hPepT1-mediated Epithelial Transport of Bacteria-derived Chemotactic Peptides Enhances Neutrophil–Epithelial Interactions

Didier Merlin,* Angela Steel,‡ Andrew T. Gewirtz,* Mustapha Si-Tahar,* Matthias A. Hediger,‡ and James L. Madara*

**Department of Pathology, Emory University School of Medicine, Atlanta, Georgia 30322; and ‡Renal Division, Harvard Medical School, Boston, Massachusetts 02115*

Abstract

Intestinal epithelial cells express hPepT1, an apical transporter responsible for the uptake of a broad array of small peptides. As these could conceivably include *n*-formyl peptides, we examined whether hPepT1 could transport the model *n*-formylated peptide fMLP and, if so, whether such cellular uptake of fMLP influenced neutrophil–epithelial interactions. fMLP uptake into oocytes was enhanced by hPepT1 expression. In addition, fMLP competitively inhibited uptake of a known hPepT1 substrate (glycylsarcosine) in hPepT1 expressing oocytes. hPepT1 peptide uptake was further examined in a polarized human intestinal epithelial cell line (Caco2-BBE) known to express this transporter. Epithelial monolayers internalized apical fMLP in a fashion that was competitively inhibited by other hPepT1 recognized solutes, but not by related solutes that were not transported by hPepT1. Fluorescence analyses of intracellular pH revealed that fMLP uptake was accompanied by cytosolic acidification, consistent with the known function of hPepT1 as a peptide H^+ cotransporter. Lumenal fMLP resulted in directed movement of neutrophils across epithelial monolayers. Solutes that inhibit hPepT1-mediated fMLP transport decreased neutrophil transmigration by $\sim$ 50%. Conversely, conditions that enhanced the rate of hPepT1-mediated fMLP uptake (cytosolic acidification) enhanced neutrophil–transepithelial migration by $\sim$ 70%. We conclude that hPepT1 transports fMLP and uptake of these peptide influences neutrophil–epithelial interactions. These data (*a*) emphasize the importance of hPepT1 in mediating intestinal inflammation, (*b*) raise the possibility that modulating hPepT1 activity could influence states of intestinal inflammation, and (*c*) provide the first evidence of a link between active transepithelial transport and neutrophil–epithelial interactions. (*J. Clin. Invest.* 1998. 102:2011–2018.)

Key words: H^+/tripeptide (fMLP) transport • hPepT1 • Caco2-BBE • neutrophil • transmigration • inflammatory bowel disease

Introduction

The fundamental roles of epithelia are: (*a*) barriers to passive diffusion and (*b*) selective transporter of nutrients, ions, and water. Recently, these classical roles have been supplemented by recognition of immune functions. Thus, transcriptional regulatory events triggered by inflammatory cytokines can lead to a phenotype switch of the epithelial surface toward immune accessory function (1). On shorter time scales (minutes), recognition of luminal pathogens by epithelial apical membranes results in polarized secretion of chemoattractants from epithelia that serve to orchestrate neutrophil movement through the underlying matrix as well as across the epithelial monolayer (2–4). Such neutrophil–intestinal epithelial cell interactions have been appropriately modeled using human intestinal epithelial cell lines (T84 and Caco2-BBE) in conjunction with human peripheral blood neutrophils (2–7). These models have been predictive of the behavior of natural epithelium in these respects (4, 7, 8). It is unknown whether classical functional attributes of epithelia such as vectorial transport of nutrients might also directly modify mucosal immune responses.

One normal transport function expressed by gut epithelial cells is the absorption of small peptides from the diet by an apical membrane peptide transporter. Recently a cDNA encoding an apical membrane protein accounting for this peptide transport capability has been cloned (hPepT1 human). hPepT1, which cotransports peptides with H^+ (9), has a broad specificity that includes many di- and tripeptides. Functional expression of hPepT1 in *Xenopus laevis* oocytes induces H^+-dependent peptide transport activity, thus recapitulating the peptide transporting activity recognized in natural gut epithelia (10, 11). hPepT1 is appropriately expressed on the apical membrane of the human gut epithelial model cell line Caco2-BBE (12, 13), thus providing an in vitro model for analyses of the biology of this transporter.

It has long been known that bacteria, such as *Escherichia coli*, release potent neutrophil chemotactic substance(s) (14). Partial characterization of culture filtrates from *E. coli* have established that at least a portion of this chemoattractant bioactivity relates to small heterogeneous peptides with blocked NH_2 groups, so-called *n*-formyl peptides (15). For example, the tripeptide formyl-Met-Leu-Phe is a major peptide neutrophil chemotactic factor produced by *E. coli* (15), and thus has been widely studied as a model of *n*-formyl peptide. Such peptides are recognized by specific G protein–coupled, seven membrane spanning integral membrane proteins on neutrophils (16). In association with lumenal bacteria in the gut, a substantial array of *n*-formyl peptides (including formyl-Met-Leu-Phe) are normally present (15). Moreover, recent studies show that in chronic disease states hPepT1 (a potential transporter of those peptides) is aberrantly expressed by colonic epithelia (17), the site at which high concentrations of *n*-formyl peptides exist. Thus *n*-formyl peptides likely influence the behavior of migrating neutrophils in the alimentary tract. Here

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Address correspondence to Didier Merlin, Emory University, Department of Pathology and Laboratory Medicine WMRB, 1639 Pierce Dr., Atlanta, GA 30322. Phone: 404-727-8537; FAX: 404-727-8538; E-mail: dmerlin@emory.edu

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we demonstrate that hPepT1 may mediate uptake of *n*-formyl peptides and this event has functional consequences on neutrophil–epithelial interactions.

Methods

Cell culture. Caco2-BBE (18), HT29-19A (19, 20), and T84 cells (21) were grown as confluent monolayers in a 1:1 mixture of Dulbecco's Vogt modified Eagle's media and Ham's F-12 medium supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer (pH 7.5), 14 mM $NaHCO_3$, and 10% new-born calf serum. Monolayers were subcultured every 7 d by trypsinization with 0.1% trypsin and 0.9 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS. For reverse transcription PCR (RT-PCR)[1] experiments, Caco2-BBE, HT29-19A, and T84 cells were plated on plastic supports (area: 9.4 cm^2) for 14 d.

