Regulatory Activation Is Accompanied by Movement in the C Terminus of the Na-K-Cl Cotransporter (NKCC1)*

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The Na-K-Cl cotransporter (NKCC1) is expressed in most vertebrate cells and is crucial in the regulation of cell volume and intracellular chloride concentration. To study the structure and function of NKCC1, we tagged the transporter with cyan (CFP) and yellow (YFP) fluorescent proteins at two sites within the C terminus and measured fluorescence resonance energy transfer (FRET) in stably expressing human embryonic kidney cell lines. Both singly and doubly tagged NKCC1s were appropriately produced, trafficked to the plasma membrane, and exhibited a Rb transport activity. When both fluorescent probes were placed within the same C terminus of an NKCC1 transporter, we recorded an 11% FRET decrease upon activation of the transporter. This result clearly demonstrates movement of the C terminus during the regulatory response to phosphorylation of the N terminus. When we introduced CFP and YFP separately in different NKCC1 constructs and cotransfected these in HEK cells, we observed FRET between dimer pairs, and the fractional FRET decrease upon transporter activation was 46%. Quantitatively, this indicates that the largest FRET-signaled movement is between dimer pairs, an observation supported by further experiments in which the doubly tagged construct was cotransfectionally diluted with untagged NKCC1. Our results demonstrate that regulation of NKCC1 is accompanied by a large movement between two positions in the C termini of a dimeric cotransporter. We suggest that the NKCC1 C terminus is involved in transport activation, with dimerization appearing to play a role in the regulatory mechanism.

Significance: Our findings contribute to a model for the conformational changes that bring about cotransporter regulation and provide a sensitive reporter of transporter activation.

The Na-K-Cl cotransporter (NKCC1)2 belongs to the family of electroneutral cation-chloride-cotransporters (CCCs) that also contains the Na-Cl and K-Cl cotransporters; the CCC family is itself part of the amino acid polyamine cotransporter (APC) superfamily. There are two isoforms of NKCC encoded by different genes as follows: the NKCC1 isoform found in most cells and highly expressed in secretory epithelia and the NKCC2 isoform that is specifically localized to the thick ascending limb of the loop of Henle in the mammalian kidney. NKCCs are involved in a wide variety of physiological processes, including intestinal secretion, renal salt and water reabsorption, regulation of blood pressure, hearing, pain perception, and other neuronal functions (1). Their importance is underscored by the fact that they are the target of loop diuretics and that mutations in NKCCs have been linked to hypertension and neurological diseases (2–4).

In common with other APC superfamily members, the transport domain of NKCCs is composed of 12 membrane-spanning helices. Recent structural studies of APCs (5, 6) have shown that the core of this domain is composed of two structurally homologous 5-helix domains with inverted symmetry across the membrane and a translocation pathway clearly evident through the middle of the helix bundle; this structural motif is shared with LeuT and several other transporter superfamilies (7). Although the monomeric structure may be sufficient for transport function, one of two APC amino acid transporters crystalized as a homodimer, with transmembrane domains 11 and 12 forming the dimerization interface. This is of immediate relevance to the related CCC transporters, because there is strong evidence that both NKCCs (8–10) and KCCs (11–13) exist as homodimers in the membrane.

Unlike most APC superfamily amino acid transporters, NKCCs and KCCs have large N- and C-terminal cytoplasmic domains. The N terminus of NKCCs is predicted to be mostly disordered (PONDR (14) and disEmbl (15) algorithms), but it contains a regulatory region, including a number of threonines whose phosphorylation state sets the activity of the transporter (16). Phosphorylation is mediated by SPAK kinase (sterile-20-related proline- and alanine-rich kinase), downstream of WNK kinases (with-no-lysine kinase) (17–20), in response to lowered cotransporter; FP, fluorescent protein; CFP, cyan FP; HEK, human embryonic kidney cells; TM, transmembrane domain.

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2 The abbreviations used are: NKCC, Na-K-Cl cotransporter; KCC, K-Cl cotransporter; CCC, cation-chloride-cotransporter; APC, amino acid polyamine cotransporter; FP, fluorescent protein; CFP, cyan FP; HEK, human embryonic kidney cells; TM, transmembrane domain.

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intracellular [Cl−] and cell volume; dephosphorylation is mediated by PP1 phosphatase (21).

The large C terminus is significantly conserved among CCCs, including NKCCs, KCCs, and a number of prokaryotic relatives. This domain is predicted to contain substantial α and β structures, and there is sufficient homology to permit a tentative alignment with a crystallized prokaryotic CCC C terminus (22); this structure shows the C-terminal domain of CCCs to be composed of a parallel β-sheet of 10 strands, connected by α-helices. The functional role of the NKCC C terminus remains poorly understood, although evidence points to phosphoregulatory modulation (23) and an essential role in dimerization (10). In addition, a key phosphoregulatory domain has been identified in the KCC C terminus (25, 26); phosphorylation of identified residues is both necessary and sufficient for inhibition of KCC activity, reciprocal to the phosphoregulatory activation by the NKCC N terminus.

