Up-Regulation of Intestinal Phosphate Transporter NaPi-IIb (SLC34A2) by the Kinases SPAK and OSR1

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Key Words
Phosphate • SLC34A2 • Oxidative stress-responsive kinase 1 • SPS1-related proline/alanine-rich kinase • WNK

Abstract
Background/Aims: SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1), kinases controlled by WNK (with-no-K[Lys] kinase), are powerful regulators of cellular ion transport and blood pressure. Observations in gene-targeted mice disclosed an impact of SPAK/OSR1 on phosphate metabolism. The present study thus tested whether SPAK and/or OSR1 contributes to the regulation of the intestinal Na\textsuperscript{+}-coupled phosphate co-transporter NaPi-IIb (SLC34A2). Methods: cRNA encoding NaPi-IIb was injected into \textit{Xenopus laevis} oocytes without or with additional injection of cRNA encoding wild-type SPAK, constitutively active T233E SPAK, WNK insensitive T233A SPAK, catalytically inactive D212A SPAK, wild-type OSR1, constitutively active T185E OSR1, WNK insensitive T185A OSR1 or catalytically inactive D164A OSR1. The phosphate (1 mM)-induced inward current (\(I_{\text{Pi}}\)) was taken as measure of phosphate transport. Results: \(I_{\text{Pi}}\) was observed in NaPi-IIb expressing oocytes but not in water injected oocytes, and was significantly increased by co-expression of SPAK, T233E SPAK, OSR1, T185E OSR1 or SPAK+OSR1, but not by co-expression of T233A SPAK, D212A SPAK, T185A OSR1, or D164A OSR1. SPAK and OSR1 both increased the maximal transport rate of the carrier. Conclusions: SPAK and OSR1 are powerful stimulators of the intestinal Na\textsuperscript{+}-coupled phosphate co-transporter NaPi-IIb.
Introduction

SPAK (SPS1-related proline/alanine-rich kinase) [1-3] and OSR1 (oxidative stress-responsive kinase 1) [4, 5] are both powerful regulators of epithelial ion transport and blood pressure [6]. The kinases are phosphorylated by WNK (with-no-K[Lys] kinases) [1, 7-10], which similarly contribute to the regulation of ion transport and blood pressure [9-17]. SPAK and OSR1 stimulate NaCl (NCC) and Na⁺,K⁺,2Cl⁻ (NKCC) co-transporters [4-8, 11, 18-26] as well as a number of ion channels [27-30]. SPAK/OSR1 are expressed in intestine [31]. Intestinal SPAK/OSR1 are both phosphorylated by treatment with forskolin, an activator of adenylate cyclase [31]. SPAK deficiency is followed by a decrease in paracellular permeability and partial resistance to inflammatory bowel disease [32]. SPAK transcription is stimulated by the transcription factors NF-kappaB and Sp1 [33]. Activators of SPAK include angiotensin II [34, 35], which upregulates the expression of the renal phosphate transporter NaPi-IIa [36]. OSR1 has similarly been shown to stimulate the renal phosphate transporter NaPi-IIa [37]. Along those lines gene targeted mice expressing WNK-resistant SPAK [38] or OSR1 [37] display subtle alterations of phosphate metabolism.

Nothing is known, however, on a putative influence of SPAK or OSR1 on the type II Na⁺-coupled phosphate co-transporter NaPi-IIb (SLC34A2), the transporter accomplishing intestinal transport of inorganic phosphate [39]. NaPi-IIb is primarily expressed in small intestine [39]. Mutations in the SLC34A2 gene may be associated with accumulation of phosphate in lung with development of pulmonary alveolar microlithiasis [40]. SLC34A2 is further expressed in the epididymis and presumably participates in the fine tuning of luminal phosphate concentration [41]. Moreover, SLC34A2 expression was observed in ovarian, papillary thyroid and breast cancer [42-44].

The present study explored, whether SPAK and/or OSR1 participates in the regulation of NaPi-IIb. To this end, NaPi-IIb was expressed in Xenopus oocytes without or with additional expression of the kinases. The phosphate induced current was determined by dual electrode voltage clamp and taken as a measure of phosphate transport.

Materials and Methods

Ethical Statement

All experiments conform with the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and were conducted according to the German law for the welfare of animals and the surgical procedures on the adult Xenopus laevis frogs were reviewed and approved by the respective government authority of the state Baden-Württemberg (Regierungspräsidium) prior to the start of the study (Anzeige für Organentnahme nach §36).

Constructs

Constructs encoding human wild-type NaPi-IIb (SLC34A2) [45], human wild-type SPAK/pGHJ, constitutively active T233E/SPAK/pGHJ, WNK insensitive T233A/SPAK/pGHJ, catalytically inactive D212A/SPAK/pGHJ [7], human wild-type OSR1/pGHJ, constitutively active T185E/OSR1/pGHJ, WNK insensitive T185A/OSR1/pGHJ and catalytically inactive D164A/OSR1/pGHJ [37], were used to generate cRNA as described previously [46, 47]. The constructs were a kind gift from Dario Alessi (University of Dundee).

