Intramolecular and Intermolecular Fluorescence Resonance Energy Transfer in Fluorescent Protein-tagged Na-K-Cl Cotransporter (NKCC1)

SENSITIVITY TO REGULATORY CONFORMATIONAL CHANGE AND CELL VOLUME*\(^2\)

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To examine the structure and function of the Na-K-Cl cotransporter, NKCC1, we tagged the transporter with cyan (CFP) and yellow (YFP) fluorescent proteins and measured fluorescence resonance energy transfer (FRET) in stably expressing human embryonic kidney cell lines. Fluorescent protein tags were added at the N-terminal residue between the regulatory domain and the membrane domain and within a poorly conserved region of the C terminus. Both singly and doubly tagged NKCC1s were appropriately trafficked to the cell membrane and were fully functional; regulation was normal except when YFP was inserted near the regulatory domain, in which case activation occurred only upon incubation with calyculin A. Quenching of YFP fluorescence by Cl\(^-\) in which case activation occurred only upon incubation with calyculin A. Quenching of YFP fluorescence by Cl\(^-\) provided a ratiometric indicator of intracellular [Cl\(^-\)]. All of the CFP/YFP NKCC pairs exhibited some level of FRET, demonstrating the presence of dimers or higher multimers in functioning NKCC1. With YFP near the regulatory domain and CFP in the C terminus, we recorded a 6% FRET change signaling the regulatory phosphorylation event. On the other hand, when the probe was placed at the extreme N terminus, such changes were not seen, presumably due to the length and predicted flexibility of the N terminus. Substantial FRET changes were observed cotemporaneous with cell volume changes, possibly reflective of an increase in molecular crowding upon cell shrinkage.

The Na-K-Cl cotransporter (NKCC)\(^3\) carries out the coupled movement of 1 Na\(^+\), 1 K\(^+\), and 2 Cl\(^-\) ions into the cell, generally serving to increase cell [Cl\(^-\)] and cell volume. NKCCs are members of the cation-chloride cotransporter family, which also includes the Na-Cl cotransporter and four K-Cl cotransporter (KCCs). One isoform, NKCC1, is found at low levels in most cells and at high levels in secretory epithelia; a second isoform, NKCC2, is specifically localized in the thick ascending limb of the loop of Henle in the kidney. NKCC1 and NKCC2 are activated by decreases in cell volume and cell [Cl\(^-\)] through phosphorylation of a regulatory domain that is present in the N terminus of both transporters (1, 2), probably by SPAK kinase (3, 4). Ion transport properties are thought to be determined by 12 transmembrane domains and a proposed re-entrant loop structure between transmembrane domains 1 and 2 (5, 6). The mechanism by which the phosphoregulatory domain in the N terminus interacts with the transmembrane domain or the large C terminus to control function is completely unknown.

Although detailed structural evidence is lacking, numerous lines of evidence support the idea that these transport proteins exist in the membrane as homodimers or higher multimers. Previous observations include dominant-negative behavior of inactive mutants (1, 5, 7–9), cross-linking studies (9, 10), yeast two-hybrid experiments (11), and immunoprecipitation experiments (8, 9, 12, 13). KCC2 dimers are apparently stabilized by a disulfide bridge (12), but this does not appear to occur in NKCCs. Aside from dominant-negative behavior, there is limited evidence for cooperative functional interaction within the NKCC dimers, although it has been proposed that the proportion of disulfide-stabilized KCC2 dimer is correlated with function during neuronal development (12).

Yellow and cyan derivatives of green fluorescent protein (YFP and CFP) have been widely used as a biological sensor pair because they are well suited as fluorescence resonance energy transfer (FRET) partners and can be genetically encoded as fusion proteins tags for a wide variety of biological molecules. FRET between CFP and YFP has been employed in numerous investigations of subunit-subunit interactions in membrane proteins (14–16) as well as in studies of conformational rearrangements of transporters and channels (15, 17, 18). In addition, engineered CFP-YFP-containing proteins are able to signal changes in intracellular [Ca\(^{2+}\)] or [Cl\(^-\)] within the cell through conformational alterations in FRET distances or, in the case of Cl\(^-\), through intrinsic properties of YFP.

In this paper we report the first utilization of the CFP-YFP pair to tag NKCC1 in a study of structure and function of the transport protein. We find that there is significant intermolecular FRET between NKCC1 molecules, clearly demonstrating homomultimeric association of the functioning transporter.
FRET in CFP- and YFP-tagged NKCC1

units. A FRET pair with YFP in the N terminus between the phosphoregulatory domain and the transmembrane domain and with CFP in the C terminus exhibits a high level of FRET which changes on transporter phosphorylation. On the other hand, FRET changes were not observed when the fluorescent protein (FP) was attached at the initial residue in the flexible N terminus. Substantial changes in intramolecular and intermolecular FRET occurred cotemporaneous with changes in cell volume, indicative of a more condensed FP-NKCC structure possibly due to increased macromolecular crowding.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Fig. 1 (below) illustrates the shark NKCC1 constructs as well as the shorthand notation we use here to describe these constructs. The shark cotransporter is functionally nearly identical to the human cotransporter with somewhat different ion affinities; it offers the advantage of availability of excellent antibodies. As shown, CFP and YFP, abbreviated C and Y respectively, were added to NKCC1 at the extreme N terminus (CNKCC and Y-NKCC) or in the N terminus near the membrane (0.83Y'-NKCC) and within a loop in the C terminus (NKCC-C and NKCC-Y); in “double” constructs two FPs were added in the N and C termini (C-NKCC-Y, Y-NKCC-C, and 0.83Y'-NKCC-C'). We also used a Cl−-insensitive Q69M mutant of YFP (19), denoted here as Y*, and in some experiments the A206K “monomeric” mutants (20) of CFP and Y*FP, denoted here C' or Y*, respectively.

