HT29-Cl.19A cells in a time-dependent manner. The full-length PepT1 cDNA was successfully cloned (GeneBank database accession No ACB71122). In addition, nuclear run-on assays indicated that EPEC strongly induced PepT1 gene transcription *in vitro* (Figure 1C). Together, these data demonstrate that EPEC transcriptionally induces PepT1 expression in HT29-Cl.19A cells.

EPEC induces PepT1 protein expression and transport activity

Western blot analysis revealed that EPEC induced a time-dependent increase in PepT1 protein expression in HT29-Cl.19A cells (Figure 1D). The total cellular amount of monocarboxylic transporter-1 (MCT-1), however, was not increased at different time points post-infection, supporting the specificity of the effect of EPEC on PepT1 expression (Figure 1D). Immunofluorescence staining revealed PepT1 expression in EPEC-infected cells, whereas no PepT1 signal was evident in uninfected cells (Figure 1E-i). Confocal microscopy of polarized HT29-Cl.19A cells at 6 h post-infection showed that PepT1 was mostly localized in the apical membranes (Figure 1E-ii). PepT1-mediated uptake of the tripeptide KPV in HT29-Cl.19A cells was increased after 4 and 6 h of infection (Figure 1F). Together, these data demonstrate that EPEC induces PepT1 protein expression and transport activity.

The transcription factor Cdx2 is crucial for EPEC-induced PepT1 expression

Previous studies have suggested that Cdx2 regulates PepT1 expression in IECs^{5, 24, 28}. We next addressed whether Cdx2 mediated EPEC-induced PepT1 expression. EPEC markedly increased Cdx2 mRNA and protein expression in HT29-Cl.19A cells (Figure 2A, B). Notably, Cdx2 bound to the human PepT1 promoter at the Cdx2⁻⁵⁷⁹-binding site as shown by EMSA (Figure 2C). The loss of the retarded protein-DNA complexes upon addition of an excess of unlabeled probe and their shift in the presence of Cdx2 antibody indicated that the binding of Cdx2 to the PepT1 promoter was sequence-specific (Figure 2C). The Cdx2-PepT1 promoter interaction was confirmed by ChIP analysis. As shown in Figure 2D, RT-PCR analysis of the Cdx2 immunoprecipitate derived from infected cells identified a sequence specific to the Cdx2⁻⁵⁷⁹-binding site, demonstrating the binding of Cdx2 to the PepT1 promoter.

We next investigated if Cdx2 was directly involved in EPEC-induced PepT1 expression. Figure 2E shows the expression of PepT1 mRNA and protein in HT29-Cl.19A cells over-expressing Cdx2. In contrast, PepT1 expression was not detected in WT HT29-Cl.19A cells or HT29-Cl.19A cells transfected with the empty vector, which exhibited low Cdx2 levels (Figure 2E). Accordingly, silencing of Cdx2 in HT29-Cl.19A cells by Cdx2 siRNA markedly reduced EPEC-induced PepT1 expression (Figure 2F). Together, these results demonstrate the importance of Cdx2 in EPEC-induced PepT1 transcription.

EPEC induces PepT1 expression in lipid rafts

We previously showed that PepT1 is localized in LR of IECs⁶. We next addressed whether PepT1 in HT29-Cl.19A cells was also targeted to LR upon infection with EPEC. Western blot and dot blot analyses revealed that the levels of PepT1 and GM1, a well-known LR marker, were increased in LR compared to other gradient fractions prepared from EPEC-infected cells (Figure 3A). GM1 co-immunoprecipitated with PepT1, but not the isotype IgG₁, in HT29-Cl.19A cells (Figure 3B). Treatment of infected cells with the cholesterol-disrupting agent mβCD caused most PepT1 and GM1 to move from the LR fraction to high-density fractions (Figure 3A). This change was accompanied by a ~70% decrease in EPEC-induced PepT1 activity, which was recovered by replenishing cell membranes with cholesterol (Figure 3C). These results suggest that EPEC induces functional PepT1 expression in the cholesterol-enriched LR of colonocytes.

