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Characterization of an oligopeptide transporter in renal lysosomes

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Abstract

Renal lysosomes play a major role in catabolism of plasma proteins. Final products of this catabolism include dipeptides and tripeptides that must be exported to the cytosol for hydrolysis. The aim of the present study was to determine whether an oligopeptide transporter is present in the renal lysosomal membrane that could mediate this export. The existence of an oligopeptide transporter was probed with the uptake of glycylglutamine (Gly-Gln) by membrane vesicles prepared from renal lysosomes. Kinetic analysis showed the presence of a single transporter with a K_m of 8.77 mM for the uptake of Gly-Gln. The Gly-Gln uptake was energized by the imposition of an inwardly directed proton gradient (pH_{out} 5.0/pH_{in} 7.3) and membrane potential (outside positive/inside negative) resulting in overshoot. The Gly-Gln uptake was inhibited by the presence of dipeptides and tripeptides, but not amino acids. Western blot analysis of lysosomal membrane proteins with Pept-1 (an oligopeptide transporter) antibody as the probe showed the presence of an immunoreactive protein. This immunoreaction was abolished when the antiserum was preabsorbed with the Pept-1 epitope (0.5 μg/ml). In conclusion, the present data show the existence of a low-affinity dipeptide transporter in the renal lysosomal membrane that appears to belong to the Pept family of transporters. The function of this transporter appears to be to prevent accumulation of dipeptides in renal lysosomes.

Keywords: Glycylglutamine; Oligopeptide transporter; Pept-1; Plasma protein; Renal lysosome

1. Introduction

We recently reported the presence of an oligopeptide transporter in hepatic lysosomal membrane [1]. A key question that follows this discovery is whether this transporter is unique to the liver lysosomes, or is it present in other lysosomes. Among the extrahepatic tissues, kidney is the most likely tissue to have a lysosomal oligopeptide transporter. Kidney is the site for large-scale degradation of plasma proteins, for example, over 8 g of plasma albumin are catabolized daily in this site [2]. After filtration across glomeruli, 99% of plasma proteins are reabsorbed via the renal proximal tubules by endocytosis [2]. After endocytosis, the plasma proteins are delivered to lysosomes for degradation [2]. The final products of lysosomal degradation of proteins include small peptides, mostly dipeptides [3].

These products must be exported to the cytoplasm in order to prevent osmotically-induced swelling and rupture of lysosomes. The objective of the present study was to determine whether renal lysosomes con-

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tain an oligopeptide transporter, and, if so, to elucidate its characteristics. The presence of a transporter was probed with the uptake of glycylglutamine (Gly-Gln) by membrane vesicles of renal lysosomes.

2. Materials and methods

2.1. Materials

Adult male rats (250–300 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Custom synthesized [*glutamine*-3,4-^{3}H]glycylglutamine (49 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Filters (type HAWP, 0.45 μm pore size) were purchased from Millipore Corp. (Bedford, MA). Cefadroxil and other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation of lysosomal membrane vesicles (LMV)

The kidneys were removed immediately after rats were killed. LMV were prepared as described previously [1]. Briefly, kidneys were cut into small pieces and homogenized in buffer 1 (250 mM sucrose, 20 mM HEPES/Tris, 1 mM EDTA, pH 7.0) containing 1 μg/ml pepstatin and 2 μg/ml leupeptin, using a Dounce homogenizer. The kidney homogenate was centrifuged for 20 min at $500\times g$ to eliminate the nuclear pellet. The resulting supernatant was centrifuged for 30 min at $20\,000\times g$ and the pellet was resuspended in buffer 2 (250 mM sucrose, 20 mM HEPES/Tris, pH 7.0). The suspension was mixed with isotonic Percoll (2.5 M sucrose and 200 mM HEPES/Tris, pH 7.0, mixed with Percoll at 1:9) in the ratio of 9:11. The Percoll mixture was centrifuged for 90 min at $40\,000\times g$. The brownish lysosomal band near the bottom of the gradient was isolated. LMV were prepared by incubation of lysosomes in buffer 2 containing 5 mM methionine methyl ester, 2 mg/ml bovine serum albumin and 2 mM magnesium chloride for 20 min at 37°C. An equal volume of ice-cold isotonic Percoll was added to the incubation mixture and the mixture was centrifuged at $35\,000\times g$ for 30 min. Purified LMV were located on the top of this dense gradient as a brownish band. Finally, LMV were diluted in preloading buffer (100 mM KCl, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.3) and used in uptake experiments. The protein concentration was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The purity of the LMV preparation was determined as described previously [1]. The activities of β-hexosaminidase, cytochrome *c* oxidase, and 5′-nucleotidase in kidney homogenate and vesicle preparation were measured by methods described previously [1].

