The Serum and Glucocorticoid Inducible Kinases SGK1-3 Stimulate the Neutral Amino Acid Transporter SLC6A19

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Key Words

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Abstract

The neutral amino acid transporter SLC6A19 (B⁰AT1) plays a decisive role in transport of neutral amino acids in the kidney and intestine. Recently, mutations in SLC6A19 were identified that result in severe neutral aminoaciduria known as Hartnup disorder. SLC6A19 expression and function is controlled by the brush-border angiotensin-converting enzyme 2 (ACE2). Beyond that the mechanisms regulating SLC6A19 function are unknown. The SLC6A19 sequence contains a conserved putative phosphorylation site for the serum and glucocorticoid inducible kinase isoforms SGK1-3, kinases known to regulate a variety of channels and transporters. The present study explored the role of SGK1-3 in the regulation of SLC6A19. As shown by two-electrode voltage clamp in the *Xenopus* oocyte expression system, leucine-induced currents in SLC6A19 expressing oocytes were activated by the protein kinases SGK1-3. The putative phosphorylation site on the transporter is not essential for SLC6A19 regulation by the kinases. As determined by quantitative immunoassay and electrophysiology, the kinases

increase SLC6A19 currents by increasing the cell surface expression of the protein without altering the affinity of the carrier. Following inhibition of carrier insertion into the cell membrane by treatment with brefeldin A (BFA), the leucine-induced current declined significantly slower in *Xenopus* oocytes expressing SLC6A19 together with SGK1 than in oocytes expressing SLC6A19 alone, a finding pointing to SGK-mediated transporter stabilization in the plasma membrane. Coexpression of ACE2 markedly increased leucine-induced currents in SLC6A19 expressing oocytes that were further enhanced by SGK1-3 kinases. In conclusion, SGK isoforms are novel potent stimulators of SLC6A19 and may thus participate in the regulation of neutral amino acid transport *in vivo*.

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Introduction

Hartnup disorder was first described in 1956 in two siblings of the Hartnup family [1] and is characterized by elevated urinary and intestinal excretion of neutral amino acids [2]. While most patients remain asymptomatic, some show transient manifestations of pellagra, cerebellar ataxia and psychosis [3]. Functional studies in brush border

membrane vesicles as well as in renal and intestinal cell lines indicated that a defective system B⁰ neutral amino acid transporter was responsible for Hartnup disorder [4-7]. A decade later, two independent groups identified mutations in the gene encoding the neutral amino acid transporter SLC6A19 (also known as B⁰AT1) that cause Hartnup disorder [8, 9]. SLC6A19 is expressed mainly in kidney and small intestine [8, 9] and has the properties of amino acid transport system B⁰ that represents the major apical neutral amino acid transporter of kidney and intestine. Expression studies in *Xenopus* oocytes revealed that the carrier is electrogenic and transports neutral amino acids in a Na⁺-dependent but Cl⁻-independent manner [9]. These functional properties are shared by the mouse ortholog of SLC6A19 (mB⁰AT1, [10]).

Although SLC6A19 kinetic properties and substrate specificity have been studied extensively [11], the mechanisms regulating SLC6A19 function are still unknown. Database analysis (ExPASy PROSITE, <http://www.expasy.ch/prosite>) of regulatory sites within the SLC6A19 amino acid sequence predicted six extracellular glycosylation sites and one intracellular consensus site for phosphorylation by protein kinases A (PKA), B (PKB) and by the serum and glucocorticoid inducible kinases SGK1-3.