EPEC induces PepT1 expression by intimately attaching to host cells through lipid rafts

Tir and intimin are known to be the virulence factors necessary for intimate attachment of EPEC¹¹. To investigate if EPEC-induced PepT1 expression requires the intimate attachment of EPEC to host cells, the effects of EPEC mutants *Δeae* and *Δtir*, which are deficient in intimin and Tir expression, respectively, on PepT1 expression were examined. Deficiency in Tir or intimin abrogated the ability of EPEC to induce PepT1 expression and transport activity (Figure 4A, B).

As LR have been shown to be important for EPEC adherence and Tir translocation²⁹, the possibility that LR are required for EPEC-induced PepT1 expression was examined. Western blot and RT-PCR analyses showed that mβCD treatment markedly reduced EPEC-induced PepT1 mRNA and protein expression in HT29-Cl.19A cells (Figure 4C, D). In addition, EPEC failed to induce PepT1 transport activity in mβCD-treated cells (Figure 4E). Suppression of PepT1 expression and activity by mβCD was effectively recovered by membrane cholesterol replenishment (Figure 4C, D, E). Collectively, these data suggest that EPEC induces PepT1 expression by intimately attaching to host cell membranes through LR.

PepT1 reduces EPEC adherence and EPEC-induced inflammation in colonocytes

To elucidate the role of PepT1 associated with LR in bacterial-epithelial interactions, EPEC adherence to HT29-Cl.19A cells overexpressing PepT1 (HT29-Cl.19A/PepT1) or the empty vector (HT29-Cl.19A/vector) was assessed. A significant decrease in EPEC adherence was evident in HT29-Cl.19A/PepT1 cells compared to HT29-Cl.19A/vector cells (Figure 5A). To further examine the involvement of PepT1 in EPEC adherence, EPEC attachment to the LR fractions prepared from HT29-Cl.19A/PepT1 or HT29-Cl.19A/vector cells was monitored in real-time using the electric cell-substrate impedance-sensing technique. Figure 5B shows the enrichment of PepT1 in the LR fraction and, to a lesser extent, the Triton X-100-soluble fractions prepared from HT29-Cl.19A/PepT1 cells and its absence in HT29-Cl.19A/vector cells. Attachment of EPEC, assessed by measuring capacitance changes, to LR from HT29-Cl.19A/PepT1 cells (Figure 5C, grey line) was delayed in comparison with that to LR from HT29-Cl.19A/vector cells (Figure 5C, black line). The time necessary for EPEC to cover half of the available electrode ($t_{1/2}$) coated with PepT1-containing LR ($t_{1/2} = 5.8 \pm 0.25$ h) was significantly increased compared to that on PepT1-lacking LR ($t_{1/2} = 4.1 \pm 0.38$ h) (Figure 5C). These data suggest that overexpression of PepT1 reduces adherence of EPEC to colonocytes.

The role of PepT1 in EPEC-induced inflammatory responses in colonocytes was next investigated. EPEC induced a stronger and faster IκB-α degradation in HT29-Cl.19A/vector cells compared to HT29-Cl.19A/PepT1 cells (Figure 5D). Furthermore, EPEC-induced phosphorylation of IκB-α, ERK1/2 and p38 kinases was delayed in HT29-Cl.19A/PepT1 cells

compared to HT29-Cl.19A/vector cells (Figure 5D). Consistent with these data was a decrease in IL-8 mRNA and protein production in HT29-Cl.19A/PepT1 cells (Figure 5E, F). Together, these findings suggest a role for PepT1 in host defense responses to EPEC infection.

***Citrobacter rodentium* induces PepT1 expression in mouse colon**

Colonic PepT1 expression upon *ex vivo* and *in vivo* infection was examined using *C. rodentium*, a murine A/E pathogen¹⁸. PepT1 mRNA and protein expression was significantly increased in cultured mouse colonic tissues by 6 h of infection (Figure 6A, B). Furthermore, colonic PepT1 mRNA and protein expression was induced in mice infected with *C. rodentium* (Figure 6C, D). These results, which are in accordance with *in vitro* data, suggest that infection with pathogenic bacteria induces PepT1 expression.

