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Functional Expressions of Endogenous Dipeptide Transporter and Exogenous Proton/Peptide Cotransporter in *Xenopus* Oocytes

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It is essential to clone the peptide transporter in order to obtain better understanding of its molecular structure, regulation, and substrate specificity. Characteristics of an endogenous peptide transporter in oocytes were studied along with expression of an exogenous proton/peptide cotransporter from rabbit intestine. And further efforts toward cloning the transporter were performed. The presence of an endogenous peptide transporter was detected in *Xenopus laevis* oocytes by measuring the uptake of 0.25 μM (10 μCi/ml) [^{3}H]-glycylsarcosine (Gly-Sar) at pH 5.5 with or without inhibitors. Uptake of Gly-Sar in oocytes was significantly inhibited by 25 mM Ala-Ala, Gly-Gly, and Gly-Sar ($p<0.05$), but not by 2.5 mM of Glu-Glu, Ala-Ala, Gly-Gly, Gly-Sar and 25 mM glycine and sarcosine. This result suggests that a selective transporter is involved in the endogenous uptake of dipeptides. Collagenase treatment of oocytes used to strip oocytes from ovarian follicles did not affect the Gly-Sar uptake. Changing pH from 5.5 to 7.5 did not affect the Gly-Sar uptake significantly, suggesting no dependence of the endogenous transporter on a transmembrane proton gradient. An exogenous H^+/peptide cotransporter was expressed after microinjection of polyadenylated messenger ribonucleic acid [poly(A)$^+$-mRNA] obtained from rabbit small intestine. The Gly-Sar uptake in mRNA-injected oocytes was 9 times higher than that in water-injected oocytes. Thus, frog oocytes can be utilized for expression cloning of the genes encoding intestinal H^+/peptide cotransporters. Size fractionation of mRNA was successfully obtained using this technique.

Key words: Cotransporter, Dipeptide, Expression, Glycylsarcosine, Oocytes

INTRODUCTION

A number of biologically active peptides are absorbed from the intestine into the blood, some of them rapidly and on a large scale. Peptides or their analogs that are active when given by mouth include a number of antibiotics, toxins, hypothalamic hormonal regulatory factors, and vitamins containing a peptide linkage, such as folic acid (pteroylglutamic acid) and pantothenic acid. Many biologically active peptides are structurally suitable for mediated uptake by the mechanisms responsible for transport of small dietary peptides (Mattews, 1987). Carnosine which is a dietary peptide as well as a putative neurotransmitter, and thyroliberin (pyro-Glu-His-Pro-NH2) can be rapidly and effectively absorbed (Mattews, 1987). Amino-β-lactam antibiotics, such as aminopenicillins and aminocephalosporins, appear to share a common uptake mechanism with ordinary dietary peptides, and some have been shown to be actively transported (Kimura, 1984; Nakashima *et al.*, 1984; Okano *et al.*, 1986a, b). Alafosfalin (l-alanyl-l-1-aminoethylphosphoric acid), an antibacterial phosphonodipeptide, has also been reported to share the intestinal transport systems for dietary peptides (Morley *et al.*, 1983). Further captopril (Hu and Amidon, 1988), angiotensin converting enzyme (ACE) inhibitors (Friedman and Amidon, 1989a, b), and renin inhibitors (Kramer *et al.*, 1990a) interact with the intestinal peptide transporter. Making poorly absorbed but therapeutically active molecules readily absorbable by incorporating molecular features required for the peptide transporter is an attractive approach.

Carrier-mediated transport of small peptides takes place in intestine, kidney, liver, brain (Banks *et al.*, 1990), skeletal muscle, red cells (Lochs *et al.*, 1990),

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and transformed cell lines. However, the structural requirements for peptide transport are not identical in all cells and tissues examined (Matthews, 1975). Small peptides are transported from the intestinal lumen into the absorptive cells by an efficient, specialized carrier-mediated process which is different from those of free amino acids in both animals and humans (Matthews, 1975, 1987; Kimura, 1984). It has been suggested that intestinal and renal transport of peptides involves co-transport with proton. Ganapathy and Leibach (1985) suggested that in the intact absorptive cell a Na^+ gradient might stimulate peptide transport indirectly by producing a proton gradient via the Na^+/H^+ exchanger.

