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The serine/threonine kinases SGK1, 3 and PKB stimulate the amino acid transporter ASCT2

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Abstract

The human Na^+-dependent neutral amino acid transporter type 2 (hASCT2/SLC1A5) plays an important role in the transport of neutral amino acids in epithelial cells. The serine and threonine kinases SGK1–3 and protein kinase B have been implicated in the regulation of several members of the SLC1 transporter family by enhancing their plasma membrane abundance. The present study explored whether those kinases modulate hASCT2. In *Xenopus* oocytes heterologously expressing hASCT2, coexpression of constitutively active ^{S422D}SGK1, ^{S419D}SGK3 or ^{T308DS473D}PKB upregulated the transporter activity. The stimulation requires the catalytical activity of the kinases since the inactive mutants ^{K127N}SGK1, ^{K191N}SGK3, and ^{T308AS473A}PKB failed to modulate the transporter. According to kinetic analysis and chemiluminescence assays, SGK1 and SGK3 modulate hASCT2 by enhancing the transporter abundance in the plasma membrane. As SGK1, 3 and PKB are activated by insulin and IGF1, the described mechanisms presumably participate in the hormonal stimulation of cellular amino acid uptake.

Keywords: Amino acid transport; Insulin; Insulin like growth factor; Kinases; Trafficking

The human Na^+-dependent neutral amino acid transporter type 2 (hASCT2, gene name SLC1A5 GenBank Accession No. NM_005628, TC# 2.A.23.3.3) is a member of the glutamate transporter superfamily which also contains the closely related (54% identity) transporter hASCT1. Both carry an overlapping but distinct set of neutral amino acids. In addition to the typical system ASC substrates (L-alanine, L-serine, L-cysteine, and L-threonine), hASCT2 also transports L-glutamine and L-asparagine at high affinity as well as some other neutral amino acids with lower affinity [1,2]. Similar to hASCT1, hASCT2 mediates Na^+-dependent obligatory exchange of substrate amino acids [1]. hASCT2 is expressed in the placenta, lung, skeletal muscle, kidney, intestine, and pancreas [3]. In kidney and intestine, the transporter is expressed in the brush-border membrane of proximal tubule cells and enterocytes, respectively [4], and contributes to transepithelial amino acid transport. Beyond that the carrier provides cells with the amino acids for metabolism.

Mechanisms regulating hASCT2 activity and expression are ill defined. During the last two years, it has been shown that ASCT2 promoter activity and ASCT2 protein expression are enhanced in the presence of glutamine [5]. Glutamine transport through ASCT2 is activated by epidermal growth factor (EGF) via a signaling pathway involving protein kinase C and mitogen-activated protein kinase MEK [6]. Cellular amino acid uptake is further regulated by insulin and insulin like growth factor (IGF), which stimulate amino acid transport through a signaling cascade involving PI3 kinase [7,8]. The downstream signaling elements of PI3 kinase include the protein kinase B (PKB) [9–11], the serum and glucocorticoid inducible kinase (SGK) 1 [12], and its isoforms SGK2 and SGK3 [13].

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SGK1–3 and PKB have been implicated in the regulation of several ion channels and transporters [14–21]. The activation of SGK1–3 requires phosphorylation at Ser422, Ser356, and Ser419, respectively, and the activation of PKB, phosphorylation at Thr308 and Ser473. Replacement of those amino acids by aspartate leads to constitutive activation of the respective kinases [13]. Destruction of the catalytic subunit by replacement of Lys127, Lys64, and Lys191 with asparagine leads to the inactive SGK1–3 mutants [13,22]. Transporters regulated by these kinases include members of the SLC1 glutamate transporter superfamily, SLC1A1 and SLC1A3 [23,24]. Thus, the present study explored whether the kinases modulate hASCT2. The present paper shows that SGK1, SGK3, and PKB upregulate hASCT2, an effect requiring their catalytical activity. The kinases enhance the maximal transport rate at least in part by increasing the abundance of the carrier in the plasma membrane.

