A Single Binding Motif is Required for SPAK Activation of the Na-K-2Cl Cotransporter

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Key Words

Membrane Transport • Phosphorylation • Stress kinases • Yeast 2-hybrid • *Xenopus* oocytes

Abstract

Background: SPAK (Ste20p-related proline alanine-rich kinase) phosphorylates and activates NKCC1 (Na-K-2Cl cotransporter) in the presence of another serine/threonine kinase WNK4 (With No lysine (K)). However, whether or not the docking of SPAK to NKCC1 is a requirement for cotransporter activation has not been fully resolved. **Methods:** We mutated both SPAK binding motifs in the amino-terminal tail of NKCC1 and tested the interaction between SPAK and NKCC1 using a semi *in vivo* yeast two-hybrid assay, ^{32}P -ATP *in vitro* phosphorylation assays, and $^{86}\text{Rb}^+$ uptake (a K^+ congener) assays in heterologously expressed *Xenopus laevis* oocytes. We also used site-directed mutagenesis to identify the principle phospho-regulatory threonine residues in the amino-terminal tail of NKCC1. **Results:** A single SPAK binding motif is necessary for isotonic NKCC1 activation. Mutation of the phenylalanine (F) residue within the motif abrogates binding and function. Phosphorylation of the cotransporter is markedly reduced in the

absence of SPAK docking to NKCC1. Truncations of internal regions of the amino-terminus of NKCC1 do not disrupt protein structure enough to affect cotransporter function. Threonine residues (T^{206} and T^{211}) are both identified as phospho-regulatory sites of NKCC1 function. **Conclusion:** We demonstrate that physical docking of SPAK to NKCC1 is necessary for cotransporter activity under both baseline and hyperosmotic conditions. We identify T^{206} and T^{211} as major phospho-acceptor sites involved in cotransporter function, with T^{206} common to two separate regulatory pathways: one involving SPAK, the other involving a still unknown kinase that is responsive to forskolin/PKA stimulation.

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Introduction

The Na-K-2Cl cotransporter-1, NKCC1, is expressed in a variety of cells where it plays a multitude of functions. The cotransporter is highly expressed on the basolateral membrane of Cl^- -secreting epithelia such as salivary, sweat and lacrimal glands as well as lung, stomach, colon, where it participates in salt/fluid secretion

(for reviews, see [1, 2]). In mouse kidney, NKCC1 is mainly found in renin-containing cells of the afferent arteriole, in the juxtaglomerular mesangium, and on the basolateral membrane of inner medullary collecting duct [3]. In the inner ear, the cotransporter is involved in the secretion of K⁺-rich endolymph [4-7]. The cotransporter is also expressed in the brain on the apical membrane of choroid plexus epithelium where it likely participates in K⁺ reabsorption [8, 9], in astrocytes where it participates in K⁺ buffering [10, 11], and in developing/maturing neurons where it modulates GABA (γ -aminobutyric acid)-synaptic transmission [12]. In sensory dorsal ganglion neurons, NKCC1 is expressed throughout adulthood where it facilitates GABA depolarization and consequently presynaptic inhibition [13, 14]. As NKCC1 is activated by cell shrinkage, in all cells where the cotransporter is expressed, it participates in cell volume maintenance and regulation [15].

Numerous studies have demonstrated that stimuli resulting in an increase of NKCC1 activity also cause an increase in phosphorylation of the cotransporter protein [16-19]. In duck red blood cells, changes in cotransport activity and concurrent phosphorylation were followed at different cell volumes (osmolarities) and a very close relationship between NKCC1 activity and ³²P incorporation was observed [20]. Furthermore, through the development of an antibody recognizing a phosphorylated form of NKCC1, Forbush's group demonstrated that in parallel to cotransporter activation there was a 4-5 fold increase in phosphorylation level of NKCC1 within 10 min of forskolin treatment [21].