Few studies on structural identification of peptide transporter have been done. Photoaffinity labeling demonstrates that a membrane protein of molecular weight 127 kDa could be a component of the intestinal transporter (Kramer, 1987; Kramer *et al.*, 1988a, 1990b). It was demonstrated that a specific interaction of the α-amino group in the substituent at position 6 or 7 of the penam or cephem nucleus, presumably with a histidine residue of the peptide transport protein, is involved in the translocation process of orally active α-amino-β-lactam antibiotics across the intestinal brush-border membrane (Kramer *et al.*, 1988b; Kato et al., 1989). Miyamoto *et al.* (1986) showed that histidyl and thiol groups are present at or near the active substrate-binding site of the rabbit renal dipeptide transporter. One or more vicinal dithiol groups are essential for the function of the renal dipeptide transporter, and these thiol groups must exist in a reduced form to maintain maximal transport activity (Miyamoto *et al.*, 1989). Kramer *et al.* (1988c) identified two binding polypeptides for β-lactam antibiotics and dipeptides with molecular weights 130 kDa and 95 kDa. Further, the transporters for β-lactam antibiotics and dipeptides in the brush-border membrane from rat kidney and small intestine are similar but not identical.

Molecular cloning and controlled expression of the transporter genes are essential to gain further insights into the biology of peptide transport. Cloning the genes encoding for the intestinal peptide transporter(s) may answer the following questions: How many distinct dipeptide transporters exist? What are their specificities? How is their expression regulated? What are the structure and functions of these membrane proteins? The cloned gene will allow us to fully characterize the molecular features of the transporter for therapeutic applications and to search for homologous genes with similar function. For example, its regulation may occur by voltage changes, binding of regulatory ions, such as H^+ or Ca^+, by chemical modification, such as phosphorylation, or by cellular trafficking, as it occurs in endocytosis or membrane fusion (Sachs and Fleischer, 1989). Transporters are universal elements regulating access of substrates to their sites of action, and therefore targets of therapeutically useful drugs.

For determining the biological properties of a transport polypeptide, it is necessary to clone the gene(s) encoding the transporter traditionally by isolating the protein for partially sequencing to obtain suitable molecular probes. The successful isolation of carrier proteins from biomembranes has so far been limited mostly to cases where the carrier is present in sufficiently high amounts (Klingenberg, 1989). In general, conventional purification procedures of such hydrophobic proteins are known to be difficult. An alternative method known as expression cloning has been introduced by Hediger *et al.* (1987a, b) to clone a Na^+/glucose transporter. Recently it has been reported that the intestinal peptide transporter in *Xenopus* oocytes was functionally expressed by the injection of exogenous poly$(A)^+$ mRNA isolated from rabbit intestinal mucosal cells (Miyamoto *et al.*, 1991). However it is essential to clone the peptide transporter in order to obtain better understanding of its molecular structure, regulation, and substrate specificity. As a preliminary experiment toward cloning the transporter, the characteristics of an endogenous peptide transporter in oocytes were studied along with expression of an exogenous proton/peptide cotransporter from rabbit intestine. In order to get an enriched fraction of mRNA from the rabbit intestine, further size fractionation was performed.

MATERIALS AND METHODS

Materials

[^{3}H]-Glycylsarcosine (39 Ci/mmol) and [^{14}C]-glucose (304 mCi/mmol) and were purchased from Amersham Co. (Arlington, IL) and were diluted as required. Collagenase A was from Boehringer Mannheim (Indianapolis, IN). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Micropipette puller, micromanipulator, beveler, and microinjector were obtained from Sutter Instrument Co. (Novato, CA).