Voltage clamp in Xenopus laevis oocytes

Xenopus oocytes were prepared as previously described [48, 49], 15 ng cRNA encoding NaPi-IIb and 10 ng of cRNA encoding wild-type, constitutively active or inactive kinase were injected on the same day after preparation of the oocytes. The oocytes were maintained at 17°C in ND96, a solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 2.5 NaOH, 5 HEPES, 5 sodium pyruvate (C₃H₃NaO₃), Gentamycin (100 mg/l), Tetracycline (50 mg/l), Ciprofloxacin (1.6 mg/l), Theophiline (90 mg/l) and pH 7.4 [50, 51]. The voltage clamp experiments were performed at room temperature 4 days after the first injection [47, 52]. Phosphate induced currents were taken as a measure of phosphate transport at a holding potential of
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-60mV [53, 54]. The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter (1322A Axon Instruments) and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [55-57]. 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. 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 [58-60]. For kinetic analysis the phosphate induced-current (Iₚᵢ) was plotted against the respective phosphate concentration (s) and maximal current (Iₘₚᵢ) as well as concentration required for halfmaximal current (kₘₚᵢ) calculated using the equation Iₚᵢ = Iₘₚᵢ ∙ s/(kₘₚᵢ + s).

**Statistical analysis**

Data are provided as means ± SEM, n represents the number of oocytes investigated. As different batches of oocytes may yield different results, comparisons were always made within a given oocyte batch. 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 or the unpaired student’s t-test, as appropriate. Results with p < 0.05 were considered statistically significant.

**Results**

The present study addressed whether the electrogenic phosphate transporter NaPi-IIb is regulated by the kinases SPAK (SPS1-related proline/alanine-rich kinase) and/or OSR1 (oxidative stress-responsive kinase 1). To this end, cRNA encoding NaPi-IIb was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type or mutant SPAK or OSR1. Dual electrode voltage clamp experiments were performed to quantify the phosphate-induced inward current (Iₚᵢ) as a measure of electrogenic phosphate transport. As illustrated in Fig. 1, Iₚᵢ was negligible in water-injected oocytes indicating that the oocytes did not express appreciable endogenous electrogenic phosphate transport. Iₚᵢ was further negligible in oocytes expressing SPAK alone. In NaPi-IIb expressing *Xenopus laevis* oocytes, however; addition of phosphate (1 mM) to the bath solution was followed by appearance of a sizable Iₚᵢ. The co-expression of wild-type SPAK in NaPi-IIb expressing *Xenopus laevis* oocytes was followed by a significant increase of Iₚᵢ.

In order to test, whether SPAK co-expression modifies the maximal Iₚᵢ and/or the affinity of the carrier, *Xenopus laevis* oocytes expressing NaPi-IIb without or with co-expression of
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SPAK were exposed to phosphate concentrations ranging from 0.1 mM to 4 mM. As shown in Fig. 2, \(I_{\text{Pi}}\) increased as a function of the extracellular phosphate concentration. Maximal \(I_{\text{Pi}}\) was significantly (\(p<0.001\)) higher in *Xenopus laevis* oocytes expressing NaPi-IIb together with SPAK (20.6 ± 1.1 nA, \(n=6\)) than in *Xenopus laevis* oocytes expressing NaPi-IIb alone (8.9 ± 0.3 nA, \(n=6\)). The concentration required for half maximal \(I_{\text{Pi}}\) (\(K_m\)) was significantly (\(p<0.05\)) higher in *Xenopus laevis* oocytes expressing NaPi-IIb together with SPAK (361 ± 66 \(\mu\)M, \(n=6\)) than in *Xenopus laevis* oocytes expressing NaPi-IIb alone (193 ± 26 \(\mu\)M, \(n=6\)).

Additional experiments were performed in *Xenopus laevis* oocytes expressing NaPi-IIb without or with SPAK mutants. As illustrated in Fig. 3, the effect of wild-type SPAK was mimicked by the constitutively active T233E SPAK but neither by the WNK insensitive T233A SPAK nor by the catalytically inactive D212A SPAK.

Similar experiments were performed to elucidate the effect of OSR1 on NaPi-IIb activity. As illustrated in Fig. 4, co-expression of wild-type OSR1 was followed by a significant increase of \(I_{\text{Pi}}\) in NaPi-IIb expressing *Xenopus* oocytes.

Further experiments again explored, whether OSR1 co-expression modifies the maximal \(I_{\text{Pi}}\) and/or the affinity of the carrier. As shown in Fig. 5, the maximal phosphate induced current was again significantly (\(p<0.001\)) higher in *Xenopus laevis* oocytes expressing NaPi-IIb together with OSR1 (14.9 ± 1.1 nA, \(n=6\)) than in *Xenopus laevis* oocytes expressing NaPi-IIb alone (6.8 ± 0.1 nA, \(n=6\)). The concentration required for half maximal \(I_{\text{Pi}}\) (\(K_m\)) was significantly (\(p<0.05\)) higher in *Xenopus laevis* oocytes expressing NaPi-IIb together with OSR1 (338 ± 92 \(\mu\)M, \(n=6\)) than in *Xenopus laevis* oocytes expressing NaPi-IIb alone (119 ± 11 \(\mu\)M, \(n=6\)).

