Original Paper

Upregulation of Na⁺-coupled Glucose transporter SGLT1 by Tau Tubulin Kinase 2

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Key Words

Glucose carrier • Tumor cells • Kinase • Electrophysiology

Abstract

The Tau-tubulin-kinase 2 (TTBK2) is a serine/threonine kinase expressed in various tissues including tumors. Up-regulation of TTBK2 increases resistance of tumor cells against antiangiogenic treatment and confers cell survival. Tumor cell survival critically depends on cellular uptake of glucose, which is partially accomplished by SGLT1 (SLC5A1) mediated Na⁺-coupled glucose transport. The present study explored whether TTBK2 participates in the regulation of SGLT1 activity. To this end, electrogenic glucose transport was determined in *Xenopus* oocytes expressing SGLT1 with or without wild-type TTBK2, truncated TTBK2^[1-450] or kinase inactive mutants TTBK2-KD and TTBK2-KD^[1-450]. TTBK2, but not TTBK2^[1-450], TTBK2-KD or TTBK2-KD^[1-450], increased membrane carrier protein abundance and electrogenic glucose transport capacity in SGLT1-expressing *Xenopus* oocytes. Thus TTBK2 is a completely novel regulator of Na⁺-coupled glucose transport.

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Introduction

The Tau tubulin Kinase 2 (TTBK2), a serine/threonine kinase [1], is expressed in various tissues including the brain [2, 3] and tumors [4]. The kinase has been shown to affect sensitivity of tumor cells against antiangiogenic treatment [4]. Loss of function mutations of the gene encoding the kinase cause the rare, inherited, autosomal dominant spinocerebellar ataxia type 11 (SCA11) [5]. The disease may result from mutations causing a premature termination of TTBK2 protein at around residue 450 (TTBK2^[1-450]) [6] leading to decreased kinase activity [5] and resulting in spinocerebellar degeneration [7]. Gene targeted mice carrying homozygous loss of function SCA11 mutations are not viable and die at embryonic day 10 [5].

The survival of both, neurons [8] and tumor cells [9-12] critically depends on the supply of glucose. The delivery of glucose is in part accomplished by the passive glucose carriers of the GLUT family [13, 14]. Beyond that glucose may be taken up by Na⁺-glucose cotransporters of the SGLT family, which mediate secondary active transport driven by the Na⁺ gradient across the cell membrane [15]. The two members of this protein family, SGLT1 and SGLT2, accomplish the concentrative cellular uptake of glucose across the apical cell membrane of epithelial cells [15]. The carrier is, however, not limited to epithelial tissues. Instead, SGLT1 has been identified in several tumor cells [16-23], which have an increased substrate demand and utilize mainly glucose for energy production [18]. In contrast to the glucose carriers of the GLUT family, SGLT1 allows cellular glucose uptake against a glucose concentration gradient and thus even at low extracellular glucose concentrations [15]. SGLT1 expression and activity may thus be particularly important for cell survival during ischemia and excessive cellular glucose utilization.

Mechanisms contributing to up-regulation of SGLT1 expression and activity in tumor cells have remained incompletely understood. Proposed mechanism include EGF receptor dependent stabilization of SGLT1 protein [17, 23] and human papilloma virus protein HPV18 E6 induced inhibition of SGLT1 protein degradation [24].

The present study explored, whether TTBK2 modifies the function of SGLT1. To this end, SGLT1 was expressed in *Xenopus* oocytes with or without additional coexpression of full length wild-type TTBK2, truncated TTBK2^[1-450], or kinase inactive mutants full-length TTBK2-KD and truncated TTBK2-KD^[1-450]. SGLT1 activity was estimated from glucose-induced current utilizing two-electrode voltage clamp and SGLT1 protein abundance in the cell membrane by both, confocal microscopy and chemiluminescence.

Materials and Methods

Quantitative RT-PCR

Experiments were performed in 8-10 week old female C57Bl/6 mice. After sacrificing the animals, brain, cerebellum, jejunum, heart and kidney tissues were immediately snap frozen in liquid nitrogen. Total RNA was isolated from mouse tissue by using Trifast Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)₁₂₋₁₈ primers (Invitrogen, Karlsruhe, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). cDNA samples were treated with RNaseH (Invitrogen, Karlsruhe, Germany). Quantitative real-time PCR was performed with the iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and iQTM Sybr Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The following primers were used (5'→3' orientation): Ttbk2 fw: CATCAAACCGTCAAACCTTCGC; Ttbk2 rev: AACGAACGTGCCCTCGAAAGC; Gapdh fw: AGGTCGGTGTGAACGGATTTC; Gapdh rev: TGTAGACCATGTAGTTGAGGTCA. The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate. Gapdh was used as internal reference.