Enhanced CFP and YFP vectors (pECFP-N1 and pEYFP-N1) were purchased from Clontech. Restriction sites were added to shark NKCC1 by the Kunkel method of site-directed mutagenesis as previously described (1) using primers shown in supplemental Table 1. FPs were cloned into NheI/BsrGI sites added in the middle of the C terminus. Original BsrGI sites, which were added at the N terminus, and into AgeI/BsrGI sites added in the middle of the C terminus. Original BsrGI sites, which were added at the N terminus, and into AgeI/BsrGI sites added in the middle of the C terminus.

Some experiments the A206K “monomeric” mutants (20) of CFP and Y*FP, denoted here C' or Y*, respectively.

NKCC and NKCC-C were prepared from 0.83Y'-NKCC-C' was prepared by transfecting NKCC-C' (hygro) with 0.83Y'-NKCC-Neo. The doubly labeled 0.83Y'-NKCC-C' was prepared from 0.83Y'-NKCC and NKCC-C' by subcloning of BstEII-BspEI fragments.

HEK Cell Expression, Analysis of Cells—Maintenance of HEK-293 cells and generation of stable cell lines have been previously described (1, 5). Individual colonies were screened for expression of FP-tagged NKCC1 by 86Rb influxes, Western blot analysis, and fluorescence. Media were supplemented with appropriate selective antibiotics: 800 μg/ml G418 (Invitrogen), 100 mg/ml hygromycin (American Bioanalytical), or 1 μg/ml puromycin (Clontech). Stable cell lines are named after the constructs they express. Cotransfectants are identified by a “+” between the two expressed constructs (e.g. C-NKCC+Y*-NKCC is a line coexpressing both C-NKCC and Y*-NKCC under different antibiotic resistances).

In some experiments we also investigated the properties of hslo K+-channels tagged with CFP and YFP, obtained as a gift from Giraldez and Sigworth (21). We transfected HEK cells and isolated a stable line coexpressing hslo-667-CFP and hslo-904-YFP as well as a cotransfectant of hslo-667-CFP with NKCC-C'. 86Rb uptake fluxes were carried out as previously described in an automated 96-well plate robotic assay (1). Two-min influxes were performed in basic medium (135 mm NaCl, 5 mm KCl, 0.5 mm CaCl2, 0.5 mm MgCl2, 0.5 mm Na2HPO4, 1 mm NaH2PO4, 15 mm HEPEs, pH 7.4) and 0.2–1 μCi of 86Rb/ml with Rb−substituting K+. NKCC1 was activated by 60 min of preincubation in solutions of various composition to lower the intracellular Cl− concentration; 86Rb influxes are >90% inhibitable by bumetanide (1). Determination of the degree of phosphorylation of the phosphoregulatory domain of NKCC1 by Western blotting and immunofluorescence of semi-thin sections were carried out as previously described (1) using J3 antibody to body to measure total shark NKCC1 (22) and R5 antibody to detect phosphorylation (23). In Western blot experiments we used equivalent numbers of confluent cells (Figs. 2 and 6) or equal aliquots of cell suspensions (fig.3, 8) and confirmed equal loading by Coomassie Blue staining of the blots.

Fluorescence Experiments—For most fluorescence experiments nearly confluent cells in 10-cm dishes were cultured overnight at room temperature. The cells were rinsed off the dish, washed, and resuspended in 1 ml of basic medium or basic medium 2-fold-diluted, both supplemented with 5 mM glucose. The cell concentration of different cell lines was adjusted by taking optical density readings at 540 nm. Fluorescence was measured in suspension in a stirred cuvette thermostatted to 22 °C (or 25 °C, Fig. 7). Approximately 200 μl of cell suspension (~5 × 107 cells) was used per fluorescence experiment, giving an optical density of 0.1–0.4 in the 2 ml of solution in the cuvette. Cells were held on ice for 1–4 h and pre-equilibrated at room temperature for 2–15 min immediately before the experiment. To activate the cotransporter, aliquots of cells were preincubated in low Cl− hypertonic medium (basic medium, but with glucose replacing all but 3 mM Cl−, diluted 2-fold with water) for 30–60 min at room temperature. In experiments involving osmolyte additions, 200 μl of cell suspension was separated into aliquots into 2-fold diluted basic medium in the cuvette, with a 200-s equilibration before other additions.

We also carried out experiments with cells grown on polylysine-coated 11 × 22-mm coverslips (Thoma). These were taken directly from culture medium and placed at a 28° angle to the illuminating beam in a modified plastic cuvette, over a stirring bar. Essentially identical results were obtained as with cells in suspension, both with regard to volume-dependent changes in NY‘CC’ and phosphorylation-dependent changes in 0.83Y‘-NKCC-C‘. The coverslip method guaranteed the freshest cells and gave lower noise in FRET measurements at some cost in
reproducibility in the number of cells from one coverslip to
another.

Fluorescence time courses and spectra were acquired on a
Fluoromax 3 fluorometer (Jobin Yvon), with excitation and
emission slits at 10 nm. For CFP excitation and emission the
monochromators were set to 420 and 475 nm, respectively; for
YFP excitation and emission the settings were 488 and 525 nm.
To measure FRET, the excitation wavelength for CFP and the
emission wavelength for YFP were used. To reduce the effect of
monochromator bleed-through of light scattered from the cell
suspension, polarizers were used in the excitation (0°) and emis-
sion beams (90°).

Fluorescence lifetime data were recorded on a Photon Tech-
nology International fluorometer (Lawrence, NJ) under the
same conditions as in the cuvette experiments. Data were fit to
a monoexponential decay after convolution with the instru-
ment response function (CFS-LS software; Michael L. Johnson,
University of Virginia, Charlottesville, VA).

