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The Peptide Transporter PEPT2 is Targeted by the Protein Kinase SGK1 and the Scaffold Protein NHERF2

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Key Words

Phosphorylation • Transport • Tansporter • Peptide • Signal transduction

Abstract

PEPT1 and PEPT2 are members of the family of proton-dependent oligopeptide transporters that mediate electrogenic uphill transport of small peptides and peptidomimetics into a variety of cells. Kinetic properties and substrate recognition sites of those transporters have been well defined previously. Little is known, however, about regulation of those transporters. Both PEPT isoforms contain putative phosphorylation sites for the serum and glucocorticoid inducible kinase SGK1 and a C-terminal PDZ binding motif that might be recognized by PDZ domains of the Na^+/H^+ exchanger regulatory factors NHERF1 and NHERF2. Thus, the present study attempted to clarify the role of SGK1 and NHERFs in the modulation of PEPT isoforms. Expression studies in *Xenopus* oocytes with subsequent electrophysiology and immunoassays revealed that SGK1 and NHERF2, but not the NHERF1 isoform specifically enhance PEPT2 function and surface abundance. The kinase is effective through phosphorylation of ^{185}Ser within the SGK1 consensus site, since disruption of this site prevented transporter modulation by the kinase. NHERF2 failed to regulate the C-terminal deletion mutant (PEPT2ΔC) indicating that the C-terminal PDZ-binding motif in PEPT2 governs transport modulation by NHERF2. Coexpression of NHE3 stimulates PEPT2 activity to a similar extent as coexpression of NHERF2. Dynasore experiments demonstrated that SGK1 and NHERF2 activate PEPT2 by stabilizing the transporter at the cell surface. In conclusion, the present results reveal two novel PEPT2 posttranslational modulators, SGK1 and NHERF2, which might regulate transport of oligopeptides and peptidomimetic drugs.

Introduction

PEPT1 (SLC15A1) and PEPT2 (SLC15A2) are members of the family of proton-dependent oligopeptide transporters that mediate electrogenic uphill transport of small (di- and tri-) peptides and peptidomimetics (including antibiotics and antiviral drugs) into a variety of cells. They represent the major route for intestinal absorption of dietary nitrogen and for renal peptide reabsorption (reviewed in [1-3]). Whilst PEPT1 is a low-affinity (K_m>1 mM) and high-capacity transporter, PEPT2 is a

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high-affinity (K_m 10–250 μM) and low-capacity transporter for the same substrates (rev. in [4]). Although PEPT1 and PEPT2 were originally discovered in the small intestine and kidney [5-7], more recent studies have localized PEPT1 and PEPT2 in various other tissues. PEPT1 is expressed in bile duct epithelial cells and in the nuclei and lysosomes of the pancreas [8, 9], whereas PEPT2 is found in brain astrocytes, epithelia of the choroid plexus, mammary gland and lung [10-12].

While various studies have addressed the mechanisms of substrate recognition and uptake, data on the transcriptional/posttranslational regulation of peptide transporters are scarce. PEPT1 transcript and protein levels are upregulated by starvation and are subject to circadian rhythm [13-16]. PEPT1 expression is downregulated upon thyroid hormone treatment of intestinal Caco-2 cells [17]. PEPT2 but not PEPT1 mRNA is upregulated in unilaterally nephrectomized rats in the remnant kidney [18]. The signaling processes underlying the regulation of PEPT1 or PEPT2 have not been explored.

PEPT1 and PEPT2 sequences contain predicted N-linked glycosylation and recognition sites for several protein kinases including PKC, PKA and SGK1 (serum and glucocorticoid inducible kinase 1). PEPT glycosylation has been demonstrated by western blotting [6]. Whether the kinases phosphorylate the transporters remained elusive. Two and five putative phosphorylation sites for PKC have been identified in PEPT1 and PEPT2 sequences, respectively. In fact, PEPT modulation by PKC has been described [19, 20]. Protein kinase A sites are present only in mouse and rat sequences, thus these sites might not be relevant for transport modulation. PEPT1 bears two and PEPT2 one putative SGK1 phosphorylation consensus sequence (R-X-R-X-X-S/T, [21]) that are conserved among various species. Since SGK1 has been reported to modulate several ion channels and transporters and its expression pattern overlaps with that of PEPT isoforms (rev. in [22]), this study aimed to elucidate the role of SGK1 in the modulation of the PEPT isoforms.

Both PEPT1 and PEPT2 contain a class I PDZ binding motif at their C-terminus (S/T-X-Φ, where Φ is a hydrophobic amino acid) [23-27] that might be recognized by PDZ domain-containing proteins. PDZ is a modular protein-protein interaction domain, first identified in the postsynaptic density PSD-95/SAP90, the *Drosophila* septate junction protein disc-large, and the tight junction protein ZO1. PDZ domain-containing proteins play a role as scaffold proteins for transporters and ion channels thus modulating their localization, surface stability and function by recruiting modulatory proteins [23, 28-30].