2.3. Gly-Gln uptake

The uptake of Gly-Gln by LMV was measured by rapid filtration technique as described previously [4]. Briefly, after preincubation with valinomycin at room temperature for 30 min, 20 μl of LMV suspension (LMV in preloading buffer) was mixed with 180 μl of transport buffer containing Gly-^{3}H-Gln. Since the composition of the transport buffers varied, each is described in the relevant figure legend. The mixtures were incubated at 23°C for various predetermined lengths of time. The uptake was terminated by injecting 200 μl of this vesicle mixture to 2 ml of ice-cold stop solution (same composition as the transport buffer, but without Gly-^{3}H-Gln) and filtered immediately (0.45 μm pore). The filters were then washed with 5 ml of ice-cold stop solution and the associated radioactivity was counted by scintillation spectrometer.

2.4. Western blot analysis

A Western blot analysis of LMV proteins was performed with Pept-1 antibody as the probe. The following technique was used to prepare Pept-1 antibody. Based on the molecular structure of rat Pept-1, a synthetic peptide (Glu-Asn-Pro-Tyr-Ser-Ser-Leu-Glu-Pro-Val-Ser-Gln-Thr-Asn-Met) corresponding to the 15 carboxy-terminal amino acids (696–710) was used as the epitope [5]. The rabbit Pept-1 antibody was generated by immunization of rabbit with this epitope, and the specificity of the antibody was confirmed by Western blot analysis with the antibody that had been preabsorbed with the epitope (0.5 μg/ml).

LMV from kidney and liver were denatured, subjected to 10% SDS-polyacrylamide gel electrophore-

sis, and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked in 10% non-fat dry milk dissolved in Tris-buffered saline containing 0.5% Tween 20 (polyoxyethylenesorbitan monolaurate) (TBST), washed with TBST, and then incubated with Pept-1 antibody (1:1000). After incubation, the membrane was washed with TBST again, incubated with the second antibody (peroxidase-conjugated goat anti-rabbit IgG, 1:2000) and then detected with ECL Western blotting system (Amersham Life Science, Arlington Heights, IL).

2.5. Calculation and statistical analysis

Kinetic constants of Gly-Gln transport were determined by applying a nonlinear regression method to the Michaelis–Menten kinetic equation using GRAFIT (Sigma):

$$V = (V_{max}[S])/(K_m + [S])$$

where V is Gly-Gln uptake in nmol/mg protein/40 s, S is external Gly-Gln concentration in mM, V_{max} is maximal Gly-Gln uptake, K_m is the concentration of S that yielded one half V_{max}. Data are given as mean ± S.E.M. of at least three replicates. Significant differences between values were determined by Student's t-test.

3. Results

3.1. Purity of LMV

Our membrane vesicle preparations showed fold depletion of both cytochrome c oxidase (a mitochondrial marker) and 5′-nucleotidase (a plasma membrane marker) (Table 1). On the other hand, there was about a 10-fold enrichment of β-hexosaminidase (a lysosomal marker) in the vesicle preparation (Table 1). These results are similar to our previous results in the liver LMV [1]. In our previous study [1], we showed 100-fold enrichment of β-glucosidase (a lysosomal membrane marker) in the liver LMV. This enrichment determination could not be performed in the present study because in kidney cells, unlike in liver cells, β-glucosidase is not present in the lysosomal fraction since it remains in the soluble fraction during LMV isolation.

3.2. Gly-Gln uptake as a function of time

Evidence suggesting the presence of an active solute transporter requires the demonstration of an 'overshoot' in membrane vesicles. An overshoot is observed when membrane vesicles transiently accumulate that solute in a concentration that is higher than its concentration in the incubation medium. To seek such evidence, we determined Gly-Gln uptake by renal LMV as a function of time in the presence and absence of an inwardly directed proton gradient and membrane potential. In previous studies we have shown that these conditions are needed for observing overshoot in transport of dipeptides such as Gly-Gln [1,4]. It should be noted that at the pH used in this experiment, Gly-Gln is in a zwitterionic form and, therefore, carries no net electrical charge.