For fMLP uptake experiments, Caco2-BBE cells were plated on plastic supports (area: 9.4 cm^2) for 14 d. For PMN transmigration assays and efflux experiments, Caco2-BBE cells were plated on the underside of collagen-coated permeable supports (area: 0.3 cm^2; pore size: 3 μM) and cultured for 14 d, thus allowing for gravitational settling of PMNs on the basolateral aspect of the monolayer as described previously (22). All experiments described in this study were performed using cells between the 20th and 55th passages.

RT-PCR of hPepT1 expression. The expression of hPepT1 in Caco2-BBE, HT29-19A, and T84 cells was determined using a RT-PCR method with oligonucleotide primers specific for hPepT1. Total RNA was isolated from confluent Caco2-BBE, HT29-19A, and T84 cells cultured on plastic supports (area: 9.4 cm^2) for 14 d with a Micro Fast track™ Kit (Invitrogen Corp., Carlsbad, CA). The yield of RNA from each preparation was determined by ultraviolet spectrophotometry. 1 μg total RNA was primed with oligo(dT) and reverse transcribed with avian myeloblastosis virus–reverse transcriptase (AMV-RT; cDNA cycle kit; Invitrogen). A dilution of the reverse transcription reaction was used as a template for amplification by PCR. After an initial denaturation at 94°C for 5 min, PCR of the samples was carried out for 35 cycles under the following conditions: denaturation at 94°C for 1 min annealing at 55°C for 2 min, and extension at 72°C for 3 min. This was followed by a final extension step at 72°C for 7 min. For detection of hPepT1, the primers specific for hPepT1 corresponded to nucleotide positions 342–360 (sense: 5′-GCA GTC ACC TCA GTA AGC T-3′) and 1592–1575 (antisense: 5′-TGA CAC AAC CGA CTT TAT-3′) of the cDNA (10) were used. PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide.

Uptake of fMLP by Caco2-BBE. fMLP uptake measurements were performed on 14-d-old cell cultures in a serum-free medium containing (in mM) 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.4 $MgSO_4$, 1.25 $CaCl_2$, 0.3 Na_2HPO_4, 5 D-glucose buffered to a pH of 7.2 with 5 NaOH. Monolayers were washed three times with the fMLP-free transport medium before incubation in 1 ml uptake medium containing 0.1 μM of [^{3}H]fMLP (specific activity: 0.08 mCi/mole) alone or in presence of 10 mM Gly, Gly-Pro, Gly-Leu, or carnosine (β-Ala-His) for 110 min at 37°C. After incubation, the medium was aspirated and the dishes were rapidly rinsed twice with 2 ml of ice-cold incubation medium. The cells were solubilized in 1 ml of 1 N NaOH, and the cell-associated radioactivity was determined by liquid scintillation counting.

Neutrophil isolation. PMNs were isolated from whole blood (anticoagulated with citrate/dextrose) obtained from normal human volunteers, using a gelatine sedimentation technique previously described in detail (22). Neutrophils were resuspended in modified HBSS devoid of Ca^{2+} and Mg^{2+} (HBSS[−]) at a concentration of 5×10^7 cells/ml (4°C) and used for subsequent experiments.

Transmigration experiments. PMN transmigration experiments were performed on inverted (basolateral to apical migration) Caco2-BBE monolayers cultured on 0.33 cm^2 permeable support as previously described (22). In brief, confluent Caco2-BBE monolayers were washed with HBSS(+) (with Ca^{2+} and Mg^{2+}; 100 μl) followed by addition of 10^6 neutrophils in 50 μl HBSS(+) to the apical reservoir (in contact with the basolateral membrane, since monolayers are inverted). Transmigration was initiated by transfer of PMNs containing monolayers to 24-well tissue culture plates containing 1 ml of 0.1 μM fMLP alone or in presence of 10 mM Gly-Leu, Gly-Pro, or Gly in HBSS(+). After incubation for 110 min at 37°C, neutrophil migration across monolayers into the chemoattractant-containing lower chambers was quantified by a myeloperoxidase assay (22).

hPepT1 expression in X. laevis oocyte. The human intestinal oligopeptide transporter hPepT1 (10, 23) was expressed in *X. laevis* oocytes by microinjection of transporter cRNA as described previously (24). cDNA was linearized by using BamHI, and cRNA was transcribed in vitro using T7 RNA polymerase (MessageMachine system; Ambion Inc., Austin, TX). Oocytes were injected with 10 ng of hPepT1 cRNA and control oocytes were injected with 50 nl of deionized water. Experiments were performed 3 d after injection.

Intracellular accumulation of fMLP and [^{14}C]Glycyl-sarcosine by Xenopus oocytes expressing hPepT1. The intracellular accumulation of radiolabeled fMLP and [^{14}C]Glycyl-sarcosine (Gly-Sar) by hPepT1 cRNA–injected oocytes was assayed in standard uptake solution composed of (in mM) 100 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 3 Hepes, 3 MES, and 3 Tris, pH 5.5. Oocytes were washed three times with substrate-free solution before incubation in 90 ml uptake solution containing 0.12 μM of [^{3}H]fMLP (specific activity: 0.08 mCi/mol) alone or in the presence of Gly-Leu (10 mM). After incubation in the uptake solution for 110 min at 25°C, oocytes were washed six times with ice-cold incubation solution containing 0.1 mM fMLP, pH 7.5. The accumulation of Gly-Sar was carried out as described previously (24). Individual oocytes were solubilized with 0.25 ml 10% SDS and the radioactivity was measured by liquid scintillation counting.

Neutrophil cytosolic Ca^{2+} measurement. Neutrophils were loaded with calcium indicator Indo-1 and cytoplasmic Ca^{2+} concentration measured as previously described (25) using a spectrofluorometer (F-4500; Hitachi Scientific Instruments, Mountain View, CA). 2×10^6 neutrophils were suspended in 960 μl of HBSS(+) and stimulated with 40 μl of 0.25 10^{-5} M fMLP alone or in presence of 25 mM Gly-Leu.

