Peptide transport in the mammary gland: expression and distribution of PEPT2 mRNA and protein

DAVID A. GRONEBERG,1 FRANK DÖRING,2 STEPHAN THEIS,2 MONIKA NICKOLAUS,2 AXEL FISCHER,1 AND HANNELORE DANIEL2

1Dept. of Pediatric Pneumology and Immunology, Charité Campus-Virchow, Humboldt-University, D-13353 Berlin; and 2Institute of Nutritional Sciences, Technical University of Munich, D-85350 Freising-Weihenstephan, Germany

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Groneberg, David A., Frank Döring, Stephan Theis, Monika Nickolaus, Axel Fischer, and Hannelore Daniel. Peptide transport in the mammary gland: expression and distribution of PEPT2 mRNA and protein. Am J Physiol Endocrinol Metab 282:E1172–E1179, 2002; 10.1152/ajpendo.00381.2001.—The lactating mammary gland utilizes free plasma amino acids as well as those derived by hydrolysis from circulating short-chain peptides for protein synthesis. Apart from the major route of amino acid nitrogen delivery to the gland by the various transporters for free amino acids, it has been suggested that dipeptides may also be taken up in intact form to serve as a source of amino acids. The identification of peptide transporters in the mammary gland may therefore provide new insights into protein metabolism and secretion by the gland. The expression and distribution of the high-affinity type proton-coupled peptide transporter PEPT2 were investigated in rat lactating mammary gland as well as in human epithelial cells derived from breast milk. By use of RT-PCR, PEPT2 mRNA was detected in rat mammary gland extracts and human milk epithelial cells. The expression pattern of PEPT2 mRNA revealed a localization in epithelial cells of ducts and glands by nonisotopic high resolution in situ hybridization. In addition, immunohistochemistry was carried out and showed transporter immunoreactivity in the same epithelial cells of the glands and ducts. In addition, two-electrode voltage clamp recordings using PEPT2-expressing *Xenopus laevis* oocytes demonstrated positive inward currents induced by selected dipeptides that may play a role in aminonitrogen handling in mammalian mammary gland. Taken together, these data suggest that PEPT2 is expressed in mammary gland epithelia, in which it may contribute to the reuptake of short-chain peptides derived from hydrolysis of milk proteins secreted into the lumen. Whereas PEPT2 also transports a variety of drugs, such as selected β-lactams, angiotensin-converting enzyme inhibitors, and antiviral and anticancer metabolites, their efficient reabsorption via PEPT2 may reduce the burden of xenobiotics in milk.

THE MAJOR SOURCE for amino acid nitrogen delivery to mammary gland epithelial cells for the synthesis of milk proteins is free amino acids. Mammary gland tissues express a multitude of transport systems for anionic (33), cationic (43), and neutral amino acids (30, 31), and their role in amino acid delivery has been characterized in different species in the past (for review see Ref. 44). However, some studies suggested that the uptake capacity for several essential amino acids could not meet the requirements of the secreting gland tissue for efficient protein synthesis (44), and it has been postulated that uptake of circulating short-chain peptides may contribute to the amino acid pool in gland tissues. The structure of protein breakdown products generated at the cell surface of epithelial cells is important for their route and extent of transepithelial transport (8). Carrier-mediated transport of di- and tripeptides plays an important role in amino acid nitrogen delivery to various mammalian tissues (26). Not only short-chain peptides that arise from the hydrolysis of systemic peptides by peptidases but also peptide-based drugs are carried by these peptide transport systems. β-Lactam antibiotics (22, 48, 49), angiotensin-converting enzyme (ACE) inhibitors (46, 47), and antiviral (16, 21) and antineoplastic (23) agents have been shown to serve as substrates of mammalian peptide transporters. In addition, δ-aminolevulinic acid (δ-ALA), an endogenous precursor of cellular porphyrin synthesis, was shown to be transported by the peptide transporters (11).