The Na^+/H^+ exchanger regulatory factors, NHERF1 and NHERF2, are PDZ domain-containing proteins that have been implicated in the targeting and stabilisation of ion channels at the plasma membrane [31-35]. Both isoforms contain two PDZ domains and an ERM binding domain at their C-termini that bind all members of the Ezrin-Radixin-Moesin-Merlin family of cytoskeletal proteins [36]. NHERF binding to the cytoskeleton allows sorting and localization of membrane proteins as well as clustering of signaling proteins in specific domains to facilitate cellular signaling. NHERF2 recruits protein kinases including PKA and SGK1 and locates them at the vicinity of membrane transporters and channels for modulation. Among others NHERF2 was found to be critically important for regulation of the Na^+/H^+ exchanger 3 (NHE3) by PKA [37, 38] and of the K^+ channel ROMK1 and the epithelial Ca^{2+} channel TRPV5 by SGK1 [39-41].

The fact that both NHERF isoforms are coexpressed with PEPT in the renal proximal tubule [42, 43] suggests that NHERFs might impact on peptide transport in the kidney. The present work attempted to clarify whether the function of the PEPT carriers is modified by the two NHERF isoforms or by SGK1 and, if so, to identify molecular mechanisms involved. Here we provide evidence that SGK1 and NHERF2 but not NHERF1 enhance PEPT2 activity by stabilizing the transporter at the plasma membrane. The effect of the kinase requires the ^{185}Ser within the SGK1 consensus site. The second PDZ domain in NHERF2 and the C-terminal PDZ-binding motif in PEPT2 are essential for transport modulation by NHERF2. SGK1 does not augment NHERF2-mediated PEPT2 activation, indicating that both regulatory proteins act independently. Taken together, our data suggest that SGK1 and NHERF2 might participate in the regulation of reabsorption of peptides and peptidomimetic drugs in the kidney by enhancing PEPT2 activity and stability at the cell surface.

Materials and Methods

Site directed mutagenesis

PEPT2 mutants lacking the putative SGK1 phosphorylation site (^{S185A}PEPT2) and the C-terminal PDZ binding motif (PEPT2ΔC) were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. Thefollowing primers were used:
^{S185A}PEPT2, s: 5′ CGG ACT AGA TAC TTC GCT GGC TTC TAC CTC GCC 3′; ^{S185A}PEPT2, as: 5′ GGC GAG GTA GAA GCC AGC

GAA GTA TCT AGT CCG 3´; PEPT2ΔC, s: 5´ CTT AGA GAC CAA GAA GTG ATG ACT CCC AGG AC 3´; PEPT2ΔC, as: 5´ GTC CTG GGA GTC ATC ACT TCT TGG TCT CTA AG 3´. All mutants were sequenced to verify the presence of the desired mutation.

Expression in Xenopus laevis oocytes

cRNA encoding wild type PEPT1, wild type or PEPT2 mutants, wild type NHERF1, wild-type NHERF2, NHERF2 lacking the first PDZ domain (NHERF2ΔP1) or NHERF2 lacking the second PDZ domain (NHERF2ΔP2) [39], wild-type NHE3 and constitutively active human ^{S422D}SGK1 [44] have been synthesized as described [45]. Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes has been described in detail elsewhere [45].

Where not otherwise specified, oocytes were injected with 15 ng of ^{S422D}SGK1 cRNA and/or 15 ng wild-type or mutants NHERF2 cRNA or H_2O on the same day after preparation of the oocytes and subsequently with 30 ng PEPT1 or PEPT2 or NHE3 cRNA.

Electrophysiology

Two-electrode voltage-clamp recordings were performed at a holding potential of -60 mV. The data were filtered at 10 Hz, and recorded with MacLab A/D-D/A converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). The control solution (superfusate/ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4. Glycyl-glycine (gly-gly) was added to the solutions at the concentrations indicated. In sodium free solution, sodium was replaced by choline. The final solutions were titrated to pH 7.4 using NaOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

