Original Paper

SPAK Dependent Regulation of Peptide Transporters PEPT1 and PEPT2

Jamshed Warsi Luo Dong Bernat Elvira Madhuri S Salker Ekaterina Shumilina
Zohreh Hosseinzadeh Florian Lang

Department of Physiology I, University of Tübingen, Gmelinstr. 5, 70276 Tübingen, Germany

Key Words

Peptide transport • Cell volume • Intestine • Knockout mice

Abstract

Background/Aims: SPAK (STE20-related proline/alanine-rich kinase) is a powerful regulator of renal tubular ion transport and blood pressure. Moreover, SPAK contributes to the regulation of cell volume. Little is known, however, about a role of SPAK in the regulation of organic solutes. The present study thus addressed the influence of SPAK on the peptide transporters PEPT1 and PEPT2. **Methods:** To this end, cRNA encoding PEPT1 or PEPT2 were injected into *Xenopus laevis* oocytes without or with additional injection of cRNA encoding wild-type, SPAK, WNK1 insensitive inactive ^{T233A}SPAK, constitutively active ^{T233E}SPAK, and catalytically inactive ^{D212A}SPAK. Electrogenic peptide (glycine-glycine) transport was determined by dual electrode voltage clamp and PEPT2 protein abundance in the cell membrane by chemiluminescence. Intestinal electrogenic peptide transport was estimated from peptide induced current in Ussing chamber experiments of jejunal segments isolated from gene targeted mice expressing SPAK resistant to WNK-dependent activation (*spak^{tg/tg}*) and respective wild-type mice (*spak^{+/+}*). **Results:** In PEPT1 and in PEPT2 expressing oocytes, but not in oocytes injected with water, the dipeptide gly-gly (2 mM) generated an inward current, which was significantly decreased following coexpression of SPAK. The effect of SPAK on PEPT1 was mimicked by ^{T233E}SPAK, but not by ^{D212A}SPAK or ^{T233A}SPAK. SPAK decreased maximal peptide induced current of PEPT1. Moreover, SPAK decreased carrier protein abundance in the cell membrane of PEPT2 expressing oocytes. In intestinal segments gly-gly generated a current, which was significantly higher in *spak^{tg/tg}* than in *spak^{+/+}* mice. **Conclusion:** SPAK is a powerful regulator of peptide transporters PEPT1 and PEPT2.

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Introduction

Cellular accumulation of nutrients involves a wide variety of carriers including the peptide transporters 1 (PEPT1) and 2 (PEPT2), which accept di- and tripeptides [1-3] as well as peptide-like drugs [1, 2]. PEPT1 is a high-capacity, low-affinity peptide transporter and PEPT2 a low-capacity, high-affinity peptide transporter [4]. PEPT1 and PEPT2 are both expressed in the renal proximal tubule [4, 5]. PEPT1 is mainly expressed in early parts of the proximal tubule, whereas PEPT2 prevails in latter parts of the proximal tubule [4]. PEPT1 is the major peptide transporter expressed in intestine [4]. PEPT2 is expressed by neurons and glial cells of cerebral cortex, olfactory bulb, basal ganglia, cerebellum and hindbrain as well as epithelial cells of the choroid plexus and ependymal cells [4]. PEPT1 is further expressed in the pancreas, bile duct and liver, and PEPT2 in the lung, mammary gland and spleen [4]. The peptide transporters are regulated by several hormones including leptin [6] and growth hormone [7].

Kinases hitherto identified in the regulation of peptide transporters include phosphoinositide (PI) 3 kinase [8], phosphoinositide dependent kinase PDK1 [8], serum & glucocorticoid inducible kinase SGK1 [9] and AMP activated kinase [10]. Kinases involved in the regulation of epithelial transport include SPAK (STE20-related proline/alanine-rich kinase) [11-13]. SPAK is regulated by with-no-K(Lys) (WNK) kinases [11, 14-17], which are powerful regulators of renal tubular ion transport and blood pressure [18-22]. Specifically, SPAK and the related oxidative stress-responsive kinase 1 (OSR1) kinase upregulate the Na^+Cl^- (NCC) and the $\text{Na}^+\text{K}^+\text{2Cl}^-$ (NKCC2) cotransporters [14, 15, 18, 23-33]. Mutations of genes encoding WNK kinases underly Gordon's syndrome, a monogenic disease leading to hypertension and hyperkalaemia [16, 17, 34, 35]. By regulation of ion transport, SPAK contributes to cell volume regulation [24, 36]. Most recent observations pointed to a role of OSR1 and/or SPAK in the regulation of Na^+ coupled phosphate transport [37, 38], and Na^+/H^+ exchanger [39], pointing to a broader role of those kinases in the regulation of epithelial transport. To the best of our knowledge, nothing is hitherto known about a role of SPAK in the regulation of peptide transporters.