Our objectives are to examine structural aspects of conformational change in NKCC1 related to regulatory events and to develop sensitive FRET-based sensors of NKCC1 regulatory conformational change. Previous work in our laboratory demonstrated that activation of NKCC1 was accompanied by a 6% FRET decrease between a YFP probe near the phosphoregulatory N terminus and a CFP probe in the C terminus; however, the utility of this sensor is limited by the fact that the presence of the tag near the phosphoregulatory domain hinders protein phosphorylation under normal physiological conditions (27). Here, we investigate NKCC1 tagged with two fluorescent proteins, both in the C terminus. Our results show that there is a large FRET decrease between these two probes when NKCC1 is activated, indicative of a large movement in the C terminus of NKCC1 that is functionally linked to cotransporter activation. The largest component of this change can be attributed to an increase in the distance between FP tags in the two C termini of the NKCC1 homodimer, suggesting an important role for C-terminal dimer interactions in the regulatory process. The dimer of singly tagged transporters signals activation with a 46% FRET decrease and is indistinguishable from the wild-type cotransporter in regulatory and kinetic behavior; it can thus be used as a reporter under normal physiological conditions. We anticipate that this genetically encoded FRET-based sensor will provide a new tool for the investigation of real time changes in the NKCC1 regulatory state and therefore contribute to the better understanding of the regulation of ion transport in native tissues.

EXPERIMENTAL PROCEDURES

cDNA Constructs—As seen in Fig. 1, we inserted CFP and YFP into two positions within the C terminus of the shark NKCC1 cotransporter (sNKCC1); these insertion sites are termed C.49 and C.80 to denote relative position within the length of the C terminus. The C.49 insertion site has been previously described (27). The shark cotransporter is functionally nearly identical to the human cotransporter with somewhat different ion affinities (28) and offers the advantage of the availability of excellent antibodies (29). For the C.49 position, insertion of CFP and YFP was accomplished by cloning the FPs into Agel/BsrGI sites added into the C terminus as described previously (27). For the C.80 position, insertion of CFP and YFP was accomplished by introducing a unique SanDI restriction site to shark NKCC1 (D1095G/D1096S/K1097Q) using QuikChange site-directed mutagenesis (Stratagene). The dual CFP/YFP construct was subsequently generated by subcloning. For all FRET pairs, both FPs were nondimerizing containing an A206K mutation, and YFP was rendered insensitive to Cl− with a Q69M mutation as described previously (27). For coexpression with separate antibiotic resistance in HEK-293 cells, final constructs were moved into pJB20 (neomycin), pcDNA3.1+ (hygromycin, Invitrogen), or pIRES3 (puromycin, Clontech) expression vectors. Using QuikChange site-directed mutagenesis (Stratagene), we also engineered a construct containing YFP with a V163S mutation for improved Cl− sensitivity (30) at position C.80 in sNKCC1.

Cell Culture and Transfection—HEK-293 cells were maintained in DMEM, 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 units/ml), at 37 °C, 5% CO2, in a humidified incubator, and transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Stable clones were selected with the appropriate selective antibodies as follows: 1 mg/ml neomycin (geneticin, Invitrogen), 200 μg/ml hygromycin (Invitrogen), or 1 μg/ml puromycin (Sigma). Cotransfectants are identified by a “+” between the two expressed constructs (e.g. C.49C + C.80Y is a cell line coexpressing C.49CFP and C.80YFP under different antibiotic resistances). All constructs were studied in stably expressing cell lines.

Western Immunoblotting—HEK cells were grown to confluence at 37 °C in 12-well tissue culture plates. Prior to sampling, cells were incubated overnight at 25 °C, as incubation at low temperature has been reported to increase plasma membrane staining of FP-tagged NKCC1 constructs (27). Cells were solubilized in 1 ml of homogenization buffer containing 1% Triton X-100, and homogenates were spun at 14,000 rpm in a microcentrifuge at 4 °C for 10 min. Supernatants were diluted in Laemmli buffer containing 100 mM DTT and also saved for protein determination using the BCA protein assay (Pierce). Approximately 2.5 μg of protein was run on a 7.5% Tris-HCl gel, transferred to nitrocellulose membrane (0.22 μm, Bio-Rad), and blocked in 5% BSA in PBS-T. Immunoblots were then incubated overnight at 4 °C with primary antibodies (anti-shark NKCC1 antibody (J3) or anti-GFP (Rockland)) diluted 1:5000 in blocking buffer, washed, and incubated with secondary antibodies (goat anti-mouse IRDye®800CW or goat anti-rabbit IRDye®680CW (Li-Cor Biosciences) diluted 1:15,000 in blocking buffer for 1 h at room temperature. Antibody binding was detected using an Odyssey Infrared Scanner (Li-Cor Biosciences).

Immunofluorescence—HEK cells were grown to 50–70% confluence on 12-mm round glass coverslips (Warner Instruments) coated with poly-L-lysine (Sigma) at 37 °C and subsequently transferred to 25 °C overnight prior to fixation. Cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 30 min, and blocked in 1% BSA in PBS for 30 min. To visualize shark NKCC1, cells were incubated with J3 antibody (1:500 in BSA/PBS) overnight at 4 °C, washed with PBS, and incubated
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with anti-mouse Alexa 488 secondary antibody (1:500 BSA/PBS; Molecular Probes) for 1 h at room temperature. For the visualization of nuclei, cells were incubated for 5 min in To-Pro-3 (1:5,000 in PBS, Invitrogen). Coverslips were mounted in Vectashield and examined at a magnification of ×63 with a Zeiss LSM 510 Meta confocal laser scanning microscope.