Isolation of oocytes

Mature female *Xenopus laevis* frogs (Xenopus One, Ann Arbor, MI) were anesthetized with 0.3% tricaine solution. Individual oocytes were isolated manually from the ovarian lobes. The oocytes were washed and stored overnight at 18°C in Modified Barth's Solution (pH 7.5) with antibiotics.

Preparation of mRNA

Total cellular RNA was isolated from intestinal mucosal cells scraped from rabbit jejunum as described by Chomcyznski and Sacchi (1987). Poly(A)$^+$ mRNA was isolated using a commercial kit (Pharmacia LKB, Piscataway, NJ) from total RNA. Only mRNA preparations yielding a clear actin (or SGLT) band in Northern blots with actin (or SGLT) cDNA is used for microinjection.

Microinjection of mRNA

Each oocyte was injected into the vegital hemisphere with 50 nl of either mRNA (1 mg/ml) or DEPC-treated water as a control. The injected oocytes were incubated up to 5 days in Ca^{2+}-containing medium (5 mM Hepes/NaOH, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) at 18℃. Collagenase treatment of oocytes was done in the 1 mg/ml concentration for 4 hrs at room temperature.

Transport measurements

Five to seven oocytes were placed in 200 µl of uptake media (pH 5.5) containing 10 µCi/ml of [^{3}H]-Glycylsarcosine (Gly-Sar, Amersham) on a multiwell dish. Oocytes were incubated for 1 h at room temperature. The incubation was terminated by adding cold buffer. Oocytes were washed several times and transferred to scintillation vials. The radioactivity associated with the oocytes was determined in a liquid scintillation counter (Beckmann LS6000).

Size fractionation of total mRNA

100 µg of mRNA was fractionated on a linear sucrose gradient (5 to 25%, w/w) by centrifugation for 17h at 34,000 rpm (Maniatis *et al.*, 1982). 0.5 ml fractions were collected and precipitated with ethanol. Fractions were pooled into 5 pools (P1 to P5) to further inject into oocytes.

RESULTS AND DISCUSSION

In order to investigate the endogenous peptide transporter non-injected oocytes were put in two different concentrations of [^{3}H]Gly-Sar in the presence of high concentrations of cold Gly-Sar as an inhibitor. As shown in Fig. 1, the 1 µCi/ml (0.025 µM) of [^{3}H]Gly-Sar concentration didn't show enough uptake into oocytes. About 4 times increase in [^{3}H]Gly-Sar uptake was observed when 10 times higher concentration (10 µCi/ml) was used, but there was a significant inhibited uptake in the presence of 25 mM cold Gly-Sar. About 50% of the endogenous uptake seems to be a capacity limited process. From the above results it can be suggested that there is a saturable and endogenous uptake of Gly-Sar in frog oocytes. Similar results were

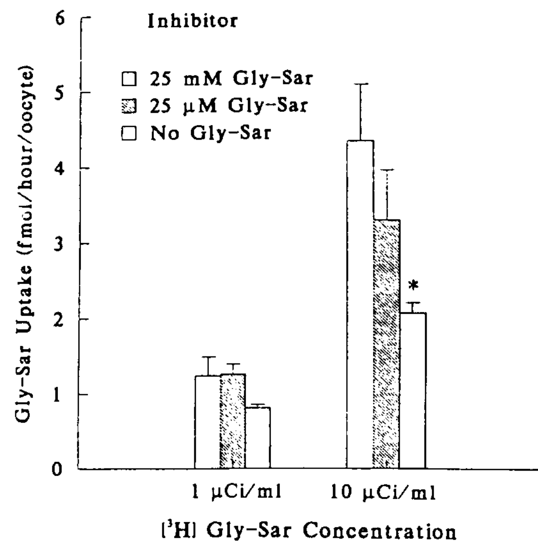

Fig. 1. Endogenous peptide uptake in the oocytes at two different concentrations of [^{3}H]Gly-Sar in the presence of high concentrations of cold Gly-Sar as an inhibitor. The 1 µCi/ml of tracer concentration was insufficient to study its uptake. Mean (± S.E.), n=3.
*p=0.017