PepT1 has a role in the protective response in mouse colon against *Citrobacter rodentium*

The role of PepT1 in host responses to *C. rodentium* infection was assessed using a PepT1 transgenic mouse model we previously developed²⁰. Figure 7A shows a decrease in *C. rodentium* adherence to cultured colonic tissues from PepT1 transgenic mice compared with those from WT mice. In addition, cultured colons from PepT1 transgenic mice produced lower mRNA and protein levels of keratinocyte-derived chemokine (KC), a potent murine neutrophil chemoattractant³⁰, compared to those from WT mice by 6 h post-infection (Figure 7B, C).

Consistent with these *ex vivo* data, colonization of *C. rodentium* in colons from PepT1 transgenic mice was reduced compared to those from WT mice (Figure 8A). Furthermore, PepT1 transgenic mice exhibited decreased neutrophil accumulation in the colon, assessed by measuring colonic myeloperoxidase (MPO) activity, compared to WT mice (Figure 8B). Examination of H&E stained colonic tissues from uninfected mice revealed no morphologic differences between WT and PepT1 transgenic groups (Figure 8C-i). Seven days after *C. rodentium* infection, increases in crypt height, an indication of hyperplasia- a distinctive characteristic of *C. rodentium* infection¹⁸, were evident in both WT and PepT1 transgenic mice (Figure 8C-i). There were relatively modest differences in morphology of colons from these mice except for a remarkable decrease in neutrophil infiltration in PepT1 transgenic mice compared to WT mice (Figure 8C-ii), which strongly correlated with the reduction of MPO activity. In addition, compared to WT mice, PepT1 transgenic mice exhibited decreased colonic KC mRNA production and attenuated serum KC levels in response to *C. rodentium* infection (Figure 8D). Colonic levels of the pro-inflammatory cytokines IL-1 β , IL-6, IL-12, TNF- α and IFN- γ in PepT1 transgenic mice were also reduced compared to that observed in WT mice (Figure 8E). Production of the anti-inflammatory cytokines IL-10 and TGF- β in the colons from PepT1 transgenic mice were increased compared to WT mice (Figure 8F), suggesting that endogenous anti-inflammatory mechanisms might be more effectively activated in PepT1 transgenic mice.

Altogether, our data suggest that PepT1 may have a role in colonic infections via modulating bacterial-epithelial interactions and inflammatory response to pathogenic bacteria.

Discussion

In the present study, we demonstrate that EPEC transcriptionally induces functional PepT1 expression in colonic epithelial cells. Previous studies have shown the effect of EPEC on membrane transport activities in IECs, such as butyrate uptake via monocarboxylic transporter-1¹⁵ and Na⁺ transport via Na⁺/H⁺ exchange isoforms¹⁶ and Cl⁻/OH⁻ exchange¹⁷. These activity changes have been suggested to be due to the redistribution of surface proteins from the apical membranes into the intracellular compartments^{15, 17}. The regulation of membrane transporters by EPEC at a transcriptional level has not yet been investigated.

Regarding pathogenic effects on PepT1 expression, only one study to date has reported the transcriptional up-regulation of PepT1 in the rat small intestine in response to *Cryptosporidium parvum* infection⁴. Under inflammatory conditions^{2, 3}, PepT1 expression in the colon has been observed, however, the underlying molecular mechanisms remain unexplored. In an effort to address this, we identified EPEC as a causal factor of PepT1 expression in human colonic HT29-Cl.19A cells, which do not express PepT1 under basal conditions². One of our important findings is that the transcription factor Cdx2 is required for EPEC-induced colonic PepT1 expression, which is in accordance with previous studies showing a role for Cdx2 in the regulation of PepT1 expression in IECs^{5, 24, 28}. With regard to pathological states, PepT1 expression was detected in the intestinal metaplastic gastric mucosa isolated from transgenic mice with stomach-specific Cdx2 expression³¹. Furthermore, a high correlation exists between PepT1 and Cdx2 expression levels in human gastric tissues developing intestinal metaplasia⁵. Together, these observations and our findings raise Cdx2 as a key transcription factor in the regulation of PepT1 expression under pathological conditions. The regulation of Cdx2 especially that is mediated by bacterial pathogen has not been broadly established. Aberrant expression of Cdx2 in *Helicobacter pylori*-associated atrophic gastritis has been reported³², but the underlying molecular mechanism is unknown. Recently, bacterial components, such as lipopolysaccharide, were suggested to up-regulate Cdx2 expression via Toll-like receptors 2 and 4 followed by NF- κ B activation in cultured biliary epithelial cells and *in vivo*³³. Importantly, our study demonstrates that EPEC is a regulator of Cdx2 expression. The signal transduction pathways that are used by EPEC to activate Cdx2, which induces PepT1 expression, are currently under investigation.