Western blotting and isolation of plasma membranes

The expression of SGK1, NHERF1 and NHERF2 was analyzed by western blotting. Briefly, oocytes were homogenized in lysis buffer containing 50 mM Tris (pH 7.5), 0.5 mM EDTA (pH 8.0), 0.5 mM EGTA, 100 mM NaCl, 1 % Triton X-100, 100 µM sodium orthovanadate and protease inhibitor cocktail (Roche, Mannheim, Germany) at the recommended concentrations. Protein was separated on a 10 % polyacrylamide gel and transferred to nitrocellulose membranes. After blocking with 5 % non-fat dry milk in PBS/0.15 % Tween 20 for 1h at room temperature, blots were incubated overnight at 4 °C with a rabbit anti-SGK1 antibody (Upstate, Waltham, MA, USA, diluted 1:2000 in PBS/0.15 % Tween 20/5 % non-fat dry milk), a rabbit anti-NHERF1 antibody (Alomone labs, Jerusalem, Israel, diluted 1:200 in PBS/5 % non-fat dry milk) or a rabbit anti-S-tag-HRP antibody (Merck, Darmstadt, Germany diluted 1:5000 in TBS/0.15 % Tween 20). Secondary peroxidase-conjugated sheep anti-rabbit IgG (diluted 1:2000 in PBS/0.15 % Tween 20/5 % non-fat dry milk) was used for chemiluminescent detection of SGK1 and NHERF1 proteins with enhanced chemiluminescent ECL kit (Amersham, Freiburg, Germany). For determination of PEPT2 expression in the plasma membrane, isolated plasma membranes were prepared as described [46]. Briefly, oocytes were homogenized in lysis buffer containing 83 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.9 and protease inhibitor cocktail (Roche, Penzberg, Germany) at the recommended concentrations and centrifuged at 1000 g at 4 °C for 10 min. The supernatant was then centrifuged at 10000 g which gave a supernatant containing cytosol, microsomes and small vesicles and a pellet containing the plasma membrane. The pellet was centrifuged again for 10 min at 10000 g to remove any residual cytosol. Pellets obtained were dissolved in SDS sample buffer, loaded into a 8 % polyacrylamide gel and transferred to a nitrocellulose membrane at 100 V for 60 min. For immunoblotting, rabbit anti-PEPT2 antibody (Santa Cruz Biotechnology, Heidelberg, Germany, diluted 1:100 in PBS/0.15% Tween 20/5% nonfat dry milk) was used to detect PEPT2. After blocking with 5% nonfat dry milk in PBS/0.15% Tween 20 for 1 h at room temperature, blots were incubated with the primary antibody at 4 °C overnight. Secondary peroxidase-conjugated sheep anti-rabbit IgG (Amersham, Freiburg, Germany, diluted 1:1000 in PBS/0.15% Tween 20/5% nonfat dry milk) was used for luminescent detection with an enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany). Band intensities were quantified using Quantity One® Analysis software (Biorad, Munich, Germany).

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All data were tested for significance using ANOVA, and only results with $P < 0.05$ were considered as statistically significant.

Results

To pursue the role of the kinase SGK1 and the scaffold proteins NHERF1 and NHERF2 in the modulation of the peptide transporters PEPT1 and PEPT2, each transporter was expressed in *Xenopus laevis* oocytes and their activity measured in the presence and absence of the constitutively active ^{S422D}SGK1 or each scaffold protein. Electrophysiological studies revealed that ^{S422D}SGK1 coexpression increased PEPT2 (149.9 ± 7.4 % of control, n = 118) but not PEPT1 (104.0 ± 8.1 % of control, n = 41) mediated transport currents. NHERF2 coexpression similarly enhanced PEPT2 activity (188.6 ± 13.9 % of control, n = 59) without significantly modifying PEPT1 currents (95.7 ± 13.4 % of control, n = 18). NHERF1 modulated neither PEPT1 (96.5 ± 10.4 % of control, n = 17) nor PEPT2 (104.8 ± 25.9 % of control, n = 13), suggesting a specific modulation of PEPT2 by NHERF2. No intrinsic substrate-induced currents were elicited in water injected oocytes or oocytes expressing ^{S422D}SGK1 or NHERF alone. Proper expression of ^{S422D}SGK1, NHERF1 and NHERF2 was assessed by western blotting of whole cell lysates (figure 1).