The molecular bases for the uptake of intact di- and tripeptides into epithelial cells are the apically located H+/peptide cotransporters PEPT1 and PEPT2. The cDNAs encoding these transporters have been identified by expression cloning and homology screening from rat (32, 39, 40), rabbit (6, 7, 15), murine (38), and human (27, 28) tissues. Both transporter proteins possess 12 membrane-spanning domains and share an identity of ~47% at the protein level (9). PEPT1 and PEPT2 mediate electrogenic uphill peptide transport by coupling of substrate translocation to movements of protons down the transmembrane electrochemical pro-
ton gradient, with the membrane potential serving as the primary driving force (8).

Whereas the high-capacity low-affinity transporter PEPT1 is expressed in enterocytes of the small intestine (1) and to a lesser extent in renal tubular cells (41, 45), the high-affinity low-capacity transporter PEPT2 is expressed in the kidney tubule (41) but also in brain and other regions of the nervous system (12, 18) and in lung (20).

Despite the proposal that dipeptide uptake by the mammary gland could meet the tissues’ demand of amino acids for protein synthesis, studies on transport of hydrolysis-resistant dipeptides in perfused rat mammary gland (44) failed to show any significant transport activity. However, preliminary data obtained in our laboratory employing RT-PCR suggested that the mRNA of PEPT2 was expressed in rat and mouse lactating gland tissue. Therefore, it was of particular interest to study the expression of the transporter PEPT2 in mammary gland tissues by RT-PCR, nonisotopic in situ hybridization, and immunohistochemistry and to demonstrate functional aspects of peptide transport in this tissue.

MATERIALS AND METHODS

Lactating Sprague-Dawley rats between days 4 and 15 of lactation with 6–11 suckling pups were used. The animals were housed under standard laboratory conditions and fed ad libitum. For each of the experiments described, samples of six animals were employed. All procedures involving animals and animal care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OD L 358.1, 1987) and have been approved by the state animal committee (T 008/01).

RT-PCR. RT-PCR was performed as described before (11). In brief, total RNA was isolated from rat mammary gland and kidney, and four samples of epithelial cells were isolated from fresh human milk (34). The RNA was digested with DNase, followed by cDNA synthesis by reverse transcription. PCR amplification was performed for 35 cycles with 94°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 1 min, and 72°C end synthesis for 10 min. PEPT2-specific primers represented nucleotides 111–134 (5’-GCTGGCTACTGAA-GCCAAATGCTTG-3’) and 437–417 (5’-AGAGGCTGTCAAGGATGTG-3’) of the protein-coding region of PEPT2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers represented nucleotides 558–579 (5’-GACCAGACTTCGATCATGACTA-3’) and 1010–990 (5’-TCCACCACCTGGTTGCTAG-3’) of the open reading frame of GAPDH. A 1:10 volume of each sample was separated on a 1% agarose gel and stained by ethidium bromide. PCR controls were performed by using H2O instead of cDNA.

PEPT2 cRNA probes. Digoxigenin (DIG)-labeled PEPT2-specific cRNA probes were produced as follows: a rat specific PEPT2 PCR fragment (nucleotides 51–290 of the open reading frame of PEPT2) was ligated into the PCRII expression vector (Invitrogen, Leck, The Netherlands). The plasmid was linearized with EcoRI (for sense probe) or NcoI (for antisense probe) and used as a template to synthesize DIG-labeled sense and antisense RNA, according to the manufacturer’s manual (Boehringer Mannheim).

In situ hybridization. Detection of PEPT2 mRNA was performed by using nonradioactive in situ hybridization as described before (19). Cryostat sections (8 μm) of rat mammary gland were mounted on Silane precoated glass slides and fixed by immersion for 10 min in 4% paraformaldehyde (PFA). Tissue sections were treated with 0.1 N HCl (10 min), washed in 1× PBS, and air dried (20 min). Each section was covered with 100 μl of hybridization buffer (50% formamide, 1× Denhardt’s, 10 mM triethanolamine, 5 mM EDTA, 6.25% dextran sulfate, 0.3 M NaCl, 1 mg/ml tRNA) containing 100 ng/100 μl PEPT2-specific DIG-labeled sense or antisense cRNA probe. After hybridization, sections were washed twice for 15 min at 60°C in 5× standard sodium citrate (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. Detection and development of hybridization signals were carried out as described above and in the manufacturer’s commercial DIG-detection kit protocol (Boehringer Mannheim). Slides were mounted in 50% glycerol in 1× PBS (pH 7.4).

