Renal Assimilation of Short Chain Peptides: Visualization of Tubular Peptide Uptake

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Purpose. Renal assimilation of short chain peptides plays an important role in systemic protein metabolism and amino acid homeostasis. The transepithelial peptide transport across the apical membrane of tubular cells is mediated almost exclusively by pH-dependent H^+-peptide symport pathways. The current study was designed to identify by visualization functional peptide transport activity along the nephron structures.

Methods. Visualization of peptide uptake was achieved by using the fluorescent dipeptide derivative D-Ala-Lys-AMCA and unlabelled cefadroxil and glycylglutamine as transport competitors to demonstrate specificity. To confirm these assays, rat specific cRNA probes were synthesized and non-isotopic high-resolution in-situ-hybridization and northern blot analysis were carried out to demonstrate the expression of the high-affinity peptide transporter PEPT2.

Results. The reporter molecule was accumulated by cells of the proximal tubulus but not in glomerular or endothelial cells. Inhibition studies revealed competitive inhibition of D-Ala-Lys-AMCA uptake by the betalactam cefadroxil and the dipeptide glycylglutamine. The control organs intestine and spleen did not show uptake of the systemically administered molecule. Non-isotopic mRNA in-situ-hybridization, using an antisense probe for rat PEPT2 confirmed uptake assays by identifying PEPT2 expression throughout segments of the straight proximal tubule at the inner cortex and outer stripe.

Conclusions. We demonstrate for the first time renal *in vivo* transport activity of a dipeptide that allows cells that participate in peptide reabsorption to be visualized. This functional assay may be used to investigate renal peptide transport mechanisms and test new compounds that are transported via proton-driven peptide transporters.

KEY WORDS: uptake assay; visualizing; peptide transport; oligopeptides; kidney.

INTRODUCTION

pH-dependent transport processes for di- and tripeptides play a central role in the transcellular reabsorption of renal amino acid nitrogen and for the plasma clearance of circulating peptides (1).

Tubular reabsorption is accomplished by apically located H^+-peptide symporters that allow cellular accumulation of peptides. The cDNAs encoding the mammalian proton-coupled peptide transporters have been cloned from rat (2), rabbit (3–5), murine (6), and human (7) tissues.

Whereas PEPT1 is mainly expressed in the small intestine (8) and to a lesser extent in kidney (9), PEPT2 is expressed in the kidney (10), the central and peripheral nervous system (11,12) and in a variety of other peripheral tissues including the respiratory system (13,14). Both peptide transporters possess 12 membrane-spanning domains and share an identity of approximate 47% at the protein level. The carrier-proteins mediate electrogenic uphill peptide transport by coupling of substrate translocation to the movement of H^+/H_3O^+ with the transmembrane electrochemical proton gradient serving as the driving force (15). In addition to short chain peptides, both carrier-proteins transport several peptidomimetics such as aminocephalosporins, aminopenicillins, bestatin, delta-aminolevulinic acid (d-ALA) and selected ACE inhibitors (1,13).

The intrarenal distribution of PEPT1- and PEPT2-mRNA has recently been demonstrated in rat using radiolabeled molecular probes and RT-PCR (9). Localization of the PEPT1-mRNA was assigned to the kidney cortex and the PEPT2-mRNA to the outer stripe of the outer medulla. Due to limited structural resolution at the cellular level in these studies, it was not possible to determine the exact cellular localization of the transporters. Immunolocalization of the transporters confined the first segment of the proximal tubule as the main site of PEPT1 expression and PEPT2 to the later segments of the proximal tubule (10).

Although peptide transport activity has been shown in renal cell lines and membrane preparations (16,17) we developed a technique that allows transport to be visualized directly and that could serve a pharmacological model to study interactions of drugs with renal peptide transport pathways.

METHODS

Adult Sprague-Dawley rats (300–500 g body weight) were allowed free access to standard laboratory chow and tap water. For each of the following techniques tissue samples of 8 animals were used. The studies were performed according to the Helsinki protocol and approved by the state ethics committee (Reg 0109/01).

Synthesis of the Reporter Molecule

As reporter molecule (D)-Ala-(L)-Lys-N-epsilon-7-amino-4-methylcoumarin-3-acetic acid (D-Ala-Lys-AMCA) was chosen. Synthesis of the conjugated dipeptide was carried out as described previously (18,19). D-Ala-Lys-AMCA was purified by a two-directional thin layer chromatography and compound concentrations were determined on the basis of its molar extinction coefficient (excitation at 318 nm, emission at 406 nm).

