Interferon- γ Increases hPepT1-Mediated Uptake of Di-Tripeptides Including the Bacterial Tripeptide fMLP in Polarized Intestinal Epithelia

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Interferon- γ causes a global phenotypic switch in intestinal epithelial function, in which enterocytes become immune accessory cells. The phenotypic switch is characterized by a down-regulation of membrane transporters and up-regulation of immune accessory molecules in intestinal epithelial cells. However, the effect of interferon- γ on the intestinal epithelia di-tripeptide hPepT1 transporter has not been investigated. In this study we demonstrate that 1) interferon- γ increases di-tripeptide uptake in dose- and time-dependent manner in model intestinal epithelia (Caco-2 BBE cell monolayers), 2) the increase in di-tripeptides induced by interferon- γ is hPepT1 mediated, 3) interferon- γ does not affect the hPepT1 expression at the mRNA and protein levels 4) interferon- γ increases the intracellular pH and consequently enhances the H⁺-electrochemical gradient across apical plasma membrane in model intestinal epithelia (Caco-2 BBE monolayers). We suggest that interferon- γ could increase the hPepT1 mediated di-tripeptides uptake in inflamed epithelial cells. Under these conditions, interferon- γ will increase the intracellular amount of such diverse prokaryotic and eucaryotic small di-tripeptides in inflamed epithelial cells. The intracellular accumulation of such di-tripeptides may be important in enterocytes becoming immune accessory cells. (*Am J Pathol* 2003, 163:1969–1977)

In intestinal epithelial cells, interferon- γ regulates expression of proteins such as major histocompatibility complex class I and II antigens,^{1–3} polymeric immunoglobulin binding receptors,^{4,5} and intercellular adhesion molecules (ICAM1),⁶ and also affects tight junctional permeability.^{7,8} More recently it has been reported that interferon- γ is able to modulate neutrophil-epithelial interactions.⁹ Furthermore, it has been reported that interferon- γ down-regulates membrane transporters such as the co-transporter NaKCl₂, Cl[−] channel CFTR, K⁺ channel(s), Na⁺/H⁺ exchangers

(NHE2 and NHE3), and Na⁺-dependent glucose absorption in T84 or Caco2 cells.^{10–12} In addition, it has also been demonstrated that interferon- γ reduces mucin exocytosis in a human colonic goblet cell line.¹³ Overall it has been suggested that interferon- γ causes a global phenotypic switch in intestinal epithelial function, in which enterocytes become immune accessory cells.¹ Although enterocytes are not conventional antigen-presenting cells, they probably play a role in antigen transport and in antigen presentation to the lamina propria lymphocytes.

One normal transport function expressed by gut epithelial cells is the absorption of small peptides from diet by an apical membrane peptide transporter. *In vivo* and *in vitro* evidence showed that the oligopeptide transport is either mostly or entirely responsible for the clearance of dipeptides but not for free amino acids and oligopeptides with more than three amino acid residues from the lumen of human intestine.¹⁴ Several investigators have provided evidence for Caco2 cell line as an appropriate cellular model for studies of solute transport by small intestine *in vitro*. The use of these cells as a model of human intestine was validated by the following studies. The studies of kinetics of uptake as function of dipeptides concentration, as in the intestinal brush border membrane vesicles (BBMV), showed the presence of a single transport system with a K_m of ~ 1 mmol/L, suggesting similarity between the functional expression of the oligopeptide transporters in Caco2 cells and in BBMV prepared from human intestine. Recently a cDNA encoding an apical membrane protein, accounting for this peptide transport capability, has been cloned (hPepT1 human).¹⁵ PepT1 was identified as an integral membrane-spanning protein that is expressed predominantly in the small intestine.^{16–18} *In vivo* and *in vitro* studies have shown that: 1) hPepT1 transports dipeptides and tripeptides but not free amino acids or peptides with more than three amino acid residues, 2) driving force of hPepT1 for uphill transport requires proton binding and the presence of an inside-negative membrane potential (as naturally occurs). More

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recently, the expression of the hPepT1 in complementary RNA-injected *Xenopus* Laevis oocytes has been studied. By measuring evoked currents, investigators found that Gly-Sar glycylsarcosine uptake was voltage-dependent over the entire range of membrane potentials tested. The apparent K_m for Gly-Sarc was 0.7 mmol/L over a pH range of 5.0 to 6.0.¹⁴ This K_m is comparable to K_m (1.1 mmol/L) of Gly-Sarc uptake by Caco2 cells.¹⁴ hPepT1 message is predominantly found in the small intestine.¹⁸ Expression was not detected in the esophagus, stomach, or colon.^{16,18} However, under inflammatory states, colonic hPepT1 expression occurs in inflammatory bowel disease.¹⁶ hPepT1 protein is expressed in Caco2-BBE cells but not in HT29-Cl.19A cells.¹⁶ These observations, with respect to hPepT1 expression, are consistent with the view that Caco2-BBE is a model small intestinal-like cell line and that HT29-Cl.19A is a model colonic-like cell line. These established *in vitro* models that mimic the normal small and the normal large intestine are used in the present study. hPepT1 co-transporters peptides with H^+ ,¹⁹ and has a broad specificity that includes many di- and tripeptides. Not only do these peptide substrates vary with respect to net charge and solubility, they also cover a wide range of molecular weights from 96.2 to 522 kd. Not all small peptides in the gut lumen are nutrient-related. For example, in association with luminal bacteria in the gut, a substantial array of N-formyl peptides (including formyl-Met-Leu-Phe) are normally present.^{20,21} We have demonstrated that hPepT1-mediated epithelial transport of bacteria-derived chemotactic peptides fMLP²² and we have shown that PepT1-mediated fMLP transport induces intestinal inflammation *in vitro* and *in vivo*.^{16,23} In addition, transport of bacterial peptides by intestinal epithelial cells influences the expression of major histocompatibility complex (MHC) class I molecules.²⁴ Overall, PepT1 may play an important role in intestinal inflammation.