Immunohistochemistry. Immunohistochemistry was carried out on 4% PFA-fixed rat mammary gland specimens. Cryostat sections (8 μm) were washed several times in PBS and preincubated for 1 h at room temperature with 2% low-fat milk powder in Tris-buffered saline + Tween 20 (TBST), pH 7.4. Sections were incubated with polyclonal anti-rabbit PEPT2 serum (10) diluted 1:1,000 in the preincubation solution overnight. As secondary antibody, an indocarboyanin antibody (1:1,000, Dianova, Hamburg, Germany) was used. Specificity of the antibody reaction was verified in parallel sections, which were incubated either with the primary antisera that had been preabsorbed with the corresponding antigenic peptide (concentration 20 μg protein/ml diluted antisera) or with only the secondary antibodies. Slides were coverslipped in carbonate-buffered glycerol (pH 8.6) and viewed using epifluorescence microscopy.

Two-electrode voltage clamp recording of transport currents mediated by PEPT2. Surgically removed Xenopus laevis oocytes were separated by collagenase treatment and handled as described previously (3). Individual oocytes were injected with 50 nl of water (controls) or 50 nl of RNA solution containing 30 ng of the PEPT2 complementary RNA (cRNA). The two-electrode voltage clamp (TEVC) technique was applied to characterize responses in inward current to substrate addition in oocytes expressing PEPT2, as described previously (3). Inward currents were measured in a buffer composed of 100 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM MES-Tris, pH 6.5, in the absence and the presence of 5 mM either free amino acids or selected dipeptides, with water-injected oocytes serving as controls. Membrane potential in oocytes was clamped to −100 mV, and 5 mM of Gly-L-Gln was used to standardize measurements between different batches of oocytes.

Immunocytochemistry. For immunocytochemistry, 4% PPA immersion-fixed X. laevis oocytes were cut into 6-μm cryostat sections, rinsed in PBS, covered with 2% low-fat milk powder in TBST, pH 7.4, for 1 h at room temperature, and then incubated with polyclonal anti-rabbit PEPT2 serum (10) diluted 1:1,000 overnight. For detection of the primary antibody, anti-mouse fluorescein-5-isocyanate antisera (1:400, Amersham, Braunschweig, Germany) was used.

Transmission electron microscopy. Six PEPT2-injected oocytes were immersion fixed by incubation with potassium phosphate-buffered 2.5% glutaraldehyde solution (pH 7.4, effective osmolarity 340 mosM) for 2 h. Subsequently, samples were postfixed with 1% osmium tetroxide, dehydrated in a graded series of alcohol, and embedded in Spurr’s medium.
Ultrathin sections of 70–90 nm were cut, transferred to 200-mesh copper grids, and contrasted with uranyl acetate and lead citrate. Microscopy was performed with a model CEM 902 Zeiss transmission electron microscope.

RESULTS

Detection of PEPT2 mRNA by RT-PCR. To demonstrate the expression of the PEPT2 mRNA in rat mammary gland, RT-PCR experiments were performed. By use of mRNA from rat kidney and mammary gland, PEPT2-specific amplification products were detected (Fig. 1). PEPT2-specific products were also identified in samples of epithelial cells isolated from fresh human milk (Fig. 1).

Distribution of PEPT2 mRNA. The tissue distribution of PEPT2 mRNA in lactating rat mammary was studied using nonisotopic in situ hybridization and high-resolution microscopy. For these experiments, DIG-labeled riboprobes that were transcribed from a rat-specific PEPT2 cDNA fragment were used and detected with an anti-digoxigenin phosphatase conjugate. PEPT2-mRNA expression was detectable in dif-

Fig. 1. Detection of peptide transporter-2 (PEPT2) mRNA in the lactating mammary gland by RT-PCR. Five micrograms of total RNA from rat mammary gland and kidney and from isolated human mammary epithelial cells were subjected to RT-PCR using specific primers for PEPT2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Products were separated by agarose gel electrophoresis and visualized with ethidium bromide to assess PEPT2 expression in the tissues.