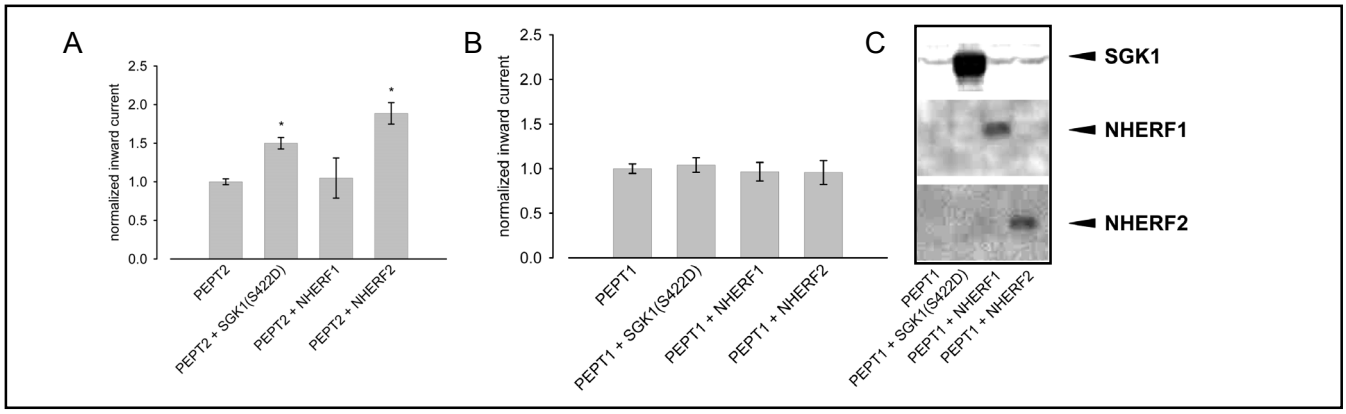

Fig. 1. Constitutively active ^{S422D}SGK1 kinase and the scaffold protein NHERF2 but not NHERF1 affect transport activity of PEPT2 in *Xenopus* oocytes. *Xenopus* oocytes were injected with wild-type PEPT1 or PEPT2 alone or together with the respective regulatory proteins (^{S422D}SGK1, NHERF1 or NHERF2). 4-5 days after cRNA injection, PEPT2 (A) and PEPT1 (B) activity was monitored by the two-electrode voltage clamp technique and expression of regulatory proteins was assessed by western blotting of whole cell lysates (C). Arithmetic means ± SEM. * indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing the transporter alone. Substrate-induced currents were normalized in each batch of oocytes to the mean value obtained in oocytes expressing the transporter alone.

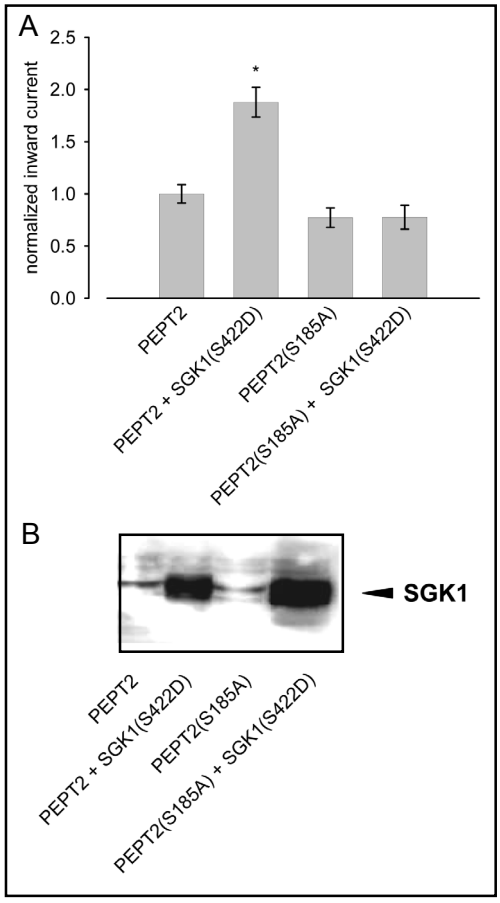

Fig. 2. PEPT2 stimulation by ^{S422D}SGK1 is abrogated upon disruption of the putative SGK phosphorylation site on PEPT2. *Xenopus* oocytes were injected with wild-type PEPT2 or ^{S185A}PEPT2 alone or together with constitutively active ^{S422D}SGK1. 4 days after cRNA injection, PEPT2 activity was monitored by the two-electrode voltage clamp technique (A). Western blotting was performed to demonstrate proper ^{S422D}SGK1 expression (B). Arithmetic means ± SEM. * indicates statistically significant difference to substrate (10 mM gly-gly)-induced currents in *Xenopus* oocytes expressing wild-type PEPT2 or ^{S185A}PEPT2 alone. Substrate-induced currents were normalized in each batch of oocytes to the mean value obtained in oocytes expressing wild-type PEPT2 alone.

The SGK1 consensus site on PEPT2 contains a Ser at position 185 that might be phosphorylated by the kinase. To address, whether SGK1 affects transport activity by phosphorylating PEPT2 at this residue, ^{185}Ser was mutated into alanine thereby disrupting the putative phosphorylation site on the transporter. Gly-gly dependent currents measured in oocytes expressing ^{S185A}PEPT2 remained unaltered upon ^{S422D}SGK1 coexpression (77.2 ± 9.4 % of control in ^{S185A}PEPT2 expressing oocytes compared to 77.6 ± 11.5 % of control in oocytes expressing ^{S185A}PEPT2 and ^{S422D}SGK1, n = 20) indicating that the kinase is effective through this site. Proper expression of ^{S422D}SGK1 in this series of experiments was again assessed by western blotting of whole cell lysates (figure 2).

In order to clarify which PDZ domain in NHERF2 is essential for the stimulatory effect, two NHERF2 deletion mutants (NHERF2ΔP1 and NHERF2ΔP2) were used. When the NHERF2 mutant deficient of the first PDZ domain (NHERF2ΔP1) was coexpressed with PEPT2, substrate-induced currents were elevated to a similar extent as following coexpression of wild-type NHERF2 (156.4 ± 14.9 % of control, n = 24). In contrast, coexpression of PEPT2 together with NHERF2 lacking the second PDZ domain NHERF2ΔP2 failed to increase PEPT2 activity (97.1 ± 10.8 % of control, n = 28, figure 3).