Live Cell Imaging—HEK cells were grown to 50–70% confluence on 30-mm glass bottom tissue culture dishes (Electron Microscopy Sciences) coated with poly-l-lysine (Sigma) at 37 °C and subsequently transferred to 25 °C overnight prior to examination. Cells were washed in regular medium containing as follows (in mM): 135 NaCl, 5 KCl, 0.5 CaCl₂, 0.5 MgCl₂, 0.5 Na₃HPO₄, 1 Na₂SO₄, 15 HEPES, pH 7.4, and 5 glucose. Cells were examined at a magnification of ×63 with a Zeiss LSM 510 Meta confocal laser scanning microscope. CFP fluorescence was imaged using an argon laser at 458 nm for excitation and a 475-nm long pass filter for detection. YFP fluorescence was imaged using an argon laser at 514 nm for excitation and a 547-nm long pass filter for detection.

³⁸Rb⁺ Influx Assay—NKCC1 transport function was examined at room temperature by ³⁸Rb⁺ influx in HEK cells as described previously (27). In brief, cells were split into 96-well poly-l-lysine-coated microplates (BD Biocoat), grown for 24 h at 37 °C, and transferred to 25 °C for overnight incubation prior to flux experiments. Before each flux assay, cells were incubated for 45 min in solutions with various compositions to lower intracellular [Cl⁻]. This was followed by a 1-min ³⁸Rb uptake period determined in regular medium containing as follows (in mM): 135 NaCl, 5 RbCl, 0.5 CaCl₂, 0.5 MgCl₂, 0.5 Na₃HPO₄, 1 Na₂SO₄, 15 HEPES, pH 7.4, and 5 glucose. Cells were solubilized in 150 μl of homogenization buffer containing 1% Triton X-100, and microplates were spun at 2500 rpm in a benchtop centrifuge at 4 °C for 10 min. Supernatants were diluted in 2× assayable buffer (20% glycerol, 4% SDS, 160 mM Tris-HCl, pH 6.8) and also saved for protein determination using the DC protein assay (Bio-Rad).

Dot Blotting—HEK cells were grown to confluence at 37 °C in 96-well tissue culture plates. Prior to sampling, cells were incubated overnight at 25 °C. After experimental manipulation, cells were solubilized in 150 μl of homogenization buffer containing 1% Triton X-100, and microplates were spun at 2500 rpm in a benchtop centrifuge at 4 °C for 10 min. Supernatants were diluted in 2× assayable buffer (20% glycerol, 4% SDS, 160 mM Tris-HCl, pH 6.8) and also saved for protein determination using the DC protein assay (Pierce). 1 μl of supernatant was manually blotted onto a nitrocellulose membrane (0.22 μm, Bio-Rad) using a multichannel pipetman. Membranes were then blocked in 5% milk in PBS-T for 30 min at room temperature and subsequently incubated with anti-phospho-NKCC antibody (R5, 1:50000) (32) overnight at 4 °C. After washing with PBS-T, blots were probed with HRP-conjugated goat anti-rabbit secondary antibody diluted 1:10,000 in 5% milk in PBS-T for 1 h at room temperature. Antibody binding was detected using chemiluminescent reagents (Pierce) in the linear range of sensitivity.

Fluorometry—HEK cells were grown to confluence on 12-mm round glass coverslips (Warner Instruments) coated with poly-l-lysine (Sigma) at 37 °C and subsequently cultured overnight at 25 °C. Prior to fluorometry experiments, coverslips were washed in regular medium containing as follows (in mM): 135 NaCl, 5 KCl, 0.5 CaCl₂, 0.5 MgCl₂, 0.5 Na₃HPO₄, 1 Na₂SO₄, 15 HEPES, pH 7.4, and 5 glucose.

Fluorescence time courses were acquired at room temperature using a fluorometer especially constructed to monitor HEK cell monolayers on 1-cm coverslips resting horizontally below a continuously flowing medium. The specifics of this instrument are as follows (further details are available upon request): chamber volume, 0.1 ml; flow rate 10 ml/min; illumination, Lxune V LEDs (Lumileds, royal blue and cyan); detection, Hamamatsu S3590 photodiodes, amplifier bandwidth 200 μs; CFP filter cube, Semrock 445/20, 494/40, dicroic 458Di; YFP filter cube, Semrock 514/3, 543/22, dicroic 520Di; CFP/YFP dicroic separation, Semrock 506Di. Excitation of CFP and YFP occurred in brief flashes (5 and 17 ms, to minimize photobleaching) 15 times/s. Fluorescence readings in CFPem and YFPem (and reference excitation) channels were taken continuously in 1-ms samples and digitized by a Picoscope 4424 (Pico Technologies) digital oscilloscope. After dark signal subtraction and reference light correction, time-averaged values for CFPex → CFPem (CC), YFPex → YFPem (YY), and CFPex → YFPem (CY) were stored in an Excel-VBA application. Time courses reported in this study are calculations of FRETnorm (below) from the unfiltered 2 sample/s data.