We previously showed that PepT1 is localized in LR membrane microdomains⁶, which are known to provide specialized lipid environments to regulate the organization and function of many membrane proteins⁷. Here, we show that EPEC induces PepT1 expression in the LRs of HT29-Cl.19A cells, and that the association of PepT1 with LRs can modulate PepT1 transport activity. These findings are of physiological importance as they demonstrate that PepT1 expressed in colonocytes upon EPEC infection is functionally active.

It has been proposed that in response to bacterial binding, signaling molecules gather at membrane LR platforms, thereby participating in bacterial adherence and invasion^{8, 9}. Accordingly, several LR-associated molecules, such as CD44, annexin II, cholesterol and GPI-anchored proteins are found at EPEC adherent site^{34, 35}. Our study demonstrates that EPEC-induced PepT1 expression requires intact LRs, together with the EPEC virulence factors Tir and intimin. Given that the binding of intimin to its receptor Tir is required for the intimate attachment of EPEC to host IECs¹¹, we speculate that EPEC induces PepT1 expression by activating signaling via molecules within LRs.

The findings that EPEC-induced PepT1 expression depends on LRs and that PepT1 expressed in colonocytes is functionally targeted to LRs led us to investigate the role of PepT1 associated with LRs in host responses to EPEC virulence. Strikingly, we found that overexpression of PepT1 in HT29-Cl.19A cells markedly reduced EPEC adherence. Furthermore, the presence of PepT1 delayed EPEC attachment to LRs as monitored in real-time using the ECIS technique. Given the importance of LRs in bacterial adherence to host IECs, these data strongly suggest a potential role for LR-associated PepT1 in bacterial-epithelial interactions. We speculate that the assembly of PepT1 molecules or their interactions with other components inside LRs may result in changes in conformation and/or composition of LRs, and consequently mollify the binding affinity of EPEC for LRs. Current efforts are centered on studying the spatial distribution of LR proteins upon expression of PepT1, especially those are required for EPEC infection. Another intriguing finding is that PepT1 attenuates EPEC-triggered pro-inflammatory responses in IECs as shown by decreases in NF- κ B and MAP kinase activation and IL-8 production. Since EPEC-induced IL-8 production in IECs depends on EPEC intimate

adherence¹⁴, the reduction of EPEC-induced inflammation might be a consequence of the decrease in EPEC adherence. Therefore, colonic PepT1 expression might be a host protective mechanism that modulates bacterial-epithelial interaction and inflammatory responses to pathogens.

Our *in vitro* data were convincingly supported by *ex vivo* and *in vivo* studies using *C. rodentium* in a mouse model of A/E pathogen infection³⁶. *Ex vivo* experiments showed that *C. rodentium* infection strongly enhanced PepT1 mRNA and protein expression in cultured mouse colon. Infection of FVB mice with *C. rodentium* also resulted in a massive increase in colonic PepT1 expression. The role of PepT1 expression in the communication of colonic epithelia with *C. rodentium* was further supported using a PepT1 transgenic mouse model, which was recently generated and characterized by our group²⁰. We found that overexpression of PepT1 in mice reduced colonic colonization of *C. rodentium* *ex vivo* and *in vivo*. Remarkably, compared to WT mice, PepT1 transgenic mice exhibited a decrease in *C. rodentium*-induced production of the murine CXC chemokine KC and other pro-inflammatory cytokines in the colon. This may account for the reduction of *C. rodentium*-induced neutrophil infiltration into the colon of PepT1 transgenic mice. However, other mechanisms might also be involved, as increased colonic anti-inflammatory cytokine production levels were observed in PepT1 transgenic mice, suggesting that endogenous anti-inflammatory mechanisms are more effectively activated in these mice compared to WT mice upon *C. rodentium* infection. Together, our studies indicate that PepT1 may participate in modulating bacterial-epithelial interactions and bacteria-induced inflammation. Further studies using other mouse models, such as PepT1 knockout mice, are required to obtain more information about the specific role of PepT1 in colonic responses to bacterial infection.