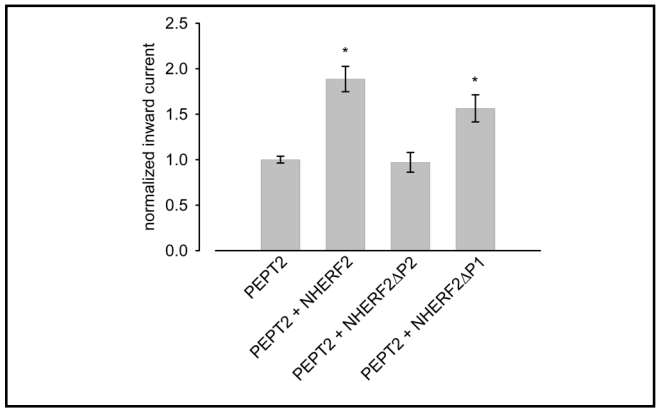

Fig. 3. The second PDZ domain in NHERF2 is required for PEPT2 activation in *Xenopus* oocytes. *Xenopus* oocytes were injected with PEPT2 alone or together with wild-type or NHERF2 mutants lacking the first (NHERF2ΔP1) or second (NHERF2ΔP2) PDZ domain. 4 days after cRNA injection, substrate-induced currents were measured. Arithmetic means ± SEM. * indicates statistically significant difference to substrate (10 mM gly-gly)-induced currents in *Xenopus* oocytes expressing PEPT2 alone. Substrate-induced currents were normalized to the mean value obtained in oocytes expressing the transporter alone.

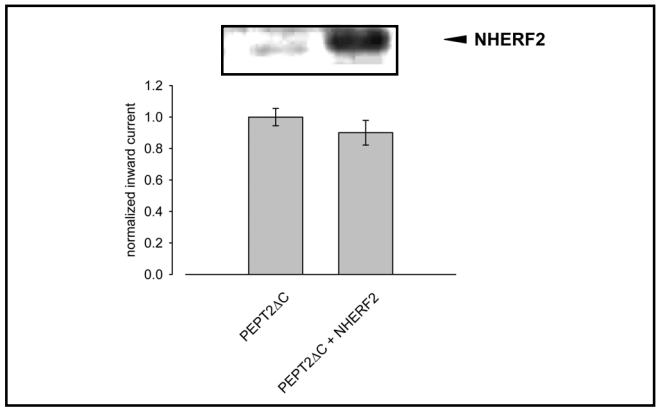

Fig. 4. C-terminal PDZ-binding motif in PEPT2 is essential for its modulation by NHERF2. *Xenopus* oocytes were injected with PEPT2ΔC alone or together with NHERF2. 4 days after cRNA injection, PEPT2 activity was assessed and western blotting performed to demonstrate proper NHERF2 expression. Arithmetic means ± SEM. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing PEPT2ΔC alone. Substrate-induced currents were normalized to the mean value obtained in oocytes expressing the transporter alone.

PDZ domains bind to a specific sequence at the C-terminus of the target protein but additional (internal) binding mechanisms have also been described [47-49]. PEPT2 bears a prototypical PDZ binding motif at its C-terminus (T-K-L). To elucidate whether the functional interaction between PEPT2 and NHERF2 involves a C- terminal PDZ-binding motif on PEPT2, PEPT2 lacking the C- terminal PDZ-binding motif (PEPT2ΔC) was expressed alone or together with NHERF2 and the transporter activity evaluated. As depicted in figure 4, NHERF2 was unable to significantly activate PEPT2ΔC currents (90.0 ± 7.8 % of control, n = 26). Thus, the C-terminal PDZ-binding motif in PEPT2 is required for the carrier stimulation by NHERF2.

Additional experiments have been performed to elucidate whether coexpression of SGK1 and NHERF2 affects substrate affinities of PEPT2. Kinetic analysis of gly-gly uptake by PEPT2 revealed that ^{S422D}SGK1 and NHERF2 significantly increase the maximal substrate induced current $I_{gly\text{-}gly}$ (V_{max}) without altering the concentration needed for halfmaximal $I_{gly\text{-}gly}$ (K_m) significantly (figure 5, table 1).

SGK1 affects several transport proteins by increasing their abundance in the plasma membrane [50-54]. To examine, whether SGK1 and NHERF2 modify the abundace of PEPT2 at the cell surface, PEPT2 protein was determined by western blotting of the isolated plasma membranes from oocytes expressing the transporter alone

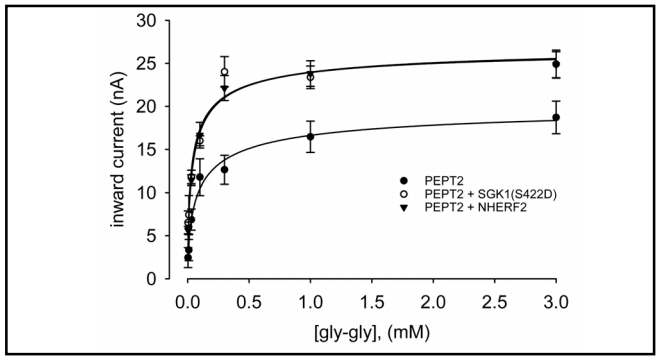

Fig. 5. PEPT2 maximal transport rate is increased by ^{S422D}SGK1 and NHERF2 without altering the transporter substrate affinity. *Xenopus* oocytes were injected with PEPT2 alone or together with constitutively active ^{S422D}SGK1 or NHERF2. 4-5 days after cRNA injection, PEPT2 activity was measured at the indicated substrate concentrations (0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 mM gly-gly). Results were normalized in each batch of oocytes to the V_{max} obtained in oocytes expressing PEPT2 alone. Data were fitted with the modified Hill equation. Arithmetic means ± SEM.