The serine and threonine kinases SGK1-3 are downstream kinases in the phosphatidylinositol 3-kinase (PI3K) signalling pathway [12]. While SGK1 and SGK3 are expressed in all tissues tested so far [13, 14], SGK2 is found only in some tissues including kidney, liver, pancreas and choroid plexus of the brain [13]. SGK1-3 require phosphorylation to become functional. The phosphorylation is triggered by a signalling cascade involving PI3K, the 3-phosphoinositide (PIP3)-dependent kinase (PDK1) and PDK2 or hydrophobic motif (H-motif) kinase [13], later shown to be mTORC2 [14, 15]. The activation of SGK1 requires its phosphorylation at ⁴²²Ser by the PDK2/H-motif kinase, followed by the phosphorylation of ²⁵⁶Ser by PDK1 that leads to its activation [12]. The equivalent phosphorylation sites for SGK2 and SGK3 are the residues ¹⁹³Thr/³⁵⁶Ser and ²⁵³Thr/⁴¹⁹Ser, respectively [13]. Mutation of ⁴²²Ser to Asp leads to the constitutive phosphorylation of ²⁵⁶Ser and thus to constitutively active SGK1. Similarly, mutation of ³⁵⁶Ser to Asp and ⁴¹⁹Ser to Asp leads to the constitutively active SGK2 and SGK3. Once activated, SGK isoforms recognize and phosphorylate Ser/Thr residues within the sequence Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr or Arg-Arg-Xaa-Arg-Ser/Thr [14]. The serine at position 100 in SLC6A19 is located within such a SGK consensus site

that is conserved among several species including human, rabbit, mouse and rat (Fig. 2A).

Several reports have demonstrated a role of SGK in amino acid transport. SGK isoforms are capable to activate the excitatory amino acid transporters EAAT1-5 (SLC1A3, SLC1A2, SLC1A1, SLC1A6 and SLC1A7), the glutamine transporter SN1 (SLC38A3) and the neutral amino acid transporter ASCT2 (SLC1A5) [16-23]. The kinases are effective by phosphorylating the carrier directly and/or by phosphorylating Nedd4-2, thus impeding the function of this ubiquitin ligase that mediates the clearance of membrane proteins from the cell membrane [16, 24, 25].

Considering the common expression of SGK isoforms and SLC6A19 in kidney and small intestine [8] and their ability to affect amino acid transport, this study aimed to clarify the role of the kinases in the regulation of SLC6A19. Two SLC6A19 accessory proteins, ACE2 and collectrin, have recently been identified that associate with and control transporter surface abundance [26-28]. Functional experiments using *ace2* and *collectrin* null mice demonstrated that SLC6A19 expression and function in small intestine and in kidney proximal tubule depend on ACE2 and collectrin, respectively [26, 27]. Thus, we further studied SLC6A19 regulation by the SGK1-3 kinases upon coexpression of those transporter accessory proteins.

Here we provide evidence that SGK1-3 activate SLC6A19 by stabilizing the transporter within the plasma membrane without altering carrier substrate affinity. The observed stimulatory effect does not require the residue ¹⁰⁰Ser within the SGK consensus site on SLC6A19, since disruption of this site did not abrogate transporter activation by the kinases. The kinases rather regulate SLC6A19 by impacting the ubiquitin ligase Nedd4-2. Similar to EAAT4 [20], Nedd4-2 downregulates SLC6A19, an effect reversed by the protein kinases SGK1-3. Coexpression of the SLC6A19 accessory protein ACE2 drastically increased leucine-induced currents in SLC6A19 expressing oocytes that were further elevated by SGK1-3 kinases. Taken together, our data suggest that SGK1-3 might participate in the regulation of neutral amino acid reabsorption by promoting SLC6A19 activity and stability at the cell surface.

Materials and Methods

Site directed mutagenesis

SLC6A19 mutants were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene,

Heidelberg, Germany) according to the manufacturer's instructions. The following primers were used:

SLC6A19(S100A), s: 5' GAG GCT ACG CAA GGG CGC CAT GGG TGT GTG GAG C 3'; SLC6A19(S100A), as: 5' GCT CCA CAC ACC CAT GGC GCC CTT GCG TAG CCT C 3'. SLC6A19(S100D), s: 5' GAG GCT ACG CAA GGG CGA CAT GGG TGT GTG GAG C 3'; SLC6A19(S100D), as: 5' GCT CCA CAC ACC CAT GTC GCC CTT GCG TAG CCT C 3'. All mutants were sequenced to verify the presence of the desired mutation.

*Expression in *Xenopus laevis* oocytes*

cRNA encoding mouse SLC6A19 [10], human SGK1, SGK2 and SGK3, constitutively active human ^{S422D}SGK1, ^{S356P}SGK2, ^{S419D}SGK3 and inactive human ^{K127N}SGK1, ^{K64N}SGK2, ^{K191N}SGK3 [12, 13], mouse ACE2 [28], human collectrin [28] and human Nedd4-2 [24] have been synthesized *in vitro* using the T7 mMessageMachine kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Oocytes were injected with 25–30 ng SLC6A19, 10 ng SLC1A6, 7.5 ng SGK1-3, 5 ng ACE2 and 10 ng collectrin cRNA or H₂O. All experiments were performed at room temperature 4–5 days after injection of the respective cRNAs [29, 30].

