Modulation of Ion Transport by Direct Targeting of Protein Phosphatase Type 1 to the Na-K-Cl Cotransporter*

Rachel B. Darman‡, Andreas Flemmer‡, and Biff Forbush¶

From the Department of Cellular and Molecular Physiology, Yale University, New Haven, Connecticut 06510 and The Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672

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The specificity of major protein phosphatases is conferred via targeting subunits, each of which binds specifically to the phosphatase and targets it to the vicinity of substrate proteins. In the case of protein phosphatase 1 (PP1), an RVxFXD motif on a targeting subunit binds to a cleft in PP1c, the catalytic subunit. Here we report that a substrate of PP1, the Na-K-Cl cotransporter (NKCC1), bears this motif in its N terminus near sites of regulatory phosphorylation and that direct binding of PP1 to NKCC1 is functionally important in determining the set point for intracellular chloride regulation. NKCC1 mutants in which the motif is destroyed or improved exhibit dramatically shifted activation curves because of a change in the rate of cotransporter dephosphorylation. Furthermore, direct interaction of NKCC1 and PP1c observed by coprecipitation of the two proteins is not seen in a mutant lacking the site. This establishes a new paradigm of phosphatase specificity, one in which a substrate protein containing an RVxFXD motif binds directly to PP1c; we propose that this may be a quite general mechanism.

A small number of protein phosphatases is responsible for dephosphorylation of the majority of cellular phosphoproteins (1, 2). The specificity of these otherwise promiscuous enzymes is conferred by targeting subunits, each of which binds specifically to the phosphatase and targets it to the vicinity of substrate proteins. For protein phosphatase 1 (PP1), a cleft in the catalytic subunit (PP1c) binds proteins that contain the consensus motif, RVxFXD, illustrated both in a direct examination of the molecular structure (3) and by the results of peptide panning experiments (4). The present study began with the observation that the N terminus of the Na-K-Cl cotransporter (NKCC1) contains a highly conserved region with the sequence RNVFVD (residues 140–145 in human NKCC1, 107–112 in shark NKCC1), posing the possibility that the cotransporter is directly targeted as a phosphatase substrate.

The Na-K-Cl cotransporter is an integral membrane protein constituting a major regulated pathway for coupled inward movement of Na⁺, K⁺, and Cl⁻ in many cell types. The cotransporter is a central element in electrolyte movement by secretory epithelia, where it functions in concert with cystic fibrosis transmembrane conductance regulator, potassium channels, and the sodium pump to bring about transcellular chloride transport (5, 6). Cotransport activity is fully controlled by direct phosphorylation of the NKCC1 protein (7–9) in response to decreases in cell volume or intracellular [Cl⁻]. Phosphorylation of NKCC1 is mediated by a kinase whose identity is still unknown, but dephosphorylation appears to occur through the action of protein phosphatase 1 (10). The phoshoregulatory region, including three identified threonine phosphoacceptors, is in the cytoplasmic N terminus of the NKCC1 (7, 11–13). Functional characteristics of cotransporter regulation are highly conserved among vertebrates from shark to human (14), consistent with conservation of the identified phosphoacceptor residues and of the RVxFD motif, which we examine here.

In this study we directly examine the hypothesis that the RNVFVD sequence in the N terminus of NKCC1 forms a functionally important binding site for PP1c. It is found that mutations at this locus dramatically alter the activation curve for Na-K-Cl cotransport, consistent with a 10-fold decrease in the rate of cotransporter dephosphorylation. On the other hand a change that “improves” the putative PP1 binding site exhibits the converse behavior, consistent with a 4-fold increase in the rate of NKCC1 dephosphorylation. In transfected HEK cells the functional consequence of removing the PP1 binding site is that the intracellular chloride concentration is maintained at a level 35% above that with wild type NKCC1, supporting the idea that a major role of the Na-K-Cl cotransporter is the regulation of [Cl⁻]. Finally, it is demonstrated through coprecipitation experiments that PP1 does indeed bind to the NKCC1 protein. Because the RVxFD motif is found in a large number of cytosolic and membrane proteins, we propose that this new paradigm of phosphatase targeting may be of very broad importance in cellular regulation.