Fig. 2. Distribution of PEPT2 mRNA expression. Distribution of PEPT2 mRNA was detected with nonisotopic in situ hybridization. Cryostat sections (8 μm) were hybridized with digoxigenin-labeled antisense (A, C, and E) or sense (D and F) PEPT2-cRNA probes. Hybridization signals were present in the epithelium (arrows) of the main and segmental ducts (A–C) and in glandular cells (arrowheads in A). Hematoxylin-eosin staining served for histological control (B). No staining with the sense probe (E and F). Scale bar, 100 μm (A and B), 45 μm (C and D), 20 μm (E and F).
ferent parts of the mammary gland of all examined tissues. In detail, the mRNA hybridization signal was localized to ductal epithelial cells along the main segmental ducts and the terminal duct lobular unit and glandules (Fig. 2). Structures surrounding the epithelial cells did not show any specific signals, nor did connective tissue or vessels show reactivity. The positive staining was reproducibly detected after antisense probe hybridizations. Hematoxylin-eosin staining served as histological control (Fig. 2). Specificity controls were performed with equivalent amounts of sense probe by using the same hybridization and washing stringency and showed no staining on alternate sections (Fig. 2). Also, omitting labeled cRNA probes from the hybridization mixture resulted in unstained sections.

Immunohistochemistry. Immunohistochemistry for PEPT2 immunoreactivity was carried out on cryostat sections of the lactating gland tissue. The incubations led to positive staining for PEPT2-like immunoreactivity in the same cells that were shown to express PEPT2 mRNA. The signal was of a nongranular type and was found in the epithelial cells of the terminal duct and glandules (Fig. 3, E and F) and was present in all sections examined. There was also a marked staining

of the ductal epithelial cells of the main and segmental ducts (Fig. 3, A–C). The signal was not related to specific subcellular structures such as nuclei or lysosomes. No signals were present in the connective tissue. The specific staining was abolished when the anti-PEPT2 serum was preabsorbed with the antigenic peptide (Fig. 3D).

Transport of selected alanyl peptides by PEPT2 when expressed in X. laevis oocytes. Subsequent to the detection of the PEPT2-mRNA and protein by in situ hybridization and immunohistochemistry, we investigated whether selected peptides that may be released from secreted proteins by hydrolysis serve as substrates of PEPT2. X. laevis oocytes expressing PEPT2 were perfused with a buffer (pH 6.5), and inward currents generated during substrate perfusion were determined in the voltage clamp mode. Control oocytes (water injected) did not show any response in inward current to the addition of the substrates. Perfusion of oocytes with 5 mM either free alanine or aspartate of lysine also failed to cause any detectable inward currents. In contrast, all alanyl peptides containing \( \text{L}-\text{enantiomers} \) (except proline-containing ones) evoked very similar inward currents of 100–150 nA when membrane potential was clamped to \(-100\) mV (Table 1). Substrate-

![Fig. 3. Localization of PEPT2-like immunoreactivity. Immunofluorescence localization of PEPT2-like immunoreactivity in the ductal epithelium (A–C) and in the terminal lobar unit and glandular cells (E and F). A control section stained for PEPT2-like immunoreactivity in the presence of the antigen peptide (D) does not show specific staining. lu, Lumen. Scale bar (in F), 80 \( \mu \)m (A, B, E, and F), 40 \( \mu \)m (C and D).](http://ajpendo.physiology.org/)

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Table 1. Transport currents induced by selected dipeptides and tripeptides in Xenopus oocytes expressing PEPT2