FRET was calculated from the CY signal, subtracting spectral crossover components (CFPem into the YFPem band and YFPex by the CFPex light) as shown in Equation 1,

\[
\text{FRET} = CY - \alpha \cdot CC - \beta \cdot YY
\]  

(1)

where α and β are instrumental constants determined using a CFP-only construct and a YFP-only construct, respectively (for example in the C.49C-C.80Y construct, these crossover components compose ~54% of the CY signal). As discussed previously (27, 33), normalization to the YFP fluorescence signal eliminates changes due to YFP fluorescence yield (including YFP photobleaching, about 10%/h with this illumination), and we have used this normalized value throughout (Equation 2).

\[
\text{FRET}_{\text{norm}} = \text{FRET}/YY
\]  

(2)

Among constructs that are tagged both with CFP and YFP, C_{TOT} ≈ YY, where C_{TOT} is the value CC would take in the absence of a FRET acceptor; FRETnorm is then directly proportional to FRET efficiency as shown in Equation 3,

\[
(C_{\text{FRET}}/C_{\text{TOT}}) \approx \text{FRET}/YY = \text{FRET}_{\text{norm}}
\]  

(3)

For presentation in this paper, the proportionality factors underlying FRETnorm have been adjusted so that FRETnorm = FRET_eff for the doubly tagged constructs. In cells cotransfected with two singly tagged CFP and YFP constructs, we correct for nonproductive dimers (CFP-CFP and YFP-YFP) and calculate a FRET interaction value (I_{FRET}) as shown in Equation 4,

\[
I_{\text{FRET}} = \text{FRET}_{\text{norm}}(1 + 1/R)
\]  

(4)

where R is the CFP/YFP ratio (27). I_{FRET} then represents the FRET efficiency obtained in a productive dimer.

In experiments presented in Fig. 8, we utilized the V163S mutant of YFP in the C.80 position of NKCC1 as a high affinity [Cl⁻] sensor at the inside surface of the plasma membrane. This
variant of YFP is strongly quenched by Cl⁻, with an affinity of 62 mM at pH 7.5 (30). At the end of each experiment utilizing this probe, we determined $F_{\text{max}}$ by permeabilizing the cells on the coverslip in a 10-min exposure to 0.004% saponin, after which YFP fluorescence responded rapidly and reproducibly to changes in bath [Cl⁻], allowing for calibration with a series of solutions containing [Cl⁻] from 0 to 280 mM (supplemental Fig. 1).

RESULTS

**NKCC1 Tagged in the C Terminus with Fluorescent Proteins**—We have recently reported that CFP/YFP reporter pairs placed within the N and C termini of NKCC1 signal a change in regulatory activation by a small (6%) FRET decrease between the two FPs (27). Here, we examined the situation when both FP probes were placed within the C terminus to see if movements in this region are associated with the activation process. Fig. 1 presents a two-dimensional schematic depiction of our doubly labeled NKCC1 construct. To increase the chance of obtaining functional constructs, we chose positions (termed C.49 and C.80 for relative position within the length of the NKCC1 C terminus) with low sequence conservation and that are predicted to have little secondary structure (Pondr (14) and dis-Emb (15)). In alignments of the C terminus to the crystallized C terminus of MaCCC (22), these regions are predicted to be loop regions not in the $\alpha$-$\beta$ sheet structure (NKCC2 (4) and MaCCC (22)).

Fig. 2 illustrates the expression, localization, and function of the FP-tagged NKCC1 constructs examined in this study. Western blot analysis of wild-type and FP-tagged NKCC1s (Fig. 2A) reveals the ~190-kDa NKCC1 protein as well as a lower band of immature poorly glycosylated cotransporters. Consistent with the size of the FP inserts, NKCC1 tagged with both CFP and YFP (Fig. 2A, lane c) is larger than NKCC1 singly tagged with CFP or YFP either in singly transfected cell lines (lanes a and b) or in lines cotransfected with two such constructs (lanes e–g); and in turn these constructs are larger than untagged NKCC1 (Fig. 2A, lanes d and h). All of these FP-tagged proteins were correctly trafficked to the plasma membrane, as demonstrated by immunostaining with the J3 antibody or by imaging of CFP and YFP fluorescence in live cells; typical results for cells expressing NKCC1 tagged with two FPs (Fig. 2B) and cells cotransfected with two singly tagged NKCC1s (Fig. 2, C–E) are shown.

Finally, cotransporter function is examined in Fig. 2F, where it is seen that FP-tagged NKCC1s exhibited levels of absolute

![Figure 1. Fluorescent protein-tagged NKCC1. A two-dimensional representation of the structure of shark NKCC1 illustrating the two insertion points for fluorescent proteins in the C terminus. Our terminology is C.49 and C.80 to denote relative position within the length of the C terminus, followed by a suffix letter, either C for CFP or Y for YFP.]

