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Properties and regulation of glutamine transporter SN1 by protein kinases SGK and PKB

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Abstract

The amino acid transporter SN1 with substrate specificity identical to the amino acid transport system N is expressed mainly in astrocytes and hepatocytes where it accomplishes Na^+-coupled glutamine uptake and efflux. To characterize properties and regulation of SN1, substrate-induced currents and/or radioactive glutamine uptake were determined in *Xenopus* oocytes injected with cRNA encoding SN1, the ubiquitin ligase Nedd4-2, and/or the constitutively active serum and glucocorticoid inducible kinase ^{S422D}SGK1, its isoform SGK3, and the constitutively active protein kinase B ^{T308D,S473D}PKB. The substrate-induced currents were enhanced by increasing glutamine and/or Na^+ concentrations, hyperpolarization, and alkalinization (pH 8.0). They were inhibited by acidification (pH 6.0). Coexpression of Nedd4-2 downregulated SN1-mediated transport, an effect reversed by coexpression of ^{S422D}SGK1, SGK3, and ^{T308D,S473D}PKB. It is concluded that SN1 is a target for the ubiquitin ligase Nedd4-2, which is inactivated by the serum and glucocorticoid inducible kinase SGK1, its isoform SGK3, and protein kinase B.

Keywords: Glutamine transport; Liver; Amino acid transport; System N; pH; Na^+; Insulin

In a wide variety of cells uptake of glutamine is accomplished by system N, a Na^+-coupled amino acid transport system [1]. Recently, SN1 has been cloned and identified as a molecular candidate for system N [2–4]. SN1 belongs to the superfamily of amino acid/auxin permeases comprising plant H^+-amino acid cotransporters, yeast vacuolar amino acid transporters, and a variety of mammalian vesicular and plasma membrane amino acid transporters [5]. Accordingly, it has received the Transporter Classification (TC)-number 2.A.18.6.3 (http://tcdb.ucsd.edu/tcdb/background.php). The human SN1 has also been classified as SLC38A3 in the HUGO nomenclature (http://www.gene.ucl.ac.uk/nomenclature/). Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. The transporter is mainly expressed in brain astrocytes and hepatocytes [6]. It is assumed that the transporter plays a prominent role in the uptake and release of glutamine into and out of these cell types. Initially, conflicting results have been reported on the electrogenicity of SN1-mediated glutamine transport. SN1 was considered to mediate an electroneutral transport of glutamine coupled to the electroneutral exchange of Na^+ and H^+ [4]. Subsequent studies, however, disclosed substrate-induced currents when SN1 was expressed in oocytes, which was taken as an indication for an electrogenic transport mechanism. [2]. The issue was resolved by demonstrating that SN1 generates substrate-induced currents, which are not coupled to substrate translocation [4,7]. However, it has not been clarified entirely whether these currents are oocyte endogenous currents, elicited by changes of the intracellular pH, or whether they reflect an inherent property of the transporter.

Glutamine transport is subjected to regulation by cell volume [8], glutamine depletion [9], insulin [10–13], insulin-like growth factor [14], and glucocorticoids [15]. However, hitherto nothing is known about the cellular mechanisms regulating the abundance and activity of SN1.

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Among the known regulators of membrane channel proteins is Nedd4-2, a ubiquitin ligase expressed in a wide variety of tissues including liver [16]. Previously, Nedd4-2 had been shown to regulate the voltage gated Na^+ channel in skeletal muscle [17] and the renal epithelial Na^+ channel ENaC [18,19]. The downregulation of ENaC by Nedd4-2 could be partially reversed by the serum and glucocorticoid dependent kinase 1 (SGK1). SGK1 was originally cloned from rat mammary tumor cells [20]. The human SGK1 has been cloned from hepatocytes as a cell volume regulated gene [21,22]. SGK1 is expressed in all human tissues studied, including pancreas, liver, heart, lung, skeletal muscle, placenta, kidney, and brain [22]. SGK1 is related to protein kinase B (PKB), which plays an important role in the trafficking of the insulin-regulated glucose transporter GLUT4 and other insulin-regulated signal transduction pathways [23].