reported by Miyamoto *et al.* (1991). They used the collagenase in order to liberate oocytes from their follicles. Unfortunately collagenase treated oocytes didn't survive many after microinjection because of fragile surfaces. Recently it was reported that by incubating oocytes with 5% horse serum in the usual saline solutions, one can increase oocyte viability (Quick *et al.*, 1992). Instead of enzymatic stripping, manual dissection of oocytes was performed under a stereomicroscope. We found that individual dissection gave much better viability than collagenase treatment (data aren't shown). Fig. 2 shows the effect of collagenase treatment on the endogenous Gly-Sar uptake with or without 25 mM cold Gly-Sar as an inhibitor. Uptake in collagenase treated oocytes is lower than that in non-treated ones. Both collagenase treated and non-treated oocytes have the same uptake in the presence of high concentration of Gly-Sar, showing about same passive uptake. Therefore manually dissected oocytes were used for further experiments.

The proton/dipeptide cotransporter requires a proton gradient during uptake (Mattews, 1987). The endogenous Gly-Sar uptake rate was examined in the different pHs ranging from 5.5 to 7.5. The pH effect on the endogenous Gly-Sar uptake in oocytes is shown in Fig. 3. There was no evident effect of proton gradient on the dipeptide transport in oocytes. A different batch of oocytes had shown similar results on the proton gradient effect, showing 10 times lower uptake rates (4.1±0.23 and 5.4±0.7 fmole/hr/oocyte for pH 5.5 and pH 7.5). We found that there was

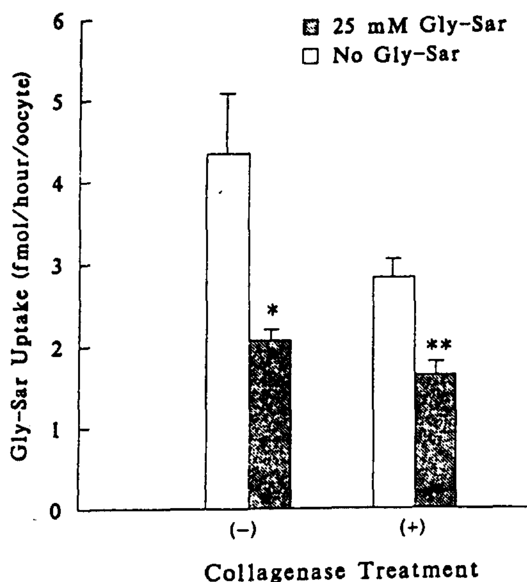

Fig. 2. Effect of collagenase treatment on the endogenous peptide uptake with or without 25 mM Gly-Sar as an inhibitor. There is no effect of collagenation, and 25 mM Gly-Sar inhibited endogenous uptake in both collagenase treated and nontreated oocytes. Mean (± S.E.) n=3 to 5 *p=0.017, **p=0.005

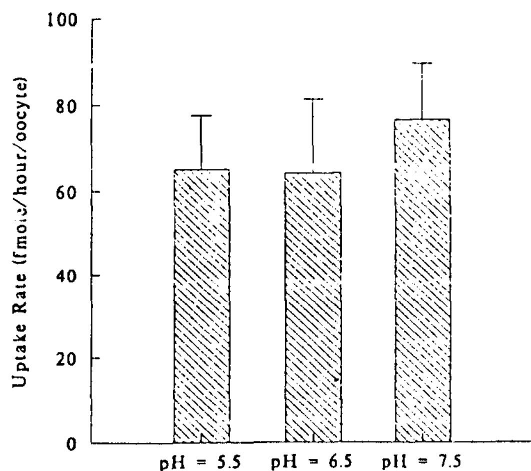

Fig. 3. The pH effect on the endogenous peptide uptake in *Xenopus* oocytes. The proton gradient does not affect the Gly-Sar uptake. Mean (± S.E.) n=10

a big seasonal change of uptake and that every batch of oocytes had a different background uptake. For experiment we had monitored the background uptake of Gly-Sar and used oocytes with a normal background (about 5 fmole/hour/oocyte).