The nomenclature used for fluorescent signals is as follows:
CC, total fluorescence seen in the donor channel (CFP excita-
tion and CFP emission wavelength); CY, total fluorescence seen
in the FRET channel (CFP excitation and YFP emission wave-
length); YY, total fluorescence seen in the acceptor channel
(YFP excitation and YFP emission wavelength). To correct for
autofluorescence of cells, background effects of added reagents,
and residual light scatter, experiments were performed twice,
with an equal amount of HEK cells expressing untagged NKCC1; the
latter readings were then subtracted from those with fluores-
cently tagged NKCC. The spectral bleed-through for our
experiments was, for instance, tagged NKCC. These experiments were performed twice,
with the exception of the SPAK binding domain (residues
40–48), the PP1 binding domain (residues 108–113), and the
phosphorylation domain (residues 167–209), has very high
function. The entire region (residues 1–246 in shark NKCC1),
tolerates the addition of various epitope tags without change of
function. The entire region (residues 1–246 in shark NKCC1),
with the exception of the SPAK binding domain (residues
40–48), the PP1 binding domain (residues 108–113), and the
phosphorylation domain (residues 167–209), has very high
indices of predicted disorder using PONDR (27) and DisEMBL
(28). We added FP tags both at the extreme N terminus
(Y-NKCC, C-NKCC) and near the first transmembrane
domain (0.83Y*-NKCC, Fig. 1). These were studied in two
major series of experiments, the 0.83Y*-NKCC experiments

4 Available evidence favors NKCC dimers (9, 10). Higher oligomers will also
follow these equations at low FRET efficiencies, in which case total FRET is
made up of the sum of the FRET values for every potential conjugate pair,
each of which is described by the same mole fraction relationship.

5 B. Forbush, unpublished information.

In the experiment shown in Fig. 6 (below) we isolated
cotransfected cell lines and picked lines with different amounts of
wild type NKCC1 (wt) and Y*-NKCC-C (doub). Assuming
transporters form dimers,4 the probability for a pairing of
Y*-NKCC-C in these lines is given by (doub/(wt + doub))2,
and the equation we fit in Fig. 6D is

\[ FRET_{\text{norm}} = FRET_{\text{norm, monomer}} + FRET_{\text{norm, dimer}} \left( \frac{1}{1 + \text{wt/doub}} \right)^2 \] (Eq. 4)

Intracellular chloride was estimated from the fluorescence
yield of YFP in cell lines expressing C-NKCC-Y (CFP and chlo-
ride sensitive YFP). Because CFP is chloride-insensitive, the
ratio YY/C_{TOT} (where C_{TOT} is the CFP fluorescence in the
absence of FRET, see supplemental Methods 1) is a measure of
changes in intracellular chloride concentration provided pH is
controlled or measured. Intracellular pH in HEK cells has been
reported to increase only 0.08 pH units on shrinkage in 200 mM
sucrose (24) and less than 0.1 pH unit on removal of extracel-
lar Cl− (25).

We used scattered light as an empirical indicator of cell vol-
ume changes. Scattered light was measured in the Fluoromax 3
with excitation and emission monochromators set to 540 nm
and with 1-nm slit, under the same conditions as in the fluores-
cence experiments. As presented, light scatter data were not
corrected for dilution of the cells upon addition of solutes.

**RESULTS**

**FP Constructs of NKCC1**—With an aim to generate func-
tional probes of NKCC function, we inserted CFP and YFP at
the N terminus of shark NKCC1 and also within the large cyto-
plasmic C terminus. The N terminus of the cation–chloride
cotransporters is poorly conserved, varies in length among spe-
cies and isoforms, is insensitive to partial truncation (26), and
tolerates the addition of various epitope tags without change of
function. The entire region (residues 1–246 in shark NKCC1),
without the exception of the SPAK binding domain (residues
40–48), the PP1 binding domain (residues 108–113), and the
phosphorylation domain (residues 167–209), has very high
indices of predicted disorder using PONDR (27) and DisEMBL
(28). We added FP tags both at the extreme N terminus
(Y-NKCC, C-NKCC) and near the first transmembrane
domain (0.83Y*-NKCC, Fig. 1). These were studied in two
major series of experiments, the 0.83Y*-NKCC experiments
being carried out subsequent to completion of most of the work with N-terminal constructs.

In contrast to the N terminus of NKCC, much of the C terminus is tightly conserved, and functional insertion of tags at the extreme C terminus has been unsuccessful. Thus, we attempted insertion of FPs in a region of the C terminus that is locally very poorly conserved among NKCCs, is absent in KCCs, and has a high degree of predicted disorder using PONDR (27) or DisEMBL (28) (Fig. 1A).

As illustrated in Fig. 1B, the fluorescence excitation and emission spectra of enhanced CFP- and YFP-tagged shark NKCC1 are similar to those of other FP constructs, consistent with appropriately folded fluorescent proteins (29); there was no detectable difference between spectra of FPs at the N terminus or within the C terminus (not shown). FP-tagged NKCCs were correctly delivered to the plasma membrane as demonstrated by immunofluorescence using the J3 antibody in semithin sections; C-NKCC-Y is illustrated in Fig. 2, in which cells incubated at room temperature for 24 h (Fig. 2A) or 25 °C (B) for 24 h before fixation. C, autocorrelation peak of images in A (solid line) and B (dotted line); autocorrelation by NIH/Scion-Image. This is a diagonal line scan of the two-dimensional function. D, Western blot of FP-tagged NKCC1s, stained with J3 antibody. 

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**FIGURE 1.** FP-tagged NKCC1 constructs. A, model of shark NKCC1 showing the positions of FP tags. Three identified phosphoregulatory threonines are designated P, B, fluorescence excitation (dashed lines) and emission spectra (heavy solid lines) recorded from C-NKCC, Y-NKCC, or C-NKCC-Y* (thin line, emission only). Shaded bars indicate the wavelengths for excitation and emission in this study: in order, CFP excitation (425 nm), CFP emission (475 nm), YFP excitation (488 nm), and YFP emission (525 nm).

**FIGURE 2.** Expression of FP-tagged NKCC1 constructs. A and B, immunofluorescence of the stable C-NKCC-Y* stained with J3 antibody recognizing shark NKCC1. Cells were grown at 37 °C (A) or 25 °C (B) for 24 h before fixation. C, autocorrelation peak of images in A (solid line) and B (dotted line); autocorrelation by NIH/Scion-Image. This is a diagonal line scan of the two-dimensional function. D, Western blot of FP-tagged NKCC1s, stained with J3 antibody.

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6 C. M. Gillen, P. Isenring, R. B. Darman, I. Gimenez, and B. Forbush, unpublished information.
good spatial correlation between Y-NKCC-C and a membrane-localized voltage-sensitive dye (30).