Experimental Procedures

NKCC1 in HEK Cells—Mutants of shark NKCC1 were prepared by the Kunkel method of single-stranded mutagenesis, as described previously (15); details are available on request. Stable transfectants were prepared in HEK-293 cells by calcium phosphate precipitation and positive selection with G418 (15). All lines were maintained in culture previously (15); details are available on request. Stable transfectants were prepared in HEK-293 cells by calcium phosphate precipitation and positive selection with G418 (15). All lines were maintained in culture in 100 μM furosemide, a rapidly reversible NKCC inhibitor (16). NKCC1 activation was achieved in these cells by 1 h of incubation in hypotonic medium containing 1.5 mM chloride (17) or in the experiments represented by Fig. 2, by preincubation in media containing various [Cl⁻] (18) and furosemide. Intracellular [Cl⁻] and cell volume were determined as type 1 and 2; PP1c, catalytic subunit of protein 1; NKCC1 and NKCC2, isoforms of the Na-K-Cl cotransporter; DTT, dithiothreitol; HEK, human embryonic kidney.
Coprecipitation experiments were performed by incubating solutions of epithelial tubules from the salt-secreting rectal gland of the dogfish shark were used in the experiments represented by Fig. 1. The rectal gland is a rich source of NKCC1 and has been utilized extensively as a model chloride-secreting epithelium (7, 19–21). As described previously (21), tubules were liberated from thin slices of the gland by incubation in collagenase and mechanical agitation and isolated by low speed differential centrifugation. NKCC1 was activated in these cells by 10 min of incubation in 50 µM forskolin, which causes the opening of chloride channels and rapid loss of cellular chloride (20).

**Dephosphorylation of NKCC1—Solubilized activated NKCC1** was obtained from transfected HEK cells (or rectal gland tubule cells; see parentheses) by addition of 0.1% Triton, 20 mM Tris-Cl (or 2% Triton, 20 mM Na-HEPES), pH 7.5, at −2°C (or 4°C) Cytochort, and agitation for 30 s (and centrifugation in a microfuge to remove debris). This solution was then diluted 11-fold (or 50-fold) in 20 mM Tris-Cl (or Na-HEPES); aliquots were incubated at 20°C with (see Fig. 1) or without (see Fig. 3) appropriate concentrations of phosphate inhibitors for 0–40 min, during which period dephosphorylation of NKCC1 occurred. Dephosphorylation was terminated at appropriate time points by addition of one-half volume of 1 M H3PO4 (or SDS transfer buffer containing 0.5 µM calyculin A), and 40-µl aliquots were dot-blotted (Milliblot-D; Millipore) in 300 µL of transfer buffer. The level of cotransporter phosphorylation at Thr184/Thr189 was determined on these blots using the anti-P-NKCC1 antibody (22) and standard Western blotting techniques using chemiluminescence detected with a cooled CCD camera.

**Transport Assay:**—Rb influxes were performed as described previously (15, 17). Transfected HEK cells were preincubated in media of various external [Cl] (with gluconate replacement) for 1 h to activate the cotransporter. Furosemide (50 µM) was included in most conditions to prevent the cotransporter from affecting cellular electrolyte composition. Maximal activation was obtained by including 0.5 µM calyculin A in the low chloride medium for the last 10 min of the preincubation.

Minimal activation was obtained by pre-incubating in 15 mM potassium RbCl (20), 1 mM CaCl2, 1 mM MgCl2, 1 mM NaH2PO4, 1 mM SO4, 0.1 mM oshain, 15 mM Na-HEPES, pH 7.4. Cells were then washed three times, and 40-µl aliquots were imaged using confocal microscopy and the Epi-Photomager screen(Molecular Dynamics). As shown previously (15, 17, 18) and confirmed in control experiments, >90% of the Rb influxes reported here are due to NKCC1 and are inhibitable by bumetaneide.