cRNA injected	K_m (mM)	V_{max} (nA)
PEPT2	0.61±0.18	20.6±3.4
+SGK1(S422D)	0.66±0.10	27.0±1.9
+NHERF2	0.60±0.13	27.5±2.7

Table 1. Kinetic parameters of PEPT2 upon coexpression of ^{S422D}SGK1 or NHERF2. Data were fitted with the modified Hill equation. Arithmetic means ± SEM.

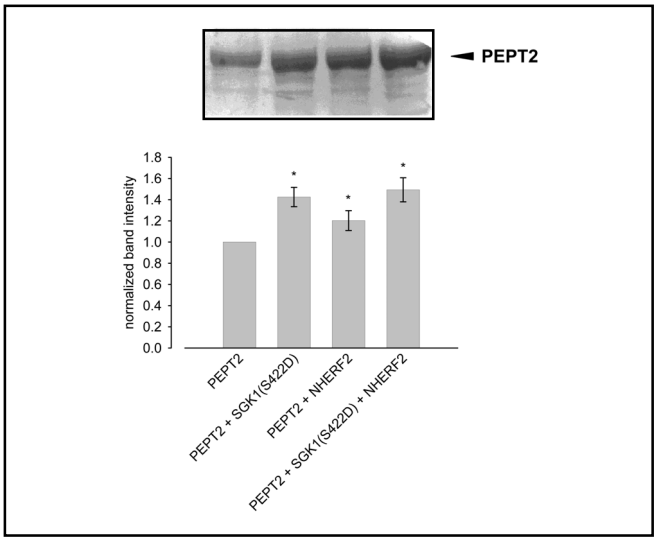

Fig. 6. ^{S422D}SGK1 and NHERF2 enhance PEPT2 protein abundance at the cell surface. *Xenopus* oocytes were injected with PEPT2 alone or together with ^{S422D}SGK1 and/or NHERF2. 4 days after cRNA injection, PEPT2 expression was determined from isolated plasma membranes. Arithmetic means ± SEM. PEPT2 band intensity from three independent experiments was normalized in each batch to the value of PEPT2 band intensity of oocytes expressing the transporter alone. Band intensities were quantified using Quantity One® Analysis software (Biorad, Munich, Germany). * indicates statistically significant difference to *Xenopus* oocytes expressing PEPT2 alone.

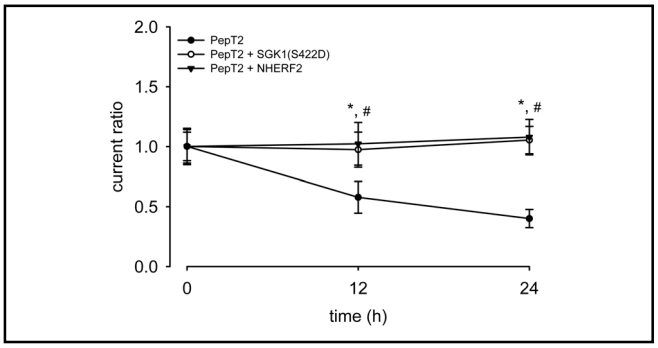

Fig. 7. NHERF2 and ^{S422D}SGK1 increase PEPT2 protein abundance at the cell surface by reducing transporter internalization. *Xenopus* oocytes were injected with PEPT2 alone or together with NHERF2 or ^{S422D}SGK1. 4 days after cRNA injection, PEPT2 activity was assessed in oocytes that had been incubated with Dynasore (1 μM) for the indicated time points. Arithmetic means ± SEM. * or # indicate significant difference between expression of PEPT2 together with NHERF2 or ^{S422D}SGK1, respectively, and coexpression of PEPT2 alone.

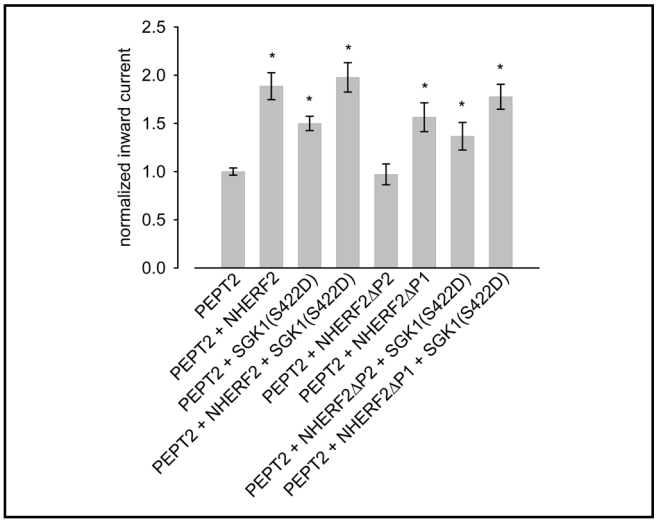

Fig. 8. ^{S422D}SGK1 and NHERF2 stimulate currents encoded by PEPT2 independently. *Xenopus* oocytes were injected with PEPT2 alone or together with ^{S422D}SGK1 with or without wild-type or NHERF2 mutants (NHERF2ΔP1 or NHERF2ΔP2). 4-5 days after cRNA injection, substrate-induced currents were measured. Arithmetic means ± SEM. * indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing PEPT2 alone. Substrate-induced currents were normalized to the mean value obtained in oocytes expressing the transporter alone.