Uptake Studies

Uptake experiments were carried out by i.v.-application of 25μM of the fluorescent dipeptide derivative D-Ala-Lys-AMCA into the dorsal caudal vein. D-Ala-Lys-AMCA was identified earlier as specific peptide transporter substrate

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ABBREVIATIONS: AMCA, 7-amino-4-methyl-coumarin-3-acetic-acid; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 1% Tween 20; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl and 0.015 M sodium citrate; TBS, tris buffered saline; TBST, tris buffered saline containing 1% Tween 20.

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(20,21). For inhibition studies, 25 μM D-Ala-Lys-AMCA and 1 mM of unlabeled glycyl-(L)-glutamine or 1mM unlabeled cefadroxil was used (Fig. 1). Controls were performed by omitting the labeled dipeptide conjugate.

Experiments were stopped after 30 min by a CO_2 -overdose. Then a perfusion with ice-cold unlabeled Eagle's minimum essential medium (MEM-21011, GIBCO, Karlsruhe, Germany) for 10 min was performed and followed by perfusion fixation with freshly prepared 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at pH 7.4 for 5 min. Kidneys, intestine and spleen were removed to serve as control tissues. The organs were subsequently immersed in a sucrose-PBS solution adjusted to 800 mosmol/kg for cryoprotection and shock-frozen in liquid nitrogen-cooled isopentane. After processing to 10 μm cryostat sections the slides were viewed using epi-fluorescence detection.

Kidney Preparation for In-Situ-Hybridization

Animals were anesthetized with pentobarbital sodium (40 mg/kg body weight i.p.) and perfused retrogradely through the abdominal aorta with freshly prepared 4% PFA in PBS at pH 7.4 for 5 min. Cryoprotection was performed as described in the uptake studies. For in-situ-hybridization, cryostat sections (8 to 10 μm thick) were thawed onto silanized glass slides.

Molecular Cloning and Preparation of Riboprobes

The PEPT2-specific cRNA riboprobe was prepared from a partial cDNA fragment of the rat PEPT2-transporter (nucleotides 51–290 of the open reading frame of PEPT2). The fragment was ligated into a PCRII expression vector (Invitrogen, Leck, Netherlands). After linearization RNA probes were synthesized and labeled by *in vitro* transcription using digoxigenin (DIG)-11-UTP. To generate an antisense probe, plasmid was linearized with NotI and then transcribed using SP6 polymerase. To obtain the sense probe (control), plasmid was linearized with EcoRI and transcribed using T7 polymerase.