**Modeling Phosphorylation and Dephosphorylation—**To compare mutant and wild type NKCC1 (see Fig. 2), we assume the following simplified model of phosphorylation and dephosphorylation in the steady state: A · k_{dephos} = (1 - A) · k_{phos}, where A is the fraction of active transporter, k_{dephos} is the rate constant of dephosphorylation, and k_{phos} is the rate constant of phosphorylation. We further assume that under any given experimental condition, k_{phos} is the same for each of our NKCC1 constructs but that k_{dephos} varies among them. Then by combining these relationships for mutant and wild types and rearranging, we have A_{mutant} = A_{wt} / (1 + [inhibitor]) where A_{mutant} and A_{wt} are fractional activities, and the ratio of A_{mutant}/A_{wt} is the ratio of k_{dephos} for mutant to wild type.

**Precipitation with Microcystin Beads—**The microcystin LR (Calbiochem) affinity matrix was prepared using the method of Moorhead et al. (23). Coprecipitation experiments were performed by incubating solubilized activated NKCC1 from HEK cells (see above) with microcystin beads (typically 0.2 mg of cell protein in 300 µL of 1% Triton, 100 mM Tris-Cl with 5 µL of packed beads) for 15 min at 20°C with and without preincubination with 4 µM microcystin. The beads were washed rapidly twice each with 0.35 M NaCl in phosphate-buffered saline and with that solution diluted 10-fold, and proteins were eluted in boiling SDS sample buffer with 40 mM DTT. In some experiments the cross-linking agent, dithiobis(succinimidyl propionate), was added for the last 10 min before lysis, potential complexes being dissociated with DTT during elution. As seen in Fig. 5, the cross-linking procedure had no detectable effect on the outcome of the experiments.

**Antibodies and Blotting—**Shark NKCC1 was detected with J4 and J7 antibodies (24); J4 recognizes an epitope in the N terminus, and J7 recognizes an epitope in the C terminus of NKCC1. Incidentally, these studies demonstrated that the RXFVD sequence comprises part or all of the epitope for the J3 monoclonal antibody (24); none of the mutants in this region were positive with J3. Cotransporter phosphorylation was determined with an anti-P-NKCC1, a polyclonal antibody raised to a peptide containing phosphorylated Thr184 and Thr189, this antibody recognizes phosphorylated versus non-phosphorylated NKCC1 with greater than 25:1 discrimination; it is linear in response compared with 32P incorporation (22). PP1c was detected with a commercial monoclonal antibody (P35220; Transduction Laboratories).

**RESULTS AND DISCUSSION**

Shark and human NKCC1 are 74% identical in overall amino acid sequence, and the regions containing the regulatory phosphoacceptors (shark 107–202) and the putative PP1 binding site (shark 107–112) are almost fully conserved between the two species (95 and 100% identity, respectively). In addition the properties of regulation of shark and human NKCC1s by [Cl], appear virtually identical in transfected HEK cells (14).

We have chosen to study shark NKCC1 because of the availability of superior antibody probes for this species and because of the ability to conduct studies in both the cell culture model and in the intact organ, the salt-secreting rectal gland of the shark.

That PP1 is responsible for regulatory dephosphorylation is suggested by previous studies of the relative effects of calyculin A and okadaic acid on Na-K-Cl co-transport (25) and NKCC1 activation (20). To further examine this relationship, we have followed the dephosphorylation of the Na-K-Cl cotransporter in solubilized cell extracts and compared the effectiveness of a number of inhibitors of PP1 and PP2A (Fig. 1). In each case inhibition of NKCC1 dephosphorylation has a K_i consistent with the action of PP1, but for most inhibitors the results are well outside the reported range for PP2A. Together these results are very strongly supportive of PP1 as the phosphatase involved in NKCC1 regulation.