Much effort has been devoted to the identification of the kinase(s) that directly phosphorylate NKCC1. In 2002, we reported a protein-protein interaction between sterile 20 protein (Ste20p)-related Proline Alanine-rich Kinase (SPAK) and NKCC1 through two putative binding motifs (R/K)Fx(V/I) (Arginine/Lysine, Phenylalanine, any residue, Valine/Isoleucine) located in the cytosolic amino-terminal portion of NKCC1 [22]. Using dominant-negative SPAK, Dowd and Forbush demonstrated a functional role for the kinase in NKCC1 function [23]. We later showed that SPAK phosphorylated and activated NKCC1 in the presence of another serine/threonine kinase WNK4 (With No lysine (K)), and that this interaction required the docking of SPAK to WNK4 [24]. Whether or not the docking of SPAK to NKCC1 was also required for activation of the cotransporter has never been fully resolved [25]. Indeed, some mutagenesis data performed in our laboratory [25] and confirmed by Dowd and Forbush (personal communication) indicated that,

although SPAK binds to the cotransporter, the binding might not be required for cotransporter activation. Unlike the Na-Cl cotransporter (NCC) or the absorptive Na-K-2Cl cotransporter (NKCC2), NKCC1 contains two putative RFXV binding motifs. Whether both motifs play a role in the regulation of NKCC1 is still unknown. In this study, we demonstrate unequivocally the requirement for kinase binding to the cotransporter, that one binding motif is sufficient to facilitate SPAK activation of the cotransporter, and that the 5' portion of the amino-terminus of the cotransporter is highly flexible, as large internal deletions do not affect NKCC1 function and the distance between the SPAK binding site and the site of phosphorylation can be variable.

In 2002, using mass spectrometry, Darman and Forbush identified three threonine residues within the N-terminal portion of the cotransporter that are phosphorylated after forskolin treatment [26]. These threonine residues, highly conserved in vertebrates, are located at positions 206, 211 and 224 in mouse NKCC1. Based on mutagenesis of each of these residues, they concluded that T²¹¹ was the major phospho-acceptor site involved in cotransporter activation [26]. In contrast to the PKA phosphorylation of the cotransporter as a result of forskolin treatment, Vitari and coworkers used mass spectrometry and mutagenesis studies to identify other threonine residues (T¹⁹⁷, T²⁰¹ and T²⁰⁶) as targets of SPAK phosphorylation [27]. Interestingly, only one residue (T²⁰⁶) is common to both phosphorylation pathways. Neither the phosphoantibody study of Darman and Forbush [26] nor the biochemical study of Vitari and coworkers [27] addressed the functional significance of each threonine phosphorylation. Therefore, we embarked on a detailed mutagenesis study of the six threonine residues (T¹⁹⁷, T¹⁹⁹, T²⁰¹, T²⁰⁶, T²¹¹, and T²²⁴) within the highly conserved 28 amino-acid segment of the mouse amino-terminus of NKCC1 and examined their phosphorylation by SPAK, and determined their functional significance. Our results indicate that T²⁰⁶ and T²¹¹ are essential phospho-regulatory sites for cotransporter function.

Material and Methods

Construction of mouse NKCC1 mutants

In this study, we used the full-length NKCC1 cDNA inserted in the *Xenopus laevis* oocyte expression vector (pBF, [25]) and the N-terminus of the cotransporter in both the GST-fusion protein vector (pGEX4T, [28]) and the yeast GAL4-binding domain vector (pGBDuc2, [22]). The amino-terminus

Fig. 1. Conservation of the N-terminus of vertebrate Na-K-2Cl cotransporters based on amino acid alignment. Red represents identical residues across all species, blue represents a block of identical residues and green represents conserved substitutions. Human (*Homo sapiens* NP_001037), chimpanzee (*Pan troglodytes* XP_526998), cow (*Bos taurus*, NP_777207), mouse (*Mus musculus* NP_033220), rat (*Rattus norvegicus* NP_113986), chicken (*Gallus gallus*, translated from EST:BU457624), frog (*Xenopus laevis*, ABN05233), shark (*Squalus acanthias* P55013), european sea bass (*Dicentrarchus labrax* ABB84251), zebrafish (*Danio rerio* NP_001002080), european eel (*Anguilla anguilla* CAD31112), tilapia (*Oreochromis mossambicus* AAR97731).