**FIGURE 2.** Expression, localization, and functional analysis of fluorescent protein-tagged NKCC1s in stable HEK-293 cell lines. A, representative Western blot of wild-type and FP-tagged NKCC1s. For each cell line, total protein in cell lysates was quantified using a BCA protein assay, and 2.5 μg of protein was run on a 7.5% SDS-polyacrylamide gel. Immunoblots were probed with J3 (anti-shark) antibody. B, confocal image of HEK cells expressing doubly tagged NKCC1 (C.49C-C.80Y). C–E, confocal images of HEK cells coexpressing singly tagged NKCCs in the same cell line (C.49C and C.80Y). F, 86Rb influx in wild-type and FP-tagged NKCC1s. 86Rb influx was determined in a 1-min 96-well microplate flux assay after 45 min of preincubation of cells in media containing various [Cl⁻]. Data are mean ± S.E. (n = 3).
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FIGURE 3. FRET efficiency in stable HEK-293 cell lines expressing fluorescent-protein tagged NKCC1s. FRET efficiency was determined from a monolayer of HEK cells on a 12-mm round coverslip incubated in a continuous flow of regular medium. Values were calibrated by comparison with two previously reported doubly labeled NKCC1 constructs, in which YFP is in the N terminus, and CFP is in the C terminus (N.00Y-C.49C (lane b) and N.83Y-C.49C (lane c) (27)). Data are mean ± S.E. (n = 4–6). For doubly tagged constructs, FRET efficiency is equal to FRETnorm (see “Experimental Procedures”). For singly tagged cotransfected cell lines, FRET efficiency is equal to I_{FRET} (see “Experimental Procedures”). I_{FRET} was calculated from the ratio of CFP to YFP (R), where R was 1.00 ± 0.03, 0.58 ± 0.01, and 0.46 ± 0.02 for the C.49C + C.80Y, C.49C + C.49Y, and C.80Y + C.80Y cell lines, respectively.

Rb influx similar to that of wild-type NKCC1, and with a similar pattern of activation by preincubation in low Cl\(^-\) media (in Fig. 7 we will point out a quantitative difference in the rate of activation of doubly tagged NKCC). In addition, insertion of a single FP tag in the C terminus of NKCC1 was found to have no detectable effect on apparent affinity for Na\(^+\), Cl\(^-\), Rb\(^+\), bumetanide, and furosemide (supplemental Fig. 2). Taken together, these results demonstrate that the FP-tagged NKCC1s examined in this study are correctly synthesized, trafficked to the plasma membrane, and exhibit the same kinetic behavior as the wild-type transporter.

FRET in Doubly and Singly Tagged NKCC1s—As shown in Fig. 3 (lane d), the CFP-YFP doubly tagged NKCC1 exhibits a high level of FRET as follows: 38% FRET efficiency as calibrated by comparison with two previously reported doubly labeled NKCC1 constructs, in which YFP is in the N terminus and CFP is in the C terminus (N.00Y-C.49C, Fig. 3, lane b, and N.83Y-C.49C, lane c) (27). Because NKCC1 exists in the membrane as a dimer (8, 10), some of this FRET arises from the interaction of intramolecular CFP-YFP pairs, and some may arise from intermolecular pairing. To isolate the component of FRET arising from intermolecular interactions, we cotransfected singly tagged NKCC1s (C.49C and C.80Y) into an HEK cell line. In addition to the C.49C + C.80Y line, we also investigated homodimeric interaction at the two positions within the C terminus (i.e. C.49C + C.49Y and C.80C + C.80Y). In all of the singly tagged, cotransfected cell lines, we observed significant FRET (Fig. 3, lanes e–g), presented as I_{I_{FRET}}. I_{FRET} represents the calculated FRET efficiency in a productive (CFP-YFP) dimer pair, correcting for fluorescence from nonproductive dimers (CFP-CFP and YFP-YFP) by using the measured ratios of CFP and YFP in the cotransfected lines (see “Experimental Procedures”). It is seen that I_{I_{FRET}} in the C.49C + C.80Y line (Fig. 3, lane e) accounts for about half of the total FRET in the corresponding doubly tagged construct (C.49C-C.80Y, lane d; 46% when the parallel rates are calculated). Using the Forster equation and the simplifying assumption that the FP probes are oriented randomly, this indicates that on average the CFP and YFP are 60 Å apart within a doubly tagged monomer and 62 Å apart between dimer C termini in C.49C + C.80Y (calculated distances within the symmetric dimers C.49C-C.49Y and C.80C-C.80Y are 68 and 64 Å, respectively).

Movement in the C Terminus of NKCC1 upon Regulatory Activation—To determine whether the two FP tags in the NKCC1 C terminus undergo movement relative to one another upon activation of the transporter, we incubated HEK cells expressing doubly tagged NKCC1 (C.49C-C.80Y) with calyculin A to inhibit protein phosphatase 1 and thus bring about full phosphorylation of the transporter (34). As shown in a representative experiment in Fig. 4, this resulted in a FRET decrease of 11 ± 0.49% (n = 4) between the two C terminus tags with a time course consistent with sNKCC1 phosphorylation and activation (16, 32). This was a surprise, for although activation of NKCCs must involve both the transmembrane domain and the phosphoregulatory N-terminal domain, the C terminus had never been implicated as part of the activation mechanism. Interestingly, the magnitude of the FRET decrease is larger than that which we previously recorded with one probe in the N terminus and the other in the C terminus (27). This is the first demonstration of movement within the C terminus of NKCC1 upon regulatory activation, and it suggests a novel involvement of the C terminus in cotransporter regulation.