As shown in Fig. 1, in the absence of a proton gradient (pH_{out} 7.3/pH_{in} 7.3) Gly-Gln concentration in LMV progressively increased during the initial 2 min and then remained unchanged for the remaining period of observation. On the other hand, in the presence of a proton gradient (pH_{out} 5.0/pH_{in} 7.3),

Table 1
Specific activity, yield, and purification of marker enzymes in LMV

Enzyme	Specific activity in homogenate	LMV		
		Specific activity	Yield (%)	Purification factor
β-Hexosaminidase	136 ± 6.85	1322 ± 182.7	8.4	9.7
Cytochrome c oxidase	3228 ± 291	199 ± 43.1	0.01	0.06
5′-Nucleotidase	162 ± 28.4	8.12 ± 6.36	< 0.01	0.05

Specific activities are given as mean ± S.D. of at least three assays. Units of enzyme activities are defined as nmol min^{-1} mg $protein^{-1}$ for β-hexosaminidase; μmol h^{-1} mg $protein^{-1}$ for 5′-nucleotidase; and Δ(log OD) min^{-1} mg $protein^{-1}$ for cytochrome c oxidase. Yield is expressed as the percentage of the total activity of the homogenate recovered in the LMV fraction, expressed as the mean of at least three assays.

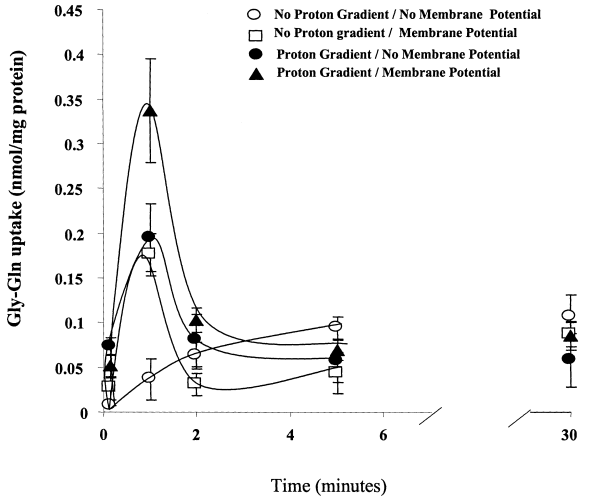

Fig. 1. Gly-Gln uptake as a function of time. Control (○): Renal LMV were preloaded with preloading buffer (100 mM KCl, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.3), preincubated with 50 μM valinomycin for 30 min at room temperature, and then incubated with the transport buffer containing 0.1 mM Gly-^{3}H-Gln, 100 mM mannitol, 100 mM KCl, 20 mM HEPES/Tris, pH 7.3. In presence of proton gradient (●): conditions were the same as above, except that the HEPES/Tris, pH 7.3, was replaced with MES/Tris, pH 5.0, in the transport buffer. In presence of membrane potential (□): conditions were the same as those in the absence of proton gradient except that the 100 mM KCl in the transport buffer was replaced with 100 mM choline chloride. In presence of membrane potential plus proton gradient (▲): conditions were the same as above except that HEPES/Tris, pH 7.3, in the transport buffer was replaced with MES/Tris, pH 5.0.

over a three-fold overshoot in Gly-Gln uptake occurred 1 min after the incubation.

As shown in Fig. 1, imposition of a membrane potential also resulted in over a three-fold overshoot in Gly-Gln uptake 1 min after the incubation. The membrane potential was generated by replacing the KCl in the transport medium with 100 mM choline chloride while maintaining the KCl concentration inside the vesicles at 100 mM. A much greater overshoot (over five-fold) than the ones observed with the imposition of proton gradient or membrane potential occurred when these driving forces were combined (Fig. 1).

3.3. Gly-Gln uptake as a function of osmolarity

To determine the extent of Gly-Gln uptake (Fig. 1) as transport into membrane vesicles, we determined Gly-Gln uptake as a function of medium osmolarity. Osmolarity was varied by addition of sucrose (0–900 mM) to the medium. The results showed a linear relationship between the rates of uptake and the reciprocal of medium osmolarities (Fig. 2). Since the osmoplot showed no Gly-Gln uptake at infinite osmolarity (Fig. 2), the uptakes shown in Fig. 1 were all transport into an osmotically reactive space.

3.4. Gly-Gln uptake as a function of concentration

To determine the kinetic constants of Gly-Gln uptake by renal LMV, the rates of uptake as a function of concentration were determined. To cover the K_m of both high- and low-affinity transporters, the concentration used ranged from 100 μM to 20 mM. These data were used to develop an Eadie–Hofstee plot (Fig. 3). Although the lower concentrations are not shown, a linear relation between v and v/s was observed over the entire range of concentration used, indicating the presence of a single transporter for Gly-Gln uptake. The calculation of kinetic constants showed that the transporter has a K_m of 8.77 ± 2.72 mM and a V_{max} of 11.00 ± 1.45 nmol/mg protein/40 s.

