Regulatory phosphorylation sites in the NH$_2$ terminus of the renal Na-K-Cl cotransporter (NKCC2)

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Giménez, Ignacio, and Biff Forbush. Regulatory phosphorylation sites in the NH$_2$ terminus of the renal Na-K-Cl cotransporter (NKCC2). Am J Physiol Renal Physiol 289: F1341–F1345, 2005. First published August 2, 2005; doi:10.1152/ajprenal.00214.2005.—Short-term regulation of members of the Na-K-Cl cotransporter family takes place by phosphorylation/dephosphorylation events. Three NH$_2$-terminal threonines have been previously identified as phosphoacceptors involved in activation of the ubiquitous/secretory Na-K-Cl cotransporter (NKCC1). In this study, we demonstrate that the corresponding threonines are also involved in the regulation of the renal Na-K-Cl cotransporter (NKCC2). The transport activity of NKCC2, exogenously expressed in *Xenopus laevis* oocytes, is shown to be stimulated by hypertonicity. Mutagenic analysis demonstrated that threonines T99, T104, and T117 comprise a regulatory domain responsible for the activation of NKCC2 in hypertonic solutions: although none of the threonines was found to be individually necessary or sufficient for regulation, the three residues together are required to obtain the full hypertonic response. Under isotonic and hypotonic conditions, NKCC2 retains 50% of its activity in the absence of phosphorylation of the threonine-regulatory domain. Selective deletions of peptide segments revealed only a minor role for the NH$_2$-terminal cytosolic domain of NKCC2 upstream of the threonine regulatory domain, including the recently identified proline alanine-rich Ste-20-related kinase-binding motif. A chimeric NKCC containing the first 104 amino acids of NKCC1 on the NKCC2 backbone behaved essentially the same as NKCC2, further arguing against a major role for this upstream region in NKCC2 regulation.

NKCC2, the kidney-specific Na-K-Cl cotransporter, is a member of the cation-chloride cotransporter superfamily that includes Na-K-Cl, Na-Cl, and K-Cl cotransporters, as well as two subfamilies of proteins of unknown function. In mammals, NKCC2 is exclusively found in the apical membrane of epithelial cells of the thick ascending limb (TAL), where it provides a pathway for the uptake of Na and Cl and is thus involved in the regulation of whole body water and electrolyte content. NKCC2 is expressed as three alternatively spliced variants each of which has different kinetic properties (7) and is found in a distinct region of the renal tubule (13).

Our understanding of the regulation of Na-K-Cl cotransporters at the molecular level is due largely to investigations of NKCC1. The secretory cotransporter has been shown to be regulated by phosphorylation of five to eight serine and threonine residues in the NH$_2$-terminal domain in response to changes in cell volume and intracellular Cl concentration (2, 10, 11). Three of these residues have been identified and studied by mutagenesis (T184, T189, and T202 in the shark NKCC1 sequence), and one of the three is absolutely essential for activity (T189) (2). The activation state of NKCC1 can be measured with a phospho-specific antibody (R5) that detects the phosphorylation state of two of these residues (4). The NKCC1 NH$_2$ terminal also contains a binding site for PP1, the phosphatase that is involved in the regulatory process: removal of this site results in a cotransporter that is much more easily activated compared with wild-type (1). The NH$_2$ terminal also contains a binding site for proline alanine-rich Ste-20-related kinase (PASK) (15). Although this high-affinity-binding site is not necessary for NKCC1 activation (1, 14), overexpression of an inactive PASK mutant effectively blocks NKCC1 regulation, providing compelling evidence that this kinase is involved (3).

Much less is known about the mechanisms of regulation of NKCC2. Expressed in *Xenopus laevis* oocytes, the renal cotransporter has a high basal level of activity and is further activated about twofold by oocyte shrinkage; a potential role of intracellular Cl concentration has not been explored. Although the PP1 binding domain found in NKCC1 is absent from the NH$_2$ terminal of NKCC2, the PASK-binding site is present and the potential phosphoregulatory domain is highly conserved; the threonine residues corresponding to the three identified phosphoacceptors in NKCC1 are all present in NKCC2 (T99, T104, and T117 in the rabbit sequence). Using the R5 antibody, we have recently demonstrated that T99 and T104 become phosphorylated during in vivo activation by vasopressin in the mouse, accompanied as well by some trafficking of NKCC2 to the plasma membrane from an intracellular compartment (6). While this constitutes strong evidence that the NKCC2 phosphoacceptor residues are responsible for activation of this transporter isoform, until now a test of this hypothesis has not been carried out. It has also been unclear whether the high level of basal activity results from a high basal phosphorylation state or whether dephosphorylated NKCC2 may simply be able to function at a high basal rate.