To become active, the wild-type SGK1 requires phosphorylation at position 422, the activation of PKB phosphorylation at Thr308 and Ser473. Replacement of those amino acids by aspartate leads to the respective constitutively active kinases ^{S422D}SGK1 [24] and ^{T308D,S473D}PKB [25]. In vivo phosphorylation and activation of SGK1 was accomplished by insulin and insulin-like growth factor IGF1.

The first objective for the present experiments was to characterize the substrate-induced currents in SN1 expressing *Xenopus* oocytes. Our results suggest that these currents are not endogenous to the oocyte, but are an inherent property of the transporter. A second objective of this study was to elucidate the influence of the ubiquitin ligase Nedd4-2 and of SGK1 and 3 and protein kinase B on the glutamine transporter SN1. To this end, cRNA encoding SN1 has been injected with or without cRNA encoding Nedd4-2 and/or SGK1, 3, and PKB into *Xenopus* oocytes.

Materials and methods

Xenopus laevis expression vectors. cRNA encoding SN1 [7], Nedd4-2 [18], constitutively active ^{S422D}SGK1 [24], human SGK3 [24], and constitutively active human ^{T308D,S473D}PKB [25] have been synthesized as described earlier [26].

Electrophysiology. Dissection of *X. laevis* ovaries, collection, and handling of the oocytes have been described in detail elsewhere [26]. Oocytes were injected with 7.5 ng cRNA of the respective kinases and/or Nedd4-2 or H_2O on the first day after preparation of the oocytes and subsequently with 10 ng SN1 cRNA. All experiments were performed at room temperature 4 days after injection. Standard 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. All substances were added to the solutions 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.

Uptake measurements. After washing twice with 4 ml ND96 buffer at pH 7.4, groups of 7–10 cRNA- or non-injected oocytes were incubated for 10 min at room temperature in a 5 ml polypropylene tube containing 100 µl of the uptake solution (ND96 added with 5 kBq ^{14}C-labelled plus 10 mM unlabelled glutamine). Transport was stopped by washing oocytes three times with 4 ml ice-cold ND96 buffer. Single oocytes were then placed in scintillation vials and solubilized in 200 µl of 10% sodium dodecyl sulphate (SDS). After addition of 3 ml scintillation fluid, their radioactivity was measured in a liquid scintillation counter (Wallac, Perkin–Elmer, Wellesley, USA).

Statistical analysis. Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least three batches of oocytes; in all repetitions qualitatively similar data were obtained. All data were tested for significance using Student's t test and only results with $P < 0.05$ were considered as statistically significant.

Results

Characterization of substrate-induced currents

Exposure of *Xenopus* oocytes expressing the rat glutamine transporter SN1 to 10 mM glutamine led to an inward current approaching -22.40 ± 2.35 nA ($n = 34$) at a holding potential of −60 mV. In water-injected oocytes glutamine did not induce a similar current (-0.67 ± 0.11 nA, $n = 6$).

As illustrated in Figs. 1A and B and Table 1, the current was highly dependent on the extracellular H^+ concentration. SN1 activity was almost abolished at pH 6 and markedly stimulated by extracellular alkalinization. The modulation of the current as a function of extracellular pH was mainly due to an alteration of the maximal current and not caused by a change of the glutamine concentration required to induce half-maximal currents ($K_{0.5}$). The drop of the maximal current at pH 6.0 was likely to result from an inability to reach I_{max} at experimentally feasible glutamine and Na^+ concentrations. This in turn was a result of the dramatic increase of the $K_{0.5}$ at pH 6.0 (Table 1).

A decrease of the holding potential enhanced glutamine-induced currents mainly by increasing the apparent maximal current without affecting the substrate affinity (Fig. 1C, Table 2). The glutamine-induced current is also sensitive to alterations of extracellular Na^+ concentration. Again, the maximal current increases as a function of extracellular Na^+ concentration (Fig. 1D, Table 3).

It has recently been proposed that substrate-induced currents in SN1 expressing oocytes are largely carried by protons [27]. In agreement with our earlier experiments [28], we found that substrate-induced currents reversed at a holding potential of −6 mV (Fig. 2A), thereby confirming that these currents are not tightly coupled to substrate transport. However, the reversal potential remained unchanged regardless of the extracellular pH.