Intracellular pH measurement in confluent monolayers of Caco2-BBE. Caco2-BBE cells grown to confluence on glass coverslips (9×21 mm) were loaded by incubation with BCECF-AM (5 μM) for 40 min at 37°C. After loading, the monolayers were rinsed three times in HBSS(+) buffer and mounted into a standard fluorescence cuvette. All solutions were preheated to 37°C. 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) (26, 27). Intracellular BCECF fluorescence was converted to pH by comparison with values from a calibration curve using a high K^+ (140 mM) and low Na^+ (4 mM) solution in a cell-free system (28).

Measurement of fMLP effluxes across the apical and basolateral membranes of Caco2-BBE monolayers. Caco2-BBE monolayers were incubated apically with 250 μl of incubation medium containing 0.1 μM [^{3}H]fMLP and basolaterally with 250 μl of unlabeled incubation medium for 110 min. At the end of the incubation period, the monolayers were rapidly washed four times on both sides with unlabeled incubation medium. The filter containing the monolayers was moved to new wells containing 250 μl of unlabeled medium and 250 μl of unlabeled medium was added to the upper reservoir. To measure apical and basolateral fMLP effluxes, 100 μl of the incubation medium was taken from both sides at different time points and replaced by 100 μl of fresh incubation medium. The radioactivity was measured by liquid scintillation counting.

Data analysis. Except where indicated, results are expressed as means±SD of at least three experiments. Statistical comparisons were carried out with Student's *t* test whereby $P < 0.05$ was considered significant.

1. *Abbreviation used in this paper:* RT-PCR, reverse transcription PCR.

Results

Expression of hPepT1 in Caco2-BBE, but not in HT29-Cl19A or T84 cells. Fig. 1 shows that hPepT1 was expressed in Caco2-BBE, but not in HT29-Cl19A or T84 cells. Since Caco2-BBE are small intestine-like cells, while T84 and HT29-Cl19A are more colonic in phenotype, these data are in accordance with previous studies demonstrating that hPepT1 is expressed in the small intestine, but not in the colon (except in chronic colitis). Others studies have shown that PepT2 (renal peptide transporters) are not expressed in mammalian intestine and in Caco2 cells (29, 30). All these results justify our choice of Caco2-BBE as a model for di- and tripeptide transport studies by hPepT1.

fMLP uptake across the apical plasma membrane in Caco2-BBE cell monolayers. As shown in Fig. 2, Caco2-BBE cells were able to take up fMLP when this solute was placed above the apical membrane. We next determined whether peptides known to be transported by hPepT1 (10 mM Gly, Gly-Pro, Gly-Leu, or carnosine [β-Ala-His]) could competitively inhibit fMLP uptake across the apical membrane. As shown in Fig. 2, fMLP accumulation was inhibited by the presence of Gly-Pro (78% inhibition), Gly-Pro (93% inhibition), and carnosine (β-Ala-His; 93% inhibition). In contrast, 10 mM glycine (not recognized by the hPepT1 transporter) did not affect fMLP uptake by Caco2-BBE cells. These results indicate that the transport pathway by which fMLP is taken up is selectively shared with other solutes that are recognized by the hPepT1 transporter.

fMLP uptake in oocytes expressing hPepT1. It is well established that the oligopeptide transporter hPepT1 transports many nonblocked di- and tripeptides, as well as a variety of peptide-related compounds (23, 24). To assess whether NH_2-blocked peptides relevant to inflammation such as fMLP are also transported by hPepT1, cRNA prepared by in vitro transcription from the cloned human cDNA was injected into *X. laevis* oocytes and their ability to transport fMLP measured. Oocytes injected with hPepT1-cRNA showed significantly greater [^{3}H]fMLP (0.12 μM incubated for 110 min with the oocytes) uptake than did water-injected oocytes (Fig. 3). In addition, the accumulation of [^{3}H]fMLP was abolished to levels similar to control oocytes with the addition of 10 mM of the competing solute Gly-Leu (recognized by hPepT1; Fig. 3). To determine the hPepT1 substrate specificity, the ability of unlabeled fMLP (0.1 and 1 mM) to compete with [^{14}C]Gly-Sar was examined. As shown in Fig. 4, hPepT1-mediated uptake of [^{14}C]Gly-Sar was inhibited by 88% in the presence of 1 mM fMLP. Taken together, these results demonstrate that fMLP is transported by hPepT1.

fMLP induced neutrophil migration across monolayers of Caco2-BBE cells. Having demonstrated that hPepT1 can recognize the bacterially derived peptide fMLP and that such recognition results in apical absorption of this peptide in hPepT1-expressing enterocytes, we next examined whether hPepT1 uptake of this PMN activating/chemoattractant peptide modified neutrophil–intestinal epithelial interactions. Physiologically directed (basolateral to apical) transepithelial migration of PMN was assessed as a function of hPepT1-mediated fMLP

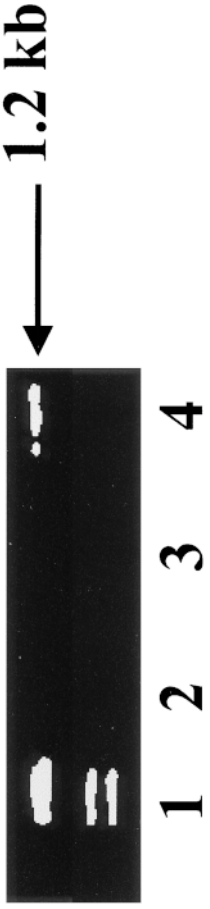

Figure 1. RT-PCR with hPepT1-specific primers. Samples isolated from HT29-19A, T84, or Caco2-BBE cells were subjected to RT-PCR using hPepT1-specific primers as described in Methods. The RT-PCR products were analyzed by agarose gel electrophoresis. The expected size of the product was 1.2 kb. Lane *1*, molecular weight markers (from bottom to top: 2,323, 1,929, 1,264); lane *2*, HT29-19A sample; lane *3*, T84 sample; lane *4*, Caco2-BBE sample. To clarify banding pattern, the image was subject to thresholding.