In this study, we analyze the regulatory role of the NH$_2$-terminal cytosolic domain of NKCC2 by mutagenesis and measurement of transport activity in *X. laevis* oocytes. We find that the high basal level of activity is not a consequence of basal phosphorylation of these residues but that the three phosphoacceptor residues together are found to be necessary for the response to hypertonic conditions. In contrast to the situation with NKCC1, where T189 is absolutely essential, no single residue in NKCC2 is by itself responsible; rather, the three together appear to be required.

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MATERIALS AND METHODS

Oocyte expression system. Defolliculated stage IV-VI X. laevis oocytes were procured as previously described (7). X. laevis toads were kept in Yale University facilities, and surgery was performed in conformity with institutional regulations (Yale University Animal Care and Use Committee). cRNA was synthesized using T7 RNA polymerase (mMessage-mMachine kit, Ambion) from linearized cDNA templates. About 70–90 ng RNA/oocyte were injected. Injected oocytes were kept for 3 days in ND-96 (in mM): 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4, supplemented with penicillin-streptomycin at 17°C.

Mutagenesis. All cDNA constructs employed in the present work were derived from wild-type cDNAs for rabbit NKCC2a and human NKCC1 cloned into an oocyte expression vector. Substitutions of single amino acids were achieved using PCR-based mutagenesis reactions (QuickChange, Stratagene). Substitutions were confirmed by sequencing a 600-bp region encompassing the mutated region. End-point mutants containing multiple substitutions were fully sequenced to rule out polymerase errors in transcription that could have resulted in amino acid substitutions outside of the targeted region. Selective deletions were obtained using PCR and primers designed to exclude the targeted sequence. Deletions were confirmed by extensive sequencing. A NKCC2/NKCC1 chimera containing the first 100 (Ncol at bp 814) amino acids of the human NKCC1 NH₂ terminus had been previously produced using a common Ncol site. Its function and regulation have been characterized in HEK cells (9).

Functional tests. We use ⁸⁶Rb uptake assays to measure the activity of NKCC2 expressed in X. laevis oocytes. All fluxes are carried out in a basic flux solution containing (in mM) 78 Na, 83 Cl, 2 K, 1 Ca, 1 Mg, 1 SO₄, 1 HPO₄, 5 HEPES, pH 7.4, and 0.1 ouabain (160 mosmol/kgH₂O) and performed at room temperature. We have shown before that under these conditions, 95–99% of the Rb uptake is bumetanide sensitive (7).

To increase the osmolarity of the flux solution while maintaining the ionic strength and ion concentrations constant, we added sucrose. On the day of the experiment, oocytes were allowed to equilibrate to room temperature, were then washed in basic flux solution, and then transferred to the uptake medium containing 20 μCi ⁸⁶Rb/ml. Isotope influx took place for the time specified (see figure legends), and was terminated by washing the oocytes in ice-cold flux medium containing 250 μM bumetanide. All solution changes took place by transferring the oocytes from one well to another in a 48-well plate, using a Pasteur pipette.

Sequential fluxes. Ion flux experiments in oocytes are subject to substantial variability not only between frogs but also between individual cells. We devised a simple method to overcome this problem and at the same time increase the yield of experiments per frog surgery. In these experiments (see Figs. 3 and 4), six to seven oocytes injected with the tested cRNA were sequentially fluxed in three solutions of different tonicities. The osmolalities in these solutions were chosen to cover the linear range of stimulation of NKCC2: 160, 260, and 400 mosmol/kgH₂O. Following each incubation in isotope-containing flux solutions of different tonicities. The osmolalities in these solutions were chosen to cover the linear range of stimulation of NKCC2: 160, 260, and 400 mosmol/kgH₂O. Following each incubation in isotope-containing flux solution while maintaining the ionic strength and ion concentrations constant, we added sucrose. On the day of the experiment, oocytes were allowed to equilibrate to room temperature, were then washed in basic flux solution, and then transferred to the uptake medium containing 20 μCi ⁸⁶Rb/ml. Isotope influx took place for the time specified (see figure legends), and was terminated by washing the oocytes in ice-cold flux medium containing 250 μM bumetanide. All solution changes took place by transferring the oocytes from one well to another in a 48-well plate, using a Pasteur pipette.