In the present study, we have investigated the effect of interferon- γ on the intestinal hPepT1-mediated di-tripeptides. Interferon- γ , as mentioned above, causes a global phenotypic switch in intestinal epithelial function, enterocytes becoming immune accessory cells. Interestingly, it is known that interferon- γ increases peptide transporter transporter-associated antigen processing (TAP) expression and peptide transport capacity in endothelial cells and in epithelial cells (personal observation, D. Merlin and X. Liu).²⁴ TAP belongs to a large family of integral membrane transporters that possess a cytoplasmic ATP-binding cassette and multiple hydrophobic regions that are thought to form a transmembrane channel.^{24,25} TAP is located predominantly in the ER, the site of MHC class I assembly, and binds transiently to nascent MHC class I heavy-chain β 2-microglobulin complexes.²⁵ Increased TAP expression leads to increased class I surface expression and decreased TAP function leads to decreased class I surface expression, suggesting that peptides transported by TAP contribute to regulation of the surface expression of class I molecules. We speculated that PepT1 transporter may play an upstream event in this context. In this study, we addressed whether interferon- γ could increase the hPepT1-mediated di-tripeptide uptake in inflamed epithelial cells. Under these conditions,

interferon- γ will increase the intracellular amount of such diverse prokaryotic and eucaryotic small di-tripeptides in inflamed epithelial cells. The intracellular accumulation of such di-tripeptides may be important in intracellular events in enterocytes becoming immune accessory cells.

Materials and Methods

Cell Culture

Caco2-BBE^{26–28} or HT29-Cl.19A cells²⁹ (between passage 20 and 40) were grown in high-glucose Dulbecco's Vogt modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 14 mmol/L $NaHCO_3$, 10% fetal bovine serum, and penicillin/streptomycin (100 U/100 μ g/ml; Invitrogen). Cells were kept at 37°C in 5% CO_2 and 90% humidity, and medium was changed every day. When confluent in 75 cm^2 flasks, cells were trypsinized and plated at a density of 5×10^4 cells/ cm^2 on collagen-coated permeable support (Transwell-Clear 1- cm^2 polyester membranes with 0.4- μ m pores; Costar, Corning, NY) for uptake and biotinylation studies.

For protein, membrane, or RNA extractions, cells were plated on 6-well cluster trays at a density of 10^5 cells/ cm^2 . HT29-Cl.19A transfected with Green Fluorescent Protein (GFP) alone or transfected with hPepT1-tagged GFP²⁰ were grown in the same medium described previously supplemented with 1.2 mg/ml G418 (Invitrogen). Cells grown on filters were used 15 days after seeding, and incubated with medium without serum and without antibiotics during the IFN- γ treatment.

Monolayer Treatments

Recombinant IFN- γ (produced in *Escherichia coli*, specific activity of 2×10 U/mg) was obtained from Genentech (San Francisco, CA) and was essentially free of endotoxin at the concentrations used. IFN- γ at indicated doses and for indicated time was added to the basolateral aspect of the monolayers in medium free of serum. Glibenclamide (ICN Biomedicals, Inc., Aurora, OH) was added both in the apical and basolateral compartment at the final concentration of 50 μ mol/L during the 15-minute uptake experiment. Phorbol 12-myristate 13-acetate (PMA) (Biomol, Inc., Plymouth, PA) was pre-incubated at the apical and basolateral sides for 2 hours at 37°C at the concentration of 1 μ mol/L then uptake experiments were performed for 15 minutes. 2-deoxy-D-glucose (50 mmol/L) with NaN_3 (3 mmol/L) (σ -Aldrich Corp, St. Louis, MO) were used to inhibit active transport during the uptake experiment. For Na^+ -free solution was prepared by replacing NaCl with equimolar choline chloride (σ -Aldrich Corp) and omission of NaH_2PO_4 .

Confocal Immunofluorescence

Caco2-BBE cells grown on filters were washed twice in Hanks' balanced salt solution (HBSS) (pH 7.4), and then fixed with 3.7% paraformaldehyde in HBSS with calcium

(pH 7.4) (HBSS⁺). Caco2-BBE cells were permeabilized with 0.5% Triton for 30 minutes at 25°C. The cells were rinsed and incubated with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) diluted 1:60 for 40 minutes. Microscopy was performed using a Zeiss epifluorescence microscope equipped with a Bio-Rad MRC600 confocal unit, computer, and laser scanning microscope image analysis software (Carl Zeiss, Jena, Germany).