The present study thus explored whether SPAK influences the function of PEPT1 and/or PEPT2. To this end, cRNA encoding the peptide transporters were injected into *Xenopus laevis* oocytes with or without cRNA encoding SPAK and peptide transport quantified by determination of peptide induced current. To define the *in vivo* significance of SPAK sensitive peptide transport regulation, peptide induced current was determined in Ussing chamber experiments of intestinal segments isolated either from gene targeted mice expressing SPAK resistant to WNK-dependent activation (*spak^{tg/tg}*) mice or from corresponding wild-type (*spak^{+/+}*) mice.

Materials and Methods

Constructs

Constructs encoding rabbit PEPT1, rabbit PEPT2 and haemagglutinin labelled PEPT2-HA [40], wild-type SPAK, WNK1 insensitive inactive ^{T233A}SPAK, constitutively active ^{T233E}SPAK, and catalytically inactive ^{D212A}SPAK [14], were used for generation of cRNA as described previously [41].

Voltage clamp in *Xenopus laevis* oocytes

Xenopus laevis oocytes were prepared as previously described [42, 43]. Where not indicated otherwise, 10 ng PEPT1 or 20 ng PEPT2 cRNA on the first day and 10 ng of wild-type SPAK, ^{T233E}SPAK, ^{T233A}SPAK or ^{D212A}SPAK cRNA were injected on the same day after preparation of the oocytes [44]. The oocytes were maintained at 17°C in ND96 solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl_2 , 1.8 CaCl_2 , 2.5 NaOH, 5 HEPES (pH 7.4) and 5 sodium pyruvate ($\text{C}_3\text{H}_3\text{NaO}_3$). pH 7.4. Gentamycin (100 mg/l), Tetracycline (50 mg/l),

Ciprofloxacin (1.6 mg/l), and Theophiline (90 mg/l) were added. Where indicated, Brefeldin A (5 μ M) was added to the respective solutions. The experiments were performed at room temperature 3 to 4 days after injection. Two-electrode voltage-clamp recordings were performed at a holding potential of -70 mV. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A(1322A Axon Instruments) converter and Clampex 9.2 software for data acquisition and analysis (Axon Instruments). The control superfusate (ND96) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 2.5 NaOH and 5 HEPES (pH 7.4). Glycine-glycine was added to the solutions at a concentration of 2 mM, unless otherwise stated. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [45, 46].

Chemiluminescence

For detection of PEPT2-HA cell surface expression, the oocytes were first incubated with primary mouse monoclonal anti-HA antibody conjugated to horseradish peroxidase (1:1000, Miltenyi Biotec Inc, CA, USA). Individual oocytes were placed in 96 well plates with 20 μ l of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer) by integrating the signal over a period of 1 sec [47]. Results display normalized relative light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol [48].

Ussing chamber experiments

All animal experiments were conducted according to the German law for the welfare of animals and according to the guidelines of the American Physiological Society and were approved by local authorities. Experiments have been performed using intestinal segments from 7-8 week old female gene targeted mice expressing SPAK resistant to WNK-dependent activation (*spak^{tg/tg}*) and in mice expressing wild-type SPAK (*spak^{+/+}*) [38]. The mice were fed a control diet (1314, Altromin, Heidenau, Germany) and had free access to tap drinking water.

For analysis of electrogenic intestinal peptide transport, jejunal segments were mounted into a custom made mini-Ussing chamber with an opening of 0.00769 cm². Under control conditions, the serosal and luminal perfusate contained (in mM): 115 NaCl, 2 KCl, 1 MgCl₂, 1.25 CaCl₂, 0.4 KH₂PO₄, 1.6 K₂HPO₄, 5 Sodium Pyruvate (C₃H₃NaO₃), 25 NaHCO₃, 5 mannitol (pH 7.4, NaOH). Where indicated, the dipeptide glycine-glycine (5 mM) was added to the luminal perfusate at the expense of mannitol (5 mM) (all substances were from Sigma, Schnellendorf, Germany, or from Roth, Karlsruhe, Germany).

In all Ussing chamber experiments the transepithelial potential difference (Vt) was determined continuously and the transepithelial resistance (Rt) was estimated from the voltage deflections (Δ Vt) elicited by imposing test currents (I_t). The resulting Rt was calculated according to Ohm's law [40].

