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FUNCTIONAL EXPRESSION OF A PEPTIDE TRANSPORTER IN *XENOPUS* OOCYTES

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ABSTRACT

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. Using the technique size fractionation of mRNA was sucessfully obtained.

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 (1).

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 (1). Amino-β-lactam antibiotics, such as aminopenicillins and aminocephalopsporins, appear to share a common uptake mechanism with ordinary dietary peptides, and some have been shown to be actively transported (2-5). Alafosfalin (l-alanyl-l-1-aminoethylphosphoric acid), an antibacterial phosphonodipeptide, has also been reported to share the intestinal transport systems for dietary peptides (6). Further captopril (7), angiotensin converting enzyme (ACE) inhibitors (8,9), and renin inhibitors (10) 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 (11), skeletal muscle, red cells (12), and in transformed cell lines. However, the structural requirements for peptide transport are not identical in all cells and tissues examined (13). Small peptides are transported from the intestinal lumen into the absorptive cells by an efficient, specialized carrier-mediated process which is different from that of free amino acids in both animals and humans (1,2,13). It has been suggested that intestinal and renal transport of peptides involves co-transport with proton. Ganapathy and Leibach (14) 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 (15-17). 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 (18,19). Miyamoto et al. (20) 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 (21). Kramer et al. (22) 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 (23). 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 (24). 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. (25,26) 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 (27). 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.

METHODS

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 (28). 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 (5mM Hepes/NaOH, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) at 18 °C.

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 (29). 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

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	--*	4.09	0.24	100
Ala-Ala	2.5	5.43	2.02	132.83
Ala-ALa	**25**	**2.29**	**0.12**	**56.12****
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****
Gly-Sar	2.5	3.58	0.17	87.66
Gly-Sar	**25**	**3**	**0.44**	**73.46****

* Concentration of Gly-Sar was 0.256 μM
** Significant ($p < 0.05$). n = 3 to 9

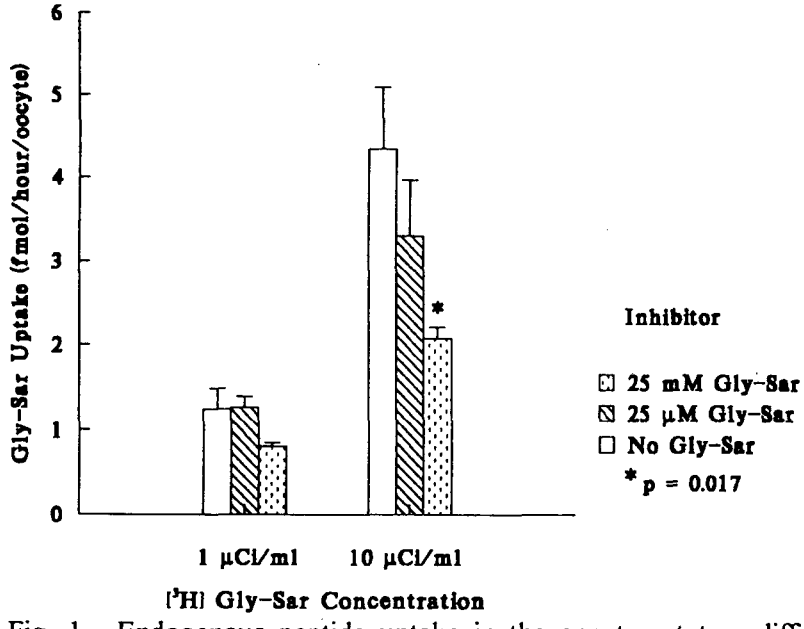

Fig. 1. Endogenous peptide uptake in the oocytes at two different concentrations of [^{3}H]Gly-Sar in the presence of high concentrations of cold Gly-Sar as an inhibitor. The 1 μCi/ml of tracer concentration was insufficient to study its uptake. Mean (± S.E.)

