Role of Cysteine Residues in the NCKX2 $Na^+/Ca^{2+}-K^+$ Exchanger: Generation of a Functional Cysteine-Free Exchanger†

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Received March 8, 2004; Revised Manuscript Received April 22, 2004

ABSTRACT: Cysteine residues play an important role in many proteins, either in enzymatic activity or by mediating inter- or intramolecular interactions. The $Na^+/Ca^{2+}-K^+$ exchanger plays a critical role in Ca^{2+} homeostasis in retinal rod (NCKX1) and cone (NCKX2) photoreceptors by extruding Ca^{2+} that enters rod and cone cells via the cGMP-gated channels. NCKX1 and NCKX2 contain five highly conserved cysteine residues. The objectives of this study were threefold: (1) to examine the importance of cysteine residues in NCKX2 protein function; (2) to examine their role in the interaction between NCKX2 and the CNGA subunit of the cGMP-gated channel; and (3) to generate a functional cysteine-free NCKX2 protein. The latter will facilitate structural studies taking advantage of the unique chemistry of the thiol group following insertion of cysteine residues at specific positions in the cysteine-free background. We generated a cysteine-free NCKX2 mutant protein that showed normal protein synthesis and processing and ~50% wild-type cation transport function. Cysteine residues were also not critical for the formation of NCKX2 homo-oligmers or NCKX2 hetero-oligomers with the CNGA subunit of the cGMP-gated channel. Our results appear to rule out a critical importance of an intramolecular disulfide linkage in NCKX2 protein synthesis and folding as had been reported before.

The $Na^+/Ca^{2+}-K^+$ exchanger (NCKX)[1] was first described as an integral membrane protein in the plasma membrane of retinal rod photoreceptors that uses both the inward Na^+ gradient and outward K^+ gradient for Ca^{2+} extrusion at a stoichiometry of $4Na^+:1Ca^{2+} + 1K^+$ (*1*, *2*). To date, four distinct human NCKX genes have been described, SLC24A1 through SLC24A4 coding for proteins NCKX1 through NCKX4, while a partial sequence for human NCKX5 has appeared in the database (*3*). NCKX1 and NCKX2 are expressed in the outer segments of retinal rod and cone photoreceptors, respectively, while in the retina, NCKX2 is also found in ganglion cells (*4*−*6*) and is widely expressed in the brain as well (*7*). In rod and cone photoreceptors, NCKX serves to extrude Ca^{2+} that enters photoreceptors in darkness through the light-sensitive, cGMP-gated channels (CNG). This functional synergy between NCKX and CNG is manifested by a direct association between NCKX and CNG in situ in bovine rod outer segments (*8*, *9*), and both NCKX1 and NCKX2 have been shown to bind to their respective CNGA1 and CNGA3 subunits when coexpressed in heterologous systems (*10*).

Both NCKX1 and NCKX2 are polytopic membrane proteins thought to consist of two sets of transmembrane-spanning segments (TMs) and two large hydrophilic loops, one at the N-terminus and one in the cytosol separating the two sets of TMs; the two sets of TMs each contain five membrane-spanning helices (*11*). The N-terminus of both NCKX1 and NCKX2 contains a cleavable signal peptide that appears to be essential for proper plasma membrane targeting (*12*). NCKX1 has been extensively studied "in situ" in retinal rod outer segments (*3*, *13*), whereas NCKX2 is currently the most studied member of the NCKX gene family in heterologous expression systems, as NCKX2 cDNAs consistently yield the best functional and protein expression (*14*). Scanning mutagenesis of the human NCKX2 has identified at least 20 residues that are important for NCKX function, including six residues that are thought to be involved in cation binding/transport (*15*). NCKX1 and NCKX2 from various species contain five highly conserved cysteine residues located in the TMs and thought to be at or close to the membrane/water interface (Figure 1); these cysteine residues are found in all NCKX1 and NCKX2 sequences currently in the database, and they are also found in the sequences of more distantly related $Na^+/Ca^{2+}-K^+$ exchangers cloned from *Caenorhabditis elegans* and *Drosophila*, respectively (*16*, *17*).