Materials and methods

Site-directed mutagenesis. The human constitutively active (^{S356D}SGK2, ^{S419D}SGK3) and inactive (^{K191N}SGK3) kinases 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: ^{S356D}SGK2 s: 5′gcattcctgggatttgattatgcgccagagg3′; ^{S356D}SGK2 as: 5′cctctggcgcataatcaaatcccaggaatgc3′; ^{S419D}SGK3 s: 5′gatgcattcgttggtttcgattatgcacctccttcag3′; ^{S419D}SGK3 as: 5′ctgaaggaggtgcataatcgaaaccaacgaatgcatc3′; and ^{K191N}SGK3 s: 5′atgctgtcaatgtgttacag3′; ^{K191N}SGK3 as: 5′ctgtaacacattgacagcat3′. The human hemagglutinin (HA)-tagged ASCT2 was generated by two-stage PCR site-directed mutagenesis using the primers ASCT2-HA s: 5′ggccgtggtggcctacgacgtaccagattacgctggcgtggcgctgg3′ and ASCT2-HA as: 5′ccagcgccacgccagcgtaatctggtacgtcgtaggccaccacggcc3′. All mutants were sequenced to verify the presence of the desired mutation.

Oocyte expression. cRNA encoding human ASCT2, human constitutively active ^{S422D}SGK1, inactive ^{K127N}SGK1 [22], constitutively active ^{S356D}SGK2, ^{S419D}SGK3 and inactive ^{K191N}SGK3, human constitutively active ^{T308DS473D}PKB [25], and inactive ^{T308AS473A}PKB [26] were synthesized in vitro as previously described [27]. Dissection of *Xenopus laevis* ovaries, collection, and handling of the oocytes have been described in detail elsewhere [27]. Oocytes were injected with 7.5 ng hASCT2, 7.5 ng ^{S422D}SGK1, ^{S356D}SGK2, ^{S419D}SGK3, ^{T308DS473D}PKB or ^{K127N}SGK1, ^{K64N}SGK2, ^{K191N}SGK3, and ^{T308AS473A}PKB. Control oocytes were injected with H_2O.

Tracer flux measurements. ^{14}C-labeled L-serine was used for uptake determination. The transport assay was performed 3 days after cRNA injection and contained 5–10 single oocytes in 0.5 ml ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.4) containing 0.5 μCi L-[^{14}C]serine and 100 μM (or the indicated amount in the kinetic analysis) of cold L-serine. For the uptake measurements in the absence of Na^+, NaCl was replaced by choline–Cl. After incubation for 30 min with L-serine (linear range of uptake), uptake was terminated by washing the oocytes four times with 3 ml ice-cold ND96. Oocytes were individually transferred into scintillation vials and dissolved by adding 200 μl of 10% SDS before the radioactivity was determined. Uptake rates were expressed as picomoles of accumulated L-serine per oocyte per minute.

Cell surface expression assay. Defolliculated oocytes were first injected with human constitutively active ^{S422D}SGK1 or ^{S419D}SGK3 cRNA (7.5 ng/oocyte), and one day later with human hemagglutinin (HA)-tagged ASCT2 (7.5 ng/oocyte). Oocytes were incubated with 1 μg/ml primary rat monoclonal anti-HA antibody (clone 3F10, Boehringer, Germany), and 2 μg/ml secondary, peroxidase-conjugated affinity-purified F(ab′)2 goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, USA). Individual oocytes were placed in 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer by integrating the signal over a period of 1 s.

Statistical analysis. Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least two batches of oocytes; in all repetitions qualitatively similar data were obtained. All data were tested for significance using ANOVA, and only results with $P < 0.05$ were considered as statistically significant.