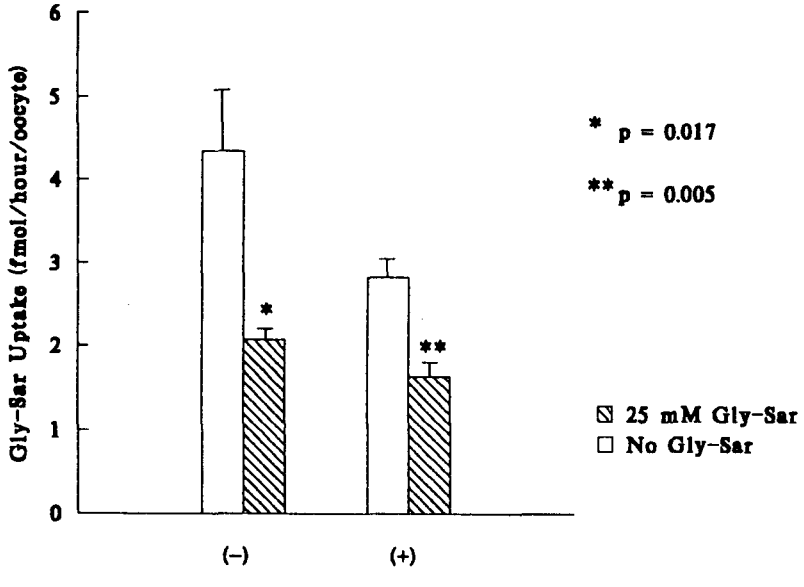

Fig. 2. Effect of collagenase treatment on the endogenous peptide uptake with or without 25 mM Gly-Sar as an inhibitor. There is no effect of collagenation, and 25 mM Gly-Sar inhibited endogenous uptake in both collagenase treated and nontreated oocytes. Mean (± S.E.) n = 3 to 5.

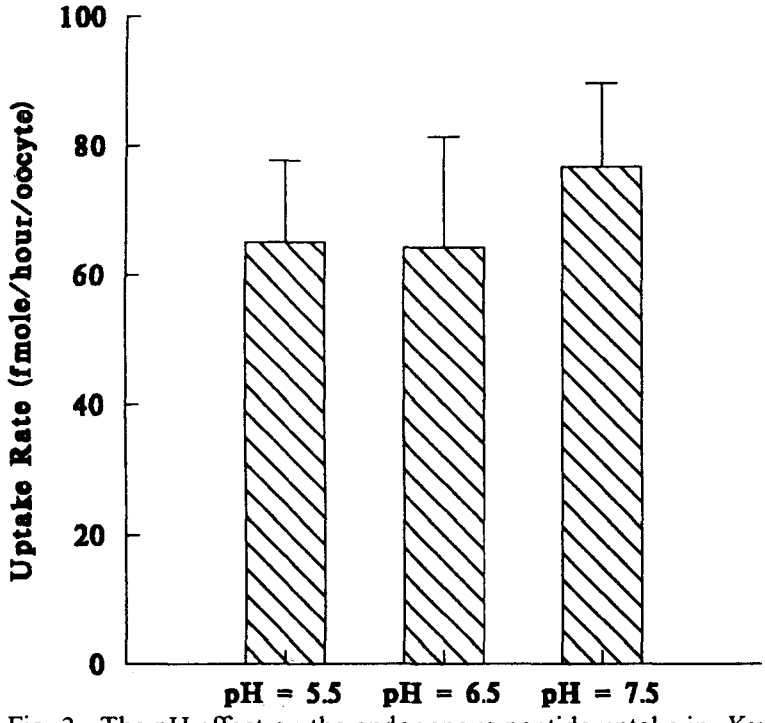

Fig. 3. The pH effect on the endogenous peptide uptake in *Xenopus* oocytes. The proton gradient does not affect the Gly-Sar uptake. Mean (± S.E.). n = 10.

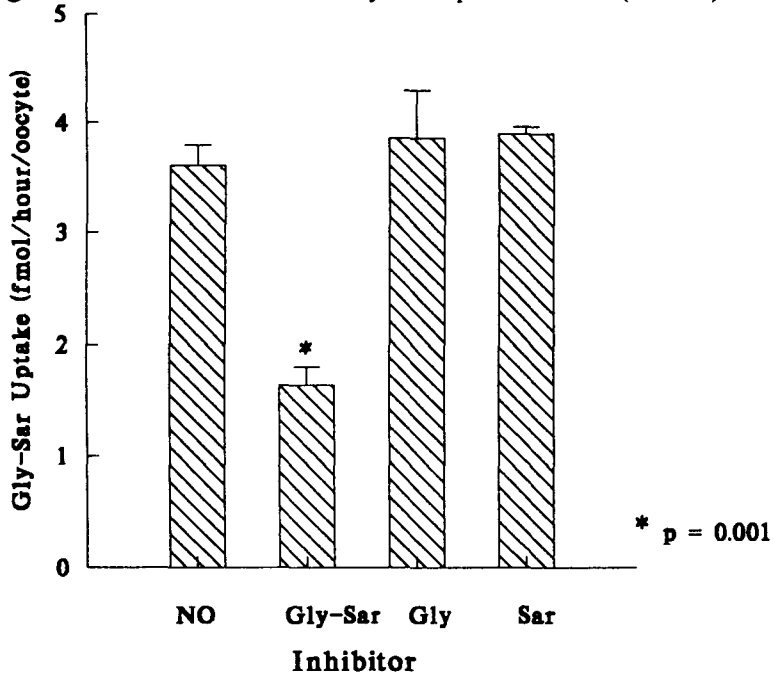

Fig. 4. Inhibition of endogenous peptide uptake in *Xenopus* oocytes. Individual amino acids did not affect the uptake, while 25 mM Gly-Sar reduced the uptake significantly. Mean (± S.E.). n = 4 to 10.