Movement Is between the C Termini of a Dimeric NKCC1—Looking specifically for movement between the C termini of dimeric NKCC1, we examined FRET in the singly tagged cotransfected cell lines during incubation with calyculin A. Remarkably, we found a very large (46 ± 4.21%, n = 4) FRET decrease upon activation of the transporter (Fig. 5A), indicating that the tagged positions within the C termini of a transporter dimer move apart relative to one another upon phosphorylation and activation; from the Forster equation, this corresponds to an 8-Å movement apart. Substantial but somewhat smaller changes were seen for the symmetrically placed CFP-YFP pairs...
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What about movement within a single C terminus of NKCC1 signaled as the intramolecular component of the FRET change seen in Fig. 4? The $I_{\text{FRET}}$ change upon activation is considerably greater in the cotransfected single constructs ($-0.096 \pm 0.1$, e.g. Fig. 5A) than the corresponding change in FRET$_{\text{norm}}$ ($-0.04 \pm 0.002$, e.g. Fig. 4) in the doubly tagged construct. This suggests that, little, if any, of the negative FRET change is due to intramolecular movement; rather the quantitative discrepancy suggests that intramolecular movement might actually contribute a positive component of FRET change upon activation of the doubly tagged construct.

If the negative FRET change in the doubly tagged construct is due principally to intermolecular FRET, it should be possible to dilute out this FRET signal by cotransfection with an excess of wild-type transporter. To test this, we performed a dilution experiment in which we transfected HEK cells stably expressing doubly tagged cotransporter (C.49C-C.80Y) with wild-type NKCC1 and isolated a number of different stable cell lines, identifying lines with various ratios of cotransfected doubly tagged and wild-type NKCC1 cotransporter. Western blot analysis was used to measure the expression ratio of wild-type to FP-tagged NKCC1 (Fig. 6A). As shown in Fig. 6B, when doubly tagged NKCC1 is diluted with wild-type cotransporter, FRET$_{\text{norm}}$ is decreased, extrapolating to a 60% decrement at high dilution. This is in reasonable agreement with the estimate available from comparison of the singly and doubly labeled constructs in Fig. 3.

Concomitant with the decrease in FRET$_{\text{norm}}$ as the double FP construct was diluted with wild-type NKCC1, we found a dramatic decrease in the magnitude of the negative FRET$_{\text{norm}}$ change that accompanies activation (Fig. 6C); the line predicted for dilution of the FP-NKCC dimer actually extrapolates to a positive value ($+0.18$) at high dilution. Indeed, in the cell line containing 74% wild-type NKCC (line number 2), we found a markedly positive FRET change upon the addition of calyculin A, as illustrated in Fig. 6D. Together, our results are consistent with a conclusion that activation of the doubly tagged NKCC results in a large decrease in intermolecular FRET representing a change in the distance between probes at positions C.49 and C.80 of an NKCC dimer. At the same time, a somewhat smaller movement takes place between the two FP tags in the same C terminus, consistent with movement of these tags toward one another.

Presence of Two FPs in Same C terminus of NKCC1 Alters Phosphorylation and Activation Kinetics—We noted above that both doubly and singly tagged constructs are fully functional and regulated in the same way as wild-type NKCC1. On closer inspection, however, we found that the doubly tagged construct was slower to activate upon incubation with calyculin A than the corresponding cotransfected singly tagged construct, as illustrated in representative FRET experiments in Fig. 7A. In four such experiments, the time at which the FRET change was half-maximal was $4.3 \pm 0.14$ and $6.0 \pm 0.30$ min in the singly and doubly tagged lines, respectively. Examining the same lines for activation of transport function by means of $^{86}$Rb influx experiments (Fig. 7B), and the increase in phosphorylation using the NKCC phosphospecific antibody R5 (Fig. 7C) (32), it is clear that the slower FRET change in doubly tagged NKCC1 is due to a lower rate of phosphorylation and that this translates as well into a lower rate of functional activation. Similar results were obtained on activation of the transporter in low [Cl$^-\$] hypotonic medium and deactivation in regular medium (supplemental Fig. 3). It is interesting that the presence of two large FP tags in the same C terminus of an NKCC1 transporter significantly impairs protein phosphorylation and activation kinetics but not basic transport function. The time courses of the phosphorylation, FRET change, and activation of transport are quite similar, consistent with schemes in which the limiting factor in the time course is the phosphorylation process and that conformational change sensed by the FRET probes and activation of transport occur relatively rapidly thereafter.
FRET Changes upon Lowering Extracellular [Cl⁻] or [K⁺] in Fluorescent Protein-tagged NKCC1s—If the observed FRET changes reported above are indeed linked to movement of FP tags in the C terminus resulting from NKCC1 regulatory events, FIGURE 6. Effect of transporter dilution on FRET norm. A, representative Western blot of HEK cell lines coexpressing wild-type and doubly tagged NKCC1 (C.49C-C.80Y). Immunoblots were probed with anti-GFP (top) and J3 (middle) antibodies. Three such blots were analyzed to obtain the wild-type/tagged-NKCC ratio in B and C. B, FRET norm as a function of time following activation with calyculin A in the number 2 cotransfected line, representative of eight such experiments.