In order to characterize the endogenous Gly-Sar uptake inhibition study was performed using individual amino acids and several dipeptides as inhibitors. Inhibition of the endogenous uptake in oocytes in the presence of individual amino acids is shown in Fig.

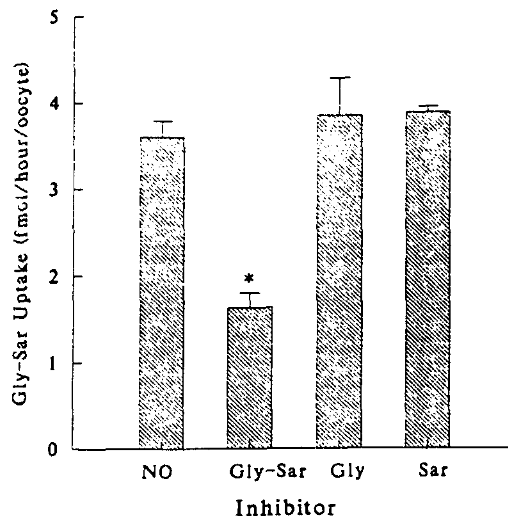

Fig. 4. Inhibition of endogenous peptide uptake in *Xenopus* oocytes. Individual amino acids did not affect the uptake, while 25 mM Gly-Sar reduced the uptake significantly. Mean (± S.E.). n=4 to 10 *p=0.001

Table I. Inhibition of endogenous Gly-Sar uptake in *Xenopus* oocytes by several dipeptides

Inhibitor	Concentration (mM)	Uptake (fmol/hour/oocyte)		% Control
		Mean	S.E.	
Control	–[a]	4.09	0.24	100
Ala-Ala	2.5	5.43	2.02	132.83
Ala-ALa	25	2.29	0.12	56.12[b]
Glu-Glu	2.5	4.46	0.18	109.05
Gly-Gly	2.5	3.62	0.59	88.59
Gly-Gly	25	3.1	0.13	75.81[b]
Gly-Sar	2.5	3.58	0.17	87.66
Gly-Sar	25	3	0.44	73.46b

[a]Concentration of Gly-Sar was 0.256 μM, [b]Significant (p<0. 05). n=3 to 9

4. There was no inhibited uptake in the presence of glycine or sarcosine, suggesting a distinct uptake mechanism from amino acid transporters. Again 25 mM cold glycylsarcosine significantly inhibited the [^{3}H]Gly-Sar uptake (p=0.001). Table I shows inhibition of the endogenous Gly-Sar uptake by several dipeptides. The uptake was inhibited with 25 mM Ala-Ala, Gly-Gly, and Gly-Sar. No inhibited uptake was detected when the concentration of inhibitors was 2.5 mM. It may be suggested that the endogenous dipeptide trasnsporter has slow kinetics to the substrates (large K_m).

In the other hand, the exogenous proton/peptide cotransporter from rabbit small intestine was expressed in *Xenopus* oocytes with 9 times higher uptake of Gly-Sar compared to water-injected oocytes at 4 days after microinjection (Fig. 5A). The results were consistent with the functional expression of the proton/peptide