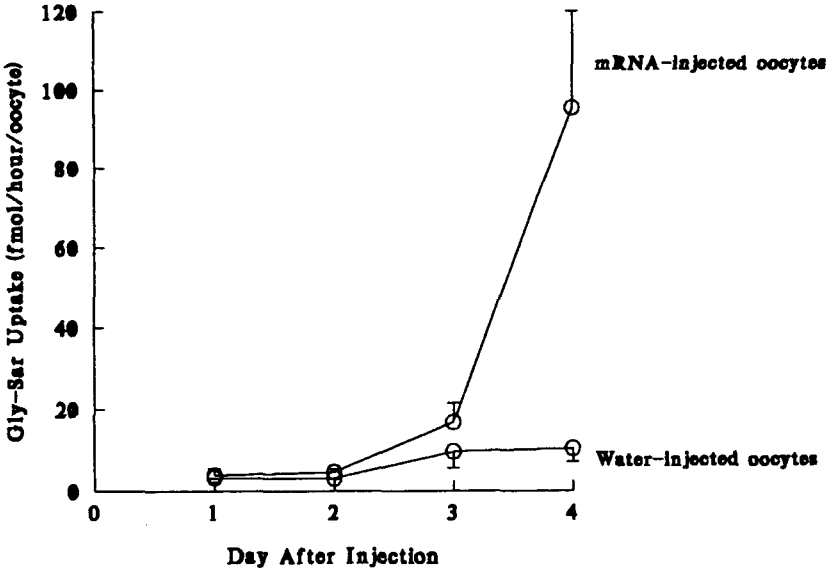

Fig. 5. Time dependence of the exogenous proton/peptide cotransporter expressed in *Xenopus* oocytes. The mRNA-injected oocytes expressed 9 times higher uptake compared to water-injected oocytes. Mean (± S.E.). n = 3 to 6.

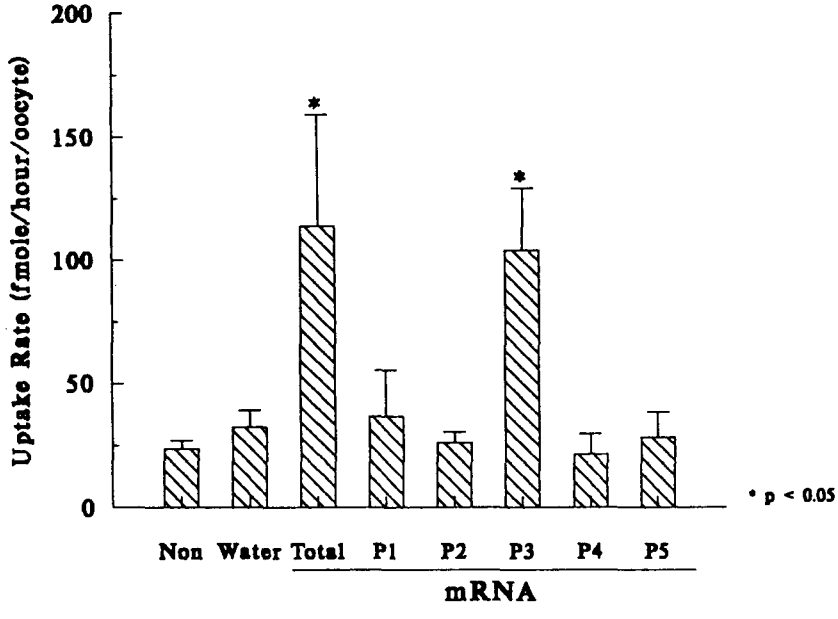

Fig. 6. Expression of exogenous proton/peptide cotransporter after microinjection of size fractionated mRNA into *Xenopus* oocytes. Approximately 10 ng of mRNA from each pool was injected and 50 ng of water or total mRNA were injected. Five days after microinjection the Gly-Sar uptake was measured. Pool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

CONCLUSIONS

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, frog oocytes can be utilized for expression cloning of the genes encoding intestinal proton/peptide cotransporters. The enriched expression of exogenous proton/peptide cotransporter was obtained after size fractionation of total mRNA of rabbit intestines.

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