Constructs

For generation of cRNA, constructs were used encoding wild-type human SGLT1 (SLC5A1) [25], wild-type human full-length TTBK2, truncated mutant TTBK2^[1-450] containing the first 450 residues of the kinase, and the kinase inactive mutants TTBK2-KD and TTBK2-KD^[1-450] containing an aspartic acid at position 163 replaced by alanine [D163A] (kindly provided by Dario Alessi). The cRNA was generated as described previously [26].

Voltage clamp

For voltage clamp analysis, *Xenopus* oocytes were prepared as previously described [27]. On the day of preparation, *Xenopus* oocytes were injected with water, 4.6 ng of cRNA encoding SGLT1 and/or 10 ng of cRNA encoding TTBK2-WT, TTBK2-KD, TTBK2-WT^[1-450] or TTBK2-KD^[1-450]. The oocytes were maintained at 17°C in ND96 solution containing: 96 mM NaCl, 4 mM KCl, 1.8 mM MgCl₂, 0.1 mM CaCl₂, 5 mM HEPES, pH 7.4, supplemented with antibiotics [28]. The two-electrode voltage clamp recordings were performed at room temperature 3 days after the injections [29] at a holding potential of -70 mV. The data were filtered at 10 Hz and recorded with a DigidataA/D-D/A converter and ClampexV.9 software for data acquisition and analysis

Fig. 1. Coexpression of wild-type TTBK2 increases SGLT1 activity. **A.** Representative original bands of *Ttbk2* (upper bands) mRNA expression in various tissues from wild-type mice. The housekeeping gene *Gapdh* (lower bands) served as a calibrator and control. **B.** Original tracings of glucose-induced currents recorded in *Xenopus* oocytes injected with water (a), expressing SGLT1 without (b) or with additional coexpression of wild-type TTBK2 (c). **C.** Arithmetic means $\pm$ SEM ($n = 11-14$) of glucose (10 mM) induced current (I_g) in *Xenopus* oocytes injected with water (H_2O , dotted bar), expressing SGLT1 without (SGLT1, white bar) or with additional coexpression of wild-type TTBK2 (SGLT1 + TTBK2-WT, black bar). *** indicates statistically significant difference ($p < 0.001$) from *Xenopus* oocytes expressing SGLT1 alone.

(Axon Instruments, Union City, CA, USA). The control 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. Glucose was added to the solutions at a concentration of 10 mM unless otherwise stated. The flow rate of the superfusion was approximately 20 ml/min, and a complete exchange of the bath solution was reached within about 10 sec [30].

Immunostaining and confocal microscopy

To visualize SGLT1 cell surface expression, oocytes were fixed in 4% paraformaldehyde for 2 h at room temperature [31]. After washing with TBS, the oocytes were permeabilized and blocked for 30 min at room temperature in 10% normal goat serum in TBS containing 0.1% TritonX-100. The primary antibody used was rabbit polyclonal anti-SGLT1 antibody (diluted 1:1000, Millipore, Billerica, MA, USA) incubated overnight at 4°C. Binding of primary antibody was visualised with a goat anti-rabbit FITC-conjugated antibody (diluted 1:1000, Invitrogen, Karlsruhe, Germany). Then, oocytes were analyzed by a fluorescence laser scanning microscope (LSM510, CarlZeiss MicroImaging GmbH, Germany) with A-Plan 10x/0.25, focused on the midline of the oocytes. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series.

Detection of SGLT1 cell surface expression by chemiluminescence

To determine SGLT1 cell surface expression by chemiluminescence [32], oocytes were incubated with rabbit polyclonal anti-SGLT1 antibody (diluted 1:1000, Millipore, Billerica, MA, USA) and subsequently with secondary goat anti-rabbit HRP-conjugated antibody (1:1000, Cell Signaling Technology, MA, USA). Individual oocytes were placed in 96 well plates with 20 μ l of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 sec. Results display normalized relative light units.