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes or intestinal segments investigated. All voltage clamp experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA (Tukey test or Kruskal-Wallis test) or t-test, as appropriate. Results with p < 0.05 were considered statistically significant.

Results

The present study explored whether SPAK participates in the regulation of peptide transport. To this end, cRNA encoding PEPT1 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type, constitutively active or inactive SPAK. Peptide transport was estimated from the current generated by addition of the dipeptide glycine-glycine to the bath solution (I_{gly-gly}).

No appreciable I_{gly-gly} was observed in water injected *Xenopus laevis* oocytes (Fig. 1Aa,B). Accordingly, *Xenopus laevis* oocytes did not express measurable endogenous electrogenic glycine-glycine transport. As illustrated in Fig. 1, addition of glycine-glycine (2mM) to *Xeno-*

Fig. 1. Coexpression of SPAK decreases electrogenic peptide transport in PEPT1-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with water (a) or expressing PEPT1 without (b) or with additional coexpression of wild type SPAK (c). B: Arithmetic means $\pm$ SEM (n = 15) of glycine-glycine (2 mM) induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with water (light grey bar), or expressing PEPT1 without (white bar) or with wild-type SPAK (dark grey). * (p < 0.05) indicates statistically significant difference from the absence of SPAK.

pus laevis oocytes injected with cRNA encoding PEPT1 was followed by appearance of $I_{\text{gly-gly}}$. Additional expression of wild-type SPAK resulted in a significant decrease of $I_{\text{gly-gly}}$ in PEPT1 expressing *Xenopus laevis* oocytes (Fig. 1).

In order to determine maximal transport rate and affinity of PEPT1, the oocytes were exposed to glycine-glycine concentrations ranging from 0.01 to 5 mM. As illustrated in Fig. 2, the increase of peptide concentration was followed by an increase of $I_{\text{gly-gly}}$ in both, *Xenopus laevis* oocytes expressing PEPT1 alone and *Xenopus laevis* oocytes expressing PEPT1 and SPAK. The increase of $I_{\text{gly-gly}}$ was, however, larger in *Xenopus laevis* oocytes expressing PEPT1 alone than in *Xenopus laevis* oocytes expressing PEPT1 with SPAK. Calculation of maximal currents utilizing the Michaelis Menten equation yielded values, which were significantly (p < 0.01) lower in *Xenopus laevis* oocytes expressing PEPT1 together with SPAK (22.4 ± 1.6 nA, n = 11-12), than in *Xenopus laevis* oocytes expressing PEPT1 alone (31.2 ± 2.4 nA, n = 11-12). Calculation of glycine-glycine concentrations required for halfmaximal current (K_M) did not yield significantly different values in *Xenopus laevis* oocytes expressing PEPT1 together with SPAK (946 ± 227 mM, n = 11-12) and in *Xenopus laevis* oocytes expressing PEPT1 alone (795 ± 82 mM, n = 11-12) (Fig. 2).

The effect of wild-type SPAK was mimicked by constitutively active T^{233E} SPAK, but not by WNK1 insensitive inactive T^{233A} SPAK and inactive kinase D^{212A} SPAK. As illustrated in Fig. 3, coexpression of T^{233E} SPAK but not coexpression of T^{233A} SPAK and D^{212A} SPAK, significantly decreased $I_{\text{gly-gly}}$ in PEPT1 expressing *Xenopus laevis* oocytes.

In order to test whether SPAK similarly regulates PEPT2, cRNA encoding PEPT2 was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type SPAK. As shown in Fig. 4, $I_{\text{gly-gly}}$ was lower in oocytes expressing PEPT2 with wild-type SPAK than oocyte expressing PEPT2 alone (Fig. 4).

Fig. 2. Coexpression of SPAK decreases maximal electrogenic peptide transport in PEPT1-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing the current induced by increasing concentrations of glycine-glycine (from 10 μ M – 5 mM) in *Xenopus laevis* oocytes expressing PEPT1 without (upper panel) or with (lower panel) additional coexpression of wild type SPAK. B: Arithmetic means $\pm$ SEM (n = 11-12) of glycine-glycine-induced current ($I_{\text{gly-gly}}$) as a function of glycine-glycine concentration in *Xenopus laevis* oocytes expressing PEPT1 without (black squares), or with (black circles) additional coexpression of wild-type SPAK.

