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Expression of human intestinal dipeptide transporter in *Xenopus laevis* oocytes

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Abstract—The human colon adenocarcinoma cell line Caco-2 retains the H^+/dipeptide cotransporter. To identify the structure of the human dipeptide transporter, we have examined the expression of the transporter in *Xenopus laevis* oocytes injected with Caco-2 poly(A)$^+$RNA, by monitoring the uptake of bestatin, a dipeptide-like anticancer agent. The bestatin uptake in the poly(A)$^+$RNA-injected oocytes was inhibited by excess glycyl-L-leucine, and showed pH dependence (optimal pH of 5.5–6.0). These observations suggest that the human intestinal dipeptide transporter can be expressed functionally in *Xenopus* oocytes.

The transport of di- and tripeptides into the intestinal epithelial cells is an active process that is mediated by the dipeptide transporter localized in the brush-border membranes [1, 2]. Studies using purified intestinal brush-border membrane vesicles have revealed that the dipeptide transporter is an electrogenic H^+-coupled cotransport system that is driven by an inward H^+ gradient and enhanced by a negative membrane potential [3, 4]. Interestingly, oral cephalosporin antibiotics can be transported in the intestinal brush-border membranes via this H^+/dipeptide cotransport system [5–7]. Information about the structure of the dipeptide transporter would lead to a better understanding of the molecular mechanisms for intestinal peptide absorption. The functional expression and cloning of a membrane transporter using *Xenopus laevis* oocytes have been demonstrated for the intestinal Na^+/glucose cotransporter [8] and for other transporters [9–11]. Miyamoto *et al.* [12] showed that the injection of poly(A)$^+$RNA prepared from rabbit intestinal mucosal cells into *Xenopus* oocytes led to a functional expression of the H^+/dipeptide cotransporter. The human colon adenocarcinoma cell line Caco-2 has recently been used as a model for studying the function of differentiated intestinal epithelial cells, including several transport activities [13–15]. We demonstrated previously that oral cephalosporin antibiotics are transported into Caco-2 cells by the H^+/dipeptide cotransporter [16]. In this study, we attempted to express the human intestinal dipeptide transport system functionally in *Xenopus* oocytes by measuring the uptake of bestatin (a dipeptide anticancer agent), which is a useful probe for the intestinal H^+/dipeptide cotransporter [17], as well as oral cephalosporins.

Materials and Methods

Caco-2 cells obtained from the American Type Culture Collection (ATCC HTB37) at passage 18 were maintained by serial passage in plastic culture dishes (100 mm) (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.). In the present study, the cells were used between the 30th and 35th passage. To measure the cellular uptake of [^{3}H]-bestatin (12.7 GBq/mmol) (Nippon Kayaku Co., Tokyo, Japan), Caco-2 cells were seeded on collagen-coated membrane filters (3 μm, 4.71 cm^2) inside Transwell™ cell culture chambers (Costar, Cambridge, MA, U.S.A.) as described previously [16]. The cell monolayers were preincubated apically or basolaterally with 2 mL of incubation medium [145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 5 mM MES* (pH 6.0) or HEPES (pH 7.4)] for 10 min at 37°. The medium was then removed and 2 mL of incubation medium containing [^{3}H]bestatin were added to the apical compartment with 2 mL of unlabeled incubation medium lacking bestatin added to the opposite side. The medium was aspirated at the end of the incubation period and the monolayers were rapidly washed twice with 2 mL of ice-cold incubation medium. The filters with monolayers were detached from the chambers, and the cells were solubilized in 0.5 mL of 1 N NaOH. The radioactivity of the solubilized cells was determined by liquid scintillation counting. The protein content of the solubilized cell monolayers was determined by the method of Bradford [18], using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratory, Richmond, CA, U.S.A.) with bovine γ-globulin as a standard.