To study the regulatory importance of the putative PP1 binding site in NKCC1, we prepared mutations of NKCC1 including alanine substitution throughout the RXFVFD motif (RANF, RVNA, AANA) the replacement of the NKCC1 sequence with the homologous sequence from NKKC2, deletion of the N terminus before G153, and replacement of the NKCC1 sequence with the sequence of a known PP1c binding peptide. Activation of the stably transfected NKCC1 mutants was accomplished by preincubation in media with a range of extra-
cellular Cl concentrations, because activity of NKCC1 is controlled via phosphorylation in response to changes in cell volume and [Cl] (18, 20). The rapidly reversible cotransporter inhibitor furosemide was included during the preincubation period to eliminate any effects of NKCC1 on cellular electrolyte balance. Fig. 2 illustrates the changes in intracellular [Cl] and volume that are brought about by these experimental preincubations. As illustrated in Fig. 2, a and b, the results of these 86Rb influx experiments demonstrated a striking shift in the activation curve of NKCC1 in each of the alanine-substituted mutants and in the N-terminal deletion. The shift is in the direction of higher NKCC activity at all Cl concentrations, consistent with a higher level of phosphorylation. The change is well fit by assuming a 10-fold decrease in the rate of dephosphorylation in a simple phosphorylation-dephosphorylation model (Fig. 2, lines).

Clearly, destruction of the RVXFD motif produces an activating shift in cotransporter regulation, consistent with a decrease in the ability of PP1 to dephosphorylate the target protein. Noting that the NKCC sequence might be “improved” relative to other RVXFD peptides, we tested the sequence KRVRFE of a known PP1c binding peptide (4). This sequence has additional basic residues at −2 and +1 and an additional acidic residue at +3 (numbering relative to valine), each of which is expected to improve the binding. Indeed this mutant proved to be extremely difficult to activate, maximum flux being attained only in the presence of the phosphatase inhibitor calyculin A. This is consistent with an increased rate of

Fig. 2. Regulation of NKCC1 function by [Cl] depends on the RVXFD site. HEK cells transfected with various NKCC1 constructs accumulated 86Rb in a 1-min flux assay after preincubation at different Cl concentrations. a, phosphorimage of 86Rb influx experiments on 96-well plates, illustrating raw data from one experiment. Each line was assayed in triplicate lanes, as indicated. b, 86Rb influx evaluated in a series of experiments similar to that in a. Values are the means ± standard errors (n = 4, except n = 3 for trunc153), obtained after normalization of each line to its maximal flux in an experiment. The line through the points for wild type NKCC1 is drawn by eye; the upper line is calculated assuming a 10-fold lower dephosphorylation rate (see “Experimental Procedures”; Φ = 0.1); the lower line is calculated assuming a 4-fold higher rate compared with wild type, c, cell [Cl] and cell volume after preincubation in the media containing various Cl concentrations. Points show means of triplicates in one experiment; four other experiments gave similar results. Lines are drawn by eye.

Fig. 3. Intracellular [Cl] and cell volume in HEK cells expressing RVXFD mutants and wild type NKCC1. Results are averages and standard errors from six experiments. [Cl], in AANA and RANF are significantly different from [Cl] in wild type and KRVRFE (p < 0.001); other differences are not significant (p > 0.05).

Fig. 4. Dephosphorylation of wild type NKCC1 and of RVXFD mutants. The values shown are duplicate assays in a single 96-well experiment. Similar results were obtained in six other experiments. The curves are least squares double exponential fits to the points (k₁ = 1.5 s⁻¹ for all, k₂ = 0.005, 0.005, 0.006, and 0.026 s⁻¹ for the three mutants and wild type, respectively. NKCC1, shark NKCC1.

Fig. 5. Coprecipitation of NKCC1 with PP1c. The efficiency of the method is illustrated by removal of PP1c from the bead supernatants (a) and release in the eluates (b), detected with anti-PP1 antibody. Precipitation is blocked by preincubation with microcystin (+ samples). c and d, coprecipitation of NKCC1 with PP1c, detection with anti-NKCC1 antibody. Results are shown from two experiments with duplicate samples, illustrative of four experiments without cross-linker (c) and five with cross-linker (d); we found no effect of cross-linker.
dephosphorylation, and the data can be modeled by a 4-fold higher rate compared with control (Fig. 2b, lower line).