Western blot analysis of the stable cell lines expressing FP-tagged NKCC1 used in this study confirmed that full-length proteins are produced (Fig. 2D). Lower bands presumably represent a minority of transporters that are not fully glycosylated (25–35% of total in different constructs); this pattern has been routinely reported for exogenous and sometimes endogenous NKCC1 but has never been definitively linked to the localization of transporter. Appropriately, singly tagged NKCC1 (second through fifth lanes and ninth through twelfth lanes) is larger than untagged NKCC1 (first lane), and the doubly tagged constructs are even larger (sixth through eighth). The same amount of protein was loaded in each lane, so this also illustrates that different HEK cell isolates express different amounts of transfected protein. Together, the results of Fig. 2 and Fig. 3 (below) demonstrate correct synthesis and cell surface delivery of FP-tagged NKCC1.

We used $^{86}$Rb influx measurements to assess the functional behavior of our CFP and YFP constructs. Fig. 3, A and B, illustrates the result of flux experiments examining representative FP transfectants as well as control NKCC1-transfected cells and untransfected HEK cells. The maximal rate of influx is similar in transfected cells with and without the fluorescent tag (Fig. 3B). This confirms the hypothesis that the regions chosen for FP insertion are not necessary for ion transport, being structurally permissive of a large insertion.

In the experiments of Fig. 3, the cotransporter was activated by changes in intracellular [Cl$^{-}$], brought about by preincubation of cells in various extracellular [Cl$^{-}$] before the flux measurements (1). It is interesting that despite the fact that the N terminus contains the phosphoregulatory machinery, the regulatory behavior of N-terminal-tagged NKCC1 is relatively unaffected by addition of FPs, seen by comparing the activation curves of the various lines in comparison to the wild type NKCC1, and tags within the C terminus also had only modest effect on NKCC1 function or activation (Fig. 3A).

In contrast to the result with N-terminal-attached probes, insertion of YFP in the distal portion of the N terminus close to the membrane domain resulted in a transporter (0.83Y'-NKCC) that was activated very little under conditions of lowered cell [Cl$^{-}$] (or by incubation in hypertonic media; not shown) but became active when phosphorylation was stabilized by calyculin A inhibition of PP1 (Fig. 3B, open circles); the same result was obtained for 0.83Y'-NKCC-C', not shown). To test if defective phosphorylation is responsible for the activation failure of the 0.83Y' insertion constructs, we examined phosphorylation using the R5 phospho-NKCC-specific antibody (23). As illustrated by the Western blot in Fig. 3C, wild type NKCC, but not the 0.83Y' construct, is phosphorylated upon lowering intracellular Cl, although both transporters become phosphorylated in the presence of calyculin A. These findings demonstrate that the inserted YFP interferes with phosphorylation of the regulatory domain under the usual activating conditions, presumably by some form of steric hindrance.

C-NKCC-Y as a Ratiometric Intracellular Chloride Sensor—Because YFP is sensitive to quenching by chloride (31), whereas CFP is not, molecular constructs tying together the two probes are useful as ratiometric Cl$^{-}$ sensors, with particular potential as genetically encoded probes (32). Fig. 4 illustrates an experiment examining the fluorescence of FP-tagged NKCCs as a function of [Cl$^{-}$] in intact cells that have been permeabilized to Cl$^{-}$ with tributyltin/nigericin (33). The YF/Ctot fluorescence ratio (where Ctot is the CFP fluorescence in the absence of FRET, see supplemental Methods 1)
in the doubly tagged construct C-NKCC-Y, therefore, gives a measure of $[\text{Cl}^-]$, and this ratiometric index is insensitive to the amount of the probe. The $K_m$ is \(~\sim 190\) mm at pH 7.2 and decreases as pH is lowered, consistent with earlier reports (Fig. 4B) (19, 31, 32).

Our objectives here are potentially impeded by $\text{Cl}^-$ sensitivity, since changes in YFP fluorescence yield are directly reflected in the absolute FRET signal. To eliminate this problem we prepared all of our YFP lines with a YFP-Q69M variant (citrine, notated here as Y*FP) which, as reported by Griesbeck et al. (19) and shown in Fig. 4A, is insensitive to chloride. We also circumvented the problem by using an index of FRET that is normalized by the YFP yield ($F\text{RET}_{\text{norm}}$) and is, thus, insensitive to changes in the YFP yield and $\text{Cl}^-$ concentration (14). In fact we found that the mathematical solution is effective by itself, as we carried out many of the remaining experiments with both YFP and Y*FP constructs with essentially identical results for $F\text{RET}_{\text{norm}}$.

**FRET in FP-tagged NKCC1 Cell Lines**—We performed fluorescence lifetime measurements with CFP in the absence and presence of a partner YFP. The average lifetime of CFP in C-NKCC and NKCC-C was $2.46 \pm 0.10$ ns ($n = 2, 2$), the average lifetime in C-NKCC-Y and Y-NKCC-C was $2.15 \pm 0.07$ ns ($n = 2, 2$), and the average lifetime in 0.83Y*-NKCC-C’ was $1.63 \pm 0.10$ (n = 4). From this, FRET efficiency ($E$) is 0.13 in C-NKCC-Y and 0.34 in 0.83Y*-NKCC-C’.

FRET efficiency for our constructs is shown in Fig. 5A, calculated from the fluorometric measurement of CC, CY, and YY fluorescence using the above values of FRET efficiency for calibration (see supplemental Methods). The fluorometric measure is fully consistent with the lifetime data in that the 0.83Y*-tagged construct exhibited 2.8 times the FRET efficiency with CFP in the C terminus compared with the corresponding molecule in which YFP is at the extreme N terminus (bars h versus b; the concordance is implicit in the finding that the same

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**FIGURE 4. Chloride-quenching of YFP fluorescence.** YFP and CFP fluorescence were monitored in cells permeabilized for protons and chloride by the addition of 50 mm nigericin and 100 mm tributyltin in a HEPES-buffered medium containing 135 mm potassium gluconate. The curves illustrate a titration with additions of NMG-Cl. A, YFP fluorescence (open symbols) and CFP fluorescence (solid symbols), corrected for dilution in C-NKCC-Y (circles) or C-NKCC-Y* (inverted triangles) at pH 6.8. B, ratio of YFP fluorescence to CFP fluorescence in Y-NKCC-C at indicated pH. Lines are from least squares curve fits ($K_{q/\text{Cl}^-} = 580, 253, 192, 153, 92$ mm and $F_{\text{norm}} = 1.71, 1.68, 1.5$-assumed, 1.3-assumed, 1.15 at pH 7.8, 7.4, 7.2, 7.0, and 6.8, respectively).