of NKCC1 contains two SPAK binding motifs separated by a poly-alanine region. We subcloned from the full-length NKCC1 cDNA, a *NarI-NcoI* fragment into pBluescript, and used unique restriction enzyme sites to selectively delete either: the first SPAK motif (amino acid residues 73 to 90: $\Delta 73-90$), the poly-alanine region between the two motifs (amino acid residues 93 to 114: $\Delta 93-114$), or the second SPAK motif (amino acid residues 116 to 161: $\Delta 116-161$). We also used QuikChange Mutagenesis (Stratagene, La Jolla, CA) with complementary sense and antisense oligonucleotides to mutate the conserved phenylalanine residue in either the first (F79A), second (F135A), or both SPAK (F79A, F135A) binding motifs. Each mutation was verified by DNA sequencing and the *NarI-NcoI* fragment was then re-inserted into the full-length NKCC1 clone in a *Xenopus laevis* oocyte expression vector (pBF), as well as into the amino-terminal portion of NKCC1 in pGEX and pGBDuc2. The six threonine phospho-acceptor sites (T¹⁹⁷, T¹⁹⁹, T²⁰¹, T²⁰⁶, T²¹¹, and T²²⁴) in the amino terminus of the mouse NKCC1 were mutated into alanine residues using complementary sense and antisense oligonucleotides and QuikChange Mutagenesis (Stratagene, LaJolla, CA). After each mutant construct was verified by DNA sequencing, a *CelII-SacI* fragment from each mutant was then re-inserted into the full-length NKCC1 clone in pBF, as well as the amino-terminal portion of NKCC1 in pGEX

and pGBDuc2.

In Vitro Kinase Assays

Wild-type and mutant forms of GST-NKCC1 and wild-type GST-SPAK fusion proteins were combined with 20 mM HEPES (pH 7.4), 2mM MnCl₂, 5 mM dithiothreitol (DTT), 2 μ M cold Na-ATP, and 8 μ Ci [γ -³²P]ATP in a 30 μ l kinase reaction and incubated at 37°C for 45min. Reactions were stopped by adding 30 μ l of 4x SDS sample buffer, followed by denaturing at 70°C for 15min. NKCC1 and SPAK proteins in the kinase reactions were separated by SDS-PAGE on a 9% polyacrylamide gel. Gels were washed 3 times for 10min each in a buffer containing 1% Na-pyrophosphate and 5% trichloroacetic acid, dried at 70°C for 5h, and phosphorylated products were visualized by autoradiography.

Yeast two-hybrid

The cytosolic N-terminal domain (residues 1-278) of wild-type and mutant forms of NKCC1 were inserted in the GAL4 binding domain yeast vector (pGBDuc2), transformed into competent PJ69-4A yeast, and placed on uracil-deficient selection media. Surviving yeast cells containing wild-type or mutated NKCC1 were then re-transformed with the regulatory domain of SPAK inserted in the pACT2 vector. These re-

Fig. 2. Schematic representation of the amino terminus of mouse NKCC1. Sequence of the first 260 amino acids of mouse NKCC1 showing the two SPAK binding motifs (solid underline) and the PP1 binding motif (open underline). The six threonine residue we mutated are indicated in bold font. The threonine residues identified by Darman and Forbush [26] are indicated by solid arrowheads, whereas the threonine residues identified by Vitari et al. are indicated by open arrowheads [27]. Numbered residues define the regions targeted for deletion.

transformed yeast were then plated on double dropout (-uracil, -leucine) plates for measuring transformation efficiency and triple dropout (-uracil, -leucine, -histidine) plates for determining protein-protein interaction. Yeast survival was assessed after 2-4 days at 30°C.

Western blot analysis

Wild-type and mutant GST-NKCC1 fusion proteins (~ 3 µg) were resolved by 9% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 2h at room temperature with 5% non-fat dry milk in TBST (150 mM NaCl, 10 mM Tris HCl, 0.5% Tween 20 [polyoxyethylene-sorbitan monolaurate]). Membranes were then subjected to a polyclonal anti-GST antibody (1:5000) in TBST/5% non-fat dry milk overnight at 4°C, washed extensively in TBST, and incubated for 1h with horseradish peroxidase-conjugated secondary antibody (1:10,000) in TBST/5% non-fat dry milk. After extensive washing, protein bands were visualized by enhanced chemiluminescence (ECL plus, Amersham Biosciences, Piscataway, NJ).

cRNA synthesis

All cDNA clones in pBF were linearized with *Mlu*I and transcribed into cRNA using Ambion's mMESSAGE mMACHINE SP6 transcription system (Ambion, Austin, TX). RNA quality was verified by gel electrophoresis (1% agarose/0.693% formaldehyde), and quantitated by measurement of absorbance at 260 nm.