The RVXFD site is 100% conserved in NKCC1 among various vertebrate species from shark to human, but the corresponding region of the renal form, NKCC2, (RISFRP) has RP instead of a favorable downstream acidic residue. Indeed, when the NKCC1 site was replaced with the NKCC2 sequence, we found that this mutant behaved similarly to others in which the RVXFD site was destroyed; regulation was shifted in favor of activation at all Cl concentrations (Fig. 2). This result must explain, at least in part, the fact that NKCC2 is constitutively more active than NKCC1 both in its native location in the renal tubule (26) and when it is expressed in Xenopus oocytes (27).

An interesting aspect of the RVXFD mutations is that they do not have all or none functional consequences. We expected that our mutants might be fully active under all conditions, but instead loss of the motif resulted in a cotransporter that is 10-fold more readily activated compared with controls. Thus the interaction appears to have evolved to fine tune the volume and Cl, regulatory system, effectively adjusting the set point to a lower cell volume and lower [Cl]. The physiological consequence of this change is illustrated in Fig. 3, where resting [Cl] in transfected HEK cells is seen to be strongly determined by the nature of the NKCC1 regulatory domain. The effect on cell volume is smaller (not significant in these experiments) consistent with the idea that NKCC1 plays a larger role in [Cl] regulation than in volume regulation (18).

The behavior of our mutants is consistent with changes in regulatory phosphorylation of NKCC. Above we modeled this as a change in the dephosphorylation rate. An alternative possibility is that a change in the phosphorylation rate is most affected by the mutations. To address this question, we examined the decrease in the level of phosphorylation of NKCC1 as a function of time in extracts of transfected HEK cells. As illustrated in Fig. 4, each of the alanine mutations results in a ~5-fold decrease in the rate of cell-free dephosphorylation compared with that of wild type NKCC1 (we have no explanation for the small rapid phase of dephosphorylation that is similar in each sample). This strongly supports the proposal that impaired interaction with PP1c is the outcome of mutations of the RVXFD site.

To look directly for interaction between PP1c and NKCC1 we utilized a microcystin affinity matrix to pull down PP1c (23). Microcystin binds to PP1 with high affinity at a site distinct from the one that binds the RVXFD sequence (3, 28). Fig. 5a illustrates the efficiency of the microcystin beads in precipitating PP1c and demonstrates that pre-incubation with excess microcystin blocks the precipitation. As shown in Fig. 5b, wild type NKCC1 is specifically coprecipitated with PP1c on the microcystin beads, as detected by NKCC1 antibodies. In an alanine mutant lacking the RVXFD consensus site, there was no detectable coprecipitation of cotransporter, but in the “improved” KRVRFED mutant, greatly increased coprecipitation of NKCC1 with PP1c was consistently observed. These results demonstrate direct binding of PP1c to NKCC1, and they show that the RVXFD consensus motif is responsible for the physical interaction.

Previous utilization of microcystin affinity methods led to the coprecipitation of numerous proteins with PP1c (29). Specific interactions have been noted between PP1 or PP2 and various potential target proteins (30–34), but a functional interaction has been demonstrated only in the case of the Bad protein (30). Our results suggest that in addition to the targeting proteins identified in earlier studies, many PP1c-associating proteins may actually be substrate proteins specifically recognized through an RVXFD motif. Similarly it appears likely that among the estimated 10% of data base proteins containing the motif (3), many more than previously suspected may have functional interactions with PP1c.

The results presented here establish a new paradigm for recognition of substrate proteins by PP1c-specific interaction between PP1c and an RVXFD motif on the final target protein. This contrasts with the conventional model in which interactions between PP1c and substrate proteins are mediated by a set of targeting proteins. To our knowledge, the present result is the first time a phosphatase-specific binding site has been identified on a substrate protein and demonstrated to be of functional significance. It seems likely that this paradigm will be found to be widely applicable to the problem of substrate recognition by protein phosphatases.

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