**FIGURE 5. FRET efficiency in stable cell lines expressing FP-tagged NKCCs.** A, FRET efficiency ($E$) was determined in hypotonic buffer in various lines as indicated. Column g, mixed in cuvette is the average of 16 measurements with cuvette mixtures of C-NKCC and Y*-NKCC ($n = 2$), C-NKCC and NKCC-Y* ($n = 8$), Y*-NKCC and NKCC-C ($n = 2$), and NKCC-C and NKCC-Y* ($n = 4$); in none of these mixed pairs was FRET significantly different from zero. B, ratio of CFP to YFP ($R$), calculated from CC/YY and $F\text{RET}_{\text{norm}}$, see supplemental Methods 1. C, interaction estimates ($I_{(\text{mix})}$) calculated from A and B. n.c., not calculated. n = 4–5 for different cell lines.
We observed significant FRET in all of our cell lines in which individually CFP-tagged and YFP-tagged cotransporters were co-expressed (Fig. 5, light gray bars). For comparison, when we examined a mixed cell suspension with CFP-tagged and YFP-tagged NKCCs, FRET\textsubscript{norm} was not significantly different from zero (labeled in upper panel, Fig. 5). Because CFP and YFP must be within less than 100 Å of one another for FRET to occur, these results show multimerization of cotransporters in the HEK cell membrane. This finding demonstrates that the intact functioning NKCC exists as a dimer or higher multimer, consistent with end-point experiments involving cross-linking studies (9, 10), dominant-negative expression effects (1, 5, 7, 9), and pulldown experiments (9).

To test the generality of responses seen with NKCC (see Fig. 10) we examined a cotransfectant of two hslo channels as well as a cotransfectant of hslo with NKCC-C'. As seen in Fig. 5A we observed significant FRET in the cotransfectant of two hslo channels (j) and a very low level of FRET when hslo was cotransfected with NKCC-C' (k).

In comparing levels of FRET in cotransfected cell lines, it is necessary to keep in mind that dissimilar levels of CFP- and YFP-tagged constructs in cotransfected cells will result in different quantitative expectations. For instance, in multimerization models with equal levels of CFP and YFP constructs, only half of the potential FRET pairs will have a CFP and a YFP, and there will be half of the maximum FRET that would occur if every CFP tag had a YFP partner. Adjusting the observed FRET according to this simple model using the CFP/YFP ratios in Fig. 5B (see “Experimental Procedures”), the interaction estimates (I\textsubscript{FRET}) in Fig. 5C are obtained.

The interaction estimate is seen to be roughly the same between N-terminal and C-terminal cotransfectants (c versus f), but compared with the double constructs (a, b, and h), I\textsubscript{FRET} is only about 60–70% in N- and C-terminal FPs (d and e), and for the 0.83Y' cotransfectant it is 32% of the double construct. This finding implies that 68% of the FRET in the 0.83Y' construct and 30–40% of the FRET signal in doubly labeled constructs with FP at the N terminus arises from intramolecular FRET. To test this conclusion, we carried out a dilution experiment in which we isolated a number of different stable cell lines from the same cotransfection, identifying lines with various ratios of cotransfected wild type NKCC1 and Y'-NKCC-C. Western blot analysis was used to measure the expression ratio of wild type to FP-tagged NKCC1 (Fig. 6A), and FRET\textsubscript{norm} was determined for each cell line (Fig. 6, B and C, will be discussed below).

As expected, the FRET level decreases as Y'-NKCC-C is diluted by the wild type transporter, but as the ratio of FP construct decreases, FRET\textsubscript{norm} extrapolates to a value well above zero (Fig. 6D). These data are fit well by a line that assumes 46% of the FRET is due to intramolecular transfer in the doubly labeled constructs, roughly consistent with the values for I\textsubscript{FRET} in the N to C combinations (Fig. 5C).

**FRET as an Indicator of Conformational Changes in NKCC1—**

We expected that intermolecular and intramolecular FRET in the FP-tagged NKCCs might change under different conditions, reflecting conformational changes in the cotransporter in different steps in the transport cycle or different conformations of active and inactive transporters. Transport-related changes would be expected to take place in the millisecond time scale, whereas regulation-related changes should take place in the time range of 5–10 min (1). To look for evidence of different transport conformations, we followed the FRET signal on var-
ious changes in extracellular ion composition or on inhibition with furosemide or bumetanide; in none of these experiments did we see changes in the FRET level consistent with transporter conformational change from predominantly “inward” to “outward facing” or vice versa (not shown). We did, however, see slower changes consistent with regulatory conformational change and less specifically with a change in cell volume.

The 0.83Y’-NKCC-C’ double construct exhibits robust FRET (Fig. 5, above), although the presence of the YFP moiety near the membrane in the N terminus apparently slows the phosphorylation event involved with transporter activation (Fig. 3). In the experiments presented in Fig. 7, we examined whether activation of the transporter during calyculin A-mediated PP1 inhibition is accompanied by a FRET change. Indeed, as shown in Fig. 7B, FRET$_{\text{norm}}$ reproducibly decreases ~6% during the period of cotransporter activation, concomitant with an increase in transporter activity measured as $^{86}$Rb influx.

The rate and extent of transport activation and of the FRET change was substantially inhibited by preincubation with the kinase inhibitor staurosporine (Fig. 7, A and B). These results indicate that during transport activation, the part of the NKCC N terminus that is close to the phosphoregulatory domain undergoes a conformational change that takes it further from the point of CFP attachment (Gln$^{952}$) in the C terminus. This is the first direct observation of a conformational change in a cation-chloride cotransporter family transporter.