Isolation of *Xenopus laevis* oocytes

Stages V-VI *Xenopus laevis* oocytes were isolated from 8 different frogs as previously described [24, 25] and maintained

at 16°C in modified L15 medium (Leibovitz's L15 solution diluted with water to a final osmolarity of 195-200 mOsm and supplemented with 10 mM HEPES and 44 µg gentamicin sulphate). Oocytes were injected on day 2 with 50 nl water containing 15 ng NKCC1 cRNA and on day 3 with 50 nl water containing 10 ng of each kinase cRNA. Control oocytes were injected with 50 nl water. ⁸⁶Rb flux measurements were performed on day 5 post-isolation.

K⁺ uptakes in *Xenopus laevis* oocytes

Groups of 20 oocytes in a 35-mm dish were washed once with 3 ml isosmotic saline (96 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES buffered to pH 7.4) and preincubated for 15 min in 1 ml same isosmotic saline containing 1 mM ouabain at room temperature. The solution was then aspirated and replaced with 1 ml isosmotic flux solution containing 5 µCi ⁸⁶Rb. Two -5 µl aliquots of flux solution were sampled at the beginning of each ⁸⁶Rb uptake period and used as standards. After 1h uptake, the radioactive solution was aspirated and the oocytes were washed 4 times with 3 ml ice-cold isosmotic solution. Single oocytes were transferred into glass vials, lysed for 1h with 200 µl 0.25N NaOH, neutralized with 100 µl glacial acetic acid, and ⁸⁶Rb tracer activity was measured by β-scintillation counting. NKCC1 flux is expressed in nanomoles K⁺/oocyte/hour.

Statistical analyses

Differences between ⁸⁶Rb uptake groups were tested by one-way analysis of variance (ANOVA) followed by multiple comparison using Student-Newman-Keuls, Bonferroni, and Tukey post tests. P < 0.001 was considered to be highly significant.

Fig. 3. Deletion of SPAK binding motifs prevents isotonic activation of NKCC1. Functional analysis of NKCC1 deletion mutants through ^{86}Rb (K^+) flux in *Xenopus laevis* oocytes. Wild-type and mutant NKCC1 RNA (15 ng) was injected on day 2, and wild-type SPAK and WNK4 (10 ng) were injected on day 3. K^+ flux was measured on day 5 in isosmotic solutions (open bars), in the presence of SPAK and WNK4 (light grey bars), and hyperosmotic solutions (black bars). Labels describe which portions of the amino terminus of NKCC1 were deleted. Bars represent means $\pm$ standard errors ($n = 20$ oocytes). Asterisk indicate highly significant difference ($p < 0.001$) from wild-type condition. The experiment was repeated twice with identical results.

Results

The cytosolic amino terminal tail of NKCC1, which participates in the modulation of cotransporter activity, contains two R/KFxV/I (SPAK binding) motifs separated by a stretch of fifteen alanine residues [22, 25]. As shown in Figure 1, these two SPAK binding domains and the region encompassing the threonine residues to the end of the cytosolic amino terminus of NKCC1 are highly conserved across vertebrate species from fish to man. Previously, we demonstrated that removal of the region ($\Delta 73$ -161) resulted in a loss of NKCC activation by the kinases SPAK and WNK4 [24]. To determine whether one (or both) binding motifs are necessary for cotransporter activation, we removed sections of the amino terminus of NKCC1 containing the first binding motif, NKCC1($\Delta 73$ -90), the second binding motif, NKCC1($\Delta 116$ -161), and the poly-alanine region between the two motifs, NKCC1($\Delta 93$ -114) (Figure 2). As demonstrated in Figure 3, neither the isosmotic activation by SPAK/WNK4 nor the hyperosmotic stimulation of the cotransporter was affected in oocytes injected with either NKCC1($\Delta 73$ -90) or NKCC1($\Delta 116$ -161). However, SPAK/WNK4 activation in isosmotic conditions was eliminated when both regions were deleted. Interestingly, both isosmotic and hyperosmotic activity of NKCC1($\Delta 73$ -90 + $\Delta 116$ -161) were reduced suggesting the importance

of SPAK-NKCC1 interaction. Because mutation of the phenylalanine residues into an alanine within the RFQV motif prevented protein-protein interaction between SPAK and WNK4 [24], we mutated the phenylalanine residues in both SPAK binding motifs of NKCC1 into alanines. The upper panel of Figure 4A demonstrates how yeast co-transformed with wild-type SPAK and wild-type NKCC1, NKCC1 (F79A), or NKCC1 (F135A) are capable of surviving on an agar selection plate lacking uracil, leucine, and histidine, whereas, yeast co-transformed with wild-type SPAK and NKCC1 (F79A&F135A) are unable to survive. Positive controls for all transformations are shown in the lower panel of Figure 4A. Co-expression of SPAK/WNK4 in *Xenopus laevis* oocytes injected with either NKCC1 (F79A) or NKCC1 (F135A) cRNA did not reduce cotransporter activation or hyperosmotic stimulation. However, simultaneous mutation of both phenylalanine residues (F79A&F135A) resulted in a significant decrease in isotonic K^+ flux and a lack of SPAK/WNK4 activation (Figure 4B).