Uptake Experiments

Cells grown on filters were washed twice with HBSS (α -Aldrich Corp.) complemented with 10 mmol/L HEPES (pH 7.4) in the basolateral compartment, and with 10 mmol/L MES (pH 6.2) in the apical compartment, and stabilized 15 minutes in the same buffers at 37°C. Then Caco2-BBE monolayers were incubated in HBSS-10 mmol/L MES (pH 6.2), containing 20 μ mol/L Gly-Sar [¹⁴C] (specific activity 50 mCi/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO) in apical compartment for 15 minutes or 2 minutes at 37°C or 4°C. The same experiments were performed with 0.150 μ mol/L fMLP [³H] (specific activity: 80 mCi/mmol, NEN Life Science Products, Inc., Boston, MA). The supernatant was then removed, and cells were washed twice with ice-cold HBSS-HEPES (pH 7.4). Cell-associated radioactivity was determined by liquid scintillation in a β counter.

Cell Surface Biotinylation

Filter-grown cells were rinsed twice with PBS supplement with 0.1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Apical sides of the monolayers were incubated with freshly prepared sulfo-succinimidobiotin (s-NHS-biotin; Pierce, Rockford, IL) diluted in PBS supplement with 0.1 mmol/L CaCl₂ and 1 mmol/L MgCl₂ (0.5 mg/ml) for 30 minutes at 4°C. The reaction was quenched with 50 mmol/L NH₄Cl, and cells were lysed with a solution of 1% (w/v) Triton X-100 in 20 mmol/L Tris, pH 8.0, 50 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.2% bovine serum albumin (BSA) supplemented with protease inhibitors (lysis buffer). After 30 minutes centrifugation (13000 $\times$ g, at 4°C), the supernatant was incubated with streptavidin-agarose (Pierce) overnight at 4°C to bind biotinylated proteins. After centrifugation, the beads were washed twice in 20 mmol/L Tris-HCl, pH 8.0. The protein solution was then boiled 5 minutes at 100°C in Laemmli buffer supplemented with 0.5% β -mercaptoethanol and Western blot analysis were performed.

Western Blot Analysis

For total protein extraction, Caco2-BBE cells pellets were homogenized at 4°C in lysis buffer supplemented with 0.1 mg/ml phenylmethylsulfonyl fluoride, 100 μ mol/L aprotinin, and 100 mmol/L NaVO₄. The homogenates were centrifuged at 15,000 $\times$ g for 30 minutes at 4°C and the supernatants collected for Western blot analysis. Protein concentration was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Briefly, 20

μ g of proteins was solubilized in electrophoresis sample buffer containing β -mercaptoethanol and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gel (Bio-Rad) and then transferred to nitrocellulose membranes. The blots were blocked overnight with 5% nonfat milk in blocking buffer, and the blots were incubated 1 hour with a 1:10,000 dilution of a rabbit polyclonal anti-hPepT1²⁰ or a goat polyclonal anti-villin (Santa Cruz Biotechnology, Santa Cruz, CA). After washing three times for 15 minutes in blocking buffer, blots were further incubated for 1 hour with the anti-rabbit horseradish peroxidase-conjugated antibody diluted 1:2000. The nitrocellulose was washed three times for 20 minutes in blocking buffer and then probed using a chemiluminescence system (ECL; Amersham Biosciences, Piscataway, NJ).

Northern Blot Analysis

Total RNA was isolated from Caco2 cells with TRI Reagent (Molecular Research Center, Inc., Cincinnati OH). Total RNA (20 μ g) was denatured by heating at 65°C in 20 mmol/L HEPES (pH 7.2), 1 mmol/L EDTA, 50% formamide, and 6% formaldehyde for 15 minutes, and subjected to electrophoresis on a 1% agarose gel containing 2% formaldehyde. Resolved RNA was transferred to a nylon membrane (PerkinElmer Life Sciences, Boston, MA) and covalently cross-linked by exposure to UV light. Hybridization was performed in a solution that contained 7% SDS, 1% BSA, 10% polyethylene glycol 8000, 250 mmol/L NaCl, 1.25 mmol/L EDTA, 125 mmol/L NaPO₄, and 1 mg of salmon sperm DNA (Ambion, Inc., Austin, TX). hPepT1 cDNA probe (2.6 kb) was labeled with [α -P³²]CTP using Rediprime II random primer labeling system (Amersham Biosciences). A GAPDH cDNA probe was used as control (Ambion, Inc.).

Intracellular pH Measurement in Confluent Monolayers of Caco2-BBE

Polycarbonate sheets (1.7-mm thick) were machined to precisely fit diagonally across standard fluorescence cuvettes (Sarstedt, Newton, NC) and included a 1.0 $\times$ 1.5-cm window cut out of the polycarbonate to incorporate the area targeted by the excitation beam of a Hitachi (Sunnyvale, CA) F-4500 fluorescence spectrometer. Transparent polyester filters (Corning Materials, Corning, NY) (0.4- μ mol/L pore size) were mounted over the window and coated with collagen. Caco2-BBE cells were plated on these filters at high density (10⁵/cm²) and allowed 7 to 10 days to become confluent polarized model intestinal epithelia. These cells and filter apparatus were then washed 3 times in HBSS, and placed into HBSS containing 5 μ mol/L 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethylester (BCECF-AM) (AM; Molecular Probes) for 60 minutes at 37°C, followed by three more washes. The bottom and side edges of the polycarbonate piece were then coated with vacuum grease, and the assembly was placed into a fluorescence cuvette. One milliliter of HBSS was subsequently