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Western blot analysis. For protein analysis purposes, oocytes were incubated in the experimental solutions for the indicated times and immediately homogenized in ice-cold anti-phosphatase solution (in mM): 15 NaCl, 30 NaF, 5 EDTA, 15 Na₂HPO₄, 15 pyrophosphate, and 20 HEPES, pH 7.2, with 1% Triton X-100 and a protease inhibitor cocktail added, using a few strokes with a 200-μl pipette tip. The homogenate was cleared by centrifugation, and the supernatants were saved for Western blot analysis. Typically, we lysed four oocytes in 80 μl of solution and loaded 6 μl of that homogenate/SDS-PAGE lane. We used the previously characterized T4 (12) and R5 (4) antibodies to detect NKCC and phosphorylated NKCC, respectively. Both antibodies were raised against NKCC1 but have been shown to recognize the renal isoform (4, 6, 12).

RESULTS

Response of NKCC isoforms to changes in medium osmolality. To study the role of the NH₂-terminal domain in regulation of NKCCs, we injected appropriate cRNAs in X. laevis oocytes and examined the dependence of ion transport activity on extracellular osmolality. As illustrated in Fig. 1A, NKCC1 and NKCC2 have quite different activation profiles. hNKCC1, as well as the endogenous X. laevis xNKCC1, is almost completely inactive at osmolarities below 200 mosmol/kgH₂O (isotonic for X. laevis) and exhibits steep activation above this osmolality, reaching maximal transport capacity at ~260 mosmol/kgH₂O. On the other hand, NKCC2-mediated influx is...
substantial under hypotonic conditions and remains relatively unchanged in isotonic medium and even in slightly hypertonic medium (230 mosmol/kgH2O). Above this point, medium osmolarity causes stimulation of ion transport, with a maximum between 300 and 400 mosmol/kgH2O. Thus, similar to previous reports (9), NKCC2 and NKCC1 have distinctly different behaviors with regard to their activation in media of various tonicities.

Three alternative splice variants of NKCC2 are deployed in segments of the TAL exposed to different osmolarities: variant F is exclusively present in the hypertonic medulla, whereas variant B is only found in the relatively hypotonic cortex; variant A is expressed in both medullary and cortical TAL cells (8). The three splice variants differ in a short stretch of amino acids, including the second transmembrane domain, which has been shown to be involved in ion binding and translocation (7, 16). As shown in Fig. 1B, the activation profile was similar for all three isoforms; we found a somewhat higher basal flux for NKCCF, in contrast to what has been previously reported with NKCC2 from the shark (5) and mouse (16). We have used NKCC2A for the balance of the experiments reported here.

We have previously reported the utilization of a phosphorylation-specific antibody to monitor two threonines residues in the NH2 terminus of NKCC1, whose phosphorylation state appears to determine the activation of the transporter (4). This phosphorylation domain is highly conserved in NKCC2, and we have reported increased phosphorylation of NKCC2 during vasopressin-induced activation of TAL transport (6). As illustrated in Fig. 2, the level of phosphorylation of both NKCC1 and NKCC2 is also modulated during activation of the cotransporter in response to cell shrinkage in oocytes.

Role of threonines T99, T104, and T117 in NKCC2 activation. We have previously identified three threonine residues in the NH2 terminus of NKCC1 whose phosphorylation appears to be the primary regulator of NKCC1 activity (T184, T189, and T202 in shark NKCC1); other evidence suggests that up to five more residues in this region of NKCC1 may also be phosphorylated, with lesser regulatory impact. To test whether the corresponding residues in NKCC2 (T99, T104, and T117) are also important for the regulatory response, we mutated each of the NKCC2 residues to alanine, individually and together, and we have reported increased phosphorylation of NKCC2 during vasopressin-induced activation of TAL transport (6). As illustrated in Fig. 2, the level of phosphorylation of both NKCC1 and NKCC2 is also modulated during activation of the cotransporter in response to cell shrinkage in oocytes.

As illustrated in Fig. 3A, the combined mutation of all three potential phosphoacceptor residues fully abolished the regulatory fraction of cotransport activity that is stimulated by incubation in hypertonic media. Surprisingly, no single threonine was found to be necessary for the activation of NKCC2, unlike the situation with NKCC1 where T189 is absolutely necessary (2). Single and double mutants exhibited a reduced response, which was statistically significant for T99A, T104A, and T104A/T117A. These results indicate that the same mechanism or a homologous mechanism is at work in hypertonic activation of NKCC2 as for NKCC1, in that the three corresponding phosphoacceptors are critically important in both isoforms.

In these experiments, a similar level of cotransporter-specific flux was observed for all of the mutants in isotonic media (data not shown). This was true as well for the triple alanine-threonine mutation, indicating that to a first approximation these residues are not essential for the baseline NKCC2 flux. We did not, however, carry out a parallel measurement of NKCC2 expression for each experiment, so we cannot rule out the possibility that there are some quantitative differences among the mutants.