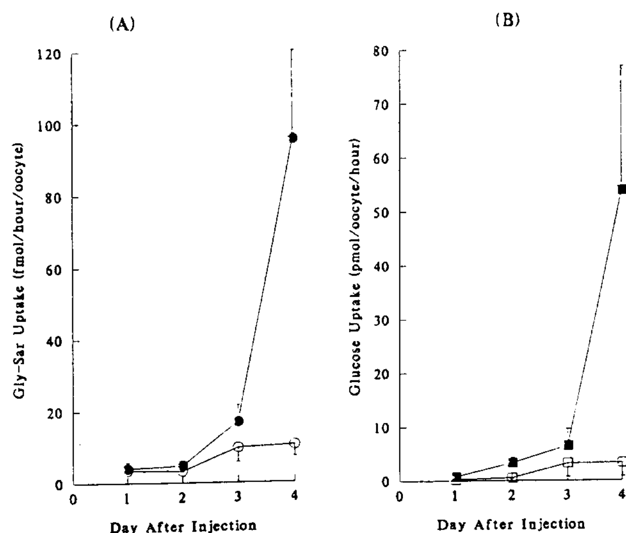

Fig. 5. Time dependence of the exogenous proton/peptide cotransporter expressed in *Xenopus* oocytes. The mRNA-injected oocytes expressed 9 times higher uptake compared to water-injected oocytes. Mean (± S.E.). n=3 to 6

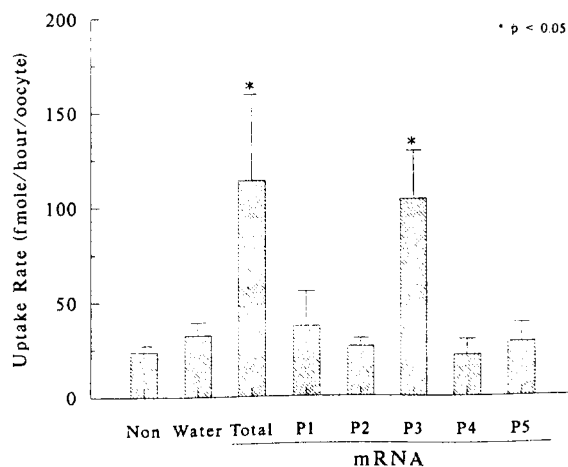

Fig. 6. Expression of exogenous proton/peptide cotransporter after microinjection of size fractionated mRNA into *Xenopus* oocytes. Approximately 10 ng of mRNA from each pool was injected and 50 ng of water or total mRNA were injected. Five days after microinjection the Gly-Sar uptake was measured. Pool 3 (P3) shows a significant expression over other pools. Mean (± S.E.). n=4 to 10

cotransporter reported by Miyamoto *et al.* (1991) but showed bigger expression in our experiments. Even though the uptake of Gly-Sar by the endogenous dipeptide transporter in water-injected oocytes exists a little, there was no problem to express the exogenous proton/dipeptide cotransporter in oocytes. Sodium/glucose cotransporter as a positive control was expressed in *Xenopus* oocytes in order to confirm the functional expression technique. As shown in Fig. 5(B), glucose uptake was significantly higher in mRNA-injected oocytes at 4 days after microinjection, verifying the technique applicable.

Size fractionation of total mRNA from the rabbit jejunum was performed and each mRNA fraction was examined for functional expression of proton/peptide cotransporter after microinjection. One of fractions, P3 showed enomous expression of the cotransporter as shown in Fig. 6. The functional expression after microinjection of 10 ng mRNA was about the same as that after microinjection of 50 ng of total mRNA. Using this enriched fraction, further investigation will be continued toward the cloning of the genes.

In conclusion the presence of an endogenous peptide transporter was confirmed by an inhibition study with several dipeptides. It is specific for dipeptides but not amino acids. Collagenase treatment of oocytes used to strip oocytes from ovarian follicles did not affect the endogenous peptide uptake. The endogenous Gly-Sar uptake was not affected by changing pH from 5.5 to 7.5. Exogenous proton/peptide cotransporter from rabbit jejunum was successfully expressed in *Xenopus* oocytes. Therefore, functional expression can be utilized for cloning of the genes encoding intestinal proton/peptide cotransporters. For one step toward the cloning, enriched expression of exogenous proton/peptide cotransporter was obtained after size fractionation of total mRNA of rabbit intestines.

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