Results

Modulation of L-[^{14}C]serine uptake by the serine and threonine kinases SGK1–3 and PKB

In order to investigate the role of the serine and threonine kinases SGK1–3 and PKB in the regulation of hASCT2 activity, hASCT2 was expressed in *X. laevis* oocytes and L-[^{14}C]serine uptake was measured. As shown in Fig. 1A, *Xenopus* oocytes injected with hASCT2 cRNA exhibited L-[^{14}C]serine uptake ~4-fold (5.48 ± 0.33 pmol/min/oocyte, $n = 26$) above water-injected control oocytes (1.34 ± 0.20 pmol/min/oocyte, $n = 25$). The uptake was significantly decreased in Na^+-free medium (2.36 ± 0.38 pmol/min/oocyte, $n = 26$) corroborating previous observations that hASCT2-mediated transport is driven by a Na^+ gradient [2]. L-[^{14}C]Serine uptake was concentration dependent and saturable (Fig. 1B). Coexpression of constitutively active ^{S356D}SGK2 failed to stimulate L-[^{14}C]serine uptake (Fig. 2). However, coexpression of the constitutively active protein kinases ^{S422D}SGK1 and ^{S419D}SGK3 stimulated L-[^{14}C]serine uptake up to 145.9 ± 9.0% of control, $n = 40$ and 155.8 ± 7.2% of control, $n = 50$, respectively. The related constitutively active ^{T308DS473D}PKB, which shares ~60% homology in the catalytic domain with SGKs and recognizes the same consensus sequence (Arg-X-Arg-X-X-Ser/Thr), also enhanced hASCT2 transport (137.8 ± 4.8% of control, $n = 46$). No significant effect on L-[^{14}C]serine uptake was observed when the kinases were expressed in water injected control oocytes ($n = 19$–21).

Effect of the serine and threonine kinase inactive mutants on L-[^{14}C]serine uptake

Regulation of hASCT2 activity depends on phosphorylation. When the catalytically inactive kinases were coexpressed in hASCT2 injected oocytes, no significant modulation of L-[^{14}C]serine uptake was observed (100.4 ± 6.6% of control, $n = 23$ in ^{K127N}SGK1

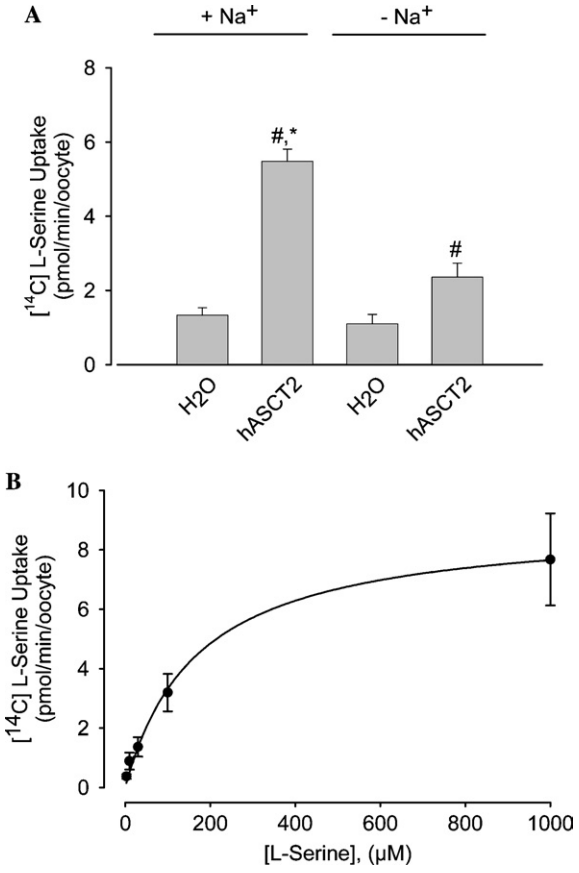

Fig. 1. L-Serine transport via hASCT2 expressed in *X. laevis* oocytes. *X. laevis* oocytes were injected with hASCT2 or with water (control oocytes). Three days after cRNA injection, labeled L-serine uptake was studied as a measure of hASCT2 activity. hASCT2-mediated L-serine uptake was dependent on Na^+ (A) and was saturable (B). Arithmetic means ± SEM. #Indicates statistically significant difference to uptake in *Xenopus* oocytes injected with water, *indicates statistically significant difference to uptake in the absence of Na^+ in *Xenopus* oocytes expressing hASCT2.