Statistical analysis

Data are provided as arithmetic means $\pm$ SEM; n represents the number of oocytes investigated. All oocyte experiments were repeated with at least three batches of oocytes and in all repetitions qualitatively similar data were obtained. All data were tested for significance by using ANOVA or t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Fig. 2. The effect of wild-type TTBK2 on SGLT1 activity is abrogated by truncation and depends on kinase activity. A. Original tracings of glucose induced currents recorded in *Xenopus* oocytes injected with water (a) expressing SGLT1 without (b) or with additional coexpression of full-length wild-type TTBK2-WT (c), of full-length kinase inactive mutant TTBK2-KD (d), of truncated wild-type TTBK2-WT^[1-450] (e) or of truncated kinase inactive mutant TTBK2-KD^[1-450] (f). B. Arithmetic means $\pm$ SEM ($n = 13-15$) of glucose-induced current (10 mM) (I_g) in *Xenopus* oocytes injected with water (H₂O, dotted bar), expressing SGLT1 without (SGLT1, white bar) or with additional coexpression of wild-type TTBK2 (SGLT1 + TTBK2-WT, black bar), of full-length kinase inactive mutant TTBK2 (SGLT1 + TTBK2-KD, grey bar), of truncated wild-type TTBK2 (SGLT1 + TTBK2-WT^[1-450], grey bar) or of truncated kinase inactive mutant TTBK2 (SGLT1 + TTBK2-KD^[1-450], grey bar). **, *** indicates statistically significant difference ($p < 0.01$, $p < 0.001$) from *Xenopus* oocytes expressing SGLT1 alone. ### indicates statistically significant difference ($p < 0.001$) from *Xenopus* oocytes coexpressing SGLT1 together with wild-type TTBK2.

Results

Similar to what has been reported earlier [3, 7], RT-PCR revealed the highest mRNA expression of *Ttbk2* in murine cerebellum (Fig. 1A). More importantly, *Ttbk2* transcript was further detectable in other tissues, with high mRNA levels in murine heart and kidney, and with lower mRNA expression in murine intestine (Fig. 1A).

Two-electrode voltage clamp experiments in *Xenopus* oocytes were performed to explore whether the Tau tubulin Kinase 2 (TTBK2) influences the activity of the Na⁺-coupled glucose carrier SGLT1. The SGLT1-mediated glucose transport rate was estimated from the glucose (10 mM) induced current (I_g). In water-injected *Xenopus* oocytes, addition of glucose to the extracellular fluid did not induce an appreciable inward current, indicating that *Xenopus* oocytes do not express appreciable endogenous electrogenic glucose transporters (Fig. 1B,C). Similarly, no significant glucose-induced current was observed in *Xenopus* oocytes expressing wild-type TTBK2 alone (data not shown). Thus, the kinase did not up-regulate an endogenous electrogenic glucose transporter. In contrast, expression of SGLT1 (SLC5A1) was followed by appearance of I_g , which reflects electrogenic entry of Na⁺ and glucose. I_g was significantly enhanced by additional coexpression of wild-type TTBK2 (Fig. 1 B,C).

Fig. 3. TTBK2 enhances the maximal current without appreciably affecting affinity. Arithmetic means $\pm$ SEM ($n = 9-11$) of I_g as a function of glucose concentration in *Xenopus* oocytes expressing SGLT1 without (SGLT1, open rombi) or with additional coexpression of wild-type TTBK2 (SGLT1 + TTBK2-WT, closed squares). The difference from the absence of wild-type TTBK2 is statistically significant ($p < 0.001$) at every glucose concentration applied.

In contrast to the effects of wild-type TTBK2, the coexpression of the truncated TTBK2^[1-450] did not significantly increase I_g . Moreover, coexpression of the full-length kinase inactive mutant TTBK2-KD and truncated kinase inactive mutant TTBK2-KD^[1-450] did not significantly modify I_g in SGLT1-expressing *Xenopus* oocytes (Fig. 2). Accordingly, TTBK2 kinase activity was required for the effects on SGLT1-induced currents.

Kinetic analysis of the glucose-induced currents in SGLT1-expressing *Xenopus* oocytes (Fig. 3) yielded a maximal current of 78.0 ± 6.2 nA ($n = 11$). Coexpression of wild-type TTBK2 significantly enhanced the maximal current to 164.9 ± 12.3 nA ($n = 9$). Calculation of the glucose concentration required for halfmaximal current (K_M) yielded 515 ± 72 μ M ($n = 11$) in the absence and 466 ± 43 μ M ($n = 9$) in the presence of TTBK2, values not significantly different. Accordingly, coexpression of wild-type TTBK2 did not significantly modify K_M but significantly increased the maximal current.

The enhanced SGLT1 activity could result from increased carrier protein abundance in the plasma membrane of *Xenopus* oocytes. To explore that possibility, immunostaining with subsequent confocal microscopy and chemiluminescence experiments were performed. As illustrated in Fig. 4A,B, the coexpression of wild-type TTBK2 resulted in a significant increase of SGLT1 protein abundance within the SGLT1-expressing *Xenopus* oocyte cell membrane. In contrast, coexpression of truncated TTBK2^[1-450] or of the full-length kinase inactive mutant TTBK2-KD and truncated kinase inactive mutant TTBK2-KD^[1-450] did not significantly modify SGLT1 protein abundance at the surface of SGLT1-expressing *Xenopus* oocytes (Fig. 4A,B).