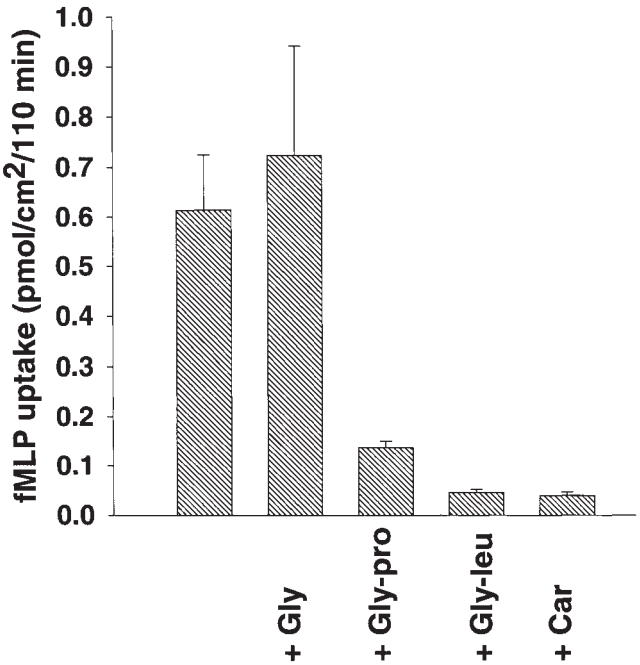

Figure 2. fMLP uptake by Caco2-BBE monolayers. Caco2-BBE monolayers were incubated for 110 min at 37°C with 0.1 μM [^{3}H]fMLP with and without 10 mM Gly-Leu, Gly-Pro, Gly, or carnosine (β-Ala-His; *Car*) added to the apical side. Thereafter, medium was aspirated, and cells were rapidly washed twice with 2 ml ice-cold incubation medium. Cells were solubilized in 1 ml of 1 N NaOH, and radioactivity was determined by liquid scintillation counting. Each column represents mean±SD of three to five monolayers.

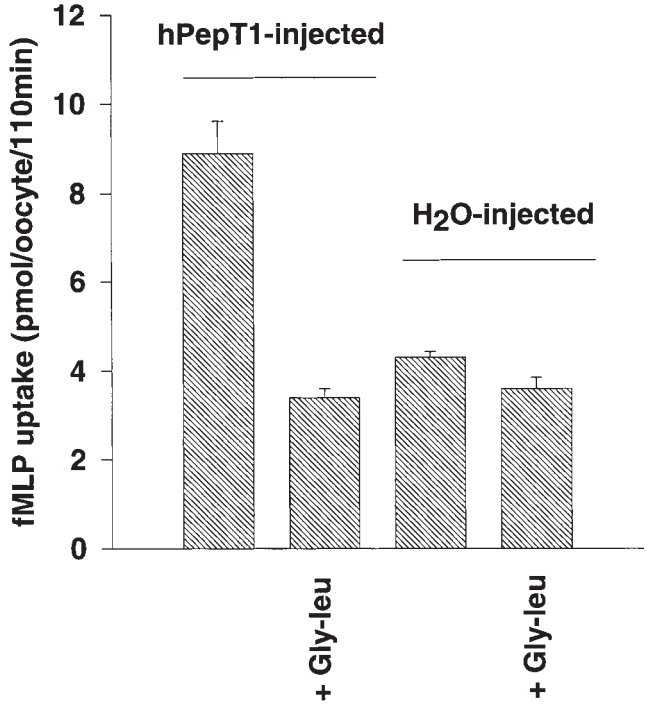

Figure 3. fMLP uptake by oocytes. *X. laevis* oocytes microinjected with either 10 ng hPepT1 cRNA or water were incubated for 110 min in uptake solution containing 0.12 μM [^{3}H]fMLP (pH 5.5) in the presence or absence of unlabeled 10 mM Gly-Leu. The oocytes were washed six times with ice-cold incubation solution, pH 7.5, and radioactivity was measured by liquid scintillation counting. The values represent the mean±SEM for six to eight oocytes from two separate experiments.

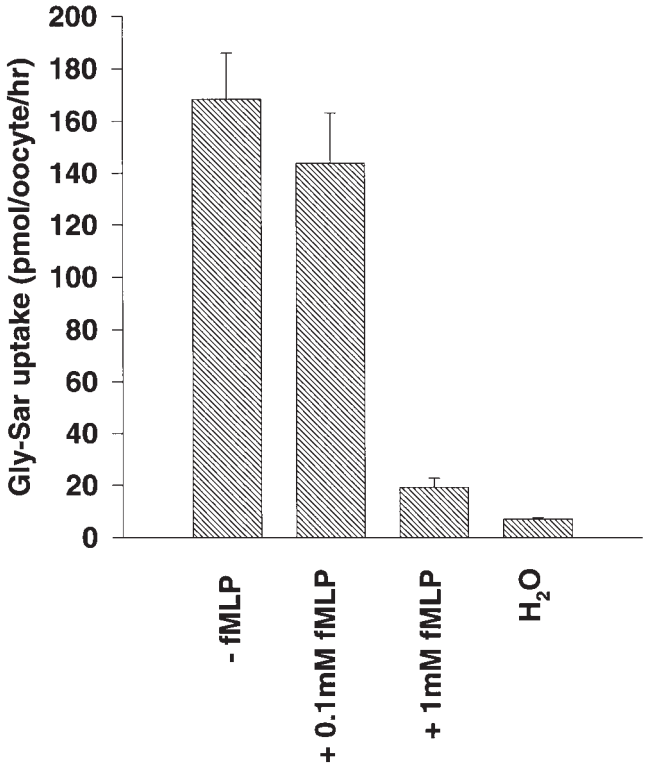

Figure 4. Inhibition of Gly-Sar uptake by fMLP in oocytes. Oocytes were injected with 10 ng of hPepT1 cRNA, or water. On day 3 after injection, the transport of 100 μM [^{14}C]Gly-Sar alone or in the presence of unlabeled fMLP (0.1 and 1 mM) was measured. The oocytes were rinsed six times in ice-cold incubation solution, pH 7.5, and the radioactivity was measured by liquid scintillation counting. The values shown are the mean±SEM for six to eight oocytes from two separate experiments.

transport. As shown in Fig. 5, in the absence of an exogenous chemottractant gradient, no significant transepithelial migration of neutrophils occurs at either pH 5.2 or 7.2. In contrast, in the presence of an fMLP gradient (i.e., 0.1 μM fMLP), active directed migration of neutrophils across epithelial monolayers is observed ($19.9 \times 10^4 \pm 3.4 \times 10^4$ versus $4.43 \times 10^4 \pm 1.17 \times 10^4$ with and without gradient, respectively). In addition, the tripeptide MLP (0.1 μM) or Gly-Leu (10 mM) did not induce a transepithelial migration of neutrophils ($18.48 \times 10^4 \pm 1.70 \times 10^4$ versus $2.55 \times 10^4 \pm 0.02 \times 10^4$ with fMLP and with 10 mM Gly-Leu in the absence of fMLP, respectively). These results confirm the ability of a physiologically directed transepithelial gradient of fMLP to support directed transepithelial migration of neutrophils. Such activity has previously been assumed to wholly represent the result of a passively established paracellular gradient of fMLP (created by diffusion of this chemoattractant peptide across the tight junction and into the paracellular spaces).