In contrast to the results with the 0.83Y’ construct, we did not see activation-related FRET changes in constructs with an N-terminal FP. Fig. 8 illustrates the result from a typical experiment in which the cell line Y*-NKCC-C was transferred from regular to low chloride hypotonic medium and vice versa. Transient changes in the FRET level were observed with these tran-

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**FIGURE 7. Changes in FRET$_{\text{norm}}$ during activation of 0.83Y’-NKCC-C’**. 
A, time course of cotransporter activation by 0.5 $\mu$M calyculin A at 25°C measured in a subsequent 1-min $^{86}$Rb influx measurement (at 22°C) with and without 10 min of pretreatment with 2 $\mu$M staurosporine as noted. Representative of three similar experiments. B and C, FRET$_{\text{norm}}$ (normalized to the value at $t = 0$) during activation with 0.5 $\mu$M calyculin A, without (B) and with (C) pre-addition of staurosporine at $t = 10$ min. Three traces each are shown from one experiment; similar results were obtained in five other experiments, two of which also employed staurosporine.

**FIGURE 8. FRET$_{\text{norm}}$ during activation and deactivation of N-terminal-labeled constructs.** The activating condition is low Cl hypotonic medium, and the deactivating condition is basal medium. A, FRET$_{\text{norm}}$ after 30 min in preincubation medium on transition to the second medium, with (light lines) or without (dark lines) staurosporine, as indicated. B, phosphorylation of NKCC, in cell suspension conditions similar to the cuvette experiments. Cells were solubilized at appropriate time points after transition from basal medium to low Cl hypotonic medium, without (B) or with (C) 2 $\mu$M staurosporine added 2 min before the change in medium. Western blotting was carried out with R5 antibody to detect phosphorylated cotransporter. Both shark C-NKCC-Y and HEK all (human, h) NKCC1 were detected.
FRET in FP-tagged NKCC1

FIGURE 10. Fractional change in FRET per change in cell volume in FP-tagged NKCCs. Data are from experiments similar to that in Fig. 8, with different cell lines, obtained by a linear fit of the change in FRET for the first two additions of NaCl + KCl (open bars), NaMg gluconate (gray bars), or sucrose (black bars). Fractional change was divided by the fractional change in cell volume determined from centrifugation of wild type NKCC1 cells in microcapses (0.08, 0.18, 0.18 per 100 mosmol for the three osmoles). Letters refer to the corresponding bars in Fig. 5. Bars labeled mon. present data for non-dimerizing (A206K) CFP and Y*FP lines; n = 4 – 9.

FIGURE 9. FRETnorm under conditions of cell shrinkage. Cells expressing Y*-NKCC-C were equilibrated in 2 ml of 2-fold diluted basal medium and 200-μl additions of either 666/333 mM NaCl/KCl, (top), 1 mM NaMg gluconate (middle), or 1 mM sucrose (bottom) were made at times marked by arrows. Red, FRETnorm, green, YY/Ctot and estimated intracellular [Cl-]; blue, light scatter, an empirical indicator of cell volume.

...sions, but they occurred in the 30-s to 1-min time range, much too slow to reflect conformational rearrangement of cotransporter states and much too fast to reflect changes in phosphorylation. When we used staurosporine to block phosphorylation of NKCC, we observed essentially the same time courses of fluorescence change (light curves in Fig. 8A). Utilizing a phosphorylation-specific antibody for NKCC in parallel experiments, it is seen in Fig. 8B that phosphorylation of NKCC takes place in the 10–20-min time range and is completely blocked by staurosporine (Fig. 8C). Incidentally, this experiment also illustrates the anomalous behavior of the native HEK cell cotransporter that we have discussed previously (23).

FRET in NKCC Responds to Changes in Cell Volume—After the results of Fig. 8, we asked if changes in cell volume might be responsible for FRET changes seen in Y*-NKCC-C. Fig. 9 illustrates a set of experiments in which we performed sequential addition of osmolyte to the medium (arrows) and monitored the fluorescence change in Y*-NKCC-C (red lines). FRETnorm rose incrementally with successive additions of osmolyte, and the result was similar whether we added NaCl + KCl, NaMg gluconate, or sucrose to bring about cell shrinkage. To see if cell volume is the variable that changes within 1 min after osmolyte addition, we monitored light scattering in parallel experiments as an empirical index of cell volume (Fig. 9, blue lines). Indeed light scattering was found to increase over 1 min after each solute addition, consistent with the conclusion that the level of FRET in FP-tagged NKCC is responsive to cell volume.

As seen in the green lines in Fig. 9, the YY/Ctot signal decreases upon NaCl + KCl additions (upper panel), demonstrating an increase in intracellular Cl− concentration concomitant with cell shrinkage within the same time period as the changes in FRETnorm and light scattering. Cl− concentration is provided on the abscissa as an estimated value, based on an assumed intracellular pH of 7.2 and the curve in Fig. 4B. The increase in cellular Cl− on shrinkage in NaMg-glucenate is small and transient compared with the increase upon shrinkage in NaCl + KCl; this presumably reflects Cl− gain in high salt media with entry mediated by NKCC1 and Cl− loss on shrinkage in organic osmolytes through the action of the K-Cl cotransporter and K+ + Cl− channels.

Fig. 10 presents the fractional change in FRET that takes place per volume change for the three osmolyte additions, cell volume being determined by measuring the cytotoxic under these conditions. There is a volume response of FRET in each of the cell lines and a rather wide range of the fractional response; it is a puzzle to us why the change with Y*-NKCC-C (b) is substantially greater than for the other constructs. The possibility that FRET changes in FP-tagged proteins are a general consequence of volume change cannot be ruled out since we observed small changes with tagged hslo K channels (j) (21) and...
FRET in CFP- and YFP-tagged NKCC1

with the cotransfectant of hslo and NKCC (k). However, the very small magnitude of the latter changes argues that the unusual properties of N-terminal-tagged NKCC1 are in some way responsible for the largest fraction of the effect.