placed into the basolateral reservoir of the model intestinal epithelia. After verifying the integrity of the grease seal by demonstrating that the HBSS did not visibly leak over to the apical aspect, 1 ml of HBSS was added to the apical reservoir of model epithelia. BCECF-loaded model epithelia was then incubated for an additional 10 minutes to permit diffusional washout of BCECF sequestered in the extracellular spaces. The HBSS (both apical and basolateral) was then aspirated out and fresh HBSS was added (1 ml HBSS at pH 7.2 to the basolateral side, 1 ml HBSS at pH 6.2 to the apical side) without disturbing the grease seal. BCECF-loaded epithelia were placed into a spectrofluorometer (thermostatted to 37°C) such that the apical surface faced (at a 45° angle) the excitation beam. A 4-mm stir bar (8-mm stir bar cut in half; Fisher Scientific Co., Pittsburgh, PA) was then placed in the aspect (apical) that was to receive addition of 10 mmol/L Gly-Sarc. Intracellular H⁺ concentration was quantified by fluorescence (excitation at 450/500 nm and emission at 530 nm) using a spectrofluorometer (F-4500 Hitachi Scientific Instruments). Intracellular BCECF fluorescence was converted to pH by comparison with values from calibration curve using a high K⁺ (140 mmol/L) and low Na⁺ (4 mmol/L) solution in a cell-free system.¹⁹

Statistics

Results are expressed as means ± SE. Statistical significance was determined using one-way analysis of variance followed by the Tukey multiple comparison test. Differences were statistically significant with *P* < 0.05.

Results

Interferon-γ Increases Di-Tripeptide Uptake in Caco-2 BBE Monolayers in a Dose- and Time-Dependent Manners

To investigate the effect of IFN-γ on di-peptides uptake, 15-day Caco2-BBE monolayers were incubated in a serum-free medium in the presence of various concentrations of IFN-γ (0.1, 1, 10, 100, 1000 IU/ml) added to the basolateral compartment for 48 hours. Uptake experiments were performed as described in Materials and Methods. As shown in Figure 1A, IFN-γ induced a concentration-dependent increase of 20 μmol/L Gly-Sar uptake. The increase of Gly-Sar uptake began to occur at 0.1 IU/ml IFN-γ concentration (17% increase), was statically significant at 1 IU/ml IFN-γ concentration (26% increase), and achieved a 128% increase at 100 IU/ml IFN-γ concentration. The IFN-γ concentration corresponding to the half-maximal stimulation of 20 μmol/L Gly-Sar uptake was 1.4 ± 0.87 IU/ml (Figure 1A, inset). We next investigated the time course of IFN-γ-induced increase in 20 μmol/L Gly-Sar uptake (Figure 1B). The increase of di-peptide uptake by Caco2-BBE monolayers was about 57% after 24 hours of treatment with 100 IU/ml IFN-γ, and 92% after 48 hours of treatment with 100 IU/ml IFN-γ, and 62% after 72 hours treatment with 100 IU/ml IFN-γ. We also demonstrate that

Figure 1. A: Interferon-γ increases di-tripeptide uptake in Caco2-BBE monolayers in a dose-dependent manner. To investigate the effect of IFN-γ on dipeptide uptake, 15-day Caco2-BBE cells were incubated in a serum-free medium in the presence of various concentration of IFN-γ (0.1, 1, 10, 20, 100 IU/ml) for 48 hours in the basolateral compartment. Uptake experiments were performed as described in Materials and Methods; 20 μmol/L [¹⁴C] Gly-Sarc was used in these experiments. IFN-γ induced a concentration-dependent increase of Gly-Sar uptake. The concentration of IFN-γ producing a half-maximal stimulation of Gly-Sar uptake was 1.4 ± 0.87 IU/ml (inset). Each bar represents mean ± SEM of three experiments performed in triplicate. * *P* < 0.05 *vs.* CTRL. **B:** Interferon-γ increases di-tripeptide uptake in Caco2-BBE monolayers in a time-dependent manner. IFN-γ (100 IU/ml) treatment induced, respectively, an increase in di-peptide uptake (uptake experiments were performed as described in Materials and Methods; 20 μmol/L [¹⁴C] Gly-Sarc was used in these experiments) of 57% after 24 hours incubation, 92% after 48 hours, and 62% after 72 hours. Values represent means ± SEM of three experiments performed in triplicate *P < 0.05 *vs.* CTRL. **C:** Interferon-γ increases fMLP uptake by Caco2-BBE cell monolayers. A two-fold increase in fMLP uptake (uptake experiments were performed as described in Materials and Methods; 0.150 μmol/L [³H] fMLP was used in these experiments) was observed after a 48-hour IFN-γ treatment (100 IU/ml) when compared to Caco2-BBE cells without interferon-γ treatment. Each bar represents mean ± SEM of three experiments performed in triplicate. *P < 0.05 *vs.* CTRL.

Figure 2. Interferon- γ (100 IU/ml) treatment for 48 hours does not affect the polarity of Caco2-BBE monolayers. Confocal microscopy of localization of actin in Caco2-BBE monolayers stained with rhodamine phalloidin (red). Horizontal sections (xy) are near the apical plasma membrane (a), and near the basolateral membrane (b) and vertical (zy) section of polarized Caco2-BBE. Villin: filter-grown Caco2-BBE monolayers were subjected to apical membrane biotinylation for 30 minutes, followed by Western blot analysis. Biotinylated proteins were subjected to 7.5% SDS-PAGE, followed by transfer to nitrocellulose membrane. The blot was immunostained with anti-villin. Caco2-BBE cell monolayers were untreated (CTRL) or treated (INF- γ : 100 IU/ml interferon- γ for 48 hours).