Inhibition of hPepT1 decreases neutrophil–transepithelial migration induced by physiologically directed fMLP gradients across Caco2-BBE monolayers. We next examined the effect of inhibition of fMLP uptake into Caco2-BBE cells on fMLP-induced neutrophil transmigration. fMLP (0.1 μM) was added to the luminal reservoir in the presence of the hPepT1-specific substrates 10 mM Gly, Gly-Pro, or Gly-Leu. Gly, which is not transported by hPepT1 and thus does not compete with hPepT1-mediated fMLP transport (see above), did not influence transepithelial migration of neutrophils ($18.1 \times 10^4 \pm 3.2 \times 10^4$ versus $15.3 \times 10^4 \pm 3.6 \times 10^4$; $n = 6$). In contrast, neutrophil–transepithelial migration was significantly attenuated in the presence of Gly-Pro ($18.1 \times 10^4 \pm 3.2 \times 10^4$ versus $9.3 \times 10^4 \pm 1.9 \times 10^4$; $n = 6$) or Gly-Leu ($18.1 \times 10^4 \pm 3.2$ versus $9.3 \pm 1.9 \times 10^4$; $n = 6$) (Fig. 6). These dipeptides transported by hPepT1 specifically inhibit hPepT1-mediated transport of fMLP.

We next ruled out the possibility that the observed attenuation of fMLP-driven transepithelial migration of PMNs did not simply reflect a direct inhibitory action of the dipeptides. For this purpose, two different experiments were carried out. First, we examined neutrophil migration across filters in the absence of epithelial monolayers. Under these conditions, neither Gly nor Gly-Leu affected neutrophil movement ($21.3 \times 10^4 \pm 1.8 \times 10^4$ versus $19.03 \pm 1.55 \times 10^4$ and $21.3 \times 10^4 \pm 1.8 \times 10^4$ versus $19.2 \times 10^4 \pm 1.57 \times 10^4$, respectively, for Gly and Gly-Leu compared to controls. All $n = 3$). Second, we assessed neutrophil cytoplasmic Ca^{2+} responses as an indicator of fMLP receptor–mediated activation. As shown in Fig. 7, resting Ca^{2+} concentration and the increase in Ca^{2+} concentration after fMLP addition were not significantly influenced by 10 mM Gly-Leu. Together, these results demonstrate that dipeptides transported by hPepT1 do not directly interfere with neutrophil motility or activation. In contrast, inhibition of fMLP

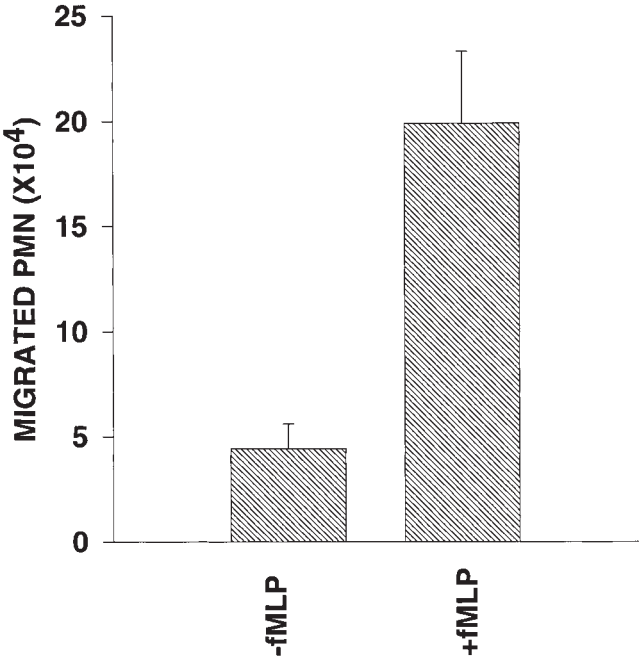

Figure 5. fMLP induces neutrophil transmigration across monolayers of Caco2-BBE cells. Transmigration assays were performed in the physiological basolateral to apical direction. Inverted Caco2-BBE monolayers were used. The transmigration assay with such inverted monolayers allows gravitational settling of neutrophils onto the filter and, subsequently, contact with the basolateral aspect of the monolayers before the initiation of migration. As described in Methods, 10^6 neutrophils were added to the apical reservoir (in contact with the basolateral membrane). The inverted monolayers were transferred to 24-well tissue culture wells containing 1 ml of HBSS(+) (pH 7.2) or 0.1 μM fMLP in HBSS(+) (pH 7.2). PMN transmigration was allowed to proceed for 110 min (37°C). Neutrophils that had transmigrated to the opposite reservoir were quantitated by myeloperoxidase assay as described in Methods. Bars represent the mean±SD of three monolayers for each condition.