In a recent study, we demonstrated *in vitro* phosphorylation of NKCC1 by incubating a GST-fusion protein of the first 260 residues on the amino-terminus of the cotransporter (GST-NKCC1) with wild-type GST-SPAK and ^{32}P -ATP [28]. In order to demonstrate the necessity of binding between SPAK and NKCC1, we

Fig. 4. Phenylalanine to alanine mutation in RFxV motif prevents isotonic activation of NKCC1. (A) Yeast transformed with different cDNA clones were plated onto agar media lacking essential nutrients: uracil, leucine, and/or histidine. For each pair, the first clone was fused to the GAL4 activating domain in pACT2, and the second clone was fused to the GAL4 binding domain in pGBDUc2. 1, SPAK (wt) + NKCC1 (wt); 2, SPAK (wt) + NKCC1 (F79A); 3, SPAK (wt) + NKCC1 (F135A); 4, SPAK (wt) + NKCC1 (F79A&F135A). Upper panel, yeast survival exists only upon interaction between the GAL4 fusion proteins translated from each clone. Lower panel, control plate lacking two essential nutrients (uracil and leucine) to demonstrate transformation efficiency. (B) Functional analysis of NKCC1 deficient mutants through ⁸⁶Rb (K⁺) flux in *Xenopus laevis* oocytes. The labels “Water” and “Wild-type” indicate the level of endogenous and heterologously expressed mouse NKCC1 activities, respectively. F79A and F135A labels indicate the phenylalanine to alanine substitutions in the first and second SPAK binding motifs, respectively. Oocytes were incubated in isosmotic (open bars), in the presence of SPAK and WNK4 (light grey bars), and hyperosmotic solutions (black bars). Bars represent means ± standard errors (n = 20 oocytes). Asterisk indicate highly significant difference (p < 0.001) from wild-type condition. The experiment was repeated twice with identical results.

created two additional NKCC1-deletion and -deficient GST-fusion proteins: NKCC1 (Δ73-161) and NKCC1 (F79A&F135A). *In vitro* phosphorylation assay exposing equivalent amounts of each GST-NKCC1 fusion protein to different concentrations of wild-type SPAK demonstrated a significant difference in the degree of ³²P-ATP incorporation between the wild-type cotransporter and both the deletion and deficient mutants (Figure 5).

Using mass-spectrometry, Darman and Forbush demonstrated phosphorylation of residues T¹⁸⁴, T¹⁸⁹, and T²⁰² of shark rectal gland NKCC1 activated by forskolin [26]. Recently Vitari and coworkers, also using mass-spectrometry, showed that SPAK targets residues T¹⁷⁷, T¹⁷⁹, and T¹⁸⁴ [27]. To reconcile these two studies, and assess the functional relevance of phosphorylation of each

Fig. 5. *In vitro* SPAK phosphorylation of wild-type and mutant NKCC1. Quantitation of ³²P phosphorylation of wild-type (WT, squares), SPAK-deletion mutant (Δ73-161, triangles) and SPAK binding deficient mutant NKCC1 (F79A& F135A, circles) as a function of increasing amounts of SPAK. Inset: autoradiography showing ³²P incorporation signals. Kinase assay was repeated twice with identical results.

Fig. 6. *In vitro* ^{32}P incorporation of wild-type and mutant forms of NKCC1. Bar graph represents the ratio between the densitometry of the ^{32}P -ATP incorporation divided by the densitometry of the protein abundance, as determined by Western blot analysis. Each bar represents mean $\pm$ SEM (n = 3-6). Representative autoradiographs of a kinase assay and its complementary Western are shown.