100 IU/ml INF- γ for 48 hours increases of 0.150 $\mu\text{mol/L}$ of the tri-peptide fMLP uptake by twofold when compared to Caco2-BBE cells without interferon- γ treatment (Figure 1C). Since IFN- γ displayed the maximal effect under these conditions, the following experiments were performed after 48 hours of treatment with 100 IU/ml IFN- γ .

Interferon- γ (100 IU/ml) Treatment for 48 Hours Does Not Affect the Polarity of Caco2-BBE Monolayers

As shown in Figure 2, the apical expression of villin, which is one of the major brush border-associated cytoskeletal proteins, was not affected by 100 IU/ml interferon- γ treatment for 48 hours in Caco2-BBE monolayer. In addition, as depicted in Figure 2, the general actin network architecture of Caco2-BBE monolayers after interferon- γ treatment (100 IU/ml for 48 hours) remains similar to untreated Caco2-BBE monolayers. In addition, the height of Caco2-BBE cells was not affected by interferon- γ as determined by confocal scanning across Caco2-BBE monolayers (untreated Caco2-BBE monolayers: $18.00 \pm 2.16 \mu\text{m}$; Caco2-BBE monolayers treated with interferon- γ (100 IU/ml for 48 hours): $18.88 \pm 2.10 \mu\text{m}$; $n = 20$).

Interferon- γ Increases hPepT1-Mediated Di-Peptide Uptake

The uptake of 20 $\mu\text{mol/L}$ [^{14}C] Gly-Sar was reduced by 70% (0.35 ± 0.03 vs. $0.1 \pm 0.02 \text{ nmol/cm}^2/15 \text{ minutes}$, $P < 0.05$) in the presence of a large amount of cold substrate (50 mmol/L Gly-Sar) in Caco2-BBE control cells as well as in IFN- γ -treated cells (0.56 ± 0.007 vs. $0.17 \pm 0.03 \text{ nmol/cm}^2/15 \text{ minutes}$, $P < 0.05$) (Figure 3). When uptake experiments were performed at 4°C or in the presence of 2-deoxy-D-glucose (50 mmol/L), the 15 minutes Gly-Sar uptake was reduced 80% (0.15 ± 0.04 vs. $0.03 \pm 0.002 \text{ nmol/cm}^2/15 \text{ minutes}$; $P < 0.05$) and 35% (0.09 ± 0.005 vs. $0.06 \pm 0.003 \text{ nmol/cm}^2/15 \text{ minutes}$; $P < 0.05$), respectively in control cells and 75% (0.27 ± 0.05 vs. $0.07 \pm 0.006 \text{ nmol/cm}^2/15 \text{ minutes}$; $P < 0.05$) and 40% (0.17 ± 0.02 vs. $0.09 \pm 0.01 \text{ nmol/cm}^2/15 \text{ minutes}$; $P < 0.05$), respectively in IFN- γ -treated cells (Figure 3). Previous studies have demonstrated that incubation with PMA, an activator of protein kinase C, results in significant inhibition of hPepT1 mediated di-peptide uptake. In our experimental conditions, pre-incubation for 2 hours with PMA (1 $\mu\text{mol/L}$) resulted in a 40% decrease in Gly-Sar uptake in control cells (CTRL) and 30% decrease in IFN- γ -treated cell (CTRL: 0.2 ± 0.01 vs. 1.2 ± 0.005

Figure 3. Interferon- γ increases dipeptides uptake hPepT1 mediated. The uptake of 20 μ mol/L [^{14} C] Gly-Sar, as described in Materials and Methods, was reduced by 70% in the presence of a large amount of cold substrate (50 mmol/L Gly-Sar) in Caco2-BBE control cells (CTRL) as well as in IFN- γ -treated cells for 48 hours [IFN- γ (48 h)]. When uptake experiments were performed at 4°C or in the presence of 2-Deoxy-D-glucose, the 15-minute Gly-Sar uptake was reduced by 80% and 35% in control cells, and by 75% and 40% in IFN- γ -treated cells for 48 hours. Uptake of Gly-Sar in Caco2-BBE cells occurred via a Na $^{+}$ -independent process in control cells and also in IFN- γ -treated cells. Pre-incubation for 2 hours with 1 μ mol/L PMA resulted in a 40% decrease in Gly-Sar uptake in control cells and 30% in IFN- γ -treated cells. Glibenclamide induced a 20% reduction in Gly-Sar uptake in control cells but failed to induce any effect in IFN- γ -treated cells. Values represent means $\pm$ SEM of three experiments performed in triplicate. In control cells, * P < 0.05 vs. CTRL; in IFN- γ -treated cells, # P < 0.05 vs. CTRL.

nmol/cm ^{2} /15 minutes, P < 0.01; IFN- γ : 0.4 ± 0.04 vs. 0.27 ± 0.02 nmol/cm ^{2} /15 minutes, P < 0.05) (Figure 3). In accordance with the literature, uptake of Gly-Sar in Caco2-BBE cells occurred via a Na $^{+}$ -independent process in control cells (0.14 ± 0.02 vs. 1.2 ± 0.03 nmol/cm ^{2} /15 minutes) and in IFN- γ -treated cells (0.31 ± 0.06 vs. 0.36 ± 0.08 nmol/cm ^{2} /15 minutes) (Figure 3). Previous studies have suggested that glibenclamide was a noncompetitive inhibitor of peptide transporters, although this agent itself is not a substrate for these transporters.³⁰ Interestingly, 50 μ mol/L glibenclamide induced a 20% reduction in Gly-Sar uptake in control cells (0.2 ± 0.01 vs. 0.16 ± 0.01 nmol/cm ^{2} /15 minutes; P < 0.05) but failed to induce any effect in IFN- γ -treated cells (0.38 ± 0.03 vs. 0.40 ± 0.04 nmol/cm ^{2} /15 minutes) (Figure 3). INF- γ treatment induces structural changes in the transporter and prevents the interaction between glibenclamide and hPepT1. Together, these results demonstrate that INF- γ increases Gly-Sar uptake mediated by an active transport system, likely the hPepT1 transporter.