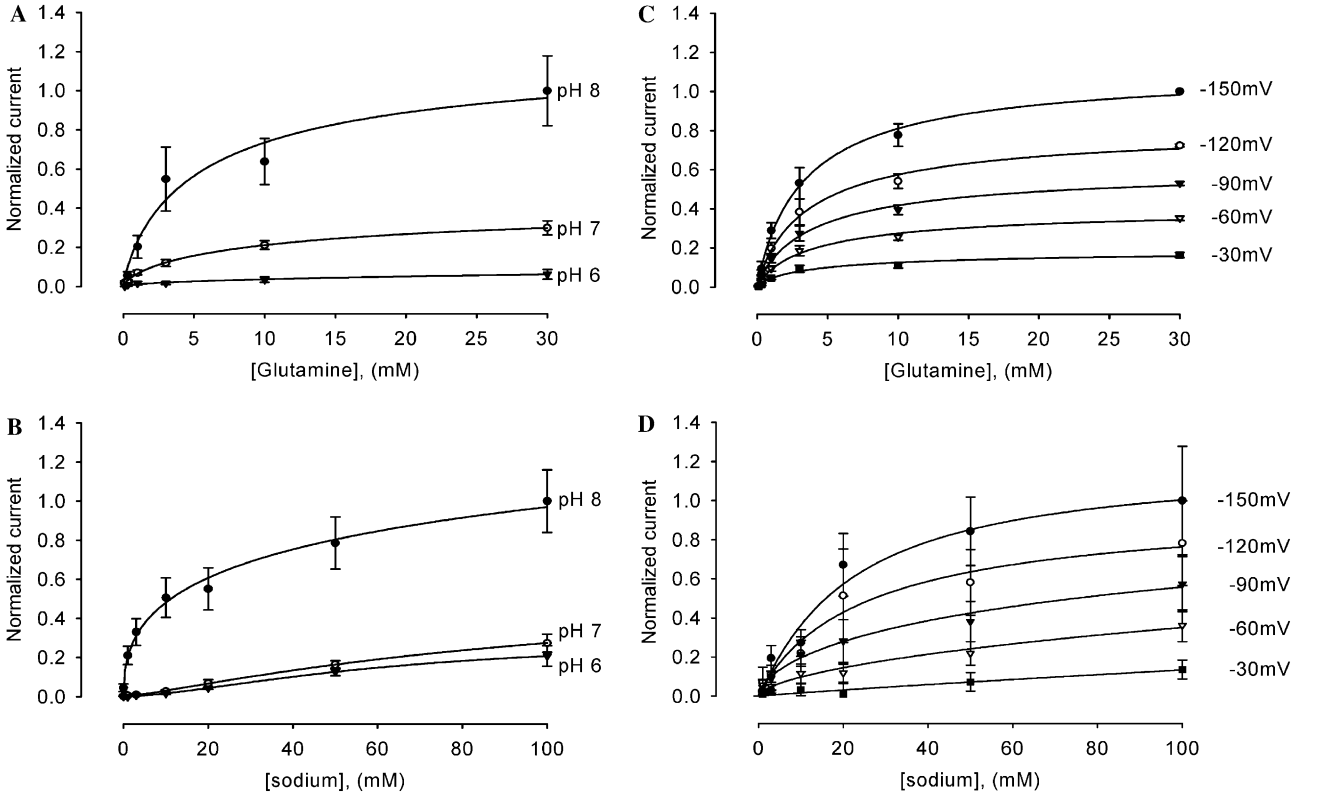

Fig. 1. Substrate and cosubstrate kinetics of SN1 currents in *Xenopus* oocytes. (A) Concentration dependence of glutamine-induced currents at different pH values. The membrane potential was clamped to −60 mV and oocytes were superfused with 100 mM sodium in the extracellular bath. (B) Dependence of glutamine-induced currents on the Na^+ concentration at three different pH values. (C) Concentration dependence of glutamine-induced transport at different holding potentials. (D) Dependence of glutamine-induced currents on the Na^+ concentration at different holding potentials. Arithmetic means ± SEM ($n = 8$–24).