Asking whether intramolecular FRET is volume-sensitive, we first note that the fractional change in the double-FP constructs (Fig. 10, a and b) is greater than that in the corresponding cotransfected single-FP constructs (d and e). This observation argues that FRET between two FPs on the same NKCC monomer is at least as sensitive to volume as that on separate monomers. A similar conclusion is obtained in the dilution experiment presented above in Fig. 6C; as Y*-NKCC-C is diluted with wild type NKCC, the fractional response does not change significantly, except for a small but significant increase that is seen in the line with the greatest amount of wild type NKCC (#12, p < 0.05, t test). Because intramolecular but not intramolecular FRET is reduced in this line and the volume sensitivity is not reduced, it appears that intramolecular FRET is indeed volume-sensitive.

Multimerization of FP-tagged NKCCs Is Not Caused by FP Dimerization—Green fluorescent protein and its derivatives have a tendency to dimerize, and this has been shown to cause dimerization of FP-tagged molecules (20). To assess this possibility in the case of NKCC, we prepared mutants of CFP- and YFP-tagged NKCC with an A206K mutation in the FP to prevent dimerization (20). Cotransfectants of these monomeric constructs (Fig. 10, bars marked mon.), fractional responses to cell volume change significantly, except for a small but significant increase that is seen in the line with the greatest amount of wild type NKCC (#12, p < 0.05, t test). Because intramolecular but not intramolecular FRET is reduced in this line and the volume sensitivity is not reduced, it appears that intramolecular FRET is indeed volume-sensitive.

DISCUSSION

FP Probes of NKCC1—The experiments presented here are the first to examine fluorescence resonance energy transfer between and within tagged monomers of NKCC1. We have engineered NKCC1 with CFP and YFP in three positions, two in the N terminus and one in a loop in the C terminus. These regions are locally poorly conserved and probably relatively unstructured within the NKCC1 protein. For two of the insertion positions there is no detectable effect of the FPs on NKCC1 function, but insertion near the phosphoregulatory domain in the N terminus resulted in a transporter that is not phosphorylated under usual activating conditions but exhibits normal transport function when phosphorylation is caused by the addition of a phosphatase inhibitor.

Intramolecular FRET was observed between FP-tagged NKCC1 pairs, and intramolecular FRET between the N and C terminus of the same NKCC1 molecule was also seen. The level of FRET changed in response to regulatory conformational change in the construct with YFP attached near the phosphoregulatory domain but not with YFP attached at the N-terminal residue. FRET changes concomitant with cell volume change were also observed in all constructs.

In fluorescence, lifetime measurements the 0.83Y’-NKCC-C’ construct exhibits a FRET efficiency of 0.34, of which ~68% is due to intramolecular transfer (Fig. 5C). Calculating from the Förster equation and a Förster radius of 49 Å for the CFP/YFP FRET pair (34), this efficiency translates to 60 Å between intramolecular CFP-YFP pairs and 70 Å between intermolecular pairs. Noting that cross-sectional dimensions for a 12-transmembrane-domain membrane protein are in the range of 20–60 Å (see e.g. Ref. 35), this indicates that the average distance between the two tags is quite large in relation to the likely size of the monomer.

Similar calculations for the N-terminal-tagged FRET pairs (Y-NKCC-C and C-NKCC-Y) yield 73 and 81 Å for intra- and intermolecular FRET, respectively. This dimension is larger than the expected size of a monomer or of the average expected distance between dimer pairs (as above, based on a 20 × 60 Å unit) but would be consistent with an N terminus that extends a considerable distance into the cytoplasm. It would not be surprising if there was a high degree of mobility of the tag, since the N terminus itself has low predicted secondary structure and may form a >160 residue flexible tether for the FP. Although the geometry is unknown, the difference in calculated average distances for Y-NKCC-C and 0.83Y’-NKCC-C indicate that the N terminus of NKCC extends at least 12 Å into the cytoplasm beyond the 0.83 attachment site nearer the membrane.

Regulatory Conformational Change—The 0.83Y’-NKCC-C’ construct reports regulatory conformational change as a 6% decrease in FRET upon phosphorylation of the cotransporter. This is the first direct observation of conformational change in NKCC and is consistent with a 1–2–Å movement of Y’FP away from the C terminus upon activation. The predicted movement is modest, as might be expected if the 0.83 insertion point (Fig. 1) is near the pivot point for movement of the phosphoryregulatory domain.

The insertion of Y’FP at the 0.83 site causes loss of regulation under normal cellular conditions, presumably either by making the phosphoacceptors less accessible to SPAK kinase (4, 36) or more accessible to protein phosphatase 1 (26). In view of the predicted flexibility of the region in NKCC as well as the flexibility of the Y’FP attachment, it seems unlikely that this is due to “kinking” of a structural domain; rather, it is likely due to steric hindrance or distortion due to the bulk of the YFP molecule. It is interesting that although this hinders phosphorylation of the regulatory domain, it apparently does not interfere with the mechanism of the regulatory switch itself, since the transporter still becomes activated when phosphorylation is promoted with calcyclin A.

It is noteworthy that the N-terminal-tagged constructs do not show significant FRET change on activation despite a reasonable expectation of movement of the entire regulatory domain. Most likely this is simply reflective of considerable distance and flexibility between the phosphoryregulatory sites and the extreme N terminus. Indeed, predictive algorithms show little structure in the 180 residues of this region, and their deletion has been found to have little functional effect on NKCC1 (26) or NKCC2 (37).
**NKCC1 Oligomerization**—The current results demonstrate for the first time oligomerization of functioning Na-K-Cl cotransporters in the HEK cell membrane using a real-time non-invasive technique. This finding is consistent with numerous lines of end-point evidence supporting homomultimerization of cation-chloride cotransporters including NKCC1, NKCC2, Na-CI cotransporter, and KCCs (1, 5, 7–13). The relatively high ratio of intermolecular to intramolecular FRET (Fig. 5) provides strong evidence that dimers or higher oligomers are the dominant, if not exclusive species in the functioning transporter. Importantly, we find that the FRET level in FP-tagged NKCC1s does not change when the transporter is activated by phosphorylation, apparently ruling out NKCC1 dimerization as part of its primary regulatory mechanism.