The effects of sulfhydryl reagents on NCKX1 transport function were previously investigated in bovine rod outer segments. Cysteine modification by Ag^+ was shown to lead to complete inhibition of NCKX1 in this preparation, while subsequent removal of Ag^+ by β-mercaptoethanol reversed inhibition and accelerated the $Na^+/Ca^{2+}-K^+$ exchanger rate (*18*). In contrast, the cysteine-alkylating reagent *N*-ethyl-

† This work was supported by an operating grant from the Canadian Institutes for Health Research to P.P.M.S. P.P.M.S. is a scientist of the Alberta Heritage Foundation for Medical Research. T.G.K. is a recipient of a studentship from the Alberta Heritage Foundation for Medical Research.

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[1] Abbreviations: NCKX, $Na^+/Ca^{2+}-K^+$ exchanger; NCX, Na^+/Ca^{2+} exchanger; Cys-less, cysteine-free; IP, immunoprecipitation; MW, molecular weight; EDTA, ethylenediamine tetraacetic acid; DOC, deoxycholate; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; DMF, dimethyl formamide; PAGE, polyacrylamide gel electrophoresis.

10.1021/bi049538y CCC: $27.50
Published on Web 05/22/2004

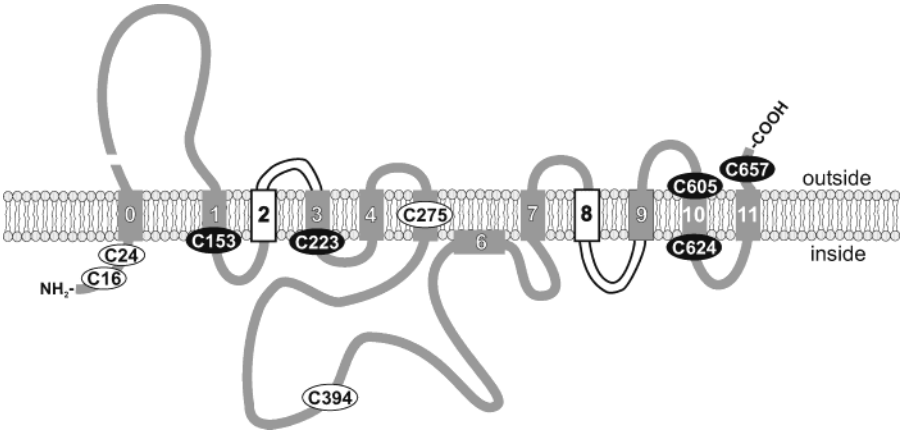

FIGURE 1: Location of cysteine residues within human NCKX2. The positions of each of the nine cysteine residues within the wild-type NCKX2 protein are indicated. C16 and C24 are located in the cleaved signal peptide (as indicated by the break). The five cysteine residues conserved in all currently available NCKX1 and NCKX2 sequences are indicated by the black background. The nonshaded sequence elements in helices 2 and 8 represent the so-called α1 and α2 repeats, respectively.