Table 1
Kinetic parameters of SN1-induced current as a function of extracellular pH and glutamine sodium concentration

Extracellular pH	I_{max} (Gln, normalized)	$K_{0.5}$ (Gln)	I_{max} (Na^+, normalized)	$K_{0.5}$ (Na^+)
8	1.18 ± 0.20	6.03 ± 1.01	1.52 ± 0.25	58.29 ± 20.28
7	0.85 ± 0.48	5.63 ± 0.77	0.69 ± 1.31	86.06 ± 29.44
6	0.07 ± 0.02	4.21 ± 2.25	0.52 ± 0.26	126.67 ± 61.57

Oocytes were injected with 10 ng SN1 mRNA and incubated for 4 days prior to the experiment. $K_{0.5}$ and I_{max} were calculated from the modified Hill equation. While the maximal current increases upon alkalinization there is no significant difference in the substrate concentration required for half-maximal current (K_m).

Table 2
Kinetic parameters of SN1-induced current as a function of membrane voltage and glutamine concentration

Membrane voltage (mV)	$K_{0.5}$ (Gln)	I_{max} (normalized)
−150	5.96 ± 3.90	1.15 ± 0.14
−120	7.05 ± 2.88	0.86 ± 0.08
−90	6.10 ± 2.22	0.62 ± 0.05
−60	6.28 ± 2.05	0.43 ± 0.03
−30	8.03 ± 2.46	0.19 ± 0.02

Oocytes were injected with 10 ng SN1 mRNA and incubated for 4 days prior to the experiment. Oocytes were superfused with 100 mM NaCI, pH 8, and glutamine was applied at concentrations ranging from 0.1 to 30 mM. $K_{0.5}$ and I_{max} were calculated from the modified Hill equation. While the maximal current increases upon hyperpolarization there is no significant difference in the substrate concentration required for half-maximal current ($K_{0.5}$) within the applied voltage range.

Table 3
Kinetic parameters of SN1-induced current as a function of membrane voltage and sodium concentration

Membrane voltage (mV)	$K_{0.5}$ (Na^+)	I_{max} (normalized)
−150	38.10 ± 12.41	1.64 ± 0.55
−120	57.84 ± 24.58	1.55 ± 0.42
−90	49.27 ± 32.80	1.26 ± 0.34
−60	62.29 ± 20.98	0.51 ± 0.30
−30	48.7 ± 28.60	0.19 ± 0.08

Oocytes were injected with 10 ng SN1 mRNA and incubated for 4 days prior to the experiment. Oocytes were superfused with 10 mM glutamine, pH 8, and sodium was applied at concentrations ranging from 0 to 100 mM. $K_{0.5}$ and I_{max} were calculated from the modified Hill equation. While the maximal current increases upon hyperpolarization there is no significant difference in the substrate concentration required for half-maximal current ($K_{0.5}$) within the applied voltage range.

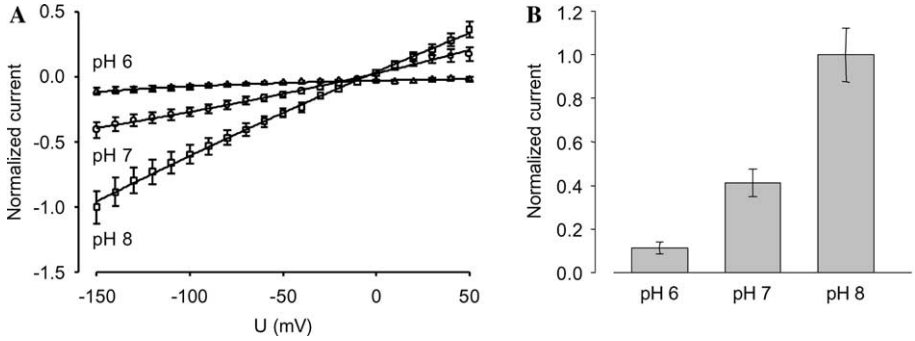

Fig. 2. pH dependence of glutamine-induced currents at different holding voltages. Current was elicited by application of 10 mM glutamine in 100 NaCl. (A) Current–voltage relations. (B) Normalized inward current at −150 mV.

Regulation of glutamine transport

As shown above substrate-induced currents showed the same characteristics as substrate fluxes and were used to study the regulation of SN1 activity. The constitutively active serum and glucocorticoid inducible kinase ^{S422D}SGK1 significantly increased glutamine-induced currents (Fig. 3). Upon coexpression of ^{S422D}SGK1, transport velocity was activated to 153.80 ± 20.32% of control. An even stronger activation of SN1 was observed upon coexpression of the highly homologous SGK3 (218.97 ± 35.13%) and protein kinase B (328.32 ± 49.42%).