Electrophysiology

Leucine (2 mM)-induced currents were analyzed by two-electrode voltage-clamp recording as a measure of SLC6A19 activity. The recordings were performed with 1xLU and 10xMGU headstages connected to a Geneclamp 500B electronic amplifier (Axon Instruments, Union City, CA, USA). The output signal was amplified 10 times and filtered at 1 kHz [31]. The analog signal was converted into digital by a Digidata 1322A (Axon Instruments, Union City, CA, USA), and data were sampled at 10 Hz using pCLAMP software (Axon Instruments, Union City, CA, USA). Oocytes were chosen that had a membrane potential of less than -30 mV [32]. Once a stable membrane potential was reached under current-clamp conditions, the amplifier was switched to the voltage-clamp mode, holding the oocytes at -60 mV [33]. The control solution contained ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4). Leucine was added to the solution at the indicated concentrations. The final solutions were titrated to the pH indicated using HCl or 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. Glutamate (1 mM)-induced currents were analyzed by two-electrode voltage-clamp recording as described above as a measure of SLC1A6 (EAAT4) activity.

Western blotting

Expression of SLC6A19, SGK, phosphorylated (active) SGK and β -actin in whole cell lysates were 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 μ M 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 1 h at room temperature, blots

were incubated overnight at 4 °C with a primary mouse anti-SLC6A19 antibody (Abnova, Taipei, Taiwan, diluted 1:500 in TBS/0.15% Tween 20/5% non-fat dry milk), a primary rabbit anti-SGK antibody (Upstate, Waltham, MA, USA, diluted 1:2000 in PBS/0.15% Tween 20/5% non-fat dry milk), a primary rabbit anti-pThr256SGK antibody (Cell Signaling Technology, Danvers, MA, USA, diluted 1:500 in PBS/0.15% Tween 20/5% non-fat dry milk) or peroxidase-conjugated mouse anti- β -actin (diluted 1:10000 in PBS/0.15% Tween 20/5% non-fat dry milk) to detect SLC6A19, SGK, phosphorylated SGK and β -actin, respectively. Secondary peroxidase-conjugated sheep anti-rabbit IgG (diluted 1:2000 in PBS/0.15% Tween 20/5% non-fat dry milk) or peroxidase-conjugated sheep anti-mouse IgG (diluted 1:1000 in PBS/0.15% Tween 20/5% non-fat dry milk) was used for chemiluminescent detection of SLC6A19, SGK, phosphorylated SGK and β -actin with enhanced chemiluminescent ECL kit (Amersham, Freiburg, Germany). β -actin was used as a loading control.

Cell surface biotinylation

For cell surface biotinylation of oocytes, 100 cells of each group were rinsed three times with ice-cold PBS buffer. The oocytes were then incubated for 30 min at 4 °C in 0.25 mg/ml Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA) diluted in ice-cold PBS buffer. After washing 4 times with ice-cold PBS, the cells were dissolved in lysis buffer containing 50 mM Tris (pH 7.5), 0.5 mM EDTA (pH 8.0), 0.5 mM EGTA, 120 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Roche, Penzberg, Germany) for 30 min on ice. The solubilized oocytes were centrifuged for 15 min at 14000 $\times$ g and then the supernatant was spun through an Immobilized NeutrAvidinTM column (Pierce, Rockford, IL, USA). After washing the column three times, the immobilized biotinylated proteins were eluted with SDS-PAGE buffer containing 50 mM DTT. The biotinylated membrane protein was separated by 10% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. After blocking with 5% nonfat dry milk in TBS (pH 7.4)/0.15% Tween 20 for 1 h at room temperature, the blots were incubated with the primary mouse anti-SLC6A19 antibody (Abnova, Taipei, Taiwan) at 4 °C overnight (dilution 1:500 in TBS/0.15% Tween 20/5% non-fat dry milk), a primary mouse anti-integrin β 1 antibody (Upstate, Waltham, MA, USA, diluted 1:1000 in PBS/0.15% Tween 20/5% non-fat dry milk) or peroxidase-conjugated mouse anti- β -actin (diluted 1:10000 in PBS/0.15% Tween 20/5% non-fat dry milk) to detect SLC6A19, integrin β and β -actin, respectively. After washing, the first antibody was detected by secondary horse anti-mouse IgG antibody conjugated with horseradish peroxidase (Cell Signalling Technology, Danvers, MA, USA) for 1 h at room temperature. Antibody binding was detected with the enhanced chemiluminescence ECL kit (Amersham, Freiburg, Germany). The cell membrane marker integrin- β 1 was used to ensure equal protein loading. The intensity of each band relative to the total plasma membrane fraction loaded per lane (integrin- β 1) was quantified by densitometry with the imaging software ImageJ (NIH). To assess that only the plasma membrane fraction had been isolated, blots were re-probed with β -actin antibodies.