As previously described the HT29-Cl.19A cell line does not express hPepT1 either at the protein or RNA level. The established HT29-Cl.19A-GFP-hPepT1 transfected cell line constitutes an excellent model to study the involvement of hPepT1 in IFN- γ -induced increase in dipeptide uptake. Western blot analysis performed with the anti-hPepT1 antiserum confirmed the presence of an immunoreactive band at approximately 115 kd, corresponding to the fused protein GFP-hPepT1 in the HT29-Cl.19A-GFP-hPepT1 cells, the presence of an immunoreactive band at 90 kd, corresponding to hPepT1 protein in Caco2-BBE cells, and was not detected in the HT29-Cl.19A-GFP cells (Figure 4A). The small basal level of 20 μ mol/L Gly-Sar uptake in HT29-Cl.19A cells was not af-

Figure 4. **A:** Apical plasma membrane delivery of GFP-hPepT1 in stably transfected HT29-Cl.19A monolayers. Filter-grown Caco2-BBE, HT29-Cl.19A-GFP, and HT29-Cl.19A-GFP-hPepT1 (clone A and C that were used for the uptake experiments) monolayers were subjected to apical domain-specific biotinylation for 30 minutes followed by Western blot analysis. The blot was immunostained with anti-hPepT1 antibody. **B:** Interferon- γ does not increase dipeptides uptake in HT29-Cl.19A that does not express hPepT1. HT29-Cl.19A cell monolayers were pre-incubated with 100 IU/ml interferon- γ or vehicle for various times (0, 24, 48 hours) to the basolateral compartment. Then uptake experiments were performed as described in Materials and Methods. The amount of Gly-Sar accumulated by HT29-Cl.19A cell monolayer radioactivity was determined by liquid scintillation counting. Values represent means $\pm$ SEM of three experiments performed in triplicate. **C:** Interferon- γ increases dipeptide uptake in HT29-Cl.19A, which express the fused GFP-hPepT1 protein. HT29-Cl.19A-GFP or HT29-Cl.19A-GFP-hPepT1 cell monolayers were pre-incubated with 100 IU/ml interferon- γ or vehicle for 48 hours to the basolateral compartment. Then, uptake experiments of 20 μ mol/L [^{14} C] Gly-Sar with or without 50 mmol/L cold Gly-Sar were performed as described in Materials and Methods. The amount of [^{14} C] Gly-Sar accumulated by HT29-Cl.19A-GFP or HT29-Cl.19A-GFP-hPepT1 cell monolayer radioactivity was determined by liquid scintillation counting. Values represent means $\pm$ SEM of three experiments performed in triplicate.

ected by IFN- γ (100 IU/ml) treatment (0 hours: 0.032 ± 0.004 vs. 24 hours: 0.029 ± 0.003 vs. 48 hours: 0.025 ± 0.002 vs. 72 hours: 0.028 ± 0.003 nmol/cm ^{2} /15 minutes) (Figure 4B). Similarly, 20 μ mol/L Gly-Sar uptake was low in control GFP transfected cells, and no further increase was observed after IFN- γ treatment (CTRL: 0.038 ± 0.002 vs. IFN- γ : 0.04 ± 0.004 nmol/cm ^{2} /15 minutes). However, in HT29-Cl.19A cells transfected with GFP-hPepT1, the basal level of Gly-Sar uptake was significantly increased compared to HT29-Cl.19A and HT29-Cl.19A-GFP and furthermore a 15% increase was observed after IFN- γ treatment (CTRL: 0.045 ± 0.001 vs. IFN: 0.051 ± 0.02 nmol/cm ^{2} /15 minutes; P < 0.05). In HT29-Cl.19A, the transfection efficiency is about 15% to 20% and explains the fact that IFN- γ modestly increases uptake of Gly-Sar by HT29-Cl.19A transfected with hPepT1. IFN- γ -induced increase in Gly-Sar uptake in HT29-Cl.19A-GFP-hPepT1 transfected cells was totally abolished by the addition of an excess of cold Gly-Sar (0.034 ± 0.002 nmol/cm ^{2} /15 minutes; P < 0.05) (Figure 4C). Altogether these data demonstrated that IFN- γ -induced increase in di-peptide uptake required the presence of hPepT1.

Figure 5. A: Interferon- γ does not change hPepT1 mRNA expression. Caco2-BBE cell monolayers were pre-incubated with 100 IU/ml interferon- γ for 0, 12, 24, 48 hours. Northern blot analysis was performed on total RNA (20 μ g/lane) of Caco2-BBE cells. Using hPepT1 probe, 3.3-kb hybridizing signal was present in Caco2-BBE cells. The same blot was stripped and re-probed with the GAPDH cDNA. **B:** Interferon- γ does not change the hPepT1 protein expression in the apical membrane of Caco2-BBE monolayers. Caco2-BBE cell monolayers were pre-incubated with 100 IU/ml interferon- γ or vehicle for various time (0, 24, 48 hours) and subjected to apical cell surface biotinylation as described in Methods. Apical biotinylated proteins were subjected to 4% to 20% SDS-PAGE followed by transfer to nitrocellulose membrane. The blot was immunostained with a rabbit anti-hPepT1 (anti-hPepT1).