We analyzed the phosphorylation site mutants for reactivity with the phospho-specific antibody R5, as illustrated by the Western blot analysis in Fig. 3B. In each mutant with a detectable R5 signal, phosphorylation was seen in hypertonic but not isotonic medium, consistent with the result in Fig. 2. For the mutant (T117A) retaining both of the immunoreactive phosphothreonines (T99 and T104), the signal was as strong as for the wild-type, showing that phosphorylation of T117 is not necessary for phosphorylation of the other two residues to occur. For constructs missing T104 (T104A and T104A/T117A), the R5 signal was greatly reduced, and for constructs without T99 (T99A and T99A/T117A) no R5 reactivity was detected. This indicates that the R5 antibody has much greater sensitivity for the diphosphorylated NKCC2 sequence than for a single phosphorylation; a similar but less dramatic difference was noted previously for NKCC1 (4). An alternative explanation would be that T99 phosphorylation is necessary to achieve phosphorylation of T104; however, this does not seem to be the
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regulated flux (Fig. 3).

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regulated flux (Fig. 3A).

Role of upstream regions in the regulation of NKCC2 by osmolarity. To further explore the regulatory importance of the NH2-terminal cystolic domain of NKCC2, we analyzed mutants possessing various regional deletions (Fig. 4). We focused on the region NH2 terminal to T99, which, with the exception of the PASK binding motif, exhibits very low homology between NKCC2 and NKCC1.

All of the mutants in Fig. 4A, except for the Δ4 mutant, exhibited similar fluxes under isotonic conditions (not shown). Oocytes injected with the Δ4 mutant were not significantly different from those of water-injected oocytes.

Figure 4B summarizes the flux data from five experiments, illustrating the fractional increase on exposure to hypertonic media. None of the deletions abrogates the hypertonicity-induced regulatory activation, demonstrating that this region of the NH2 terminus is not necessary for activation of NKCC2. There is some loss of regulatory response, particularly at 260 mosmol/kgH2O, with the Δ2a (P = 0.172) and Δ2c mutants (P = 0.045); this region was inspected in three segments after preliminary experiments (results not shown) showed decreased regulation in a larger Δ2 deletion (Y48–Y78). This result suggests that, as has been found for NKCC1(2), phosphorylated residues upstream of the three identified phosphoacceptors may partially contribute to the regulatory response, although they are not by themselves sufficient to mediate the response.

The Δ1 construct (L3–L57) is particularly interesting because it eliminates the PASK-binding site identified by Piechotta et al. (15). We have shown that the activity of PASK is essential for upregulation of NKCC1 (3) and NKCC2 (Gimenez I, Dowd B, and Forbush B, unpublished observations). Hence it is quite surprising that the Δ1 construct has a significantly increased upregulation (P = 0.021) in hypertonic media compared with the wild-type NKCC2. This extends the paradox of the PASK-binding site; i.e., it has been previously shown that deletion or mutation of the corresponding site in NKCC1 has little or no effect on regulation of that isoform (1, 3, 14) (Dowd B, Gimenez I, and Forbush B, unpublished observations) despite the demonstrated role of PASK.

Functional differences between NKCC1 and NKCC2 do not rely on different NH2-terminal sequences. As illustrated above (Fig. 1), there are striking differences between the activation curves for NKCC1 and NKCC2, NKCC2 being active in isotonic media and responding less steeply to a hypertonic stimulus. To determine whether these differences could be explained by binding sites or structure in the NH2 terminus, we revisited a construct (h1r2A0.7) in which much of the NH2 terminus of NKCC2 (104 aa) is substituted with the NH2 terminus from NKCC1 (Fig. 5A). As observed previously in HEK-293 cells (9), the h1r2A0.7 chimera exhibits robust flux activity in isotonic media (actually higher than that of NKCC2, P < 0.05), unlike NKCC1. In addition, we see here that h1r2A0.7 has an activation profile very similar to NKCC2. This result clearly demonstrates that the region NH2 terminal to the critical phosphorylated residues does not contain the determinants of the NKCC1-NKCC2 regulatory differences.

DISCUSSION

In this study, we examine the role of the NH2-terminal cytosolic domain in the regulation of NKCC2, the renal isoform of the Na-K-Cl cotransporter. We demonstrate that, when expressed in X. laevis oocytes, NKCC2-mediated transport rates can be activated by increasing medium osmolarity and that this stimulation is mediated by phosphorylation of three threonines located in the NH2-terminal cytosolic domain. These threonines have been previously identified as key elements in the regulation of NKCC1, the secretory isoform of the Na-K-Cl cotransporters; among these three, one (T189 in sNKCC1) is absolutely essential and the other two play modulatory roles (2). As discussed by Darman and Forbush (2), at least three to four additional yet-unidentified phosphoacceptors in the NH2 terminus of NKCC1 are not essential but may further modulate the response.