Statistics

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

Results

To address the role of the SGK1-3 kinases in the regulation of the neutral amino acid transporter SLC6A19, the carrier was expressed in *Xenopus laevis* oocytes and its activity measured in the presence and absence of the kinases. As apparent from electrophysiology, SGK1 coexpression increased leucine-induced currents (by 30.05 ± 18.28 %, $n = 19$). Coexpression of SGK2 and SGK3 similarly enhanced SLC6A19 activity (by 43.44 ± 14.34 %, $n = 18$ and by 40.17 ± 18.75 %, $n = 18$, respectively, Fig. 1A). The observed effect was mimicked by the constitutively active kinases. In *Xenopus* oocytes expressing ^{S422D}SGK1, ^{S356D}SGK2 or ^{S419D}SGK3, leucine-induced currents mediated by SLC6A19 were elevated by 53.95 ± 6.83 % ($n = 104$), 40.55 ± 8.38 % ($n = 56$) and 50.75 ± 11.74 % ($n = 59$) (Fig. 1B), respectively. Negligible leucine-induced currents were elicited in water injected oocytes or oocytes expressing the constitutively active kinases alone. To investigate whether SLC6A19 activation requires functional SGKs, the carrier was expressed in *Xenopus laevis* oocytes and its activity measured in the presence and absence of the catalytically inactive kinases ^{K127N}SGK1, ^{K64N}SGK2 or ^{K191N}SGK3. None of the kinases was capable to regulate SLC6A19 activity (96.50 ± 3.74 % of control, $n = 16$, ^{K127N}SGK1, 91.64 ± 5.32 % of control, $n = 14$, ^{K64N}SGK2, and 94.90 ± 3.67 % of control, $n = 21$, ^{K191N}SGK3). In order to demonstrate proper expression of cRNAs injected, western blots were performed from whole cell lysates at the same day as oocytes were used for electrophysiological experiments. As depicted in Fig. 1C all proteins investigated were appropriately expressed. As previously alluded to SGK kinases are activated upon phosphorylation by PDK1. To demonstrate functionality of injected kinases, membranes were probed with an antibody recognizing the phosphorylated (active) form of SGK (anti phosphoThr256-SGK). Fig. 1C shows that both wild-type and constitutively active kinases were phosphorylated in the oocyte expression system. In those experiments β -actin was used as loading control.

The SLC6A19 sequence contains a Ser at position 100 which is conserved among several species (Fig. 2A) that might be phosphorylated by the kinases. To test