Additional experiments were performed in *Xenopus laevis* oocytes expressing NaPi-IIb without or with OSR1 mutants. As a result, the effect of wild-type OSR1 was mimicked by constitutively active T185E OSR1 but neither by the WNK insensitive T185A OSR1 nor by the catalytically inactive D164A OSR1 (Fig. 6).

A final series of experiments explored the effect of simultaneous co-expression of SPAK and OSR1 on \(I_{\text{Pi}}\). As a result, the electrogenic current in NaPi-IIb expressing *Xenopus laevis* oocytes tended to be slightly higher in oocytes co-expressing both, SPAK and OSR1 together than in oocytes co-expressing either SPAK or OSR1 alone. However, the difference of \(I_{\text{Pi}}\) between oocytes co-expressing both, SPAK and OSR1, and the co-expressing either SPAK or OSR1 alone did not reach statistical significance (Fig. 7).

**Fig. 2.** Co-expression of SPAK increases maximal electrogenic phosphate transport in NaPi-IIb expressing *Xenopus laevis* oocytes. A: Representative original tracings showing the current induced by increasing concentrations of phosphate (from 0.1 mM to 4 mM) in *Xenopus laevis* oocytes expressing Napi-IIb without (upper panel) or with (lower panel) additional coexpression of wild-type SPAK (dashed line indicates zero current). B: Arithmetic means ± SEM (\(n=6\)) of \(I_{\text{Pi}}\) as a function of logarithmic phosphate concentrations in *Xenopus laevis* oocytes expressing NaPi-IIb without (white circles), or with (black circles) additional co-expression of wild-type SPAK.
Discussion

The present study reveals that SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) are both powerful positive regulators of the intestinal phosphate transporter NaPi-IIb. Co-expression of either SPAK or OSR1 increases the phosphate-induced inward current (I_p) in NaPi-IIb expressing oocytes. SPAK and OSR1 are both effective in large part by increase of the maximal transport rate. Co-expression of the kinases decreased the apparent affinity of NaPi-IIb. The simultaneous co-expression of both kinases SPAK and OSR1 does not show an additive effect. This outcome could be explained by the expression of SPAK and OSR1 in different cells relative to the same tissue or organ. Similar to wild type SPAK, constitutively active T233E SPAK increases the phosphate induced current.
in NaPi-IIb expressing *Xenopus* oocytes. In contrast, neither the WNK insensitive T233A SPAK nor the catalytically inactive D164A OSR1 significantly modified the phosphate induced current. The effect of wild type OSR1 is similarly mimicked by the constitutively active T185E OSR1, but not by WNK insensitive T185A OSR1 or by the catalytically inactive D164A OSR1. These observations suggest that SPAK and OSR1 are activated by phosphorylation at the WNK phosphorylation site and that they are effective by phosphorylating target molecules. The observations do, however, not necessarily reflect direct phosphorylation of the NaPi-IIb carrier protein by SPAK and OSR1. Instead, the kinases might phosphorylate and thus modify the function of other NaPi-IIb regulating signaling molecules. Kinases previously shown to participate in the regulation of NaPi-IIb activity include AMP activated kinase (AMPK) [61], B-RAF [45], serum & glucocorticoid inducible kinase SGK1 [62], and mammalian target of rapamycin (mTOR) [63].
In view of the regulation of NaPi-IIb by SPAK and OSR1, the two kinases contribute to the orchestration of phosphate metabolism. Observations in gene targeted mice indeed reveal that SPAK [38] and OSR1 [37] affect phosphate metabolism and OSR1 modifies Napi-IIa activity expressed in the proximal tubule [37]. However, the effects of SPAK [38] and OSR1 [37] on phosphate metabolism are not limited to regulation of NaPi-IIa and NaPi-IIb.

SPAK and OSR1 foster cellular KCl uptake by stimulating NaCl co-transporters and Na⁺,K⁺,2Cl⁻ co-transporters and by inhibiting KCl co-transporters [4-8, 11, 18-26], effects leading to cell swelling [64-66]. Stimulation of Na⁺ coupled phosphate transport is similarly expected to increase cell volume, as it leads to cellular uptake of Na⁺ and phosphate as well as to depolarisation of the cell membrane, which favours entry of negatively charged chloride. Due to the low transport rate and the limited availability of phosphate in extracellular fluid, activation of NaPi-IIb is, however, not expected to rapidly swell cells.

**Conclusion**

NaPi-IIb is markedly up-regulated by the kinases SPAK and OSR1, an effect presumably contributing to the orchestration of phosphate metabolism.

**Disclosure Statement**

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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