or together with ^{S422D}SGK1 and/or NHERF2. The western blots demonstrate that both regulatory proteins indeed enhanced the abundance of PEPT2 protein at the oocyte surface (142.5 ± 9.1 % of control in ^{S422D}SGK1 expressing oocytes, 120.3 ± 9.3 % of control in NHERF2 expressing oocytes and 149.4 ± 11.4 % of control in oocytes coexpressing ^{S422D}SGK1 and NHERF2, n = 3, figure 6).

In addition to the scaffolding function, NHERF2 regulates endocytosis and/or exocytosis. NHERF2 could thus enhance PEPT2 plasma membrane expression by increasing PEPT2 insertion into the plasma membrane or reducing its internalization. To differentiate among both mechanisms, PEPT2-mediated currents were recorded in oocytes exposed to the Dynamin inhibitor Dynasore, which blocks endocytosis. Dynasore (1 μM) incubation of oocytes expressing PEPT2 led to a decrease in transport activity in oocytes expressing PEPT2 alone when compared to oocytes expressing PEPT2 together with NHERF2. Similar results were observed in Dynasore experiments performed with oocytes expressing PEPT2 along with ^{S422D}SGK1. These data suggests that SGK1 and NHERF2 stabilize cell surface expression of PEPT2 (figure 7).

SGK1 is capable to interact with the second PDZ domain in NHERF2 [55]. Further experiments were therefore performed to study the possibility that SGK1 and NHERF2 synergize to stimulate PEPT2. Coexpression of constitutively active ^{S422D}SGK1 with wild-type NHERF2 did not further activate PEPT2 (197.7 ± 15.2 % of control, n = 43, figure 8). Moreover, coexpression of ^{S422D}SGK1 with NHERF2 lacking the second PDZ

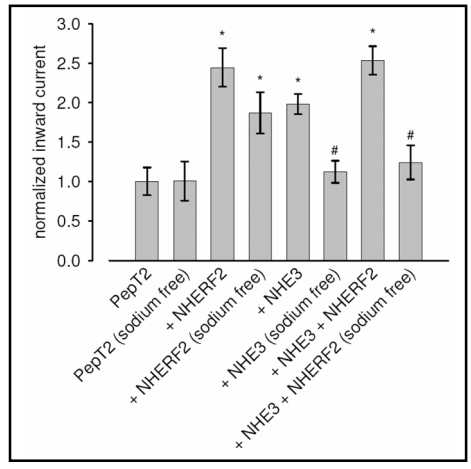

Fig. 9. NHE3 stimulates currents mediated by PEPT2. *Xenopus* oocytes were injected with PEPT2 with or without NHE3 and/or NHERF2. 4-5 days after cRNA injection, substrate-induced currents were measured in the presence and absence of sodium. Arithmetic means ± SEM. * indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing PEPT2 alone. # indicates statistically significant difference to substrate-induced currents under sodium free conditions of the same group. Substrate-induced currents were normalized to the mean value obtained in oocytes expressing the transporter alone.

domain (NHERF2ΔP2) still stimulated PEPT2 (136.7 ± 14.3 % of control, n = 28), indicating that SGK1 and NHERF2 do not synergize in PEPT2 regulation.

A functional interaction between peptide transporters and the sodium proton exchanger NHE has been described earlier [56]. To investigate if PEPT2 is upregulated by NHE3 in the *Xenopus* oocyte expression system we coexpressed both proteins with or without the NHERF2 scaffold protein. As obvious from figure 9, PEPT2 coexpression with NHE3 or NHE3 together with NHERF2 in oocytes increased PEPT2 activity significantly (198.3 ± 12.6 % of control, n =13 and 253.6 ± 17.8 % of control, n =12, respectively). Furthermore, while substrate induced currents are completely sodium independent in oocytes expressing exclusively PEPT2, the peptide transporter becomes partially dependent on the sodium ion when coexpressed with NHE3 or NHE3 together with NHERF2 (44.3 ± 4.7 %, n =13 and 51.7 ± 6.7 %, n =12 of total current fraction, respectively).