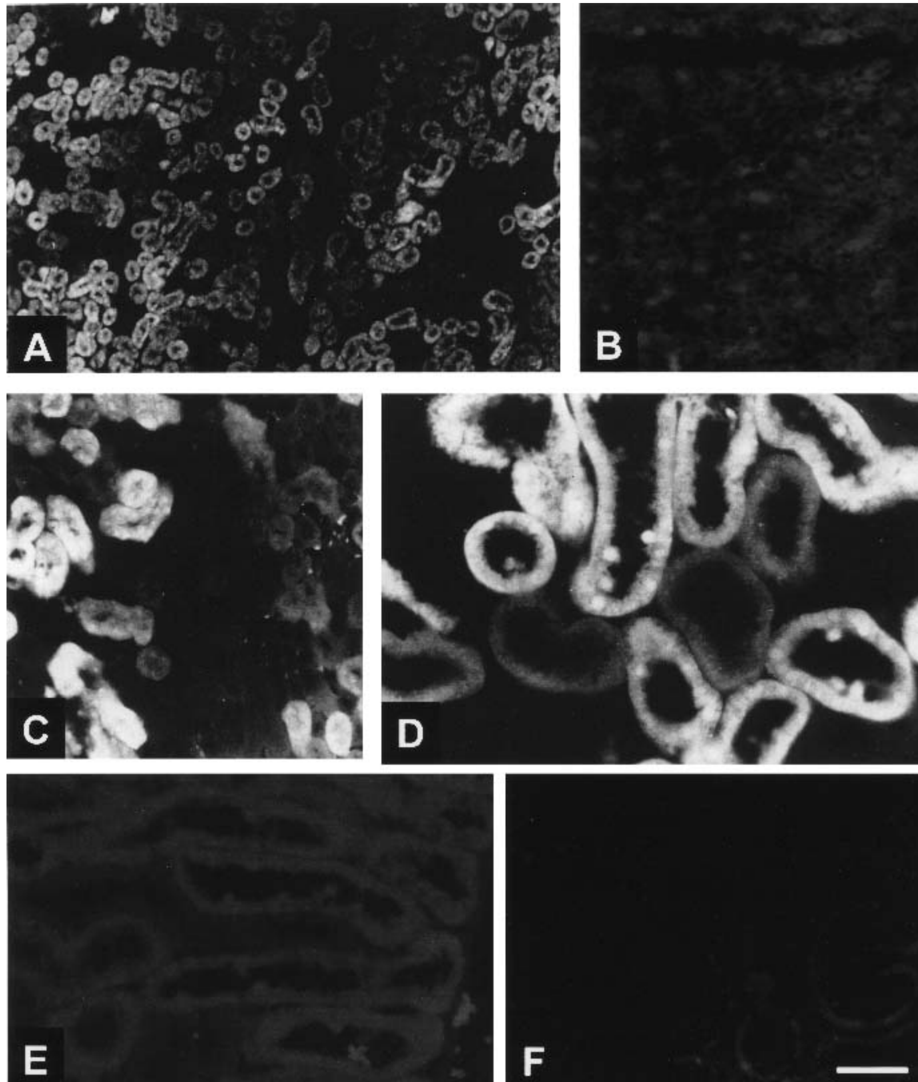

Fig. 1. Substrates for uptake studies: For uptake studies, the fluorophore-labeled dipeptide derivative (D)-Ala-(L)-Lys-AMCA was used. For competitive substrates, unlabeled glycyl-(L)-glutamine, and cefadroxil were used.

In-Situ-Hybridization

Detection of PEPT2-mRNA was performed by using non-isotopic in-situ-hybridization (22). After cutting, tissue sections were treated with 0.1 N HCl (10 min) washed in 1× PBS and air dried (20 min). Each section was then covered with 100 μl prehybridization buffer (Each section was covered with 100 μl prehybridization buffer (50% formamide, 1× Denhardt's, 10 mM triethanolamine, 5 mM EDTA, 6.25% dextransulfate, 0.3 M NaCl, 1mg/ml tRNA) for 1 to 2 h at 40°C in a moist chamber. After removal of the prehybridization buffer, the slides were incubated for 12 to 16 h with 40 μl of the hybridization buffer (50% formamide, 1× Denhardt's, 10 mM triethanolamine, 5 mM EDTA, 6.25% dextransulfate, 0.3 M NaCl, 1mg/ml tRNA) containing 40 ng of PEPT2-specific digoxigenin labeled sense or antisense cRNA probe. After hybridization, sections were washed twice for 15 min at 60°C in 5× SSC and twice for 15 min at 65°C in 1× SSC, followed by two 15 min washes at 60°C in 0.1 × SSC. Subsequently, the sections were treated with 20 μg/ml RNase A to remove unhybridized single stranded RNA. The detection was started by rinsing in buffer I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 10 min at room temperature (rt) and by blocking of unspecific binding sites with modified blocking medium (2% normal sheep serum, 0.5% bovine serum albumine, 3% Triton X-100 in Buffer I (Boehringer Mannheim, Germany)) for 30 min at rt. After removal of the blocking medium, the sections were incubated with 40 μl sheep-anti-digoxigenin-alkaline-phosphatase conjugate (1:500 diluted in blocking medium) for 12 h at 4°C in a moist chamber. Sections were then rinsed twice in Buffer I for 15 min and equilibrated in Buffer III (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5) for 2 min at rt. Detection of the signal was performed by incubation with 150 μl chromogen solution per slide (45 μl nitroblue tetrazolium (75 mg/ml in 70% dimethylformamide) and 35 μl 5-bromo-4chloro-3-indolyl-phosphate (50 μg/ml in 100% dimethylformamide) in 10 ml Buffer II) in a moist chamber at 4°C. Slides were checked for color development after 1 to 15 h. The reaction was stopped by rinsing the slides in 10 mM Tris HCl with 1mM EDTA, pH 8.0 for 15 min. After washing in PBS for 2 × 15 min the slides were coverslipped with phosphate buffered glycerol, pH 8,4 and examined with an Olympus BX 60 microscope.