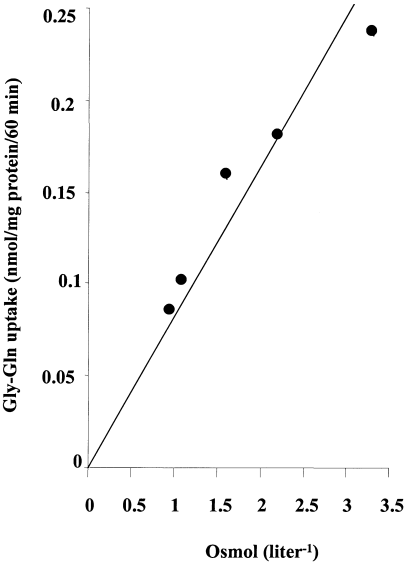

Fig. 2. Gly-Gln uptake as a function of osmolarity. Renal LMV were preloaded with preloading buffer (100 mM KCl, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.3) preincubated with 50 μM valinomycin for 30 min at room temperature, and then incubated with the transport buffer containing 0.1 mM Gly-^{3}H-Gln, 100 mM mannitol, 100 mM KCl, 20 mM MES/Tris, pH 5.0, and 0–900 mM sucrose for 60 min.

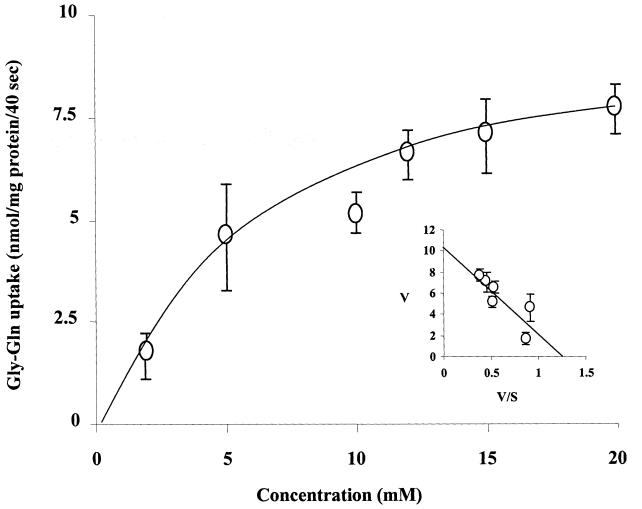

Fig. 3. Gly-Gln uptake as a function of substrate concentration. Renal LMV were preloaded with preloading buffer (100 mM KCl, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.3), preincubated with 50 μM valinomycin for 30 min at room temperature, and then incubated with the transport buffer containing 2–20 mM Gly-^{3}H-Gln, 100 mM mannitol, 100 mM choline chloride, and 20 mM MES/Tris, pH 5. The insert shows Eadie–Hofstee transformation of the data; V is the rate of Gly-Gln uptake (nmol/mg protein/40 s) and S is the concentration of Gly-Gln in the medium (mM).

3.5. Inhibition of Gly-Gln uptake

Inhibition profile is commonly used to determine the substrate specificity of a transporter. We, therefore, determined inhibition of lysosomal uptake of Gly-Gln by representative amino acids (glycine and glutamine), dipeptides (Gly-Gln, Gly-Sar, Gly-Pro), tripeptides (Gln-Gln-Gly, Gly-Pro-Ala), and a peptidomimetic drug (cefadroxil). Glycine and glutamine had no effect on Gly-Gln transport, but the others greatly inhibited this transport (Table 2).

3.6. Western blot analysis of the oligopeptide transporter

To investigate whether the transporter in renal lysosomes belonged to the family of oligopeptide transporters recently cloned, we carried out Western blot analyses of renal LMV with an antibody against Pept-1 (Fig. 4). Pept-1 is the brush-border membrane oligopeptide transporter in the intestinal and renal epithelium [6,7]. The results showed the presence of a Pept-1 immunoreactive protein in renal LMV. The

Table 2
Inhibition of Gly-Gln uptake

Substrate	Uptake (%)
Gly-Gln (control)	100
Gly-Gln	0
Gly-Sar	16
Gly-Pro	0
Gly-Gly-Gly	20
Gly-Pro-Ala	0
Glycine	100
Glutamine	100
Cefadroxil	32

Renal LMV were preloaded with preloading buffer (100 mM KCl, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.3), preincubated with 50 μM valinomycin for 30 min at room temperature, and then incubated for 40 s with the transport buffer containing 0.1 mM Gly-^{3}H-Gln, 100 mM mannitol, 100 mM choline chloride, 20 mM MES/Tris, pH 5, and a possible inhibitor of Gly-Gln uptake at concentration of 30 mM.

specificity of this protein was established by its disappearance when the antiserum was preabsorbed with the Pept-1 epitope.