of these threonine residues, we used site-directed mutagenesis to substitute an alanine for each of the corresponding mouse threonine residues (T¹⁹⁷, T²⁰¹, T²⁰⁶, T²¹¹, and T²²⁴) and an additional threonine residue (T¹⁹⁹). With each NKCC1 mutant, we performed *in vitro* ^{32}P phosphorylation assays with wild-type SPAK (Figure 6). Alanine substitution of residues T¹⁹⁹, T²¹¹, and T²²⁴ did not reduce the level of substrate phosphorylation. Mutation of residues T¹⁹⁷ or T²⁰⁶ into alanines resulted in 33% reduction in ^{32}P incorporation individually, and 66% when combined. Although Vitari had shown that T²⁰¹ is a target of SPAK by mass spectrometry [27], we found that alanine substitution of T²⁰¹ did not alter the level of phosphorylation alone or when combined with either T¹⁹⁷, T¹⁹⁹, or T²⁰⁶. However, mutating T²⁰¹ into alanine in conjunction with T¹⁹⁷ and T²⁰⁶ resulted in ~95% reduction in ^{32}P incorporation. Because phosphorylation of a residue does not necessarily translate into a functional effect, we expressed each threonine mutant form of NKCC1 in *Xenopus laevis* oocytes (Figure 7). Alanine substitution of residues T¹⁹⁷, T¹⁹⁹, T²⁰¹, and T²²⁴ did not significantly reduce the level of K⁺ flux in the presence of SPAK and WNK4 (grey bars) or under hyperosmotic conditions (black bars). In contrast, alanine substitution of T²⁰⁶ and T²¹¹ resulted in a non-functional cotransporter, as K⁺ flux

is similar to that of water-injected oocytes [24]. Combination of the T206A mutation with other threonine mutations (such as the triple and quadruple mutants shown in Figure 6) resulted in a similar lack of cotransporter function (data not shown).

Since our *in vitro* phosphorylation experiments showed an ~33% reduction in ^{32}P incorporation following alanine substitution of T¹⁹⁷ and T²⁰⁶, we mutated these two residues singly and together into glutamic acid residues to mimic phosphorylation (Figure 8). Contrary to our expectation that these mutations would yield an activated cotransporter, we found no significant difference in the activity of NKCC1 (T197E) and a loss of cotransporter function with the mutated NKCC1 (T206E). Because alanine substitution of residue T²¹¹ results in a non-functional cotransporter (Figure 7), we replaced T²¹¹ with a serine, a conserved phospho-acceptor residue. In this case, we observed a functional cotransporter under isosmotic and hyperosmotic conditions that could not be activated in the presence of SPAK and WNK4 (Figure 8). Finally, based on the similarity between SPAK and TAO2 (Thousand And One amino acid protein kinase 2), we previously suggested that targets of SPAK phosphorylation required a hydrophobic residue immediately following the site of phosphorylation [28].

Fig. 7. Alanine substitution of specific threonine residues inhibits NKCC1 activity. Amino acid sequence delineates the six threonine residues targeted for alanine substitution. K⁺ flux in *Xenopus laevis* oocytes expressing wild-type and mutant forms of NKCC1 incubated in isosmotic (open bars), in the presence of SPAK and WNK4 (light grey bars), and hyperosmotic solutions (black bars). Labels below bars describe which threonine residues of the amino terminus of NKCC1 were mutated into alanine residues. Bars represent means $\pm$ standard errors (n = 20 oocytes). Diamond indicates a significant difference (p<0.01) from wild-type condition. Asterisks indicate highly significant difference (p < 0.001) from wild-type condition. Experiments were repeated twice with identical results.

Fig. 8. Additional mutagenesis of NKCC1. K⁺ flux in *Xenopus laevis* oocytes expressing wild-type and mutant forms of NKCC1 incubated in isosmotic (open bars), in the presence of SPAK and WNK4 (light grey bars), and hyperosmotic solutions (black bars). Labels below bars describe which residues of the amino terminus of NKCC1 were mutated. Bars represent means $\pm$ standard errors (n = 20 oocytes). Asterisk indicate highly significant difference (p < 0.001) from wild-type condition. Experiments were repeated twice with identical results.

To address this possibility, we targeted the P+1 residues of T²⁰⁶ and T²¹¹ (F²⁰⁷ and M²¹², respectively) for alanine substitution (Figure 8). Consistent with the hypothesis that T²⁰⁶, but not T²¹¹, is a target of SPAK phosphorylation, mutation of residue F²⁰⁷ into an alanine resulted in a non-functional cotransporter, whereas alanine substitution of the hydrophobic methionine immediately following T²¹¹ did not alter cotransporter function.