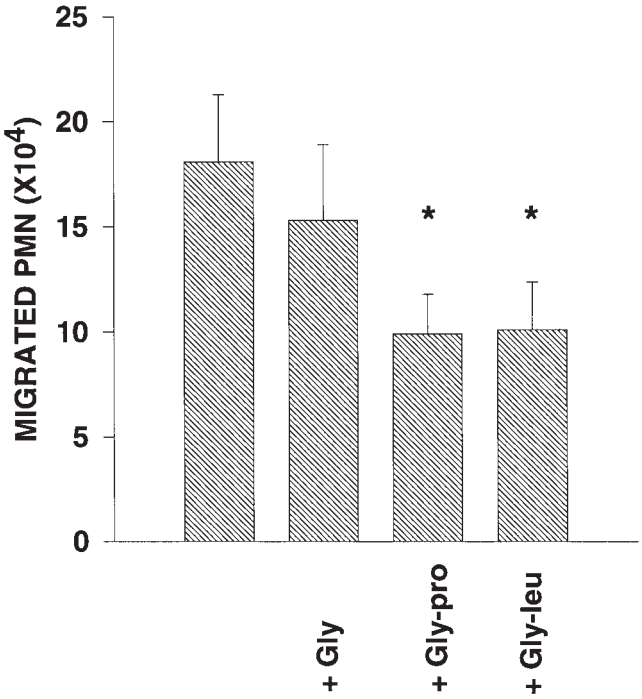

Figure 6. Inhibition of fMLP accumulation in Caco2-BBE cells decreases neutrophil transmigration across Caco2-BBE cell monolayers. Transmigration assays were performed as in Fig. 5. 10^6 PMNs were added to the apical reservoir (in contact with the basolateral membrane). The inverted monolayers were transferred to 24-well tissue culture wells containing 0.1 μM fMLP alone or with 10 mM Gly-Leu, 10 mM Gly-Pro, or 10 mM Gly in 1 ml HBSS(+) (pH 7.2). Neutrophil transmigration was allowed to proceed for 110 min (37°C). Neutrophils that had transmigrated to the opposite reservoir were quantitated by myeloperoxidase assay as described in Methods. Bars represent the mean±SD of six monolayers for each condition. $*P <$ 0.05 versus control.

transport mediated by hPepT1 substantially modified the ability of neutrophils to move across epithelial monolayers.

Finally, we performed neutrophil transmigration on inverted T84 monolayers that do not express hPepT1 (Fig. 1). Neutrophil–transepithelial migration was not influenced by addition of the hPepT1 substrates Gly-Leu ($23.6 \times 10^4 \pm 1.2 \times 10^4$ versus $25.0 \times 10^4 \pm 3.2 \times 10^4$; $n = 3$) in these monolayers. These data demonstrate that the dipeptide Gly-Leu does not affect neutrophil transmigration in a cell line not expressing hPepT1.

fMLP induces intracellular acidification of Caco2-BBE cell monolayers. Since hPepT1 transports specific substrates via an H^+-coupled mechanism, if PepT1-mediated transport of fMLP occurs as indicated by the above data, this event should result in measurable fMLP-dependent cytosolic acidification of intestinal epithelial cells. As demonstrated by Fig. 8, fMLP exposure (100 μM fMLP) to the apical chamber of Caco2-BBE intestinal epithelial cells results in rapid acidification (apical medium clamped at pH 7) comparable to that induced by 10 mM Gly-Leu. The amino acid glycine (10 mM) had no effect (Fig. 8). In this particular experiment, Caco2-BBE cells were grown on glass and are probably not fully polarized. However, these data still demonstrate that hPepT1 mediates this intracellular acidification in Caco2-BBE cells since the basolateral oligopeptide transporter does not transport dipeptides at pH 7.0.

An inwardly directed pH gradient, which drives hPepT1 transport, increases fMLP-driven neutrophil transmigration across monolayers of Caco2-BBE cells. hPepT1-mediated solute transport is coupled with the downhill movement of H^+ across the apical brush border membrane and into the cytoplasm (see above). Also as shown above, inhibition of hPepT1-mediated fMLP transport attenuates transepithelial migration of neutrophils. We thus tested whether enhanced hPepT1-mediated transport of fMLP would similarly enhance transepithelial–neutrophil migration. To enhance hPepT1-mediated absorption of fMLP, we clamped an inwardly directed H^+ gradient across the apical membrane. As shown in Fig. 9, lowering the luminal pH to 5.2 increased the transepithelial migration of neutrophils by ~ 70% ($18.2 \times 10^4 \pm 2.0 \times 10^4$ versus $30.9 \pm 5.6 \times 10^4$; $n = 6$). Furthermore, both the neutrophil migration at pH 7.2 as well as the enhanced neutrophil migration at luminal pH 5.2 were inhibited ~ 50% by addition of 10 mM of the hPepT1 competing solute Gly-Leu ($18.2 \times 10^4 \pm 2.0 \times 10^4$ versus $9.7 \pm 3.3 \times 10^4$ at pH 7.2 and $30.9 \times 10^4 + 5.6 \times 10^4$ versus $17.2 + 4.7 \times 10^4$ at luminal pH 5.2; $n = 6$). In aggregate with results provided above, these results indicate that both

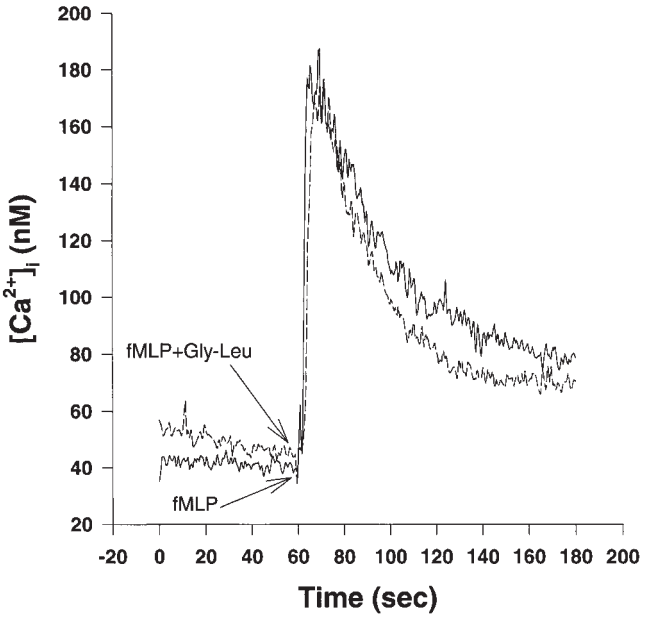

Figure 7. Gly-Leu does not prevent the change in neutrophil cytoplasmic-free Ca^{2+} concentration induced by fMLP. *Arrows*, addition of fMLP (2.5 μM) alone (*solid lines*) or fMLP (2.5 μM) in the presence of 10 mM Gly-Leu (*dotted lines*) to the cuvette containing neutrophils in suspension. $[Ca^{2+}]$ was measured by indo1 epifluorescence (see Methods). 10 mM Gly-Leu alone did not affect the resting $[Ca^{2+}]$ (not shown). The tracing is representative of results with eight different neutrophil suspensions.