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Fig. 4. Functional analysis of deletions in the NH2 terminus of NKCC2. A: diagram illustrating the positions of the amino acids deleted in each construct. The proline alanine-rich Ste-20-related kinase (PASK)-binding site and threonines T99, T104, and T117 are noted. B: activation of 86Rb influx expressed as the ratio of flux at 400 mosmol/kgH2O to that at 160 mosmol/kgH2O in wild-type NKCC2 and deletion constructs (n > 4; *P < 0.05 denotes the difference between wild-type and mutant). Data are not shown for the Δ4 mutant, for which fluxes were not different from those of water-injected oocytes.

Fig. 5. Analysis of an NKCC1/NKCC2 chimera (h1r2A0.7). A: chimera replaces the first 104 amino acids of NKCC2 with an NKCC1 sequence. B: osmolarity curves for 86Rb influx in water-injected oocytes (xNKCC1) or oocytes injected with hNKCC1, rbNKCC2, or h1r2A0.7 (n = 5).
A major difference between NKCC1 and NKCC2 uncovered in the present study is that no single residue among the critical threonines was found to be necessary for the activity of NKCC2. This is in marked contrast to the results obtained for NKCC1, where replacements in T189 render the protein completely inactive. Not only is the NKCC2 protein able to transport when each of the threonines is replaced independently, the stimulation by hypertonicity is maintained as well. Only when at least two of the three threonines were replaced did we observe a significant change in the response to hypertonicity.

On the other hand, the conformational change that activates the protein in response to hypertonic stimulation requires the presence of at least one of the three threonines. Our data do not lead to a model of additive interaction among the threonines; rather, there seems to be some degree of redundancy. Among the combinations tested, T104 and T117 are apparently the most relevant in the regulatory process. Phosphorylation of NKCC2 threonines must result in conformational changes and probably in changes in domain interactions, perhaps between the two cytosolic domains or between the NH₂ terminus and the short intracellular loops of the transmembrane domain. Our results indicate that in NKCC2 these changes can be induced by phosphorylation of any of the three studied threonine residues in the NH₂ terminus.

The present experiments indicate that the region NH₂ terminal of the three-threonine region in NKCC2 is of lesser importance. There is some effect of deletion of residues within Y48–Y78 (Δ2), suggesting that there may be additional modulatory phosphoacceptors there, but they are clearly not essential and do not play a large role in hypertonicity-induced activation. Interestingly, the PASK-binding domain is not required, as its deletion actually increases the regulated fraction of transport. This would be a very surprising result were it not already known that, paradoxically, the PASK site in the NH₂ terminus of NKCC1 has very little effect on regulation (1, 14) (Dowd B, Gimenez I, and Forbush B, unpublished observations), despite clear evidence that PASK activity is required for phosphorylation of NKCC1 (3).

NKCC2 differs markedly from NKCC1 in having a high basal rate of transport. We previously proposed that this difference may be caused by the occurrence of a PPI-binding site in NKCC1 that is absent in NKCC2. The presence of this site results in a shift to a lower level of NKCC1 phosphorylation under any given condition and thus a shift in the activation profile. It may be noted, however, that our own data were not in strong support of our hypothesis, because none of the PPI-site mutants exhibited high levels of transport under basal conditions. The results in Fig. 5 clearly demonstrate that the absence of a PPI-binding site from the NKCC2 sequence is clearly not the key to high constitutive activity, because chimeric replacement of the NKCC2 NH₂ terminus with that of NKCC1 retains the high basal flux level. Indeed, the results shown here make it clear that this NKCC1-NKCC2 difference is not conferred by residues upstream of the three-threonine region.

In the present study, we have confirmed the universality of the NH₂-terminal phosphorylation sites that determine the activation state of Na-K-Cl cotransporters. Phosphorylation of the three threonines previously identified in studies on NKCC1 regulation is shown to play a critical role in the activation of NKCC2. Quite possibly, these residues are also necessary for activation of the Na-CI cotransporter (NCC), because the corresponding region of that transporter is highly homologous to the NKCCs. We also investigated the role in NKCC2 regulation of the region in the NH₂-terminal cytosolic domain NH₂ terminal to the three-threonine region and found that it does do not appear to contain important regulatory domains. In addition, we found that this upstream region is not responsible for the marked differences between basal activation levels of NKCC1 and NKCC2.

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