maleimide caused only a slight inhibition of $Na^+/Ca^{2+}-K^+$ exchange transport in bovine rod outer segment membranes, while some thiol-based cross-linking reagents resulted in a slight increase in transport rate (*19*). A recent study reported that mutagenesis of two of the above five conserved cysteine residues in NCKX2 cloned from rat brain led to the complete absence of NCKX2 protein expression in HEK293 cells, and it was concluded that these two cysteine residues are involved in an intramolecular disulfide linkage required to stabilize the NCKX2 protein (*20*). The human cone NCKX2 shares over 89% conservation in amino acid sequence with that of the rat brain NCKX2. Furthermore, sequence variation between the two types of NCKX is found largely in the two large hydophilic loops with near complete conservation within the transmembrane regions containing the two cysteine residues in question. Here, we report mutagenesis of all nine cysteine residues of the human cone NCKX2 individually, in several combinations, and finally, all nine residues combined to yield a cysteine-free (Cys-less) NCKX2. NCKX2 protein expression was not affected in any of these cysteine mutants, while replacement of all nine cysteine residues by serine resulted in a Cys-less NCKX2 that showed ~50% wild-type $Na^+/Ca^{2+}-K^+$ exchange function in two different expression systems. A Cys-less NCKX protein permits insertion of single or pairs of cysteine residues for topological and structural studies that take advantage of the unique reactivity of its thiol group.

EXPERIMENTAL PROCEDURES

Generation of Cysteine-Free Human Cone NCKX2. The single-cysteine mutants and, ultimately, the cysteine-free human cone NCKX2 mutant were made in the Myc-tagged short splice variant of the human retinal cone NCKX2 cDNA described before (*15*) (accession #AAF25811). In all cases, cysteine residues were mutated to serine residues. Mutagenesis was carried out using cassettes with upper and lower primers upstream and downstream of the restriction sites used to clone the fragment. There are nine cysteine residues in the wild-type human cone NCKX2: C16, C24, C153, C223, C275, C394, C605, C624, and C657 (note residue numbering in this and in our previous studies reflects residues in the full-length, untagged human NCKX2).

The vector for the C605S mutant was prepared by cutting the wild-type human cone NCKX2 cDNA with BsrG I at two sites located at bases 1299−1304 (amino acid residue 434) and bases 1757−1762 (amino acid residue 557) along with BamH I, which cut in the 3′-polylinker region. This removed the wild-type BsrG I fragment from bases 1300−1757 (amino acids 434−557), which was cloned in upon completion of the cysteine-free mutant. The C605S-containing fragment was then cloned as a BsrG I/BamH I insert. This C605S mutant was used as a template to generate a C605S/C657S mutant insert, which was then cloned in using the same BsrG I and BamH I restriction sites. The C605S/C657S mutant construct was used as a template for generation of a C605S/C624S/C657S fragment, which again was cloned using the BsrG I and BamH I sites.

To generate the C153S mutant, an Xho I site was introduced into the wild-type human cone NCKX2 cDNA at bases 379−384 (amino acid residue 127). This construct was used as a template to generate the C153S fragment. The C153S fragment was then cloned using the Xho I site and Xba I site located at bases 679−684 (amino acid residue 227). The resulting mutant was used as the template for making a C153S/C223S insert, which was cloned using the same Xho I and Xba I sites. C275S was cloned into the C153S/C223S mutant using the Xba I site and the BsrG I sites after dropping out the wild-type human cone NCKX2 BsrG I fragment.

The fragment containing C153S/C223S/C275S was then cloned into the C605S/C624S/C657S clone using a 5′-polylinker Apa I site and the BsrG I site. The resulting mutant was used as a template for PCR amplification of a C153S/C223S/C275S/C394S insert, which was cloned into the C153S/C223S/C275S/C605S/C624S/C657S mutant construct using the introduced Xho I site and the BsrG I site. C16S and C24S were PCR amplified with overlapping primers using the C153S/C223S/C275S/C605S/C624S/C657S mutant as a template. The C16S/C24S fragment was cloned into the C153S/C223S/C275S/C394S/C605S/C624S/C657S mutant using the polylinker Apa I site and an Avr II site located at bases 226−231 (amino acid residue 76). The wild-type human cone NCKX2 BsrG I fragment was then reinserted to create a completely cysteine-free human cone NCKX2

mutant. All PCR-generated fragments were thoroughly sequenced after insertion to ensure that no unwanted mutations were generated through PCR errors. Plasmid DNAs were prepared using the EndoFree Plasmid Maxi Kit system from Qiagen (Mississauga, Ontario, Canada).