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<tr>
<th>Peptide, 5 mM</th>
<th>Current, %I_{Gly-Gln} (112 ± 23 nA)</th>
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<tr>
<td>L-Ala-L-Ala</td>
<td>112.5 ± 3.5</td>
</tr>
<tr>
<td>L-Ala-D-Ala</td>
<td>71.2 ± 1.8</td>
</tr>
<tr>
<td>D-Ala-L-Ala</td>
<td>88.5 ± 8.8</td>
</tr>
<tr>
<td>D-Ala-D-Ala</td>
<td>4.7 ± 4.3</td>
</tr>
<tr>
<td>D-Ala-L-Pro</td>
<td>21.2 ± 6.0</td>
</tr>
<tr>
<td>L-Ala-D-Pro</td>
<td>3.0 ± 2.0</td>
</tr>
<tr>
<td>L-Ala-L-Ala-L-Ala</td>
<td>108 ± 2.4</td>
</tr>
<tr>
<td>L-Ala-L-Asp</td>
<td>115.2 ± 3.8</td>
</tr>
<tr>
<td>L-Lys-L-Ala</td>
<td>119.6 ± 6.2</td>
</tr>
<tr>
<td>L-Asp-L-Ala</td>
<td>93.1 ± 6.5</td>
</tr>
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Transport currents were recorded in oocytes clamped to a membrane potential of −100 mV and perfused with either buffer (pH 6.5) or buffer containing 5 mM substrate. To compensate for variations in the peptide transporter-2 (PEPT2) expression rate between batches and experiments, all data are expressed as currents in percentage of that in response to 5 mM Gly-Gln (I_{Gly-Gln}). Transport currents are given as means ± SE of 3–5 experiments in ≥2 different batches of oocytes.

The present study demonstrates the expression of the high-affinity peptide transporter PEPT2 mRNA and protein in the lactating mammary gland of the rat. Using RT-PCR, we were able to detect PEPT2 expression in the glandular tissues of the rat as well as in extracts from epithelial cells isolated from human milk. The localization of both PEPT2 mRNA by nonradioactive in situ hybridization and PEPT2 protein allowed identification and determination of the exact cellular site of the transporter in glandular epithelial cells.

When rat lactating mammary tissue was perfused with hydrolysis-resistant dipeptides such as D-[3H]Phe-L-Gln and D-[3H]Phe-L-Glu, much lower transport rates than the rate for free L-[3H]Gln were observed. In addition, uptake of D-[3H]Phe-L-Glu was not inhibited by competing dipeptides such as L-Leu-L-Ala. This suggests that no significant basolateral transport of dipeptides occurs in the mammary gland. The lack of any basolateral staining for immunoreactivity of the PEPT2 protein confirms that there is no molecular basis for transport of intact dipeptides from the circulation into the mammary tissue. However, the PEPT2 mRNA is expressed at high levels in the gland, and the protein is localized to the apical side of the epithelial cells of the terminal duct and glandules as well as the main and segmental ducts.

The luminal expression of PEPT2 suggests that its role in the mammary gland could be similar to that in renal tubules, where it contributes to amino acid reab-