Discussion

Kinases phosphorylate proteins at specific residues such as serines, threonines and tyrosines. The substrate recognition site generally consists of only a few amino acids and therefore can be found in a large number of

proteins. Protein kinase A (PKA), for instance, is known to phosphorylate serine or threonine residues located two positions downstream of an arginine. As the probability of finding a RXT/S site is approximately 0.005, RXT/S can be found in abundance in many proteins. Substrate recognition is therefore specified by accessory proteins which target the kinase to the substrate. For example, there are more than 50 different A-kinase anchoring proteins or AKAPs [29] which provide mechanisms of specificity for PKA to its individual substrates (Figure 9A, upper panel). The first examples of phospho-

Fig. 9. Schematic models of kinase/substrate interactions and SPAK/WNK4 regulation of NKCC1 activity. (A) upper panel illustrates kinase specificity as determined by both substrate recognition and an accessory anchoring protein (e.g. A-kinase anchoring protein (AKAP)); lower panel depicts SPAK interaction with NKCC1 utilizing a specific docking site separate from the primary site of substrate recognition. (B) NKCC1 is depicted situated in the plasma membrane in three states (inactive, inactive bound to SPAK, and active bound to phosphorylated SPAK). SPAK interacts with either WNK4 or NKCC1 at the putative RxFV motif, but not simultaneously.

regulation of other proteins requiring the direct binding or docking of the enzyme to their substrate, were identified in MAP kinases [30-32]. In 2002, we reported a novel docking site for two mammalian Ste20-related serine/threonine kinases (SPAK and OSR1) on several cation-chloride cotransporters, including two distinct sites on NKCC1 [22]. Our present study demonstrates that these two binding sites are both functional and that only one site is necessary for *in vivo* activation of the cotransporter by SPAK/WNK4. Indeed, removal (Figure 3) or phenylalanine to alanine mutations (Figure 4) of the two sites resulted in the significant loss of cotransporter stimulation. Furthermore, docking of the kinase to the cotransporter markedly enhances *in vitro* substrate phosphorylation by SPAK (Figure 5), indicating that SPAK and NKCC1 participate in a binding equilibrium involving the docking site. The fact that a previously published NKCC1 mutant, which contained alanine substitutions of the phenylalanine and valine residues in the first and

second RxFV motifs, respectively, exhibited no yeast 2-hybrid protein-protein interaction, but still demonstrated activation by SPAK/WNK4 [25], indicates some kinase interaction was likely still occurring *in vivo*. In fact, in a recent paper Vitari and coworkers demonstrated that a peptide carrying the valine to alanine mutation only partially prevented the phosphorylation of GST-NKCC1 by GST-OSR1, indicating the existence of a weak interaction between the kinase and a RFQA motif-containing peptide [27].

Since our truncation data (Figure 3) also show that the physical distance between the RxFV docking site and the substrate phosphorylation site does not matter for cotransporter function, variability in length indicates a high degree of freedom or flexibility of the N-terminal portion of the cotransporter's amino terminus. With the exception of the two SPAK binding domains and the region containing the regulatory threonines, the 5' portion of the N-terminus is very poorly conserved in vertebrate evolution

from shark to man. Clearly, areas of low conservation imply low functional significance, whereas the three highly conserved regions indicate their importance in NKCC1 function. In fact, the conserved region containing the regulatory threonines also participates in the activation of NKCC2 [33] and NCC [34]. Although the reason for the presence of two docking sites in NKCC1 is still unknown, it might be related to the fact that the second motif overlaps with a PP1 binding motif. This overlap suggests a possible competition between the two enzymes, thus the presence of a second SPAK binding domain guarantees interaction of the kinase with the cotransporter.

Two recent studies investigating the phosphorylation of NKCC1 identified different threonine residues as targets of kinase activity [26, 27]. We therefore mutated each of the mouse corresponding threonine residues (T¹⁹⁷, T¹⁹⁹, T²⁰¹, T²⁰⁶, T²¹¹, and T²²⁴) and examined their level of ³²P incorporation and significance to the function of the cotransporter. Consistent with Vitari's findings [27], we observed that mutation of T¹⁹⁷, T²⁰¹, and T²⁰⁶ together resulted in a highly significant reduction in ³²P incorporation. However, some intriguing findings were made with the T201A mutant. Indeed, when mutated alone into an alanine or in conjunction with T197A, T199A, or T206A we did not observe any change in ³²P incorporation relative to wild-type or individual mutations of T197A, T199A, or T206A. Because residue T²⁰¹ was identified as a phospho-acceptor site by mass spectrometry and solid phase Edman sequencing [27], we propose that the single T201A mutation results in the transfer of a phosphate to an adjacent threonine (e.g. T²¹¹), and that event is unable to occur due to modification of peptide structure when residues T¹⁹⁷ and T²⁰⁶ are mutated into alanines.