Transient Expression of Human Mutant and Myc-Tagged Cone NCKX2 cDNAs in Insect Cells and in HEK293 Cells. A lepidopteran insect cell expression system was used for transient transfection of BTI-TN-5B1-4 (High Five; Invitrogen, Burlington, Ontario) insect cells with the various mutant human cone NCKX2 cDNAs as described previously (*16*). High Five cells were subcultured at 28 °C in HyQ-IPL-41 insect cell medium (Hyclone, Logan, UT) supplemented with 0.35 g/L $NaHCO_3$, 2.6 g/L tryptose phosphate, 9.0 g/L sucrose, 0.069 mg/L $ZnSO_4 \cdot 7H_2O$, 7.59 mg/L $AlK(SO_4)_2 \cdot 12H_2O$, 10% heat-inactivated fetal bovine serum (Invitrogen, Burlington, Ontario), and penicillin−streptomycin−fungizone (Invitrogen, Burlington, Ontario). The various cysteine NCKX2 mutants were also subcloned into pcDNA3.1 and expressed in HEK293 cells as described before (*21*).

Measurement of $^{45}Ca^{2+}$ Uptake via Reverse $Na^+/Ca^{2+}-K^+$ Exchange. K^+-dependent $^{45}Ca^{2+}$ uptake was measured in sodium-loaded High Five cells transiently transfected with the various mutant human cone NCKX2 cDNAs as previously described (*16*). Background $^{45}Ca^{2+}$ uptake was monitored by using untransfected cells or cells transfected with empty vector.

Fluorometric Assay for Na/Ca−K Exchange Function. HEK293 cells were transiently transfected with NCKX cDNA and subsequently loaded with the Ca^{2+}-indicating dye fluo-3 as described previously (*21*). After fluo loading, cells were washed and the final pellet was resuspended in a medium containing 150 mM NaCl, 1.5 mM $CaCl_2$, 2 mM KCl, 0.25 mM sulfinpyrazone, and 20 mM Hepes (pH 7.4) and kept at room temperature. Transfected HEK293 cells were diluted 20−40-fold into a cuvette in a medium containing 150 mM LiCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 0.1 mM EDTA. The cuvette was placed in a Series 2 SLM-Aminco fluorometer equipped with a thermostated cuvette housing and a stirrer. Fluo-3 fluorescence was monitored after the addition of various agents to the cuvette under constant stirring as described in the text; further details on instrumentation and procedures have been published elsewhere (*22*).

Immunoprecipitation of NCKX and CNGA Subunits. To examine the importance of cysteine residues in the NCKX−NCKX and NCKX−CNGA interaction, High Five cells were cotransfected with cDNAs encoding Myc-tagged NCKX2 (either wild-type or Cys-less) and NCKX or CNGA cDNAs containing a 40 amino acid sequence containing the 6H2 tag as described previously (*10*). Two days after transfection, cells were harvested and washed two times in a 150 mM NaCl, 20 mM Hepes, 80 mM sucrose, 200 μM EDTA, pH 7.4, solution by centrifugation at 300*g* for 4 min. Membranes were solubilized in RIPA buffer containing 1% Triton X-100, 0.5% DOC, 140 mM NaCl, 25 mM Tris pH7.5, 10 mM EDTA, and one protease inhibitor tablet/10 mL (Complete, Mini, Roche Diagnostics, Laval, Quebec), and sedimented for 5 min at 20 000*g* at 4 °C. The protein concentration in the supernatant was determined with the Bradford assay. Solubilized membranes (400−1600 μg of protein) were diluted nine times in TBS (25 mM Tris, 150 mM NaCl, pH 7.4) and then incubated for 2 h at 4 °C with 5 μL of 6H2 monoclonal antibody (0.5 mg/mL, Novus, Littleton, CO) or 3 μL of polyclonal Myc antibody (1 mg/mL, Cell Signaling, NEB Pickering, Ontario). Subsequently, 40 μL of a 25% slurry of protein A/G PLUS−Agarose beads (Santa Cruz Biotech, Santa Cruz, CA) was added, and the mixture was incubated overnight at 4 °C under continuous mixing. The precipitate was collected by centrifugation at 1000*g* for 5 min, and washed four times with 1 mL of RIPA/TBS cocktail (9:1 ratio). Finally, the immunoprecipitate was extracted with sample buffer (62.5 mM Tris−HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 41 mM DTT, and 0.03% (w/v) phenol red), and proteins were separated on a 8.5% Laemmli gel with a 4% stacking gel. Western blotting was carried out by blocking the nitrocellulose in TBST + 10% skim milk for 2 h at room temperature. The membrane was then incubated overnight at 4 °C with a 1:2000 dilution of anti-[c-Myc]-peroxidase monoclonal antibody (Roche Diagnostics, Laval, Quebec) in TBST (100 mM NaCl, 0.05% Tween 20, 10 mM Tris, pH 8.0) + 5% skim milk. After incubation, the membranes were washed four times in 1.5X TBST. Detection was carried out using the Phototope-HRP detection kit from NEB. The membranes were finally exposed to Kodak Biomax ML film (Eastman Kodak Company, Rochester, NY).