Fig. 1. SGK isoforms affect transport activity of SLC6A19 in *Xenopus* oocytes. *Xenopus* oocytes were injected with SLC6A19 alone or along with wild-type (A) or constitutively active kinases ^{S422D}SGK1, ^{S356D}SGK2 or ^{S419D}SGK3 (B) or solely with water as control (n.i.) with or without constitutively active kinases. 4-5 days after cRNA injection, leucine (2 mM) induced currents were determined as a measure of SLC6A19 activity. (C) 4-5 days after cRNA injection, SLC6A19 and SGKs expression was monitored by Western Blotting as described in Materials and Methods. β -actin was used as a loading control. Arithmetic means $\pm$ 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. SGKs stimulate SLC6A19 activity independently of the putative SGK phosphorylation site on the transporter by influencing the ubiquitin ligase Nedd4-2. Multiple sequence alignment of human, rabbit, mouse and rat SLC6A19. The putative SGK consensus site is boxed (A). *Xenopus* oocytes were injected with wild-type SLC6A19, ^{S100A}SLC6A19 or ^{S100D}SLC6A19 alone or together with constitutively active ^{S422D}SGK1 (B) or with wild-type SLC6A19 alone or together with the ubiquitin ligase Nedd4-2 (C). The activity of the excitatory amino acid transporter SLC1A6 (EAAT4) was assessed in parallel as described in Material and Methods as a positive control for Nedd4-2 activity (C). To address for reversal of Nedd4-2 effects by the SGK1-3 kinases, *Xenopus* oocytes were injected with wild-type SLC6A19 alone or together with the ubiquitin ligase Nedd4-2 with or without constitutively active kinases (D). Arithmetic means $\pm$ SEM. * indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing wild-type SLC6A19, ^{S100A}SLC6A19 or ^{S100D}SLC6A19 alone. # indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing wild-type SLC6A19 together with the ubiquitin ligase Nedd4-2. Substrate-induced currents were normalized in each batch of oocytes to the mean value obtained in oocytes expressing wild-type transporter (SLC6A19 or EAAT4) alone.

whether this residue is essential for SLC6A19 regulation by ^{S422D}SGK1, ¹⁰⁰Ser was mutated into alanine or into aspartate thereby disrupting or mimicking, respectively, the putative phosphorylation of the transporter. Leucine-dependent currents measured in oocytes expressing ^{S100A}SLC6A19 were elevated upon ^{S422D}SGK1 coexpression, however to a lesser extent than in wild-type SLC6A19 (137.79 ± 4.23 % of control, $n = 14$, Fig. 2B). As compared to the leucine-induced current in oocytes expressing wild type SLC6A19 (100 %), the current approached 83.08 ± 2.85 % ($n = 39$) in ^{S100A}SLC6A19 expressing oocytes. The phosphorylation mimicking mutant, ^{S100D}SLC6A19, presented similar activity in the absence of coexpressed kinases (98.40 ± 3.95 % of control ($n = 15$)) and a similar stimulation (141.85 ± 5.62 % of control, $n = 13$), by SGK1 coexpression as wild-type SLC6A19. Accordingly, phosphorylation at this serine is not required for the stimulation of SLC6A19 by SGK1.

Previous reports demonstrated that SGK1 regulates various amino acid transporters by influencing Nedd4-2 [16, 17, 20, 22], an ubiquitin ligase that mediates the clearance of membrane proteins from the cell surface [16, 24, 25]. To determine whether Nedd4-2 is involved in SLC6A19 modulation, SLC6A19 transporter activity was evaluated in oocytes expressing SLC6A19 alone or together with the ubiquitin ligase. In parallel the activity of the excitatory amino acid transporter SLC1A6 (EAAT4) was assessed in the presence and absence of the ubiquitin

ligase Nedd4-2 and used as a positive control for Nedd4-2 activity [20]. As a result the leucine-induced currents significantly decreased upon coexpression of the ubiquitin ligase (76.39 ± 7.41 % of control, $n = 79$, figure 2C). SGKs negatively regulate Nedd4-2 by phosphorylating the ubiquitin ligase [24]. To assess whether the kinases might reverse SLC6A19 inhibition by the ubiquitin ligase, Nedd4-2 effects on SLC6A19 activity were determined when expressed alone or upon SGK1-3 coexpression. As depicted in figure 2D, SGK1-3 coexpression reversed the downregulation of SLC5A19 by Nedd4-2 (105.38 ± 7.89 % of control, $n = 13$, ^{S422D}SGK1, 110.01 ± 4.80 % of control, $n = 13$, ^{S356D}SGK2, and 85.38 ± 3.12 % of control, $n = 13$, ^{S419D}SGK3).