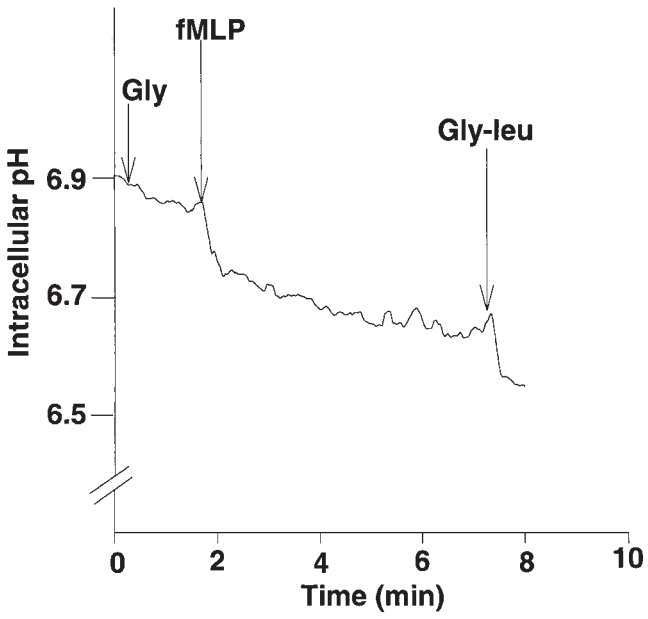

Figure 8. fMLP-induced intracellular acidification in Caco2-BBE cell monolayers. Gly, fMLP, and Gly-Leu at 100 μM were added to the apical surface (at pH 7.0) of BCECF-loaded Caco2-BBE cell monolayers (see Methods). The figure represents a single experiment representative of three separate experiments.

negative and positive changes in hPepT1-mediated absorption of fMLP exhibit corresponding negative and positive alterations in fMLP-driven migration of neutrophils across Caco2-BBE monolayers.

Intracellular accumulation of fMLP mediated by hPepT1. Previous studies have shown the existence of a distinct dipeptide transporter in the basolateral membranes of Caco2 in comparison with the hPepT1 cotransporter in their apical membranes. We have compared the transport of fMLP across the apical and basolateral membrane of Caco2-BBE cell monolayers. After incubating Caco2-BBE monolayers with [^{3}H]fMLP (0.1 μM) for 110 min, we found that the fMLP efflux across the apical plasma membrane of Caco2-BBE was $\sim$ 10 times greater than the fMLP efflux across the basolateral membrane (Fig. 10). These last results suggest that (*a*) fMLP entry across apical and exit across the basolateral membranes is mediated by active and facilitated transport systems respectively, and (*b*) the transcellular fMLP transport is likely to be rate limited by its transport at the basolateral membrane.

Discussion

The nutritional aspects of oligopeptide transport by epithelial cells have been extensively characterized in recent years (31). Enzymatic hydrolysis of diary proteins formed by 20 amino acids could cause the release of 400 different dipeptides and 8,000 different peptides. Not only will these peptide substrates vary with respect to net charge and solubility, they also cover a wide range of molecular weights from 96.2 Da (di-Gly) to 522.6 Da (tri-Trp). It has been reported that hPepT1 is predominantly (or entirely) responsible for apical transport of a large variety (if not all) di- and tripeptides and related peptide mimetics. This diversity of substrate recognition led us to consider whether hPepT1 could transport peptides with *n*-formyl blocks and, if so, whether transport of such bacterially derived chemotactic peptides influenced neutrophil–epithelial interactions. fMLP is a major physiological chemotactic peptide released by bacteria, such as *E. coli* and, as such, has been widely used as a model for *n*-formyl peptide–mediated activation of neutrophils. Here we show that hPepT1 actively transports fMLP and such transport modifies interactions of hPepT1-expressing epithelial cells with neutrophils.

The model *n*-formyl peptide fMLP is transported across the apical plasma membrane of Caco2-BBE cells and into *Xenopus* oocytes expressing hPepT1. As would be expected for a hPepT1-mediated transport event, both in oocytes and Caco2-BBE cells, fMLP transport is competitively inhibited by known hPepT1 dipeptide substrates, but not by free amino acids. Others, using a variety of oligopeptides (31), have shown that little oligopeptide terminal hydrolysis occurs at the brush border, resulting in 90% of the corresponding solute being transported into the cytosol as intact peptide. Coupled with our findings, these studies would suggest that intestinal epithelial cells that express hPepT1 could accumulate significant chemotactic peptide quantities in the cytosol if such peptides were present at significant luminal concentrations. It is less clear how the absorbed oligopeptides ultimately move across the basolateral membrane to complete nutrient absorption, but it appears this event does occur and may be mediated by basolateral oligopeptide transport systems yet to be identified (32). Our results suggest that the transcellular fMLP transport is likely to be rate limited by its transport at the basolateral membrane.

It appears that PepT1-mediated uptake of n-formyl peptides may have biological influences on neutrophil interactions