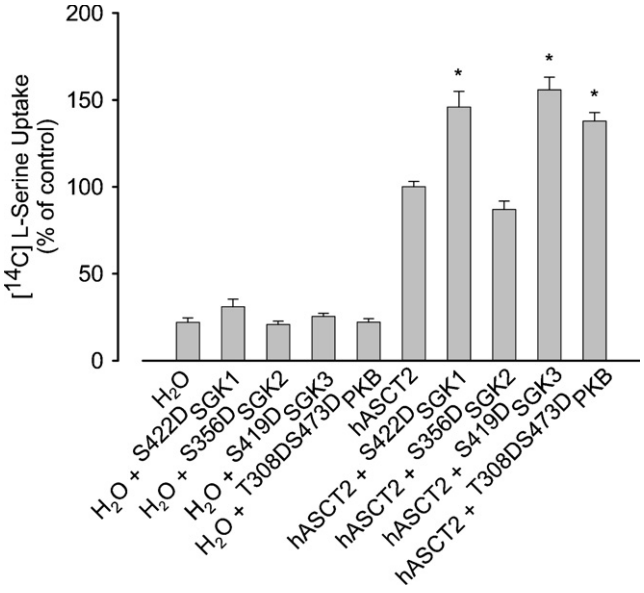

Fig. 2. Stimulation of hASCT2 transport by constitutively active ^{S422D}SGK1, ^{S419D}SGK3, and ^{T308DS473D}PKB but not by ^{S356D}SGK2. *X. laevis* oocytes were injected with H_2O or hASCT2 with or without constitutively active ^{S422D}SGK1, ^{S356D}SGK2, ^{S419D}SGK3 or ^{T308DS473D}PKB. Three days after cRNA injection, labeled L-serine uptake was studied. hASCT2-mediated L-serine uptake was modulated by ^{S422D}SGK1, ^{S419D}SGK3, and ^{T308DS473D}PKB but not by ^{S356D}SGK2. The kinases did not significantly modify L-serine uptake when expressed alone. Arithmetic means ± SEM.*Indicates statistically significant difference to uptake in *Xenopus* oocytes expressing hASCT2 alone.

expressing oocytes, 88.9 ± 10.3% of control, $n = 9$ in ^{K191N}SGK3-expressing oocytes, and 106.1 ± 5.3% of control, $n = 50$ in ^{T308AS473A}PKB-expressing oocytes) indicating that the catalytic function of SGK1, SGK3, and PKB is required for hASCT2 upregulation (Fig. 3).

hASCT2 kinetic properties upon coexpression of constitutively active ^{S422D}SGK1 and ^{S419D}SGK3

To elucidate whether ^{S422D}SGK1 and ^{S419D}SGK3 upregulate hASCT2 by modifying the transporter kinetic properties, hASCT2 substrate affinity was measured in the presence and absence of ^{S422D}SGK1 or ^{S419D}SGK3. The hASCT2-mediated L-serine uptake was measured at 3–1000 μM in the standard uptake solution and plotted against L-serine concentration. As shown in Fig. 4 and Table 1, ^{S422D}SGK1 and

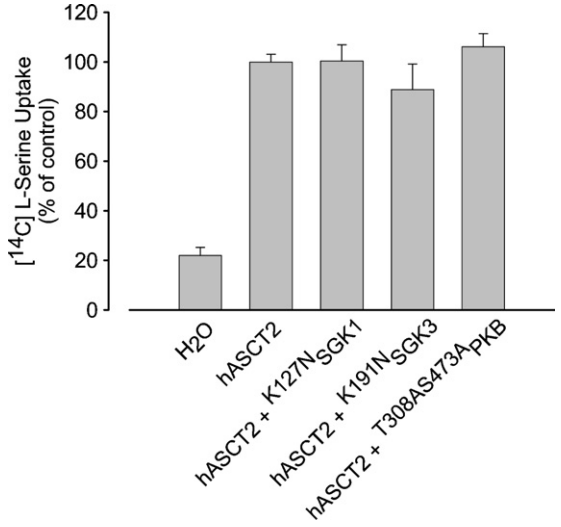

Fig. 3. The inactive kinases ^{K127N}SGK1, ^{K191N}SGK3, and ^{T308AS473A}PKB do not significantly modify hASCT2 activity. *X. laevis* oocytes were injected with hASCT2 with or without inactive ^{K127N}SGK1, ^{K191N}SGK3 or ^{T308AS473A}PKB. Three days after cRNA injection, labeled L-serine uptake was measured. All inactive kinases failed to significantly modify hASCT2 activity. Arithmetic means ± SEM.