Increase of Di-Peptide Uptake Induced by IFN- γ Is Independent of hPepT1 mRNA and Protein Expressions

We performed northern and western blot to determine the total amount of PepT1 mRNA and hPepT1 protein. No change in hPepT1 mRNA was observed after IFN- γ -treated cells compared to control cells (Figure 5A). Furthermore, Figure 5B shows no increase in hPepT1 protein in the apical membrane after various periods (24 to 48 hours) of IFN- γ treatment compared to control cells.

Increase in Gly-Sar Uptake Induced by IFN- γ Is Mediated by an Augmentation of the Basal H^+ -Electrochemical Gradient Across Apical Plasma Membrane in Caco2-BBE Monolayers

The role of the transmembrane H^+ gradient on IFN- γ -induced increase in Gly-Sar uptake was further analyzed by studying the influence of apical extracellular pH on the uptake in control and IFN- γ -treated cells. Changing the pH of the medium from 7.4 to 5.4 stimulated 20 μ mol/L Gly-Sar uptake fivefold in control cells (CTRL: pH 7.4, 0.086 ± 0.003 vs. pH 5.4, 0.462 ± 0.04 nmol/cm²/15 minutes; $P < 0.001$) and sixfold in IFN- γ -treated (100 IU/ml for 48 hours) cells (IFN- γ : pH 7.4, 0.168 ± 0.01 vs. pH 5.4, 1.06 ± 0.12 nmol/cm²/15 minutes; $P < 0.001$) demonstrating the requirement of an inwardly directed H^+ gradient across the cell membrane in control as well as in IFN- γ -treated cells. Moreover, for each of the apical extracellular pH studied, the IFN- γ treatment induced a two-fold increase in Gly-Sar uptake (Figure 6A). We next hypothesized that the increase in the maximal transport rate could be due to alterations of the PepT1 driving

Figure 6. A: IFN- γ -induced increase in Gly-Sar uptake is pH sensitive. Changing the pH medium from 7.4 to 5.4 stimulated 20 μ mol/L Gly-Sar uptake, as described in Materials and Methods, fivefold in control cells and sixfold in IFN- γ -treated cells. Values represent means $\pm$ SEM of three experiments performed in triplicate. In control and in IFN- γ -treated cells (* $P < 0.05$ vs. CTRL). **B:** Interferon- γ increases H^+ -electrochemical gradient across apical plasma membrane in Caco2-BBE monolayers. Bar graph representing the intracellular pH in untreated or interferon- γ -treated (100 IU/ml for 48 hours) Caco2-BBE monolayer before and after the addition of 10 mmol/L Gly-Sar to the apical surface (at pH 6.2) of BCECF-loaded Caco2-BBE monolayers. Values represent means $\pm$ SEM of three experiments performed in triplicate. * $P < 0.05$ vs. CTRL. **C:** Bars showing the Δ pH_i induced by Gly-Sar in untreated (CTRL) and in interferon- γ -treated (100 IU/ml for 12, 24, and 48 hours) Caco2-BBE monolayers. Values represent means $\pm$ SEM of three experiments performed in triplicate. * $P < 0.05$ vs. CTRL.

force. Since the transmembrane pH gradient across the apical plasma membrane is required for the H^+ -coupled uptake of oligopeptide mediated by PepT1, IFN- γ may stimulate Gly-Sar absorption indirectly by alternating of the proton driving force.

We therefore examined if IFN- γ altered the intracellular pH (pH_{in}) in Caco2 cells, which is a critical determinant of the driving force of PepT1. The measurement of pH_{in} of Caco2-BBE cells revealed that IFN- γ (100 IU/ml for 48 hours) treatment induced increase in basal pH_{in} (6.7 ± 0.025 vs. 6.8 ± 0.025 ; $P < 0.05$). Moreover the addition of 10 mmol/L Gly-Sar in the apical side of the monolayer caused an expected decline of pH_{in} in control (6.7 ± 0.025 vs. 6.6 ± 0.029 ; $P < 0.05$) as well as in IFN- γ -treated cells (6.8 ± 0.025 vs. 6.6 ± 0.025 ; $P < 0.05$) (Figure 6B). Figure 6C is showing the Δ pH_i induced by 10 mmol/L Gly-Sar in untreated (CTRL) and in IFN- γ -treated Caco2-BBE monolayers. In addition, we investigated the time course of IFN- γ -induced increase of Δ pH_i induced by 10 mmol/L Gly-Sar. As shown in Figure 6C, Δ pH_i induced by 10 mmol/L Gly-Sar occurs at 100 IU/ml IFN- γ for 12 and was statistically significant at 100 IU/ml IFN- γ for 24 hours and achieved a maximal increase at 100 IU/ml IFN- γ for 48 hours.