In conclusion, our study demonstrates that: i) EPEC transcriptionally induces functional PepT1 expression in LRs of colonocytes, ii) EPEC induces PepT1 expression by intimately attaching to host cell membranes through LRs, iii) the transcription factor Cdx2 is crucial for EPEC-induced PepT1 expression, and iv) PepT1 associated with LRs has a role in bacterial-epithelial interaction and bacteria-induced intestinal inflammation. Our findings reveal a novel mechanism underlying the regulation of colonic PepT1 expression/function under pathological conditions and point out the potential contribution of this transporter to host defense mechanisms in response to pathogen infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. EPEC induces PepT1 expression and transport activity in HT29-Cl.19A cells
(A) PepT1 promoter activity assessed by measuring luciferase activity at different time points of EPEC infection (normalized to *Renilla* luciferase activity). **(B)** PepT1 mRNA expression assessed by RT-PCR. **(C)** *In vitro* transcription run-on assay performed on nuclei isolated from uninfected and infected cells. **(D)** Expression of PepT1 and monocarboxylic transporter-1 (MCT-1), used as a control, assessed by Western blot. **(E)** Representative confocal microscopy images of immunofluorescence staining for PepT1 in non-polarized HT29-Cl.19A cells on cover slips (i) or polarized cells on filter supports (ii). Bars, 10 μm. **(F)** PepT1-mediated [³H] KP_V uptake in HT29-Cl.19A monolayers. Data are means ± S.E.M of three determinations. (***P* < 0.005; ****P* < 0.001) vs uninfected cells.

Figure 2. Cdx2 is important for EPEC-induced PepT1 expression

HT29-Cl.19A cells were infected with EPEC and assessed for Cdx2 expression by RT-PCR (A) and Western blot (B). The binding of Cdx2 to PepT1 promoter was analyzed by EMSA (C) and ChIP assay (D). EMSA was performed using a Cdx2⁻⁵⁷⁹-specific probe. Specificity of complexes was verified using 200-fold excess of unlabeled probe and by supershift experiment using Cdx2 antibody. ChIP was performed using Cdx2 antibody and Cdx2⁻⁵⁷⁹-specific primers. (E) Cells were transfected with Cdx2 plasmid (HT29-Cl.19A/Cdx2) or the empty vector (HT29-Cl.19A/vector). Cdx2 and PepT1 expression levels in wild-type HT29-Cl.19A, HT29-Cl.19A/Cdx2 and HT29-Cl.19A/vector cells were analyzed by RT-PCR and Western blot. (F) HT29-Cl.19A cells transfected with scramble or Cdx2 siRNA for 24 h were

infected with EPEC for 6 h. Cdx2 and PepT1 expression was assessed by Western blot. All experiments were repeated three times.

Figure 3. EPEC induces PepT1 expression in lipid rafts of HT29-Cl.19A cells

(A) Cells were infected with EPEC for 6 h, treated or not with 10 mM methyl-β-cyclodextrin (mβCD), lysed, and subjected to sucrose-gradient fractionation. Equal total protein amounts of the gradient fractions were analyzed for PepT1 and GM1 distribution by Western blot and dot blot, respectively. (B) EPEC-infected cell lysates were immunoprecipitated using PepT1 antibody or the isotype IgG₁. Immunoprecipitates were analyzed by dot blot to detect GM1. (C) EPEC-infected cells were treated with mβCD and replenished or not with cholesterol. PepT1-mediated [³H]KPV uptake was measured. Values are means ± S.E.M of three determinations. **P* < 0.05; ***P* < 0.005.