RESULTS

Functional Consequences of Mutagenesis of Cysteine Residues of NCKX2. Figure 1 illustrates the position of each of the nine cysteine residues present in the human NCKX2 protein: the first two are located in the cleavable signal peptide, one (Cys394) is located in the large cytosolic loop and has been shown to be the most reactive cysteine residue toward thiol-based cross-linking of NCKX2 (*10*), while the remaining six cysteine residues are located within the two sets of transmembrane-spanning segments (TMs). Five of the six cysteine residues located in the TMs are conserved in all NCKX1 and NCKX2 sequences known to date and are shown with the black background in Figure 1. As the signal peptide is cleaved in the functional NCKX2 protein present in the plasma membrane (*12*), we did not mutate the fist two cysteine residues individually. Each of the other seven cysteine residues was individually mutated to a serine residue, and the seven different mutant NCKX2 proteins were expressed in High Five cells and assayed for transport function by measuring K^+-dependent ^{45}Ca uptake in Na^+-loaded cells, a simple quantitative assay for NCKX function (*15*).

NCKX2 protein expression levels were assessed by Western blotting with the Myc monoclonal antibody, as all NCKX2 constructs contained the Myc tag inserted in the N-terminus (*11*). The Western blot inset in Figure 2 illustrates that none of the individual cysteine substitutions had any effect on mutant NCKX2 protein expression. Except for the Cys394Ser, located in the large cytosolic loop, the other six individual cysteine replacements caused a reduction in NCKX2 transport activity when compared with wild-type, with Cys153Ser being the most deleterious substitution (~40% wild-type NCKX2 transport function). Wild-type NCKX2 and all the mutant NCKX2 proteins studied here ran as a doublet on SDS-PAGE; we previously showed that