Northern Blot Analysis

A total of 10 μg RNA prepared from rat kidney was separated by agarose gel electrophoresis and transferred onto nylon membranes (Boehringer, Mannheim). Hybridization with the digoxigenin-labeled PEPT2-specific cRNA-probe was carried out overnight at 65°C in the presence of 50% deionized formamide, 5× SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS and 2% blocking reagent (Boehringer Mannheim). After hybridization, membranes were washed twice for 15 min at 65°C in 2× SSC containing 0.1% SDS and twice for 15 min at 65°C in 0.5× SSC containing 0.1% SDS. For detection of the DIG-labeled hybrids, membranes were washed briefly in PBS (1×), blocked with blocking reagent (Boehringer; 1h at r.t.) and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (diluted 1: 400; 2h at room temperature). Unbound antibody was removed by two washing steps in 100 mM maleic acid, 150 mM NaCl and 0.3% Tween (pH 7.4). Subsequent development was performed according to the manufacturer's digoxigenin detection kit for glycoconjugate and protein analysis protocol (Boehringer Mannheim; developing times 2 to 4 h).

RESULTS

Uptake Studies Employing the Fluorophore-Conjugated Dipeptide

For localization and visualization of peptide transport activity *in vivo* in renal tissues the reporter molecule D-Ala-Lys-AMCA was synthesized (Fig. 1) and administered by intravenous injection.

Pronounced fluorescent staining of parts of the outer and inner cortex and outer stripe was obtained. The inner medulla, renal capsule and papillary surface did not show fluorescence accumulation indicating a lack of transport. Inspection under high magnification allowed the cellular localization of uptake activity. In the parts of the outer and inner cortex and outer stripe fluorescence signaling was found which was localized to cells of the proximal tubule (Fig. 2). A maximal accumulation of fluorescence in the tubular cells was obtained 30 min after administration of the reporter molecule. Interstitial cells, glomerular and macula densa cells did not show any D-Ala-Lys-AMCA fluorescence (Fig. 2). Also, all vascular structures were non-reactive throughout the kidney. Control organs such as intestine and spleen did not show any fluorescence from D-Ala-Lys-AMCA after its intravenous administration.

For inhibition studies, solutions containing D-Ala-Lys-AMCA and higher concentrations (1mM) of unlabeled glycyl-(L)-glutamine or cefadroxil were injected. Higher amounts of both unlabeled Glycyl-(L)-glutamine as well as cefadroxil completely inhibited D-Ala-Lys-AMCA uptake (Fig. 2). Additional controls omitting the labeled dipeptide also prevented fluorescence accumulation.

In-Situ-Hybridization

Expression of PEPT2-mRNA in adult rat kidney was examined using non-radioactive in-situ-hybridization. For these experiments, DIG-labeled riboprobes were transcribed from a PEPT2-specific fragment. Incubation with the antisense PEPT2-hybridisation probe lead to labeling of tubular structures: The signals were localized to the inner cortex and outer medulla, while in the inner medulla or papillary surface epithelium, no staining was observed. High resolution interference contrast microscopy allowed to localize the distribution of the hybridization signals on a cellular level. PEPT2-mRNA was expressed in cells of the proximal straight tubule. Also some podocytes in the glomerula showed hybridization signals. Other structures were non-reactive. The positive staining was reproducibly detected after hybridizations with the antisense probe (Fig. 3 A, B). Control hybridizations with equivalent amounts of sense-probe using the same hybridization and washing stringency were unstained on alternate sections (data not shown). Omission of labeled cRNA-probes from the hybridization mixture also resulted in unstained sections identical to results obtained when RNA was digested before hybridization.