^{S419D}SGK3 increased hASCT2 activity by enhancing the maximal transport rate, V_{max}, without affecting hASCT2 substrate affinity.

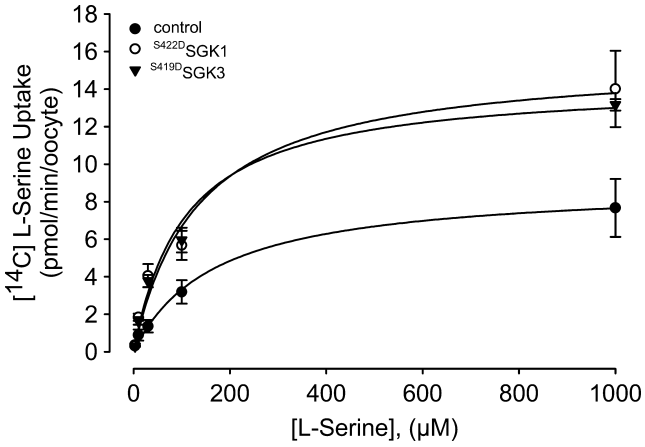

Fig. 4. hASCT2 maximal transport rate is enhanced by ^{S422D}SGK1 and ^{S419D}SGK3. *X. laevis* oocytes were injected with hASCT2 with or without constitutively active ^{S422D}SGK1 or ^{S419D}SGK3. Three days after cRNA injection, L-serine uptake was analyzed at the indicated substrate concentrations. ^{S422D}SGK1 and ^{S419D}SGK3 significantly increased V_{max}. Arithmetic means ± SEM.

Table 1
Kinetic parameters of hASCT2 upon coexpression of ^{S422D}SGK1 or ^{S419D}SGK3

Injected cRNA	K_m (μM)	V_{max} (pmol/min/oocyte)
hASCT2	169.67 ± 70.90	8.95 ± 1.18
hASCT2 + ^{S422D}SGK1	134.25 ± 36.84	15.64 ± 1.44
hASCT2 + ^{S419D}SGK3	107.12 ± 12.96	14.39 ± 0.53

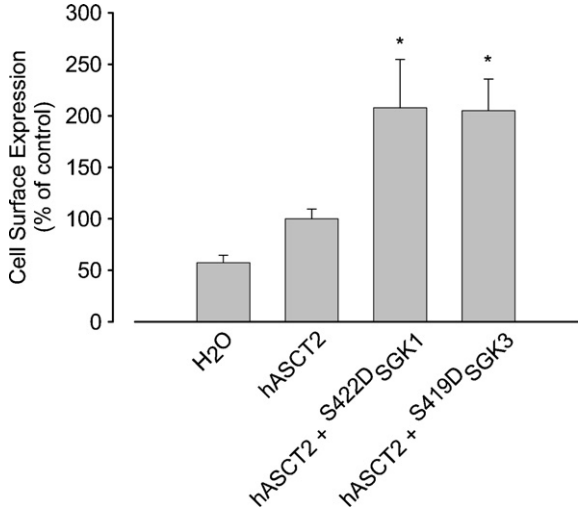

Fig. 5. hASCT2 plasma membrane abundance is enhanced by ^{S422D}SGK1 and ^{S419D}SGK3. Surface expression of hASCT2 was assessed by chemiluminescence in oocytes expressing hASCT2 alone and in oocytes expressing hASCT2 together with ^{S422D}SGK1 or ^{S419D}SGK3. Arithmetic means ± SEM,*indicates statistically significant difference between oocytes injected with hASCT2 alone and those injected with hASCT2 together with ^{S422D}SGK3 or ^{S419D}SGK3.