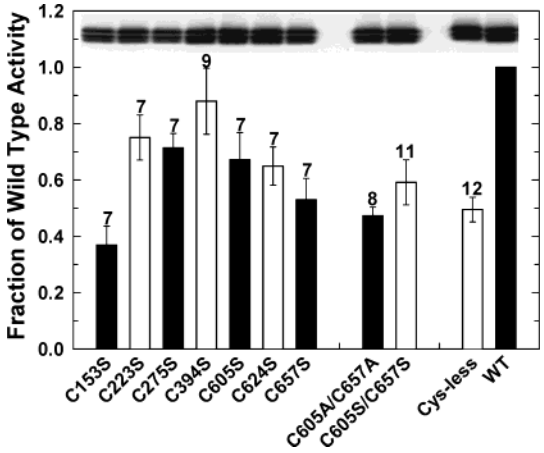

FIGURE 2: Effect of substitution of endogenous cysteine residues on NCKX2 protein synthesis and NCKX2 function when expressed in High Five cells. The indicated NCKX2 mutants were expressed in High Five cells, and NCKX2 function was measured by $^{45}Ca^{2+}$ uptake via reverse exchange as described in Methods. Results were normalized with respect to $^{45}Ca^{2+}$ uptake observed for wild-type (WT) NCKX2. Results shown are average uptake levels ± standard error of the mean with the number of separate experiments at the top of the bar. The Western blot inset at the top shows typical protein expression levels observed for the indicated NCKX2 mutants and wild-type NCKX2. Temperature: 25 °C.

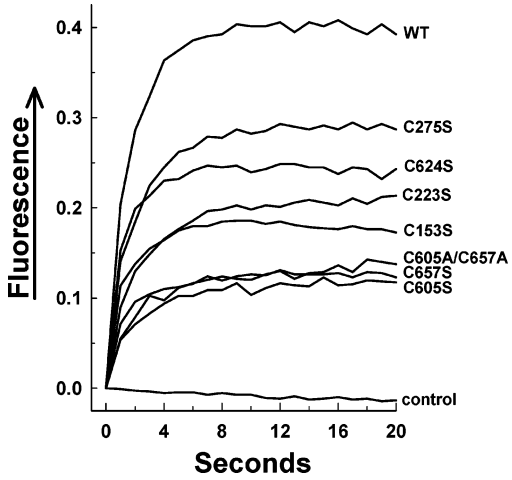

FIGURE 3: Effect of substitution of endogenous cysteine residues on NCKX2 function when expressed in HEK293 cells. The indicated NCKX2 mutants were expressed in HEK293 cells, and NCKX2 function was measured as K^+-dependent Ca^{2+} uptake via reverse exchange as indicated by increases in fluo-3 fluorescence (as described in Methods and in text). NCKX2 function was initiated by addition of 20 mM KCl at time zero. WT represents cells transfected with wild-type NCKX2, while control represents cells transfected with empty vector. Temperature: 25 °C.

the lower band represents cleavage of a signal peptide that appears to be critical for plasma membrane targeting (*12*). It is clear that the cysteine substitutions had no effect on this process.

Analysis of NCKX2 Cysteine Mutants in HEK293 Cells with a Fluorometric Assay. Our results are different from those obtained by Cai et al., who found a complete abolition of NCKX2 protein expression when mutating the equivalent of residues Cys605 and Cys657 in rat NCKX2 (*20*). In the latter study, Fura-2 imaging and Western blot analysis of transfected HEK293 cells were used to examine substitution of endogenous cysteine residues of rat NCKX2. To address the possibility that NCKX2 expression in High Five cells differs from that in HEK293 cells, we examined functional expression of our NCKX2 cysteine mutants in HEK293 cells using fluorometric methods. Figure 3 illustrates our fluo-3 assay for NCKX function when HEK293 cells were transfected with the different NCKX2 cysteine mutants. First, fluo-loaded HEK293 cells were transferred to a cuvette containing a buffered LiCl solution without any Na^+ or Ca^{2+}. Ca^{2+} was then raised to 100 μM, which is sufficient to saturate the external Ca^{2+} binding site of NCKX2 ($K_d \approx 2$ μM (*23*)), but in the absence of external K^+, this Ca^{2+} addition did not result in any noticeable increase in intracellular free Ca^{2+} as judged by the absence of any time-dependent change in fluo-3 fluorescence (not illustrated). Finally, 20 mM KCl was added, which resulted in a fast rise in fluo-3 fluorescence when cells were transfected with wild-type NCKX2 (Figure 3, top trace); the increase in fluo-3 fluorescence indicates the rapid increase in cytosolic Ca^{2+} via reverse $Na^+/Ca^{2+}-K^+$ exchange. Untransfected cells or cells transfected with empty vector never showed any K^+- and Ca^{2+}-dependent increase in fluorescence indicating the absence of any $Na^+/Ca^{2+}(-K^+)$ exchange activity endogenous to HEK293 cells (Figure 3, bottom trace). As observed in the High Five cell expression system, all single Cys to Ser substitutions of cysteine residues located in the TMs resulted in mutant NCKX2 proteins that showed a reduction of $Na^+/Ca^{2+}-K^+$ exchange activity when compared with wild-type NCKX2; however, in all cases, significant transport activity was still observed. In the case of HEK293 cells, transport activity was the most reduced for the Cys605Ser and Cys657Ser substitutions. The results shown in Figure 3 represent results of a single experiment, representative for at least five other experiments. A comparison of the NCKX2 activity observed for the different cysteine mutants compared to wild-type NCKX2 was more difficult in the case of HEK293 cells, as mutant NCKX2 protein expression levels in the HEK cells (as judged by the anti-Myc blot) were considerably more variable (not illustrated) compared with the very consistent expression in High Five cells (e.g., Figure 2), similar to what we have observed and discussed before (*12*).