Four of the six threonine to alanine substitutions in the N-terminus of NKCC1 (T¹⁹⁷, T¹⁹⁹, T²⁰¹, and T²²⁴) did not alter cotransporter function. These results suggest that while each of these residues are targets of phosphorylation by SPAK or a PKA-sensitive kinase, their role in modulation of NKCC1 activity is only marginal. However, alanine substitution of T²⁰⁶ or T²¹¹ resulted in a lack of function, indicating their individual importance to cotransporter activity. Many protein kinases depend on hydrophobic flanking residues immediately following the specific site of substrate phosphorylation (P site) [35]. We have previously identified phospho-acceptor residues T²⁰⁶ and T²¹¹ as having P+1 hydrophobic residues [28]. Mutation of the P+1 residues (F207 and M212) had completely opposite effects on NKCC1 function.

Whereas the F207A mutation resulted in a non-functional cotransporter, similar to the T206A mutant, the M212A mutation did not alter the activity of NKCC1, under isotonic control conditions, co-expressed with SPAK and WNK4, or under hypertonically-stimulated conditions. The effect of these two mutations on cotransporter activity reveals several insights into NKCC1 regulation. Clearly, T²⁰⁶ with its hydrophobic phenylalanine at position 207 is a key target of the P+1 pocket of SPAK, whereas T²¹¹/M²¹² is not. Second, the lack of hyperosmotic stimulation of both T206A and F207A NKCC1 mutants indicate the importance of this P+1 motif to the volume sensitivity of NKCC1. Whether or not the kinase activity of SPAK is directly (or indirectly) involved in the hyperosmotic stimulation remains to be determined. Third, the NKCC1 (T211A) mutation (Figure 7) could indicate that the mutation is structurally detrimental to the function of the cotransporter. In addition, Darman and Forbush have shown that although the glutamic acid substitution of T²¹¹ provides a negative charge, it does not properly mimic the actual phosphorylation of the threonine [26]. Finally, the substitution of T²¹¹ into a serine residue conserves the isosmotic cotransporter function, and reduces both the SPAK/WNK4 stimulation and the hyperosmotic activation by approximately one third, indicating that phosphorylation of T²¹¹ is critical in the regulation of NKCC1.

As we previously demonstrated that the binding of WNK4 to SPAK is required for NKCC1 activation [24], we propose a model in which free cotransporter is in equilibrium with cotransporter binding either inactive or active SPAK at one or both of its docking sites (Figure 9B). Under isosmotic conditions, only a fraction of cotransporters are phosphorylated by SPAK and this fraction accounts for the basal activity of the cotransporter. Whether or not WNK4 is able to form a complex with SPAK and the cotransporter is still under investigation. Earlier, we presented some evidence arguing against the dimerization of SPAK, a mechanism by which WNK4 could be associated to NKCC1 while using identical SPAK interaction domains [28]. In a recent paper, Anselmo and coworkers demonstrated two OSR1 molecules interacting using co-immunoprecipitation, leaving open the possibility that the Ste20 kinases might scaffold WNK4 to the cotransporter [36]. However, in the absence of evidence for a NKCC1-SPAK-WNK4 complex, in the current model, we propose that WNK4 binds and activates SPAK in the cytosol through phosphorylation of its activation loop and residue S³⁸³ in its regulatory domain [37]. When bound to the

cotransporter, at one of the two docking sites, activated SPAK would phosphorylate T¹⁹⁷, T²⁰¹, and T²⁰⁶ at a separate substrate recognition site, leading to activation of the cotransporter (Figure 9A, lower panel).

Taken together, our data demonstrate that SPAK binding (or docking) to the N-terminus of NKCC1 is a pre-requisite for cotransporter activity under both baseline and hyperosmotic conditions. Whereas physical binding is necessary for cotransporter activation, the distance between the docking site and the phosphorylation sites appears to be unimportant. Finally, we identified T²⁰⁶ and T²¹¹ as major phospho-acceptor sites involved in

cotransporter function, with T²⁰⁶ common to two separate regulatory pathways: one involving SPAK, the other involving a still unknown kinase that is responsive to forskolin/PKA stimulation.

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