Discussion

The present observations reveal a completely novel function of the Tau tubulin Kinase 2 (TTBK2), i.e. regulation of the Na⁺-coupled glucose carrier SGLT1. The kinase up-regulates SGLT1 activity by increasing the SGLT1 protein abundance in the cell membrane. Moreover, mutations that truncate the C-terminus noncatalytic domains of TTBK2 protein (TTBK2^[1-450]), shown previously to be involved in spinocerebellar degeneration [7], did not modify the activity and protein abundance of the Na⁺-coupled glucose carrier.

The TTBK2 sensitive regulation of SGLT1 may be relevant for tumor cells, as both, TTBK2 [4] and SGLT1 [16-23] are expressed in tumor cells and influence the survival of those cells.

Tumor cells are dependent on delivery of glucose to cover their excessive demand for this substrate [18]. Even in the presence of glucose, tumor cells degrade glucose to lactate and thus utilize only a small fraction of the energy which could be generated by oxidative degradation of glucose [9-12]. At least in some tumor cells, the uptake of glucose with facilitative glucose carriers alone presumably fails to supply sufficient amounts of glucose. SGLT1 couples the uphill transport of glucose to Na⁺ entry down its electrochemical potential gradient

Fig. 4. Coexpression of wild-type but not of inactive mutants TTBK2 increases SGLT1 protein abundance in the cell membrane of SGLT1-expressing *Xenopus* oocytes. A. Confocal images reflecting SGLT1 membrane protein abundance in *Xenopus* oocytes injected with water (H₂O), expressing SGLT1 without (SGLT1) or with additional coexpression of wild-type TTBK2 (SGLT1 + TTBK2-WT), of full-length kinase inactive mutant TTBK2 (SGLT1 + TTBK2-KD), of truncated wild-type TTBK2 (SGLT1 + TTBK2-WT^[1-450]) or of truncated kinase inactive mutant TTBK2 (SGLT1 + TTBK2-KD^[1-450]). B. Arithmetic means $\pm$ SEM (n = 53-78) of the chemiluminescence reflecting SGLT1 protein abundance in the cell membrane of *Xenopus* oocytes injected with water (H₂O, dotted bar), expressing SGLT1 without (SGLT1, white bar) or with additional coexpression of wild-type TTBK2 (SGLT1 + TTBK2-WT, black bar), of full-length kinase inactive mutant TTBK2 (SGLT1 + TTBK2-KD, grey bar), of truncated wild-type TTBK2 (SGLT1 + TTBK2-WT^[1-450], grey bar) or of truncated kinase inactive mutant TTBK2 (SGLT1 + TTBK2-KD^[1-450], grey bar). *** indicates statistically significant difference (p<0.001) from *Xenopus* oocytes expressing SGLT1 alone. ### indicates statistically significant difference (p<0.001) from *Xenopus* oocytes coexpressing SGLT1 together with wild-type TTBK2.

across the plasma membrane and is thus able to accomplish cellular glucose uptake when extracellular glucose concentrations are below those of the cell [15]. Accordingly, decrease of the extracellular glucose concentration compromises cellular glucose uptake through the facilitative glucose carriers more severely than cellular glucose uptake by Na⁺-coupled glucose transport. In contrast to glucose uptake by GLUT carriers, the Na⁺-coupled uptake of glucose requires, however, the maintenance of the Na⁺ gradient across the cell membrane, a function of ATP-consuming Na⁺/K⁺ ATPase. Na⁺ entry without Na⁺ extrusion by the Na⁺/K⁺ ATPase leads to cellular K⁺ loss with subsequent depolarisation of the cell membrane, Cl⁻ entry and cell swelling [33]. As the Na⁺/K⁺ ATPase extrudes 3 Na⁺ ions for one ATP, the energy needed for subsequent extrusion of the cotransported Na⁺ is, however, only a small fraction of the energy gained, even if glucose is utilized for ATP generation by glycolysis without oxidative degradation.

TTBK2 may be only one of several mechanisms regulating glucose uptake into tumor cells and other tissues. According to previous observations, SGLT1 activity is regulated by several kinases including protein kinase A (PKA) [34, 35], protein kinase C (PKC) [34, 35], serum- and

glucocorticoid-inducible kinase [25] and AMP-activated protein kinase [36]. Moreover, SGLT1 is up-regulated by the human papilloma virus protein HPV18 E6 [24]. Similar to TTBK2, the kinases and HPV18 E6 regulate SGLT1 activity by modifying the abundance of carrier protein within the plasma membrane.

In conclusion, the present study discloses that TTBK2 is a powerful regulator of Na⁺-coupled glucose transport and thus participates in the regulation of cellular glucose uptake into epithelia, tumor cells and further SGLT1 expressing tissues.

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