FIGURE 7. Time course of regulatory activation of fluorescent protein-tagged NKCC1s with the phosphatase inhibitor calyculin A. A, representative experiments showing FRET (normalized to value at t = −2 min) during cotransporter activation in doubly tagged (gray line) and cotransfected singly tagged (black line) NKCC1s. B, time course of NKCC1 activation by 500 nM calyculin A measured in a subsequent 1-min 86Rb influx assay in wild-type NKCC1 and FP-tagged NKCC1s. Values are expressed relative to the 20-min time point. Data are mean ± S.E. (n = 3). C, time course of NKCC1 phosphorylation by 500 nM calyculin A in wild-type and FP-tagged NKCC1s as measured by dot blotting with the phospho-specific antibody R5. Values are expressed relative to the 20-min time point. Data are mean ± S.E. (n = 3).
Movement in the C Terminus of NKCC1 upon Activation

In this study we have shown that FRET between CFP and YFP tags in the C termini of NKCC1 cotransporters is a sensitive reporter of regulatory movements in the transport protein. We found that when the two probes were placed in the same C terminus, a high efficiency of energy transfer is seen and that 11% of this FRET signal is sensitive to the regulatory state of the cotransport protein (34, 35).

FIGURE 8. Regulatory activation and deactivation of NKCC1 in cells containing cotransfected C.49C and C.80Y on lowering extracellular [Cl] or [K]. A, I_{FRET} (normalized to value at t = 0 min) during cotransporter activation and deactivation on manipulation of extracellular [Cl⁻]. B, intracellular [Cl⁻] (mM) during cotransporter activation and deactivation on manipulation of extracellular [Cl⁻] in HEK cells expressing an improved YFP-based [Cl⁻] sensor. The activating conditions are low Cl⁻/hypotonic medium and 500 nm calyculin A, and the deactivating condition is regular medium. C, I_{FRET} (normalized to value at t = 0 min) during cotransporter activation and deactivation on manipulation of intracellular [K⁺]. D, intracellular [Cl⁻] (mM) during cotransporter activation and deactivation on manipulation of extracellular [K⁺] in HEK cells expressing an improved YFP-based [Cl⁻] sensor. The activating conditions are 0 K⁺/isotonic medium and 500 nm calyculin A, and the deactivating condition is regular medium. E, time course of cotransporter activation (black) and deactivation (gray). For activation, cells were preincubated for 6 min in regular medium and subsequently incubated in low Cl⁻/hypotonic medium for the time plotted on the abscissa. For deactivation, cells were preincubated for 60 min in low Cl⁻/hypotonic medium and subsequently incubated in regular medium for the time plotted on the abscissa. A 1.6-min ⁸⁶Rb influx was then carried out in regular flux medium. Values are expressed relative to the 0- or 20-min time points. Data are mean ± S.E. (n = 3).

DISCUSSION

Movement in the C Terminus of NKCC1 upon Regulatory Activation—In this study we have shown that FRET between CFP and YFP tags in the C termini of NKCC1 cotransporters is a sensitive reporter of regulatory movements in the transport protein. We found that when the two probes were placed in the same C terminus, a high efficiency of energy transfer is seen and that 11% of this FRET signal is sensitive to the regulatory state of the transport protein. Further investigation revealed that the majority of the observed change in FRET during protein regulation in this construct could be attributed to changes in intramolecular interactions between the two members of an NKCC1 homodimer, rather than to a change in the distance between the two probes in a single molecule. Thus, when CFP and YFP were placed on different NKCC1 molecules and cotransfected in
Movement in the C Terminus of NKCC1 upon Activation

HEK cells, there was a very large fractional FRET change (46%) upon protein activation.

Our previous understanding of the regulatory mechanism in NKCC was that phosphorylation of between 3 and 8 threonine residues in the N terminus somehow brings about a structural change allowing transport to proceed through a transport pathway in the transmembrane domain (16). The results presented here make it clear that the C terminus is also involved in the regulatory event and suggests that the C terminus may very well play a key structural role in the regulatory process. This hypothesis is strengthened by the recent finding that K-Cl cotransporters (KCCs), which are regulated reciprocally to NKCCs, are de-activated by phosphorylation of threonine residues within the C-terminal domain (26). One possible scenario, at this time completely speculative, is that the NKCC C terminus has an inhibitory interaction with the intracellular loops connecting helices in the transmembrane domain and that the phosphorylated N terminus somehow interacts with either the C terminus or the TM domain to disrupt this inhibitory interaction.

Although in most cases the structural details are not clear, there are now many examples of transport proteins that are regulated by large conformational changes involving N or C termini. In a FRET study similar to this one, Bykova et al. (36) found a large conformational change in CLC-0 associated with slow gating of the channel. A similar mechanism of regulation has recently been proposed for the Na⁺/betaine symporter, BetP (37, 38). In this transporter, transport activity is thought to be controlled by a “molecular switch” involving electrostatic interactions between the surrounding lipid membrane, the N and C termini, and cytoplasmic loop 8 (37).

Role of Homodimerization in NKCC1 Regulation—There is now extensive evidence that NKCCs and KCCs exist in the membrane primarily, if not always, as dimers (8, 9, 11, 27). The dimer interface may be tentatively inferred to be in the C-terminal part of the protein from recent crystallographic studies; one of two related ACP superfamily amino acid transporters crystallizes as a dimer, with TMs 11 and 12 forming the interface (5); and the soluble C terminus of the prokaryotic cation-chloride cotransporter, MaCC (22), also exhibits a dimer interface (10, 22). This hypothesis is also consistent with cross-linking studies that illustrate a critical role of the C-terminal domain in dimeric associations (10, 22).