Figure 4. EPEC-induced PepT1 expression requires Tir, intimin and intact host lipid rafts
(**A, B**) HT29-Cl.19A cells were infected with WT EPEC or EPEC mutants deficient in Tir (Δtir) or intimin (Δeae) for 6 h. Western blot analysis of PepT1 expression (**A**) and PepT1-mediated $[^3\text{H}]$ KPV uptake (**B**). (**C, D, E**) Cells were treated with 10 mM mβCD and replenished or not with 2 mM cholesterol prior to EPEC infection. PepT1 expression and activity were assessed by RT-PCR (**C**), Western blot (**D**) and uptake experiments (**E**). Values are means $\pm$ S.E.M of three determinations. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Figure 5. PepT1 associated with lipid rafts attenuates EPEC adherence and EPEC-induced inflammation in HT29-Cl.19A cells

(A) EPEC adherence to HT29-Cl.19A cells over-expressing PepT1 (HT29-Cl.19A/PepT1) or the empty vector (HT29-Cl.19A/vector) at 4 h post-infection. (B) Western blot analysis for PepT1 distribution in the sucrose-gradient fractions. (C) EPEC adherence to lipid raft-coated electrodes monitored in real-time using the electric cell-substrate impedance sensing technique. The time required for EPEC to cover half of the available electrode ($t_{1/2}$) was determined. (D) Degradation of IκB-α and phosphorylation of IκB-α, ERK1/2 and p38 were assessed by Western blot. IL-8 mRNA and protein levels were analyzed by real-time RT-PCR (E) and ELISA (F). Values are means ± S.E.M of three determinations. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Figure 6. *Citrobacter rodentium* induces PepT1 expression in mouse colon

(A, B) Cultured mouse colonic tissues were infected with *C. rodentium* for 6 h and analyzed for PepT1 expression by real-time RT-PCR (A) and Western blot (B). (C, D) For infection of mice, food was withdrawn and drinking water was replaced with *C. rodentium* suspension in 20% sucrose distilled water overnight. Mice were euthanized 7 days after infection. PepT1 expression in mouse colon was analyzed by real-time RT-PCR (C) and Western blot (D). Data are means $\pm$ S.E.M. * $P < 0.05$; *** $P < 0.001$. All experiments were repeated twice with $n = 9$ (A, B) or $n = 10$ (C, D) per condition for each experiment.

Figure 7. PepT1 overexpression reduces *C. rodentium* adherence and *C. rodentium*-induced cytokine production in cultured mouse colons
 Cultured colonic tissues from WT and PepT1 transgenic mice were infected *ex vivo* with *C. rodentium* for 6 h. (A) *C. rodentium* adherence to cultured colonic tissues. KC mRNA and protein levels were determined by real-time RT-PCR (B) and ELISA (C), respectively. Data are means ± S.E.M. **P* < 0.05; ***P* < 0.005; ****P* < 0.001. All experiments were repeated twice with n = 9/group/condition for each experiment.

Figure 8. PepT1 transgenic mice exhibit decreased susceptibility to *C. rodentium* infection compared to WT mice

WT and PepT1 transgenic mice were infected with *C. rodentium* and euthanized at 7 days post-infection. (A) *C. rodentium* colonization levels in the colon. (B) Neutrophil infiltration into the colon quantified by measuring MPO activity. (C-i) Representative H&E-stained distal colonic sections. Original magnification, $\times 20$. (C-ii) Neutrophils were identified by their distinctive nuclear morphology and counted in 10 crypts per colon. The mean number of neutrophils per crypt was calculated for each colon observed. (D-i) KC mRNA levels in the colon determined by real-time RT-PCR. (D-ii) KC levels in serum determined by ELISA. Colonic mRNA levels of pro-inflammatory (E) and anti-inflammatory (F) cytokines

determined by real-time RT-PCR. Data are means $\pm$ S.E.M. $*P < 0.05$; $**P < 0.005$; $***P < 0.001$. All experiments were repeated twice with $n = 9$ /group/condition for each experiment.