Fig. 3. The effect of SPAK is mimicked by active T^{233E} SPAK but not by inactive D^{212A} SPAK or WNK insensitive T^{233A} SPAK. **A:** Representative original tracings showing glycine-glycine (2 mM) - induced current ($I_{gly-gly}$) in *Xenopus laevis* oocytes injected with water (a), expressing PEPT1 alone (b) or with additional co-expression of SPAK (c) or with constitutively active T^{233E} SPAK (d), or with catalytically inactive D^{212A} SPAK (e) or with WNK1 insensitive T^{233A} SPAK (f). **B:** Arithmetic means $\pm$ SEM (n = 8-10) of glycine-glycine (2 mM) - induced current ($I_{gly-gly}$) in *Xenopus laevis* oocytes injected

with water (light grey bar) expressing PEPT1 without (white bar) or with wild-type SPAK (dark grey bar) or with constitutively active T^{233E} SPAK (black bar), or with catalytically inactive D^{212A} SPAK (middle grey bar) or with WNK insensitive T^{233A} SPAK (light grey bar). ** (p<0.01) indicates statistically significant difference from the absence of PEPT1.

Fig. 4. Coexpression of SPAK decreases electrogenic peptide transport in PEPT2-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with water (a) or expressing PEPT2 without (b) or with additional coexpression of wild-type SPAK (c). B: Arithmetic means $\pm$ SEM (n = 12) of glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with water (light grey bar), or expressing PEPT2 without (white bar) or with wild-type SPAK (dark grey bar). **($p < 0.01$) indicates statistically significant difference from the absence of SPAK.

Fig. 5. SPAK coexpression decreases PEPT2 protein abundance in the *Xenopus laevis* oocyte cell membrane. Arithmetic means $\pm$ SEM (n = 96-115) of PEPT2-HA protein abundance determined by chemiluminescence in *Xenopus laevis* oocytes injected with water (light grey bar) or expressing PEPT2-HA alone (white bar), or expressing PEPT2-HA together with wild-type SPAK (dark grey bar). *($p < 0.05$) indicates statistically significant difference from *Xenopus laevis* oocytes expressing PEPT2-HA alone (Kruskal-Wallis test).

The down-regulation of $I_{\text{gly-gly}}$ in PEPT1 or PEPT2-expressing *Xenopus laevis* oocytes following coexpression of SPAK could have resulted from a decline of protein abundance in the cell membrane. Thus, chemiluminescence was employed to quantify the carrier protein abundance in the cell membrane. As illustrated in Fig. 5, coexpression of wild-type SPAK resulted in a significant decrease of the PEPT2 protein abundance in the cell membrane of PEPT2 expressing *Xenopus laevis* oocytes.

Additional experiments were performed to determine the stability of $I_{\text{gly-gly}}$ following disruption of new carrier protein insertion into the cell membrane by Brefeldin A (5 μ M). As illustrated in Fig. 6, following addition of Brefeldin A, $I_{\text{gly-gly}}$ did not decline faster in *Xenopus laevis* oocytes expressing PEPT1 together with SPAK than in *Xenopus laevis* oocytes expressing PEPT1 alone. Thus, SPAK decreased $I_{\text{gly-gly}}$ by a mechanism other than accelerating carrier clearance from the cell membrane.

In order to define the *in vivo* significance of SPAK sensitive regulation of peptide transporters, peptide induced current was determined in Ussing chamber experiments of intestinal segments isolated from 16-week-old female gene targeted mice carrying WNK insensitive SPAK ($spak^{tg/tg}$) or corresponding wild-type mice ($spak^{+/+}$). As illustrated in Fig. 7, addition of glycine-glycine (5 mM) to the luminal perfusate generated a transepithelial current ($I_{\text{gly-gly}}$), which was significantly higher in intestinal epithelium from $spak^{tg/tg}$ mice than from $spak^{+/+}$ mice.

Fig. 6. Coexpression of SPAK does not accelerate the decline of electrogenic peptide transport in the presence of Brefeldin A in PEPT1 expressing oocytes. Arithmetic means $\pm$ SEM ($n = 10-13$) of glycine-glycine (2 mM) - induced current ($I_{\text{gly-gly}}$) in *Xenopus laevis* oocytes injected with cRNA encoding PEPT1 without (PEPT1, white bars) or with (PEPT1+SPAK, grey bars) wild-type SPAK and exposed to 5 μ M Brefeldin A for the indicated time periods. **($p < 0.01$) indicates statistically significant difference from the absence of SPAK; ###($p < 0.01$) indicates statistically significant difference from the absence of Brefeldin A.