**Fig. 4. Morphology of Xenopus laevis oocytes expressing PEPT2.** Immunocytochemistry for PEPT2 resulted in intense and nongranular immunosignaling, which was localized to the microvilli of the plasma membrane (A). Transmission electron microscopy of X. laevis oocytes that were injected with PEPT2 cRNA demonstrated intact microvilli of the plasma membrane, which was localized below the vitelline membrane, and intact subapically localized cortical (arrow) and pigment granules (arrowhead) and yolk (Y) platelets (B). Scale bars, 25 μm (A) and 270 nm (B).
sorption by transporting filtered or proteolytically re-
leased di- and tripeptides back into the epithelial cells. 
Because milk contains a variety of proteases, including 
kalikrein (29), cathepsins (25), and plasmins (4), pro-
etolysis of secreted proteins within the gland releases 
peptide fragments and free amino acids. Intramam-
mary hydrolysis has been studied in bovine glands, in 
particular during involution, and plasmin has been 
identified as the major enzyme responsible for the 
deradation of caseins, mainly β-casein (4). Partial 
hydrolysis of milk proteins by co-secreted proteases 
would release peptide fragments including di- and tri-
peptides, and those may be reabsorbed by PEPT2 into 
epithelial cells. In addition, specific hydrolysis of bio-
logically active proteins, such as binding proteins for 
insulin-like growth factors in human milk, has been 
reported (14) that also would release short-chain pep-
tides. These peptides either could be submitted to hy-
drolysis by surface-bound aminopeptidases (5, 13) to 
yield free amino acids or may be transported in intact 
form into the epithelial cells. Analysis of human or 
bovine milk has not yet identified di- and tripeptides 
as normal constituents, although free amino acids are 
found and their concentration changes with the state of 
lactation (2). It is not known currently whether these 
amino acids are secreted into milk or produced by 
intramammary proteolytic processing of short-chain 
peptides or proteins. Taken together, the presence of 
PEPT2 in the apical membrane of epithelial cells in 
various areas of the secreting mammary gland sug-
gests that this protein contributes to the removal of di-
and tripeptides that are either secreted into the ductal 
lumen or released by proteolysis. Because PEPT2 dis-
plays a high affinity for substrate binding and has the 
capability for sequence-independent transport of all di-
and tripeptides, a very efficient removal of these sub-
strates from the secretions can be achieved. To demon-
strate the capability of PEPT2 for transport of a variety 
of di- and tripeptides that could be released by partial 
hydrolysis of secreted proteins in the gland, we deter-
mained peptide-induced inward currents in voltage-
clamped X. laevis oocytes perfused with a series of 
alanyl peptides representing peptides with different 
chain length and side chains. In addition, alanyl dipep-
tides containing either L- or D-enantiomers of amino 
acids were employed. Substrate-evoked inward cur-
rents revealed enantioselective transport of the di- and 
 tripeptides with very similar transport rates for most 
of the physiologically relevant dipeptides containing 
L-enantiomers of amino acids, including those contain-
ing charged side chains. This analysis of function of 
PEPT2 confirms its capability for sequence-independent 
transport of most, if not all, possible di- and tripeptides derived by hydrolysis of proteins or oli-
gopeptides in mammalian milk.

Because PEPT2 has also been shown to transport a 
variety of peptidomimetics, including aminocephalo-
sporins, penicillins, and ACE-inhibitors such as captopril have been detected in breast milk after oral administration to lactating mothers 
(24), their concentrations in milk are comparatively 
low. Regardless of the route of transport of this drug 
across the gland into milk, the presence of PEPT2 in 
the epithelial cells would provide an efficient pathway 
for reabsorption of the peptidomimetics back into the 
gland tissue to reduce the burden of the milk.

We have previously demonstrated that δ-ALA is a 
substrate of both PEPT2 and PEPT1 (11). There are 
several physiological and clinical implications of this 
finding for the mammary gland. δ-ALA’s therapeutic 
relevance is based on its use as a substrate for photo-
dynamic therapy (37), which is based on the accumu-
lation of porphyrins and consecutive photoactivation to 
induce tissue necrosis and apoptosis after administra-
tion of δ-ALA (36). Besides the use of δ-ALA for the 
treatment of breast cancer (17, 35), epithelial neo-
plasms of other tissues like the lung, colon, and urinary 
bladder are addressed by this new therapeutic princi-
ple (37). By demonstration of PEPT2 expression in 
epithelial cells of the mammary gland, we provide the 
first molecular and morphological basis for the trans-
port of δ-ALA in breast tissues. Further investigations 
have to elucidate the expression levels of δ-ALA trans-
porter and define the possible therapeutic value of 
induced transporter expression in neoplasms.

In conclusion, this is the first study that examines 
the expression of PEPT2 in the lactating mammary 
gland. We found a high expression level of PEPT2 
mRNA in the epithelial cells of the ducts and glands, 
demonstrated corresponding immunohistochemical 
data on the expression of PEPT2 protein, and corre-
lated these morphological data to functional analysis 
on model peptides using two-electrode voltage clamp 
recording. Our study suggests PEPT2 as a potentially 
important peptide-transporting system in the mam-
mary gland that could play a role in amino acid nitro-
gen handling, in reducing drug disposition in the milk, 
and in the transport of δ-ALA for treatment of epithe-
lial neoplasms by photodynamic therapy.

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