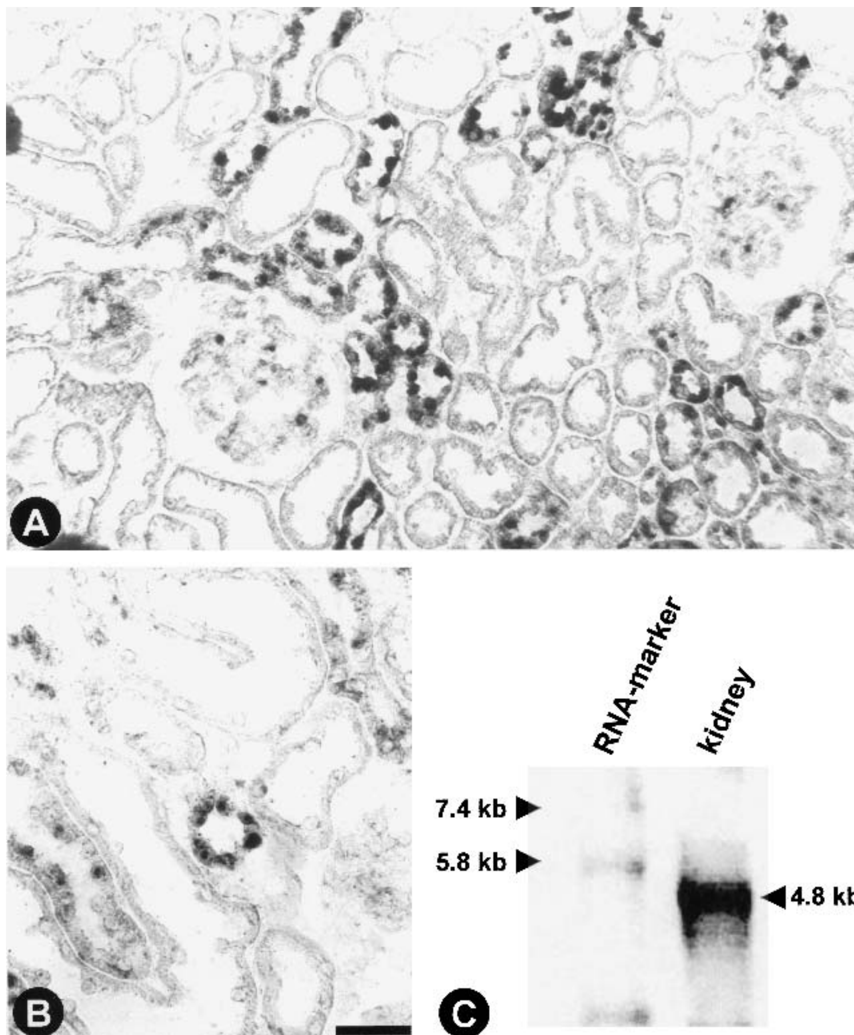

Fig. 2. Uptake studies: I.v.-application of 25 μM (D)-Ala-(L)-Lys-AMCA lead to an uptake that was restricted to epithelial cells of the proximal straight tubule and of the outer cortex (A) that was inhibited when rats were perfused with higher concentrations of unlabeled dipeptide (B). High resolution demonstrated uptake into S1 to S3 segments of the proximal tubule (C and D). No signal as a result of adding 1 mM of unlabeled cefadroxil (E). Control without the use of the marker molecule (F) also did not result in fluorescence signals. Scale bar = 25 μm (D), = 35 μm (C, E, F), = 125 μm (A, B).

Northern Blot

Northern Blotting studies were carried out to test the PEPT2-specific digoxigenin-labeled cRNA probe. The size (4.8 kb) of the hybridization signal obtained from rat kidney proofed the specificity of the probe (Fig. 3C)

DISCUSSION

Intravenously administered di- and tripeptides rapidly disappear from plasma by glomerular filtration followed by efficient tubular reabsorption (23). Numerous studies have shown that peptide transport activity with a specificity for di- and tripeptides is found on the apical side of renal epithelial cells whereas the peritubular site obviously lacks a peptide transport pathway (24). With the molecular identification and characterization of these transporters (1) it is now generally accepted that renal uptake of peptides is mediated by the apical pH-dependent peptide transporters PEPT2 and PEPT1 expressed in cells of cortex and medulla (25). Apart from the large variety of endogenous substrates, namely di- and tripeptides that reach the tubules by filtration or that are released by enzymatic hydrolysis by brush border peptidases, the renal peptide transport systems are also of pharmacological importance. Various peptidomimetic drugs utilize these transporters for reabsorption and the peptide carriers consequently contribute to the pharmacokinetics of the drugs. Recent studies have determined the essential minimal structural determinants for substrate recognition and transport for both peptide transporters that also explain why certain peptidomimetics are bound and transported (26,27).

The present study utilized a novel approach to visualize renal peptide transport in-situ and to localize the expression of PEPT2-mRNA at higher resolution. The uptake studies allowed renal cell subpopulations to be identified that possess peptide transport activity. The fluorescent dipeptide conjugate D-Ala-Lys-AMCA was chosen as a reporter molecule