In contrast, coexpression of the ubiquitin ligase Nedd4-2 led to a dramatic downregulation of the SN1-mediated current to 23.06 ± 4.74% of control ($n = 21$, Fig. 4). The additional coexpression of ^{S422D}SGK1 abolished the effect of Nedd4-2 expression, i.e., ^{S422D}SGK1 reverses the effect of Nedd4-2 (69.73 ± 23.12%). SGK3 and PKB were also capable of reversing the inhibitory effect of Nedd4-2 (173.73 ± 35.98% and 184.66 ± 38.63%, respectively) (Fig. 4).

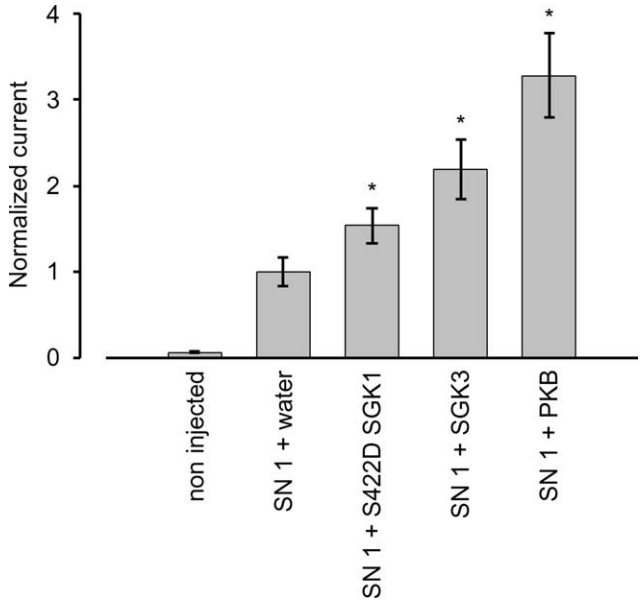

Fig. 3. Stimulation of glutamine-induced currents by the constitutively active serum and glucocorticoid inducible kinase ^{S422D}SGK1, its isoform SGK3 and protein kinase B. Arithmetic means ± SEM, * indicates statistically significant difference to current in *Xenopus* oocytes expressing SN1 alone.

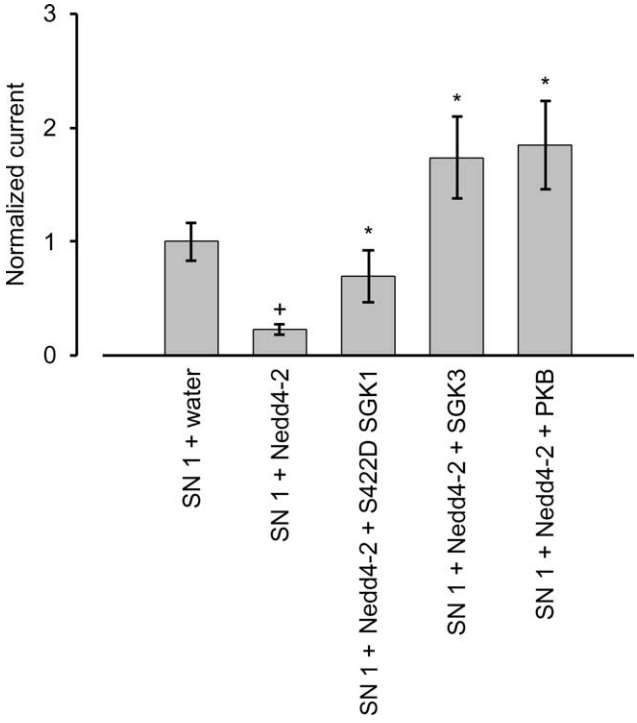

Fig. 4. Downregulation of glutamine-induced current by coexpression of SN1 together with the ubiquitin ligase Nedd4-2 and reversal of the effect by the constitutively active serum and glucocorticoid inducible kinase ^{S422D}SGK1, SGK3, and the protein kinase B. Arithmetic means ± SEM, + indicates statistically significant difference to current in *Xenopus* oocytes expressing SN1 alone. * indicates statistically significant difference to current in *Xenopus* oocytes expressing SN1 and Nedd4-2.