hASCT2 plasma membrane abundance upon coexpression of constitutively active ^{S422D}SGK1 and ^{S419D}SGK3

To explore whether plasma membrane abundance is modified upon coexpression of ^{S422D}SGK1 and ^{S419D}SGK3, hASCT2 cell surface expression was analyzed by chemiluminescence. As depicted in Fig. 5, hASCT2 surface abundance was approximately 2-fold higher in oocytes coexpressing ^{S422D}SGK1 (207.8 ± 46.8% of control, $n = 44$) and in oocytes coexpressing ^{S419D}SGK3 (205 ± 30.6% of control, $n = 54$) than in oocytes expressing hASCT2 alone ($n = 55$). Thus, the observed increase of hASCT2 activity upon coexpression of ^{S422D}SGK1 and ^{S419D}SGK3 correlates with altered hASCT2 abundance in the cell membrane.

Discussion

Similar to other members of its family, hASCT2/SLC1A5 is stimulated by the serine and threonine kinases SGK1, SGK3, and PKB [23,24], an effect requiring the catalytical activity of the kinases. SGKs and PKB modulate transport through several mechanisms. While SLC1A1 is modulated directly by phosphorylation at the SGK/PKB phosphorylation site at Thr482 and indirectly by phosphorylating and thus inhibiting the ubiquitin ligase Nedd4-2, SLC1A3 is only regulated by the latter mechanism [23,24]. Since the hASCT2 transporter does not bear a SGK/PKB consensus site in its sequence or a proline–tyrosine (PY) motif, a target structure for Nedd4-2, the kinases might be effective by phosphorylating an intermediate molecule that regulates hASCT2. Alternatively, the kinases might phosphorylate hASCT2 at a non-consensus site. Regardless of the mechanism involved, the kinases modulate the members of the SLC1 family by increasing the transporter abundance in the plasma membrane as indicated by kinetic analysis and/or chemiluminescence [23,24].

Regulation of hASCT2 activity by phosphorylation has recently been reported [6]. Epidermal growth factor (EGF) stimulates the transporter through a signaling cascade involving protein kinase C and mitogen-activated protein kinase MEK. As described here for SGK1 and SGK3, EGF activates transport by increasing the maximal transport rate without altering the transporter affinity. In addition, EGF leads to increased transporter transcription and number of functional copies of transporter units [6].

hASCT2 has been reported to be a retrovirus receptor [28]. This finding is reminiscent of the cationic amino acid transporter 1 (CAT1, gene name SLC7A1) which was originally identified as a viral receptor [29,30]. CAT1 is, however, not modulated by the serine and threonine kinases SGK1, 3 and PKB [31], an observa-

tion pointing to the specificity of SGK and PKB. hASCT2 is used by the RD114/type D interference group of viruses to infect host cells. Upon infection the viruses reduce the transport of amino acids specific for hASCT2 which might contribute to the pathogenicity of these viruses [28]. Impaired transport of specific neutral amino acids essential for cellular metabolism might compromise viral replication and cell proliferation. Recently, it has been shown that N-linked glycosylation of ASCT2 determines its retroviral receptor function [32]. An *N*-deglycosylated mutant derivative of ASCT2 is active in mediating infectivity and membrane fusion by the HERV-W envelope [33]. Furthermore, hASCT2 is needed for internal multiplication of the opportunistic pathogen *Legionella pneumophila* [34]. Pharmacological (2-aminobicyclo[2,2,1]heptane-2-carboxylic acid, BCH) and biological (siRNA) inhibition of hASCT2 reduced hASCT2 replication in monocytes [34]. Thus, the regulation of hASCT2 might be relevant for intracellular growth of the pathogen.

In conclusion, the present observation discloses three novel modulators of hASCT2 activity, the kinases SGK1, SGK3, and PKB which are effective by enhancing hASCT2 plasma membrane abundance. The kinases may participate in the regulation of cellular nutrient uptake following stimulation of cells with insulin and growth factors as well as during infection with intracellular pathogens.

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