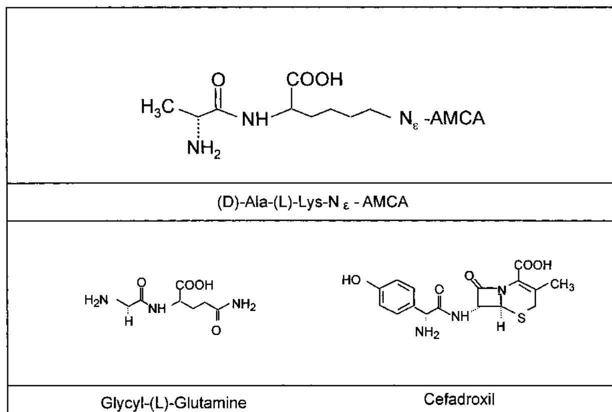

Fig. 3. Detection of PEPT2 mRNA in rat kidney by in-situ-hybridization and northern blot analysis: 8 μm cryostat sections of rat kidneys were subjected to non-isotopic in situ hybridization. PEPT2-mRNA hybridization signals were localized to epithelial cells of the proximal straight tubule (A–B). There was also weak staining of some podocytes (A). Samples of total RNA (10 μg) from rat kidney were separated by agarose gel electrophoresis, blotted and hybridized with a specific PEPT2 antisense probe (C). Scale bar = 35 μm (A), = 25 μm (B).

since it was shown previously to be a good transporter substrate (20,21). This molecule has the advantage of high enzymatic stability due to the NH2-terminal peptidase resistant D-Ala residue. Peptide transport activity was visualized by D-Ala-Lys-AMCA fluorescence accumulation in tubular cells of the proximal tubule in the outer and inner cortex and the outer stripe. The fluorescent staining was not restricted to sites were the PEPT2-mRNA (later segments of the proximal tubule) was localized, indicating that in the first segment of the proximal tubule, PEPT1 is mediating D-Ala-Lys-AMCA uptake. In this respect, PEPT1 expression was shown for the S1 segment by immunohistochemistry, microdissection-RT-PCR and radioactive low-resolution in-situ-hybridization in previous studies (9,10). Also, a previous report has demonstrated the uptake of D-Ala-Lys-AMCA by PEPT1 in the murine intestine (28).

The specificity of D-Ala-Lys-AMCA uptake by the renal peptide carriers was demonstrated by almost complete inhibition of fluorescence accumulation by the two competitors. Cefadroxil and glycyl-(L)-glutamine are good substrates of PEPT1 and PEPT2 and by their higher concentrations can competitively block uptake of D-Ala-Lys-AMCA. Passive uptake of the reporter by diffusion was excluded by lack of any staining in other structures of the kidney.

The functional uptake studies were extended by in-situ-hybridization studies using non-isotopic probes for PEPT2. So far, the renal distribution of PEPT2-mRNA was only assessed by low-resolution isotopic in-situ-hybridization (9). By contrast, the renal cellular PEPT2 and PEPT1 protein expression was already demonstrated in detail by immunohistochemistry (10). The limited structural information of isotopic in-situ-hybridization was expanded by the present non-isotopic in-situ-hybridization and high-resolution interference contrast microscopy. PEPT2-mRNA signals were found mainly in cells of the later proximal tubular segments. These results confirmed the in-situ uptake studies and verified the previously reported mRNA data (9,10) on a cellular level. An interesting finding was the expression of PEPT2 in endothelial cells of some venules in the inner cortical region and in a few cells of some but not all glomerula. PEPT2-mRNA was also found in some intraglomerular and endothelial cells. However, in these cells a functional uptake of D-Ala-Lys-AMCA was not detectable. This could suggest an intracellular localization of the carrier-protein that does not participate in transmembrane transport. In this respect, recent findings on peptide transport activity in lysosomes associated with peptide transporter immunoreactivity already suggested intracellular localizations of peptide carriers (29,30).

In conclusion, the present study shows by in-situ uptake studies the function of renal peptide transporters in removal of a fluorescent dipeptide conjugate from circulation with its high level accumulation in the proximal tubule. The distribution of transport activity corresponds partly with the in-situ-hybridization pattern for expression of PEPT2, except in the early segment of the proximal tubule, where PEPT1 has been localized in earlier studies suggesting that D-Ala-Lys-AMCA is taken up via both transporters. Previous functional and kinetic studies in peptide transporters in a variety of *in vitro* settings find their *in vivo* correlate here by direct visualization of the transport process in the proximal tubule. Together with recent findings on the substrate specificity of peptide transporters, our findings provide a new approach for studies on renal uptake mechanism for short chain peptides and peptide based drugs. The uptake studies using D-Ala-Lys-AMCA might be particularly useful to study the interference of drugs and other compounds that utilize PEPT1 or PEPT2 for uptake into renal epithelial cells or that inhibit peptide transport activity.

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