To exclude that an increase in glutamine-induced currents may reflect the activity of an intrinsic pH-sensitive channel (as protons are cotransported by SN1), we also performed [^{14}C]glutamine uptake experiments to characterize glutamine transport directly (Fig. 5). With

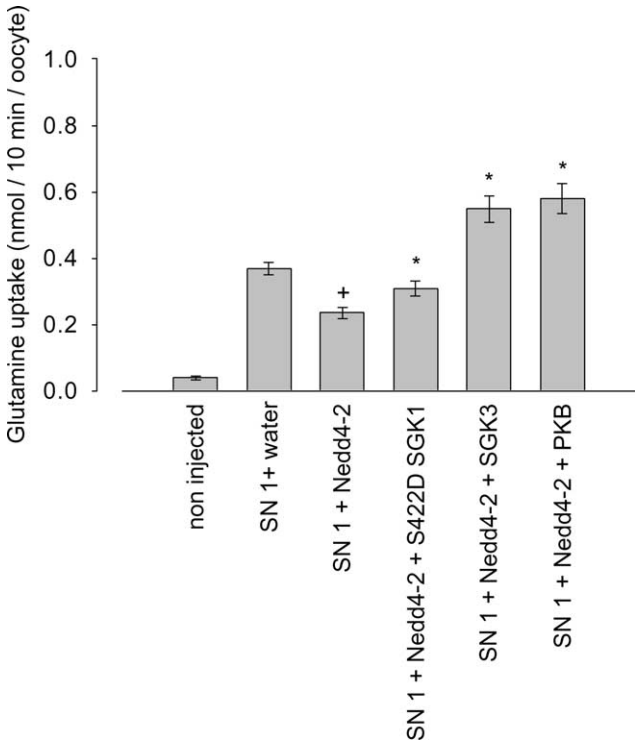

Fig. 5. Downregulation of glutamine uptake in SN1 expressing *Xenopus* oocytes by the ubiquitin ligase Nedd4-2 and reversal of the effect by the constitutively active serum and glucocorticoid inducible kinase ^{S422D}SGK1, SGK3, and the protein kinase B. Arithmetic means ± SEM, + indicates statistically significant difference to uptake in *Xenopus* oocytes expressing SN1 alone. * indicates statistically significant difference to current in *Xenopus* oocytes expressing SN1 and Nedd4-2.

labelled substrate in the medium, qualitatively similar results were obtained as in the electrophysiological experiments (Fig. 5). Expression of SN1 leads to glutamine uptake that was increased up to tenfold over background to 367.88 ± 18.29 pmol/10 min/oocyte compared to 38.99 ± 6.41 pmol/10 min/oocyte of non-injected cells. The significant reduction upon coexpression of Nedd4-2 (63.81 ± 4.48% of control, $n = 26$–48) was reversed by further coexpression of either ^{S422D}SGK1 (83.85 ± 5.81%), SGK3 (149.09 ± 10.90%), or PKB (157.48 ± 11.85%). These effects confirm, that modulation of glutamine-induced currents indeed reflects SN1 transport activity.

Discussion

The glutamine transporter SN1 is considered to co-transport glutamine together with $1Na^+$ in exchange with $1H^+$ [7] and thus to mediate electroneutral transport. The present observations confirm, however, the generation of inward currents paralleling the transport of glutamine [2,4,7]. The glutamine-induced currents are a function of extracellular glutamine and Na^+ concentrations. The kinetic parameters are similar to those determined by flux experiments [7]. Substrate-induced currents reverse at holding potentials more positive than −10 mV, confirming that they are not coupled to substrate translocation. Two mechanisms can be envisaged that generate these currents. First, alkalinization of the oocyte cytosol activates endogenous pH-regulated ion channels or second, the ion channel is an intrinsic property of the transporter. The match of kinetic constants determined by substrate-induced currents with those determined in flux experiments [7] and the parallel activation of currents and substrate fluxes by coexpression of protein kinases SGK1, SGK3, and PKB suggests that the conductance is an inherent property of the SN1 transporter, as suggested recently [27,28].