Total RNA was isolated from Caco-2 cells cultured for 7 days by using a cesium chloride/guanidinium thiocyanate (Fluka Chemie AG, Buchs, Switzerland) centrifugation method [19]. Briefly, Caco-2 cell monolayers (10 dishes) were disrupted by spreading 4 M guanidinium thiocyanate solution on the culture dishes. The lysates were layered onto a CsCl cushion (5.7 M cesium chloride, 0.1 M EDTA, pH 7.5) and total RNA was pelleted by centrifugation (himac CP70G, RPS40T rotor, Hitachi Co., Tokyo, Japan) for 20 hr at 85,000 *g* at 20°. Poly(A)$^+$RNA was purified by oligo(dT)-cellulose (Collaborative Research Inc., Bedford, MA, U.S.A.) chromatography [20]. Approximately 40 μg of poly(A)$^+$RNA were yielded by this method. Ovarian lobes were dissected from a female *Xenopus laevis*

Table 1. Effect of inhibitors on bestatin uptake by Caco-2 cell monolayers

Inhibitor	Bestatin uptake (nmol/mg protein/hr)	% of control
Control	16.77 ± 0.42	100
Bestatin	2.41 ± 0.06	14
Glycyl-L-leucine	1.06 ± 0.09	6
Cephradine	11.62 ± 0.20	69

Caco-2 monolayers grown in Transwell™ chambers were incubated for 1 hr at 37° with medium containing [^{3}H]-bestatin (1 mM, 37 kBq/mL) added to the apical side (2 mL, pH 6.0) in the absence or presence of 10 mM of the inhibitor. Unlabeled incubation medium (2 mL) was added to the basolateral side (2 mL, pH 7.4). Following incubation, the monolayers were washed twice with ice-cold incubation medium on both sides, the cells were solubilized in 0.5 mL of 1 N NaOH and the cell-associated radioactivity was determined.

Each value represents the mean ± SE of three monolayers.

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethane sulfonic acid.

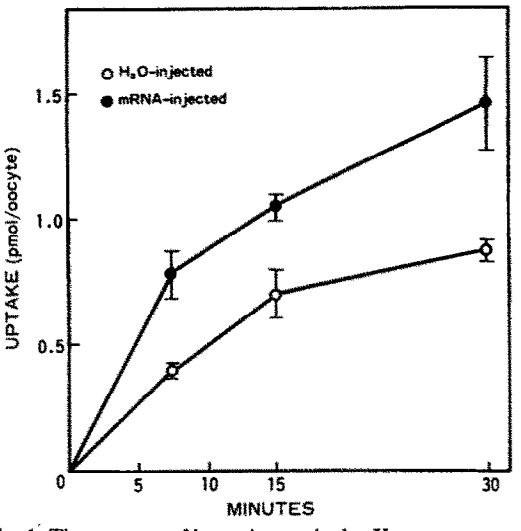

Fig. 1. Time course of bestatin uptake by *Xenopus* oocytes injected with either water or Caco-2 cell poly(A)$^+$RNA. Oocytes were injected with 50 nL of Caco-2 poly(A)$^+$RNA (50 ng) (●) or water (○). After incubation at 18° for 4 days, the oocytes were incubated with [^{3}H]bestatin (30 μM, 0.37 MBq/mL) at 37°, and radioactivity associated with individual oocytes was counted. Each point represents the mean ± SE of six oocytes.

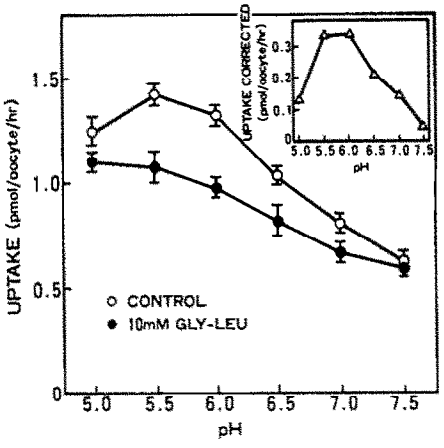

Fig. 2. pH dependence of bestatin uptake by oocytes injected with Caco-2 poly(A)$^+$RNA. Oocytes were injected with 50 nL of Caco-2 poly(A)$^+$RNA (50 ng) and were incubated at 18° for 6 days. The oocytes were then incubated for 1 hr at 25° with [^{3}H]bestatin (30 μM, 0.37 MBq/mL) in the absence (○) or presence (●) of 10 mM glycyl-L-leucine. MES (5 mM) was used to buffer the incubation medium between pH values 5.0 and 6.0, and HEPES (5 mM) was used to buffer medium between pH values 6.5 and 7.5. Following incubation, the radioactivity associated with individual oocytes was measured. Each point represents the mean ± SE of five oocytes. Inset shows the curve obtained by subtracting bestatin uptake in the presence of glycyl-L-leucine from the uptake in the absence of the inhibitor.