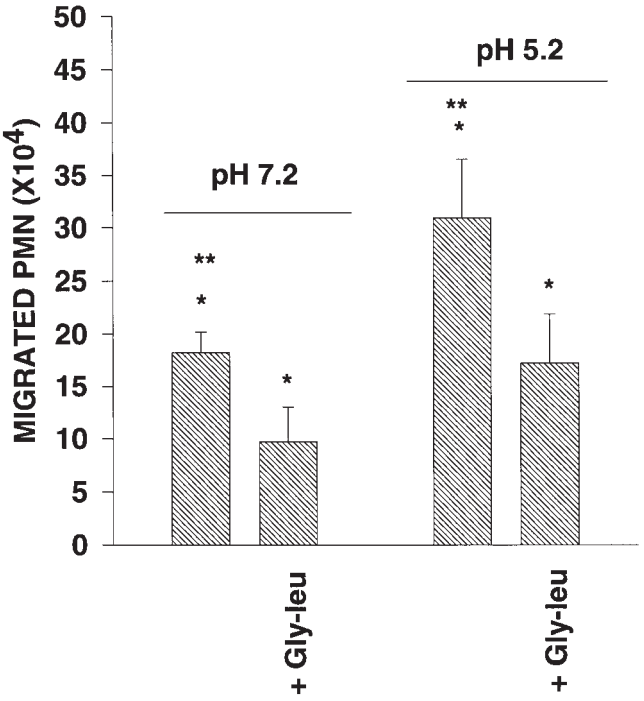

Figure 9. An inwardly directed pH gradient enhances neutrophil transmigration across Caco2-BBE cell monolayers. Transmigration assays were performed as in Fig. 5. 10^6 PMNs were added to the apical reservoir (in contact with the basolateral membrane). The inverted monolayers were transferred to 24-well tissue culture wells containing 0.1 μM fMLP 1 ml HBSS(+) at either pH 5.2 or 7.2. Neutrophil transmigration was allowed to proceed for 110 min (37°C). Neutrophils that had transmigrated to the opposite reservoir were quantitated by myeloperoxidase assay as described in Methods. Bars represent the mean±SD of six monolayers for each condition. $*P <$ 0.05 versus control (at pH 7.2 control versus Gly-Leu and at pH 5.2 control versus Gly-Leu); $**P < 0.05$ control at pH 7.2 versus control at pH 5.2.

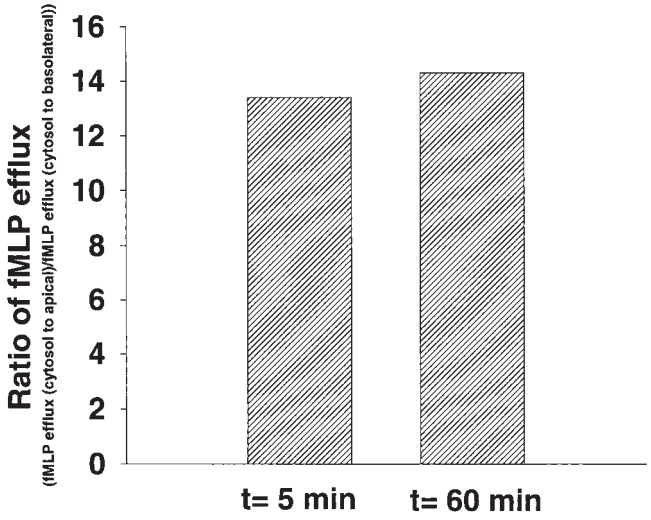

Figure 10. fMLP efflux across apical and basolateral membranes of Caco2-BBE monolayers. Effluxes of [^{3}H]fMLP across both apical and basolateral borders was measured by first loading monolayers with [^{3}H]fMLP for 110 min. The monolayers were washed (four times) with fresh medium. Loss of cellular [^{3}H]fMLP into the apical and basolateral chambers was determined after 5 and 60 min as described in Methods. Data are expressed in ratio of fMLP efflux (fMLP efflux [cytosol to apical]/fMLP efflux [cytosol to basolateral]). Bars represent the mean of four monolayers for each time point.

with epithelial monolayers. For example, inhibition of fMLP uptake through hPepT1 attenuated the fMLP-driven transepithelial migration of neutrophils by 50%. Similarly, enhancing hPepT1-mediated epithelial absorption of fMLP increased transepithelial migration of neutrophils. The most straightforward interpretation of these observations is that active transepithelial transport of fMLP favorably modifies the transepithelial gradient of the chemoattractant such that more efficient movement of neutophils across epithelial cells occurs. However, our results also suggest the possibility that intracellular uptake of *n*-formyl peptides results in rapid signals that lead to modifications of the basolateral membranes with which the neutrophils interact during transepithelial migration.

Assuming that the observed influences of hPepT1-mediated fMLP absorption on neutrophil–epithelial interactions represent the effects on active transcellular fMLP absorption, how might such effects occur? Intercellular tight junctions lie at the apex of the paracellular spaces of polarized columnar epithelial cells and represent the rate-limiting barriers to passive diffusion of solutes between cells (33). Given the known sieving characteristics of tight junctions, small peptides such as fMLP should passively diffuse across the tight junctions in limited amount so as to create an exceedingly steep transjunctional gradient of chemotactic peptide. Parallel transcellular active transport of solute (i.e., hPepT1-mediated transport of fMLP) by translocating peptide across the lateral membrane from the tight junction to the interface with the basolateral membrane ($\sim$ 20 μm in length) would serve to lengthen and decrease to slope of the resulting paracellular gradient. The data observed, including the coupling of efficiency of neutrophil transmigration to rates of hPepT1 transport of fMLP, are consistent with this speculative possibility.

fMLP has been demonstrated to be a major *n*-formylated peptide of the human colonic lumen (16) and the total *n*-formyl peptide content of the human colon provides an fMLP equivalent concentration of $\sim 10^{-7}$ M (34). This later concentration is within the range of fMLP concentrations that provide gradients that maximally influence PMN migration when used in the current model system. It is likely that *n*-formyl peptide concentrations are substantially lower in the small intestine than in the colon, in parallel with the lower mass of prokaryotes present in this former site in humans. Interestingly, hPepT1 expression normally is restricted to the small intestine in which bacterial populations are far fewer in number. However, recent studies indicate that colonic expression of hPepT1 does occur in certain chronic colitis (17), and it is thus possible that transepithelial sensing of such chemotactic peptides could thus be influenced by signals that result in the transcriptional regulation of hPepT1 expression. Also interesting in this regard is the clinical syndrome of bacterial overgrowth in the human small intestine. Additionally, it is now well recognized that bacterial pathogens can orchestrate neutrophil movement toward and across intestinal epithelia by activation of proinflammatory pathways of epithelial origin (2, 3). However, such epithelial orchestration of these events is unique to pathogens such as *Salmonella typhimurium* and requires bacterial contact

with the apical membrane (2, 3, 7). In contrast, increased numbers of nonpathogenic bacteria simply present in the lumen of the human small intestine leads to an inflammatory state characterized by transepithelial migration of neutrophils. It is possible that transepithelial transport of *n*-formylated peptides by hPepT1 as well as paracellular diffusion of these peptides could play a role in the pathobiology of such small intestinal disorders.

In summary, we describe for the first time that activation of normal transport molecules on the surface of polarized intestinal epithelial cells can modify subsequent interactions with neutrophils. To the extent that hPepT1-mediated transport of fMLP and the subsequent effects of this transport on epithelial–neutrophil interactions can be competed with normal hPepT1 substrates, these data also make the first specific molecularly defined connection between an active transport process, intestinal inflammation, and nutrition.

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