Kinetic analysis reveals that increasing Na^+ concentrations increase mainly the maximal transport rate without significantly altering the affinity of the carrier. In other words, the binding of Na^+ does not influence the affinity to glutamine. This observation is in agreement with a mechanism in which glutamine binds first and Na^+ second as proposed previously [7]. Remarkably, this indicates that the order of binding is not a conserved feature in the SLC38 family. At variance with SN1, Na^+ binds before glutamine to the related system A isoform ATA1 [28–30].

The present experiments further confirm that similar to glutamine transport [7] and glutamine-induced alkalinization [7] the glutamine-induced current is stimulated by alkaline extracellular pH and inhibited by acidic extracellular pH. The present experiments further reveal that, like the Na^+ concentration, extracellular pH mainly affects the maximal current without significantly altering the affinity of the carrier to glutamine. The reduction of the maximal current is likely to result from a dramatic drop of the affinity for Na^+ caused by acidic pH. This confirms our earlier observation that the pH dependence is not caused by the proton antiport, but is a result of a pH-sensitive modifier site that is a hallmark of all members of the SLC38 family (see [31] for a review). The pH sensitivity of the carrier may be of physiological relevance, as hepatic glutamine uptake and use for urea synthesis are stimulated by alkalosis [32].

Most importantly, the present paper discloses a completely novel mechanism of regulation of SN1. The carrier appears to be a target for the ubiquitin ligase Nedd4-2, which ubiquitinates the carrier protein thus preparing it for internalization and degradation. The regulation of SN1 may be particularly important for the regulation of glutamine transport in liver, muscle, and brain [32,33]. Nedd4-2 has most recently been described to be a target of the serum and glucocorticoid inducible kinase SGK1 and its isoforms [18,19]. SGK1 phosphorylates Nedd4-2 and thus impairs binding of the ubiquitin ligase to its target proteins.

The regulation of SN1 by SGK1 may be important for the regulation of hepatic glutamine uptake by

glutamine depletion [9]. SGK1 is known to be upregulated by removal of amino acids in the medium and downregulated by addition of amino acids [22]. Moreover, transcription of SGK1 is regulated by cell volume [22,34] and may well participate in the regulation of glutamine transport by cell volume [8]. In addition, SGK1 may contribute to the regulation of glutamine transport by hormones. Glutamine uptake is upregulated by glucocorticoids [15] which stimulate the expression of SGK1 [20]. Moreover, glutamine uptake is stimulated by insulin [10–13] and insulin-like growth factor IGF1 [14]. Both insulin and IGF1 activate SGK1 through a signalling cascade involving PI3 kinase and PDK1 [24,35].

A number of further hormones and cytokines upregulate the expression of SGK1 [36]. Oxidative stress has similarly been shown to upregulate the expression of SGK1 [24]. In addition oxidative stress also activates the expressed SGK1 [24]. Whether or not those triggers of SGK1 expression or activity modify glutamine transport has not been explored to our knowledge.

SGK1 has previously been shown to stimulate ion channels including the epithelial Na^+ channel ENaC [37–40] and the voltage gated K^+ channel Kv1.3 [41–43]. At least in the case of the Kv1.3, the function of SGK1 is shared by the isoforms SGK2 and SGK3 [41]. Similar to SGK1, the isoforms SGK2 and SGK3 are stimulated by insulin and the insulin-like growth factor IGF1 [24]. Moreover, as the related protein kinase B shares the consensus sequence of SGK1, it may similarly be involved in the regulation of Nedd4-2 activity. It remains to be established, which of these signal transduction cascades regulates SN1 activity in the cellular background.

In conclusion, substrate-induced currents in SN1 expressing oocytes are dependent on glutamine, Na^+, and H^+ concentrations in the extracellular fluid and parallel glutamine transport. It is thus likely that the observed conductance is an inherent property of the transporter and reflects transport activity. The SN1-mediated current is markedly downregulated by coexpression of Nedd4-2, an effect reversed by additional coexpression of SGK1. Thus, the ubiquitin ligase Nedd4-2 and the serine/threonine kinase SGK1 represent novel, powerful regulators of glutamine transport which are likely to participate in the regulation of cellular amino acid uptake into cells.

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