Discussion

Interferon- γ is a soluble factor released by mucosal activated immune cells and plays an important role in intestinal epithelial cell functions. It is well known that interferon- γ down-regulates key intestinal epithelia membrane transporters such as NaKCl₂, Cl⁻ channel CFTR, K⁺

channel(s), Na^+/H^+ exchangers (NHE2 and NHE3), Na^+ -dependent glucose absorption.^{10–12} In contrast interferon- γ up-regulates the intestinal immune functions such as MHC class I and II, adhesion molecules such as CD98, and ICAM-1.^{6,31} Interestingly, interferon- γ up-regulates at the mRNA and protein levels the intracellular transporter TAP (transporter-associated antigen processing) (personal observation, D. Merlin and X. Liu) and peptide transport capacity in endothelial cells.²⁴ TAP consists of two proteins, TAP1 and TAP2, located predominantly in the ER, which play an important role in providing antigenic peptides for presenting MHC class I.²⁵

Interestingly, the di-tri-peptide transporter hPepT1 that is normally not expressed in colonic epithelial cells is expressed under inflammatory states.¹⁶ Mucosal surfaces of the colonic epithelial cells are constantly exposed on the luminal surface to foreign antigens, while at the same time intimately associated with immune system via lymphoid tissue at the basolateral surface. In association with luminal bacteria in the colon, a substantial array of N-formyl peptides (including N-formyl-Met-Leu-Phe) and other small di-tripeptide bacteria products are normally present. In normal colon that does not express hPepT1, these small peptides are not transported into the cytoplasm of colonic epithelial cells. However, in disease states such as inflammation, hPepT1 is up-regulated and consequently luminal N-formyl peptides and other di-tripeptides are potentially transported by hPepT1. It is likely that N-formyl peptide concentrations are substantially lower in the small intestine than in colon, in parallel with a lower mass of prokaryotes present in this site in humans. However, in diseases such as the clinical syndrome of bacterial overgrowth in the human small intestine, N-formyl peptide concentration is likely to be increased and may be transported by hPepT1.

In the present study we demonstrate that under our experimental conditions interferon- γ does not affect the cell polarity and increases the transport activity of hPepT1-mediated di-tripeptides in polarized cells expressing hPepT1. In contrast, interferon- γ had no effect on di-tripeptides uptake in epithelial cells that do not express hPepT1. In addition, interferon- γ does not affect hPepT1 expression at either RNA or the protein levels. Together these results suggest that under conditions of inflammation, colonic epithelial cells express hPepT1¹⁶ and that hPepT1-mediated di-tripeptide uptake, including fMLP, is increased by interferon- γ .

We demonstrate that intracellular pH in Caco2-BBE monolayers is increased by interferon- γ treatment and this enhances the H^+ electrochemical gradient across the intestinal apical plasma membrane. In addition, we show that the time course of IFN- γ -induced increase in 20 $\mu\text{mol/L}$ Gly-Sar uptake and in intracellular pH is similar. It has been reported that the major regulatory mechanisms for intracellular pH are located in the basolateral membrane of the enterocytes. We speculate that interferon- γ decreases the activity of an acid loader transporter and consequently increases the intracellular pH. One candidate could be the $\text{Cl}^-/\text{HCO}_3^-$ exchanger that is located on the basolateral membrane of epithelial cells.³² Furthermore, our results demonstrate that under interferon- γ

treatment, $\text{H}^+/\text{di-tripeptide}$ absorption across the human intestinal epithelium is likely not regulated via a functional Na^+/H^+ exchanger since interferon- γ down-regulates expression of Na^+/H^+ exchangers, NHE2, and NHE3, in Caco2-BBE¹⁰ and that in accordance with the literature we found that uptake of Gly-Sar in Caco2-BBE cells occurred via a Na^+ -independent process in control cells and in IFN- γ -treated cells.

Interferon- γ that is present at high levels in inflammatory bowel disease (IBD) tissues causes a phenotypic shift by enterocytes preparing them for a role in host defense. In this context, hPepT1-mediated uptake of small antigenic peptides may play a role in the enterocytes becoming immune accessory cells. For example, small N-formyl peptides are known to be bacterial products, and are supposed to be marginally present in the cytoplasm of eucaryotic cells. In normal conditions the endogenous supply of N-formyl peptides in eucaryotic cells is limited by the amount gleaned from degradation of mitochondria. N-formyl-peptides cannot be transported from the mitochondria to the cytoplasm, because no such transporter has been described as of yet. N-formyl-peptides can be considered antigenic molecules by eucaryotic cells (because it contains an N-formylated N terminus). Presence of intracellular prokaryotic products, considered as antigenic by eucaryotic cells, by itself may lead to signals that influence surface expression of immune effector cells. In addition, it has been shown that the mouse MHC class I molecules, including H2-M3, present fMLP to the cell surface in a TAP-dependent or independent manner.³³ It has been reported that TAP is important, but not absolutely required, for transporting N-formylated peptides into the ER. TAP, however, is essential for maintaining the intracellular pool of M3.³⁴ Interestingly, as mentioned above, TAP is up-regulated under condition of intestinal inflammation; however, a human homologue of H2-M3 has yet to be reported. Lack of sequence homology to M3 in the human does not preclude functional equivalence as seen in the case of MHC class Ib molecules Qa1 and HLA, which bind leader peptides from MHC class Ia molecules in mice and humans, respectively.

In conclusion, hPepT1 may play a role in inflammation by providing a large spectrum of small eucaryotic and prokaryotic di-tripeptides to the intracellular compartment of enterocytes. The intracellular accumulation of such peptides may be important in the enterocytes becoming immune accessory cells.

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