SGK kinases modulate the kinetic properties and/or abundance of several transport proteins in the plasma membrane (rev. in [14]). In order to clarify whether coexpression of SGK1 or SGK3 activate SLC6A19 by varying the kinetic properties of the carrier, SLC6A19 substrate affinities were determined in the absence and presence of the kinases. Kinetic analysis of leucine-induced currents by SLC6A19 revealed that ^{S422D}SGK1 and ^{S419D}SGK3 significantly increased the maximal substrate induced current $I_{\text{leu}} (V_{\text{max}})$ without significantly modifying the concentration required for halfmaximal $I_{\text{leu}} (K_m)$ (Fig. 3A, B, Table 1). The estimated concentrations required for halfmaximal current (K_m) were 1.11 ± 0.31 mM, $n=13$ (expression of SLC6A19 alone), 1.18 ± 0.23 mM, $n=12$ (SLC6A19 + ^{S422D}SGK1) and

Fig. 3. S^{422D} SGK1 and S^{419D} SGK3 increase SLC6A19 maximal transport rate without modifying the transporters substrate affinity. *Xenopus* oocytes were injected with SLC6A19 alone or together with constitutively active S^{422D} SGK1 or S^{419D} SGK3. Substrate-induced currents were normalized in each group of oocytes to the mean current value evoked with 10 mM leucine in oocytes expressing wild-type SLC6A19 alone (A) or to the current value evoked with 10 mM leucine in the same oocyte (B). 4-5 days after cRNA injection, SLC6A19 activity was measured at the indicated substrate concentrations. Data were normalized to the maximum value of the respective group and were fitted with the modified Hill equation. Arithmetic means $\pm$ SEM.

1.17 ± 0.21 mM, $n=12$ (SLC6A19 + S^{419D} SGK3), respectively.

In a further series of experiments a quantitative immunoassay was performed to elucidate, whether the kinases modify SLC6A19 expression at the cell surface. SLC6A19 protein abundance was determined by cell surface biotinylation and by western blotting of the biotinylated plasma membranes from oocytes expressing the transporter alone or together with constitutively active S^{422D} SGK1, S^{419D} SGK3 or inactive kinases K^{127N} SGK1 or K^{191N} SGK3 (Fig. 4A). As evaluated by densitometry, SLC6A19 surface abundance was higher in oocytes coexpressing S^{422D} SGK1 (129.01 ± 3.46 % of control, $n = 3$) and S^{419D} SGK3 (176.01 ± 18.33 % of control, $n = 3$) but not in oocytes coexpressing the inactive kinases K^{127N} SGK1 (108.33 ± 23.48 % of control, $n = 3$) and S^{191N} SGK3 (78.33 ± 3.48 % of control, $n = 3$, Fig. 4B).

Enhanced SLC6A19 plasma membrane expression might result from increased carrier insertion into the

Fig. 4. S^{422D} SGK1 and S^{419D} SGK3 but not kinase inactive mutants K^{127N} SGK1 and K^{191N} SGK3 augment SLC6A19 protein abundance at the plasma membrane. *Xenopus* oocytes were injected with SLC6A19 alone or together with S^{422D} SGK1, S^{419D} SGK3, K^{127N} SGK1 or K^{191N} SGK3. 4-5 days after cRNA injection, SLC6A19 cell surface expression was determined by biotinylation and by immuno-blotting as described in Materials and Methods. Original western blot (A) and quantification by densitometry (B). Arithmetic means $\pm$ SEM. * indicates statistically significant difference to *Xenopus* oocytes expressing the carrier alone. The cell membrane marker integrin- β 1 was used to ensure equal protein loading. The intensity of each band relative to the total plasma membrane fraction loaded per lane (integrin- β 1) was quantified by densitometry with the imaging software ImageJ (NIH). To assess that only the plasma membrane fraction had been isolated, blots were re-probed with β -actin antibodies. Whole cell lysates from oocytes expressing SLC6A19 were used as a positive control for β -actin immunodetection.

cRNA injected	Km (mM)	n
SLC6A19	1.11 ± 0.31	13
SLC6A19 + SGK1(S^{422D})	1.18 ± 0.23	12
SLC6A19 + SGK3(S^{419D})	1.17 ± 0.21	12

Table 1. Coexpression of S^{422D} SGK1 and S^{419D} SGK3 does not modify the transporters substrate affinity. Kinetic properties of leucine-induced currents. Km was calculated by the modified Hill equation on the basis of functional data depicted in figures 3a and b.