Since our previous studies have shown the presence of an oligopeptide transporter in hepatic LMV [1], we also carried out Western analyses of hepatic

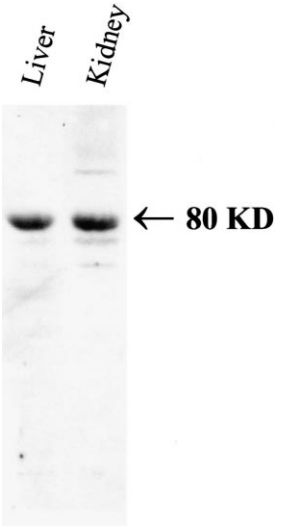

Fig. 4. Western blot analysis of the transporter. Equal amounts (100 μg) of protein from renal and hepatic LMV were loaded on 10% SDS-polyacrylamide gel. The Pept-1 antibody was used to probe the oligopeptide transporter in all the above membrane preparations. The peroxidase-conjugated goat anti-rabbit IgG was used for the detection of bound antibodies and the blot was visualized by chemiluminescence on X-ray film.

LMV with antibody against Pept-1 as the probe. The results showed the presence of a Pept-1 immunoreactive protein.

4. Discussion

The present results indicate that liver is not unique in having a lysosomal oligopeptide transporter since kidneys appear to have a similar transporter. The similarity between the hepatic and renal lysosomal transporters is suggested by the following comparison. Both are low affinity transporters (K_m in the mM range). Both are energized by an inwardly directed proton gradient and membrane potential resulting in overshoot (Fig. 1). Both have a similar inhibition profile, namely inhibition by dipeptides and tripeptides, but not by amino acids (Table 2). The question of whether the oligopeptide transporter is expressed only in the lysosomes of cells with high rates of protein degradation (for example, liver and kidney) or ubiquitously expressed in cell lysosomes cannot be answered until a systematic tissue study is performed.

A major difference exists between liver and kidney in the uptake of dipeptides circulating in plasma. The apical membrane of renal cells contains two transporters for the uptake of dipeptides, which are filtered across glomeruli [7], while the sinusoidal membrane of hepatic cells does not have any transporter for the uptake of dipeptides [8,9]. As a result, renal cells accumulate dipeptides circulating in plasma, while hepatic cells do not participate in this metabolic process [10]. Therefore, renal lysosomes, unlike hepatic lysosomes, could play a substantial role in the metabolism of circulating dipeptides. However, our data (here and with hepatic LMV) suggest that the function of the lysosomal oligopeptide transporter is to export rather than to import dipeptides. For example, conditions such as proton gradient and membrane potential, which normally exist across lysosomal membrane, favor export and not import of dipeptides. In fact, in vitro incubation studies have shown that (a) lysosomes release oligopeptides [11], and (b) if lysosomes import dipeptides, they are likely to swell and rupture [12].

Another novel finding of the present study was that the lysosomal oligopeptide transporter belongs to the Pept family of transporters recently cloned. Besides Pept-1, the cloned transporters include Pept-2, which is located on the brush-border membrane of renal tubular cells [7]. In view of a substantial homology among the oligopeptide transporters, we speculated that a Pept-1 antibody would recognize the transporter in lysosomes. We chose Pept-1 over Pept-2 antibody because Pept-1, like the lysosomal transporter, is a low affinity transporter while Pept-2 is a high affinity transporter. This speculation proved to be productive with our finding of a protein in the membrane of renal and hepatic lysosomes that would disappear if antiserum was preabsorbed with Pept-1 epitope. However, we do not believe that the lysosomal oligopeptide transporter is actually Pept-1, because Pept-1 gene expression is absent in the liver [5,13]. Using the same strategy as we have done in the present experiment, others [14] have found the presence of Pept-1 immunoreactive protein in the lysosomes of acinar cells of the pancreas. However, whether the lysosomes in the pancreatic acinar cells are capable of peptide transport was not studied. Therefore, the significance of Pept-1 immunoreactive protein in the lysosomes of acinar cells remains to be determined.

In conclusion, we suggest that the main function of the oligopeptide transporter in renal lysosomes is to provide an active mechanism for the completion of the final stage in the catabolism of circulating proteins. Dipeptides and tripeptides, produced from this catabolism, are transferred from a region of low peptidase activity (lysosome) to a region of high peptidase activity (cytosol). This suggestion brings a new dimension to the concept of catabolism of plasma proteins in health and in disease.

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