The present results demonstrate that fluorescent proteins at C.49 and C.80 positions within the C terminus of an NKCC1 homodimer move apart from one another upon activation of the cotransporter based on the 46% FRET change, and we estimate that this movement averages 8 Å. However, it is likely that we underestimate the significance of the FRET change for two reasons. 1) Although we do seem to be able to “saturate” the activation mechanism with regard to time (Fig. 7) and calyculin A concentration (data not shown), we cannot rule out the possibility that at any time there may be a fraction of transporters in the inactive state. 2) It is possible that a small fraction the observed “dimer” FRET is due to random collisional events in the membrane. It is difficult to make a reliable estimate of the magnitude of this effect, but based on the random collisions between CFP-tagged hslo channel and YFP-tagged NKCC (see Fig. 5 in Ref. 27), we estimate that as much as 5% of our dimer FRET could be due to random collisions. Thus, the regulatory change may actually result in somewhat more than 8 Å movement. However, the extreme possibility of complete dissociation of the dimer halves appears to be ruled out by the fact that smaller changes in intermolecular FRET are observed when the probes are placed at other positions in N and C termini (Fig. 5 of this study and see Ref. 27).

As a working model we propose a stable NKCC homodimer with a primary interaction domain in TMs 11 and 12, by homology to the related amino acid transporter AdiC (5). We suggest that in the inactive state the C termini also interact at a dimerization motif homologous to that in MaCC (22) and that in this state they also engage the intracellular loops of the transmembrane domain, inhibiting transport. A homology model of these relationships is presented in Fig. 9, left. Our FRET data show that positions within the C termini get further apart upon activation, and we propose that this is because they dissociate from each other and from the TM domain, releasing inhibitory control of the transport activity of the TM domain (Fig. 9, right). A finished model must also include the N terminus, which is the key site of regulatory phosphorylation (shown schematically in Fig. 9). Somehow, through mechanisms still to be elucidated, phosphorylation of threonine residues in the N terminus must be translated into the conformational change in the C terminus and activation of transport in the transmembrane domain.

These fluorescent molecules are attached to the NKCC C terminus in large loop regions that are not contained in MaCC (22) and are generally predicted to be disordered. Our attempts to engineer FP constructs within regions with predicted structure have resulted in misfolded protein.³ As a consequence, we cannot make any prediction about the attachment geometry in Fig. 9 (shown with dotted lines), and molecular triangulation using the Forster distances is not likely to be particularly informative as to cotransporter structure.

³ M. Y. Monette and B. Forbush, unpublished observations.
Individually, the fluorescent tags reported here do not appear to have significant effects on transporter biosynthesis, delivery, function, regulation, or kinetics presumably because they are inserted in regions that are not predicted to be part of the α-β structure that makes up the core of the C terminus and that exhibit a low level of sequence conservation. Interestingly, however, we observed that by comparison with the singly tagged constructs and with the wild-type transporter, the doubly tagged transporter exhibited a significantly lower rate of FRET change and of cotransporter activation (Fig. 7). Because the rate of phosphorylation is similarly affected, the effect is not due to a change in the coupling mechanism linking phosphorylation of the N terminus to activation of function. Rather, the decreased activation rate may reflect a steric effect on kinase accessibility, due to the combined interference of four relatively large FP tags in the NKCC1 dimer (for relative size, see Fig. 9).

Genetically Encoded Sensors of Intracellular [Cl\(^-\)] and NKCC1 Regulatory State—NKCC1 serves as the major Cl\(^-\) entry pathway into most vertebrate cells. It is clear that NKCC1 transport activity is switched on by direct phosphorylation of the N terminus in response to decreases in intracellular Cl\(^-\) and cell shrinkage, both in heterologous expression systems and in intact epithelia (24, 31, 34). However, although the broad outlines are clear, a thorough analysis of NKCC1 regulation in intact tissue is restricted by the difficulty in measuring parameters such as intracellular [Cl\(^-\)] and the activation state of NKCC1. The FP-NKCC1 biosensors presented here should be invaluable in addressing these physiological questions.

This investigation presents a sensitive FRET-based sensor of NKCC1 regulation, consisting of FP-tagged NKCC1 in which YFP and CFP tags are located in the C terminus of different NKCC1 molecules of a homodimer (C.49C + C.80Y). We demonstrate the utility of this probe by showing the following. 1) The addition of an FP tag in the C terminus of NKCC1 has no detectable impact on protein production, localization, or function. 2) This probe exhibits a large change (46%) in FRET signal with two independent stimuli known to cause protein phosphorylation and activation. 3) The time course of the change in FRET signal is consistent with the timing of NKCC1 activation. Together, these results support the use of this FRET-based sensor for the detection of NKCC1 regulatory state. In addition, we are able to use the YFP-based [Cl\(^-\)] sensitivity (V163S) (30) to detect changes in intracellular [Cl\(^-\)] within the physiological range, targeted with NKCC1. In future work, we plan to develop transgenic mouse models with these genetically encoded biosensors to allow for real time monitoring of intracellular [Cl\(^-\)] and NKCC1 activation in NKCC1-expressing cells under physiological and pathological conditions.

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