Fig. 6. SGK isoforms further activate SLC6A19 when coexpressed with the accessory protein ACE2 but not collectrin. *Xenopus* oocytes were injected with ACE2 alone, SLC6A19 alone or along with ACE2 with or without constitutively active kinases S^{422D}SGK1, S^{356D}SGK2 or S^{419D}SGK3 (A) and with the same batch of oocytes with collectrin alone, SLC6A19 alone or along with collectrin with or without constitutively active kinases S^{422D}SGK1, S^{356D}SGK2 or S^{419D}SGK3 (B) or solely with water as control (n.i.). 4-5 days after cRNA injection, leucine (2 mM) induced currents were determined as a measure of SLC6A19 activity. Arithmetic means $\pm$ SEM. * indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing the transporter alone. # indicates statistically significant difference to substrate-induced currents in *Xenopus* oocytes expressing the transporter together with the accessory protein ACE2. Substrate-induced currents were normalized in each batch of oocytes to the mean value obtained in oocytes expressing the transporter alone.

plasma membrane or reduced internalization. To discriminate between those two possibilities, SLC6A19 activity and cell surface expression were determined in oocytes expressing the transporter with or without S^{422D}SGK1 and incubated during different time points with Brefeldin A, which inhibits cellular secretory mechanisms and thus

insertion of the carrier protein into the cell membrane. When retrieval of SLC6A19 from the cell surface is delayed by SGKs, the rate of inhibition of the transport-linked current by Brefeldin A should be lower than in control oocytes, implying that the pool of functional SLC6A19 transporters in the plasma membrane is stabilized by SGKs. Brefeldin A (5 μ M) exposure of oocytes expressing SLC6A19 led to a decrease in transport activity and surface abundance that was significantly faster in oocytes expressing SLC6A19 alone as compared to oocytes expressing the carrier together with ^{S422D}SGK1, suggesting that the kinase stabilizes the transporter at the cell surface (Fig. 5A-D).

SLC6A19 function in small intestine and in kidney proximal tubule depends on ACE2 and collectrin, respectively [26, 27]. To address whether the kinases control SLC6A19 activity when coexpressed with those accessory proteins, leucine-induced currents were evaluated in oocytes coexpressing SLC6A19/ACE2 or SLC6A19/collectrin with or without SGK1-3 kinases. Coexpression of ACE2 or collectrin drastically increased SLC6A19 activity by ~38-fold (n = 11, Fig. 6A) and ~20-fold (n = 21, Fig. 6B) respectively. SGK1-3 significantly stimulated leucine-induced currents in SLC6A19 and ACE2-expressing oocytes (113.67 ± 4.82 %, n = 9, ^{S422D}SGK1, 118.11 ± 7.61 %, n = 9, ^{S356P}SGK2, and 112.50 ± 4.53 %, n = 10, ^{S419D}SGK3, as compared to expression of SLC6A19 and ACE2 alone, Fig. 6A). No significant activation was observed in SLC6A19 and collectrin-expressing oocytes (Fig. 6B).

Discussion

At present very little is known about the regulation of SLC6A19 activity and expression. Although several glycosylation and phosphorylation sites have been predicted within the SLC6A19 sequence, it is hitherto unknown whether these sites play a role for carrier modulation. SLC6A19 bears a putative SGK recognition site, the transporter might thus be targeted by the SGK kinases (SGK1-3). In this study we provide evidence that SGK1-3 indeed activate SLC6A19 function. The stimulatory effect by the kinases does not require a serine residue at position 100 within the putative SGK phosphorylation site on SLC6A19, since a serine-to-alanine or serine-to-aspartate mutation at this site did not significantly abolish transporter regulation by the kinases.

Previous reports demonstrated that SGK1 regulates amino acid transport by phosphorylating the carrier di-

Fig. 7. Multiple sequence alignment of SLC6A19 amino acid sequence. The putative SGK consensus site is boxed. Note that the putative SGK consensus site is not conserved among species.

rectly and/or by phosphorylating Nedd4-2 thus reducing the affinity of the ubiquitin ligase to its target proteins. As coexpression of Nedd4-2 did significantly affect SLC6A19 transporter activity, SGK isoforms might activate SLC6A19 indirectly via inhibition of the ubiquitin ligase. Our data on the reversal of Nedd4-2 effects by the kinases supports this assumption.