Fig. 7. Glycine-glycine-induced current is increased in intestinal segments from SPAK-deficient mice. A: Original representative tracings illustrating the effect of 5 mM glycine-glycine on the transepithelial potential difference across jejunal segments from (A) SPAK corresponding wild-type mice (*spak^{+/+}*) and (B) gene targeted mice expressing SPAK resistant to WNK-dependent activation (*spak^{tg/tg}*). Arrows indicate addition of Gly-Gly. Throughout the experiments test currents of 1 μ A were applied to allow determination of transepithelial resistance. B: Arithmetic means $\pm$ SEM ($n = 5$) of glycine-glycine (5 mM) induced current ($I_{\text{gly-gly}}$) in jejunum from *spak^{+/+}* (white bar) and *spak^{tg/tg}* (black bar) mice. * indicates statistically significant ($p < 0.05$) difference from wild-type mice (Students t-test).

Discussion

The present study reveals that SPAK (STE20-related proline/alanine-rich kinase) down-regulates the peptide transporters PEPT1 and PEPT2. Coexpression of SPAK, of constitutively active ^{T233E}SPAK, but not of catalytically inactive ^{D212A}SPAK or WNK1 insensitive inactive ^{T233A}SPAK was followed by a decrease of electrogenic dipeptide transport in PEPT1 expressing *Xenopus laevis* oocytes. As the catalytically inactive ^{T233A}SPAK was not effective, the effect apparently requires kinase activity. The effect on PEPT2 resulted at least in part from a decrease of PEPT2 protein abundance in the cell membrane. The experiments with Brefeldin A suggest that SPAK was not effective by enhancing PEPT1 carrier retrieval from the cell membrane and are compatible with the assumption that SPAK delayed carrier insertion into the cell membrane.

The down-regulating effect of SPAK on the peptide transporters is in seeming contrast to the up-regulation of the Na^+Cl^- (NCC) and the $\text{Na}^+\text{K}^+\text{2Cl}^-$ (NKCC2) cotransporters [14, 15, 18, 23-33]. Presumably, the mechanisms involved in the regulation of the peptide transporters are distinct from those regulating NCC and NKCC. SPAK influences NCC and NKCC at least in part by direct phosphorylation of the carrier protein [33]. The mechanism underlying SPAK sensitivity of the peptide transporters remains to be identified. The *in vivo* significance of the observations in *Xenopus laevis* oocytes is underscored by the observation that the peptide induced current was higher in *spak^{tg/tg}* than in *spak^{+/+}* mice. The possibility should be kept in mind, though, that SPAK deficiency may indirectly modify intestinal peptide transport.

The peptide transporters significantly contribute to intestinal nutrient uptake [2, 49], and the majority of dietary nitrogen is taken up by PEPT1 [50]. Thus, SPAK sensitivity of PEPT1 is expected to impact on intestinal nutrient transport.

At least in theory, the impact of SPAK on intestinal peptide transport may be particularly relevant for altered intestinal transport in intestinal inflammation, which involves altered intestinal SPAK [51] and PEPT1 [3] activity. In the absence of inflammation, PEPT1 expression is restricted to small intestine [3]. PEPT1 is, however, highly expressed in colon following inflammatory bowel disease (IBD) [3].

SPAK interacts with several kinases, such as p38 kinase, Jun activated kinase (JNK), protein kinase C theta (PKCtheta), with no K kinase (WNK) and myosin light chain kinase (MLCK), kinases participating in the signalling of cell differentiation, cell transformation, cell proliferation, and cytoskeleton architecture [51]. Whether or not SPAK modifies cellular peptide uptake during differentiation, cell transformation and/or cell proliferation, remains to be shown. Whether sensitivity of SPAK activity to cell volume [24, 36] impacts on the regulation of peptide transporters, similarly remains to be shown.

Peptide transporters mediate the transport of several drugs including beta-lactam antibiotics, angiotensin-converting enzyme inhibitors, antiviral drugs, and anti-cancer agents [2, 49, 52-55]. Peptide transporters are thus not only important for cellular nutrient uptake but as well for cellular drug uptake [1, 10, 56, 57]. The transporters are, for instance, expressed in pancreatic cancer cells [58] and prostate cancer cells [59] and the carriers have been considered potential targets for tumor therapy [59]. Whether or not drug uptake into tumor cells is sensitive to SPAK, remains to be shown.

Conclusion

The present observations reveal a powerful effect of SPAK on the activity of the peptide transporters, PEPT1 and PEPT2. As shown for PEPT2, the kinase is effective by decreasing carrier protein abundance in the cell membrane. SPAK sensitive regulation of peptide transporters may be relevant for cellular peptide and xenobiotic uptake in intestine and presumably other polarized or non-polarized cell types.

Disclosure Statement

The authors state that they do not have any conflicts of interest.

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