Discussion

Two SGK1 recognition sites have been predicted in PEPT1 and one in PEPT2. Both PEPT isoforms also bear a class I PDZ binding motif at their C-terminus, a recognition sequence for PDZ domain-containing proteins. Whether these sites are used for PEPT modulation was hitherto not known. SGK1 is an ubiquitously expressed kinase. In the kidney, SGK1 is found in the aldosterone-sensitive distal nephron (ASDN) and at moderate levels in the glomerulus and in the proximal tubule (rev. in [57]). The PDZ domain-containing proteins NHERF1 and NHERF2 are expressed, among other tissues, in the renal proximal tubule, where PEPT1 and PEPT2 have also been identified [42, 43, 58]. The common expression of SGK1, the NHERFs and the PEPT isoforms suggested a possible regulation of the oligopeptide transporters by the protein kinase and the scaffold protein. In the present study we identified PEPT2 as a specific target of SGK1 and NHERF2. Whereas SGK1 elevated PEPT2-mediated transport, PEPT1 activity was unaffected by the kinase. These results could be explained by the fact that amino acids neighbouring the phosphorylation consensus site may affect the ability of the kinase to phosphorylate its targets [59].

NHERF1 and NHERF2 contain two tandem PDZ domains [36]. While both PDZ domains in each NHERF isoform share high structural homology, they bind different targets (rev. in [60]). Some proteins specifically associate with one of the two isoforms [55, 61-64]. Here we identified PEPT2 as a specific target for NHERF2. According to our results NHERF2 activates the transporter through its second PDZ domain and the C-terminal PDZ-binding motif in PEPT2.

Both PEPT1 and PEPT2 are proton-dependent oligopeptide transporters. Thus, SGK1 and NHERF2 might affect PEPT2 indirectly through altering the cytosolic pH. The fact that only PEPT2 is regulated and that deletion of the putative phosphorylation site in PEPT2 abrogates the stimulatory effect of the protein kinase rules out this possibility. *In vivo*, however, NHERF2 might bridge PEPT2 with the Na^+/H^+ exchanger 3 (NHE3) that mediates the exchange of Na^+ and H^+ across the plasma membrane [65]. This coupling might provide PEPT2 with a sufficient proton gradient. In fact, functional linkage of PEPT2 and NHE1 and/or NHE2 has been described in primary cultures of astrocytes to permit optimal uptake of dipeptides [56]. This model is supported by our data that demonstrate that PEPT2 becomes partially sodium dependent upon coexpression of NHE3. This also suggests a partial indirect regulation of PEPT2 by NHERF2 via NHE3 at least in cells expressing both, the peptide transporter and the sodium proton exchanger isoform 3.

NHERF2 acts as a scaffold protein to localize protein kinases in the vicinity of ion channels/transporters to enable their modulation [37, 38, 40, 41, 55, 66, 67]. NHERF2 enhances the K^+ channel ROMK and the epithelial Ca^{2+} channel TRPV5 by scaffolding SGK1 that

phosphorylates and thereby activates the channel [40, 41, 68]. In this study we demonstrate that SGK1 and NHERF2 independently affect PEPT2. NHERF2 association with PEPT2 occurs via its second PDZ domain which has also been implicated in the binding to SGK1. The fact that the kinase is capable to modulate PEPT2 when coexpressed with NHERF2 lacking the second PDZ domain strengthens the concept that these regulatory proteins act independently. The lack of further increase in PEPT2 activity when SGK1 and NHERF2 were coexpressed together compared to expression of each regulatory protein alone with the transporter might be due to maximal stimulation of PEPT2 by any of the regulators alone.

SGK1 modulates transporters at least in part by enhancing their protein abundance at the cell surface. Our data suggest that SGK1 and NHERF2 similarly increase PEPT2 cell surface expression. The PDZ domain-containing protein PDZK1 has been reported to associate with PEPT2. Similar to NHERF2, the scaffold protein impacts PEPT2 activity by augmenting the transporters maximal velocity and surface expression [69].

An additional function of NHERF2 is the regulation of endocytosis and/or exocytosis (rev. in. [70]). NHERF2 could thus enhance PEPT2 plasma membrane expression by increasing PEPT2 insertion to the plasma membrane or by inhibiting internalization. In this study we demonstrate that NHERF2 increases PEPT2 activity by slowing the transporter internalization from the cell surface.

Taken together, this study indicates that SGK1 and NHERF2 enhance PEPT2 activity and expression by stabilizing the transporter in the plasma membrane. The kinase is presumably effective through phosphorylation of ^{185}Ser within the SGK1 consensus site and NHERF2 through its second PDZ domain and the C-terminal PDZ-binding motif in PEPT2. Our data suggest that SGK1 and NHERF2 might participate in the regulation of PEPT2 in part by controlling PEPT2 surface expression. Mice with targeted PEPT2 inactivation display increased urinary excretion of dipeptides and reduced reabsorption of peptidomimetic drugs [71-73]. Thus, mutations of PEPT2 at these regulatory sites might affect peptide reabsorption and uptake of peptidomimetic drugs. Moreover, a SGK1 gene variant leading to enhanced SGK1 activity [74-76] as well as glucocorticoids or stress stimulating SGK1 expression [22], may be paralelled by enhanced activity of PEPT2 and the respective acceleration of transport of di- and tripeptides and peptidomimetic drugs.

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