SGK1 is expressed in the kidney [34]. The kinase is found in the aldosterone-sensitive distal nephron (ASDN) and at moderate levels in the glomerulus and in the proximal tubule [14]. In the small intestine, SGK1 has been localized in villus enterocytes. The common expression of SGK1/3 and SLC6A19 suggests a possible regulation of neutral amino acid transport by the protein kinases *in vivo*. As a matter of fact, intestinal amino acid transport (75 %) and SLC6A19 abundance (60%) in brush border membrane proteins prepared from kidney were both decreased in PDK1 hypomorphic mice (PDK1^{hm}) that express 10 - 25 % of PDK1 [35]. Since PDK1 is a kinase upstream of SGK1-3, the impaired renal and intestinal amino acid transport in PDK1^{hm} mice might be attributed in part to impaired activation of SGK1-3 as suggested by the data presented in this study.

In vivo ACE2 and collectrin are essential for proper SLC6A19 expression in small intestine and in kidney proximal tubule, respectively [26, 27]. Co-immunoprecipitation experiments in *Xenopus* oocytes and in isolated brush border membrane proteins from small intestine and kidneys demonstrated that ACE2 and collectrin physically associate with SLC6A19 [26, 28]. Furthermore, SLC6A19 is absent in small intestine from *ace2* null mice whilst the transporter is normally expressed in kidney. Inversely, SLC6A19 is not expressed in kidney from *collectrin* null mice whereas it is present in small intestine [26]. Interestingly, while SGK1-3 activate SLC6A19 when coexpressed with ACE2, the kinases do not further increase leucine-induced currents in SLC6A19/collectrin co-expressing oocytes. The presence of a pu-

tative SGK phosphorylation site on the accessory proteins does not seem to play a role in SLC6A19 regulation by the kinases, since ACE2 does not bear any SGK consensus site on its sequence and a putative phosphorylation site on collectrin is not conserved among species (Fig. 7). Further studies are necessary to clarify the selective activation of SLC6A19 by SGK1-3 when co-expressed with the accessory protein ACE2. Recently, ACE2 and collectrin were reported to differentially interact with Hartnup mutations [26]. The mechanism by which several SLC6A19 mutants specifically associate with the accessory proteins and thus modulate the transporter activity is yet unknown. Nonetheless, these accessory proteins may allow selective regulation of amino acid transport in kidney and intestine. Camargo et al. [26] further suggested that the differential functional interaction of mutant SLC6A19 transporters with ACE2 and collectrin in small intestine and in kidney contributes to the phenotypic heterogeneity of Hartnup disorder.

Cellular amino acid uptake is regulated by insulin and insulin like growth factor (IGF), which stimulate amino acid transport through a signalling cascade involving PI3 kinase. As SGK1 and SGK3 are activated by both stimuli, the kinases might be involved in PI3 kinase signalling enhancing amino acid uptake [35].

SGK1 modulates transporters at least in part by enhancing their protein expression at the cell surface (rev. [14]). Our data indicate that SGK1 and SGK3 similarly increase SLC6A19 by augmenting the transporters maximal velocity and surface expression. Several reports suggest that SGK1 enhances abundance of the target membrane proteins by delaying protein internalization from the

cell surface [36, 37]. The observations in the presence of brefeldin A indeed suggest that SGK1 upregulates SLC6A19 by stabilizing the transport protein within the plasma membrane.

At least in theory, defective regulation of the transporter could lead to amino aciduria. Loss-of-function mutations in *slc6a19* [8, 9] lead to Hartnup disorder, resulting from reduced neutral amino acid resorption in the kidney and intestine [2, 38].

Collectively, this study identified the SGK1-3 kinases as novel modulators of the neutral amino acid transporter SLC6A19. The kinases activate SLC6A19 function and expression by stabilizing the transporter in the plasma membrane without modifying carrier substrate affinities. The observed stimulatory effect does not require the residue ¹⁰⁰Ser within the SGK consensus site on SLC6A19, since disruption of this site did not prevent transporter activation by the kinases. The kinases rather regulate SLC6A19 by impacting the ubiquitin ligase Nedd4-2. Furthermore, the kinases control SLC6A19 activity when coexpressed with the transporter *in vivo* accessory protein ACE2. Our data suggest that SGK1 and SGK3 might participate in the regulation of SLC6A19 by controlling the carrier surface abundance.

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