(Hamamatsu Seibutsu Kyozai Co., Shizuoka, Japan). Individual oocytes were isolated by gentle agitation in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, 0.1 mg/mL gentamicin sulfate, pH 7.5] [21] containing 1.0% collagenase (Type IA, Sigma Chemical Co., St Louis, MO, U.S.A.) for 1 hr at room temperature (23–25°) followed by a 30–60 min incubation in 100 mM K_2HPO_4 and 0.1% bovine serum albumin (fraction V, Sigma) [22]. Then, oocytes were maintained in modified Barth's solution at 18°, and 24 hr later healthy looking oocytes were injected with 50 nL of poly(A)$^+$RNA or water by a semiautomatic injector (Narishige Scientific Instrument Lab., Tokyo, Japan). The oocytes were incubated at 18° in modified Barth's solution for 4–6 days. Uptake of bestatin was assayed by placing two oocytes in 0.1 mL of radioactive uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM MES, pH 6.0). To enhance the uptake rate, oocytes were incubated at a higher temperature than 18° [23]. After incubation for an appropriate period, oocytes were washed rapidly four times with 1 mL of ice-cold incubation medium. Individual oocytes were transferred to scintillation vials, solubilized in 0.5 mL of 10% (W/V) SDS, and counted in

Table 2. Bestatin uptake by *Xenopus* oocytes injected with either water or Caco-2 poly(A)$^+$RNA

Inhibitor	Bestatin uptake (pmol/oocyte/15 min)	
	Water-injected	Poly(A)$^+$RNA-injected
Control	0.70 ± 0.10 (100)	1.27 ± 0.29 (100)
Bestatin	ND	0.72 ± 0.08 (57)
Glycyl-L-leucine	0.71 ± 0.05 (101)	0.75 ± 0.06 (59)
Cephradine	ND	0.74 ± 0.03 (58)
Glycine	ND	1.18 ± 0.10 (93)

Oocytes were injected with 50 nL of Caco-2 poly(A)$^+$RNA (50 ng) or water. After incubation at 18° for 4 days, the oocytes were incubated with [^{3}H]bestatin (30 μM, 0.37 MBq/mL, pH 6.0) in the absence or presence of 10 mM of the inhibitor for 15 min at 37°, and radioactivity associated with individual oocytes was counted.

Each value represents the mean ± SE of six oocytes. Value in parenthesis represents % of control. ND, not determined.

10 mL of scintillant fluid (ACS II, Amersham International, Amersham, U.K.).

Results and Discussion

We examined the ability of Caco-2 cells to accumulate bestatin via the H^+/dipeptide cotransporter. As summarized in Table 1, [^{3}H]bestatin uptake by Caco-2 monolayers was inhibited greatly by excess amounts of unlabeled bestatin, glycyl-L-leucine and cephradine, indicating that these cells express dipeptide transport activity. It is therefore suggested that Caco-2 cells are a good source for isolating the mRNA that encodes the dipeptide transport protein.

As shown in Fig. 1, bestatin uptake was greater in oocytes injected with poly(A)$^+$RNA isolated from Caco-2 cells than in those injected with water. To determine whether the increased activity of bestatin transport in the poly(A)$^+$RNA-injected oocytes was due to expression of dipeptide transporter, we examined the effect of dipeptides and cephradine on bestatin uptake. As summarized in Table 2, bestatin uptake in poly(A)$^+$RNA-injected oocytes was markedly inhibited by the presence of excess unlabeled bestatin, glycyl-L-leucine and cephradine, but not by the presence of glycine. In contrast, the uptake in water-injected oocytes was not affected by the presence of glycyl-L-leucine.

In addition, we studied pH dependence of bestatin uptake in oocytes injected with Caco-2 cell poly(A)$^+$RNA. As illustrated in Fig. 2, the expressed bestatin uptake showed pH dependence with an optimal pH of 5.5–6.0, a similar pH profile of bestatin uptake having been found in the intestinal brush-border membranes [17]. Preliminarily, we also observed that bestatin uptake across the apical membranes of Caco-2 cells showed an optimal pH of 6.0 (unpublished data). Thus, the dipeptide transporter localized in the apical membranes, i.e. H^+/dipeptide cotransporter, may be expressed in the oocytes injected with Caco-2 cell poly(A)$^+$RNA. Additional studies are required to characterize the expressed dipeptide transporter, in comparison with the cellular mechanisms and regulation of dipeptide transport in Caco-2 cells.

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