All previous studies of cation-chloride cotransporter multimerization have been consistent with or have specifically supported a dimer model, although some have suggested the possibility of higher oligomers. In the present study we find FRET between all combinations of tagged transporters (N to C, N to N, and C to C). At this point it is not possible to infer the degree of multimerization from our observations, and in general the FRET approach appears less useful in unraveling stoichiometries of homomultimeric proteins than it has been for determining the stoichiometry of heteromultimeric channels when the total number of subunits is known (38).

NKCC1 makes up about 2% of the membrane protein in secretory epithelia (39, 40), and there is a similar amount of NKCC1 in transfected HEK cells, which implies that there are less than 500 transporters per μm². At these densities random collisions of transporter monomers would be expected to contribute FRET in the range of \(E = 0.01\) (Ref. 41 and Fig. 5), which is indeed the level of FRET we found in a cotransfection of a tagged hslO channel and NKCC-C’ (Fig. 5A, bar k). The evaluation of the contribution of random collisions to resonance energy transfer measurements can be difficult to determine (42), but since the YFP acceptor concentration in our transfectants is similar to that in the hslO cotransfectant,² we do not expect random collisions to be a major contributory factor. In particular, the fact that intermolecular FRET is competed by untagged transporter (Fig. 6) is consistent with competition for oligomeric partners but is not consistent with random collisional FRET.

**NKCC1 Clomeleon**—We have demonstrated here that the doubly labeled YFP-NKCC1-CFP can be used as an intracellular Cl⁻ sensor by virtue of the fact that YFP fluorescence is quenched by chloride. Because YFP and CFP are encoded as part of the same NKCC1 construct, the fluorescence yields of the two fluorophores provides a ratiometric measure that is insensitive to cotransporter expression levels. The ability to genetically encode this sensor will enable construction of a mouse model in which it should be possible to continuously monitor intracellular Cl⁻ levels in secretory epithelia. This is an extension of the “clomeleon” concept of cytoplasmic Cl⁻ sensor (32) with the advantage that the sensor would be specifically localized in high concentrations at the intracellular aspect of the basolateral membrane in secretory cells. In the future, enhancement of the Cl⁻ affinity can be attained by mutation of a single residue (YFP-V163S) to yield a YFP with \(K_d(\text{Cl}^-)\) in the physiological range (43, 44).

**Volume Sensitivity of the FRET Signal**—A surprising finding in the current work is that the amount of FRET within and between NKCC1 monomers is sensitive to cell volume. Several lines of evidence support cell volume as the relevant variable. 1) FRET changes are dependent on extracellular osmotic strength whether salt, organic salt, or sucrose is used, and the FRET change is roughly proportional to cell shrinkage (Fig. 10). 2) FRET changes are temporally consistent with cell volume change, as determined by light scattering and intracellular [Cl⁻] measurements (Fig. 8). 3) FRET changes are temporally inconsistent with transporter conformational change or phosphorylation events. 4) FRET changes are virtually abolished by permeabilization of the cells with streptolysin O.⁸

What does the volume sensitivity of FRET represent? One suggestion is that homooligomeric interactions are directly affected by stretch of the cell membrane, i.e. that oligomers are actually pulled apart or crowded together by attachment to cytoskeletal or membrane-embedded elements. This otherwise attractive hypothesis lacks a quantitative physical foundation, and it also fails to explain the finding that observed intramolecular FRET changes are also observed (Figs. 6C and 10). In addition, we have found no effect on the FRET response of a 1-h preincubation at 37°C with any of 4 cytokskeletal inhibitors: latrunculin B (25 μM), cytochalasin C (20 μM), cytochalasin D (20 μM), and jasplakinolide (5 μM) (data not shown).

A second possibility is that changes in macromolecular crowding are responsible for our observed FRET changes. “Macromolecular crowding” is used to express the fact that a significant fraction of cellular volume is comprised of macromolecules and that the resulting steric exclusion has major effects on protein structures and reaction rates (45, 46). Cell shrinkage increases macromolecular crowding, and it is possible that crowding leads to a more condensed FP-tagged NKCC1 structure and restricts the movement of flexible probes, keeping them near the cell membrane and each other. A more condensed state is indeed the direction predicted by theoretical models of molecular crowding (47). The observation that FRET change is smallest in the C terminus to C terminus pairs (Fig. 10f) is consistent with the idea that the largest part of this phenomenon is due to the movement of FPs attached to a flexible N terminus.

An intriguing possibility is that the factors which alter FRET during cell volume changes, be they molecular crowding or other physical interactions, result in differential NKCC phosphorylation and, thus, regulation of the Na-K-Cl cotransporter. The mechanisms by which cell volume regulate cellular processes remain for the most part elusive, and macromolecular...

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² The approximate YFP levels in our constructs relative to hslo-667-YFP in the NKCC-C’ cotransfected (k in Fig. 5) are 2.3, 2.4, 1.3, 0.6, 1.9, 1.4, 0.4, 0.8, and 0.2 for constructs labeled a, b, c, d, e, f, h, i, and j in Fig. 5.
crowding has been seen as a strong candidate for the first step in the signaling process (48, 49). However, although crowding or other physical factors may directly affect NKCC1, there is also strong evidence that the activities of upstream modulatory kinases (50, 51), particularly WNK (with no lysine) kinases (52–54), are determined by changes in intracellular [Cl−] and cell volume.

**Conclusion**—The present study introduces singly and doubly tagged FP derivatives of NKCC1 and examines their utility as fluorescence and FRET-based probes of parameters relating to transport. The Cl−-sensing ability of YFP renders these molecules useful as Cl− sensors; we have also found that some of the FRET pairs exhibit a substantial change in response to change in cell volume. With YFP attached near the phosphoregulatory domain, one of the constructs signals cotransporter activation, although the presence of the probe hinders activation under normal conditions. It is anticipated that further development will provide genetically encoded probes of both intracellular [Cl−] and of cotransporter regulatory state that would be expressed at high concentrations in secretory and absorptive epithelia.

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