Alanine versus Serine Substitutions. Our results shown in Figures 2 and 3 differ fundamentally from those obtained by Cai et al., who showed a complete loss of protein expression (and therewith function) when the residues equivalent to Cys605 and Cys657 were mutated in rat NCKX2 (*20*). In the latter study, cysteine residues were replaced by alanine, while in our study cysteine residues were replaced by serine. We used serine substitutions in view of observations made with the NCX1 Na^+/Ca^{2+} exchanger where in some cases Cys to Ala substitution led to an almost complete loss of Na^+/Ca^{2+} exchange transport, whereas with Cys to Ser substitutions, NCX1 transport function was retained (*24*). To examine whether the discrepancy between our results and those of Cai et al. (*20*) were due to the different substituents used, we generated both the Cys605Ser/Cys657Ser and Cys605Ala/Cys657Ala double mutants and examined protein and functional expression in both HEK293 cells and High Five insect cells. Figures 2 and 3 show that both double mutants showed at least 30% of the wild-type NCKX2 function and normal NCKX2 protein expression when expressed in insect High Five cells.

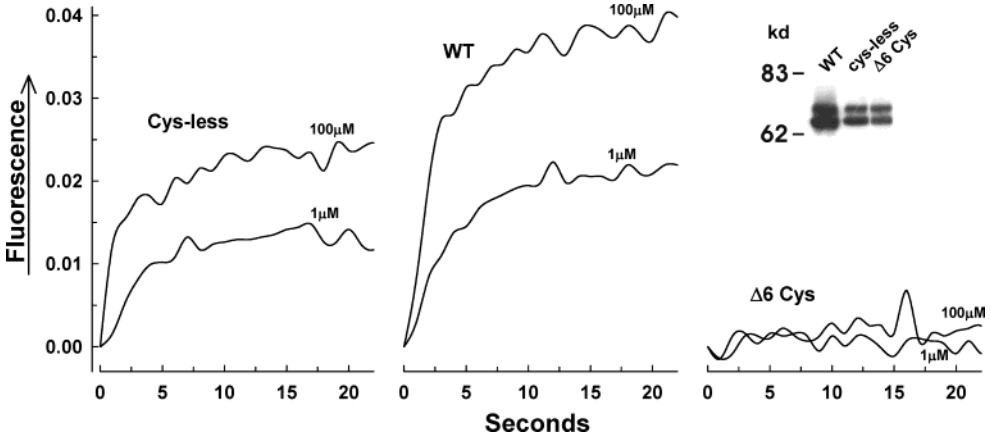

FIGURE 4: Functionally competent Cys-less NCKX2. Cys-less NCKX2 (left panel), wild-type NCKX2 (middle panel), and NCKX2 in which the six cysteine residues located in the TMs were replaced by serine (Δ6 Cys) were expressed in HEK293 cells. NCKX2 function of these three Myc-tagged NCKX2 constructs was measured as K^+-dependent Ca^{2+} uptake via reverse exchange as indicated by increases in fluo-3 fluorescence (as described in Methods and in text). Reverse $Na^+/Ca^{2+}-K^+$ exchange was initiated by addition of 20 mM KCl at time zero. The insert in the right-hand panel shows a Western blot with the Myc antibody of the protein samples obtained after this experiment. Temperature: 25 °C.

Generation of a Cysteine-Free or Cys-Less NCKX2. In our hands, individual substitution of each of the highly conserved cysteine residues in human NCKX2 yielded mutant proteins that retained significant NCKX2 transport function. Next, we made several types of multiple cysteine to serine substitutions with the ultimate goal of removing all nine cysteine residues (Cys-less) and obtaining a functional and Cys-less NCKX2 protein for further structural studies. With one exception, all the multiple cysteine substitutions showed $Na^+/Ca^{2+}-K^+$ exchange activity and unaltered protein expression levels when expressed in High Five cells. Only when all six cysteine residues located within the two TMs were replaced by serine (Δ6 Cys) was little or no $Na^+/Ca^{2+}-K^+$ exchange activity (1 ± 2% of the wild-type activity, $n = 9$) (here and below: average value ± standard error of the mean) observed, although protein expression was not affected in either High Five insect cells or HEK293 cells (see also Figure 4). Curiously, when the remaining three cysteine residues were mutated to serine as well, the resultant Cys-less human NCKX2 protein showed significant $Na^+/Ca^{2+}-K^+$ exchange activity (50 ± 4% wild-type NCKX2 activity, $n = 12$), while protein expression was not affected (Figure 2). As the first two cysteine residues are part of the cleaved signal peptide and not present in the mature protein, we reinserted Cys394 in the large cytosolic loop to examine whether this residue plays a critical role. However, this proved not to be the case. In seven experiments, the Cys-less activity was compared with that in which Cys394 was reinserted in the Cys-less background: activities of the Cys-less and the Cys-less/Ser394Cys were 52 ± 3% and 49 ± 3% compared to wild-type NCKX2. We also examined functional activity of these two multiple cysteine mutants in HEK293 cells with essentially the same result. This assay is illustrated here in Figure 4 for HEK293 cells expressing either wild-type NCKX2 (middle panel), the Cys-less mutant (left panel), or the Δ6 Cys NCKX2 construct (right panel). The experimental paradigm is the same as that described for Figure 3. Addition of 20 mM KCl at time zero led a rapid increase in free cytosolic Ca^{2+} due to Ca^{2+} influx via reverse $Na^+/Ca^{2+}-K^+$ exchange in cells expressing wild-type NCKX2 or cells expressing the Cys-less NCKX2, but not in cells expressing the Δ6 Cys NCKX2 construct. This result is consistent with the results obtained in High Five cells as discussed above. The experiment illustrated in Figure 4 was carried out at two different free external Ca^{2+} concentrations of 1 and 100 μM, respectively. In our fluo-3 assay for NCKX2 function, half-maximal NCKX2 activity was consistently observed at ~1 μM free external Ca^{2+} concentration. Replacing all nine endogenous cysteine residues with serine had no clear effect on the affinity of NCKX2 for extracellular Ca^{2+} (the same was observed for all the individual cysteine replacements; data not shown). In some experiments, NCKX-mediated Ca^{2+} influx showed a biphasic kinetics (e.g., Figure 4), less prominent in other experiments (e.g., Figure 3). The reason for this is unclear.

Do Cysteine Residues Play a Role in NCKX−NCKX and NCKX−CNG Interactions? We have recently shown that heterologously expressed NCKX1 and NCKX2 proteins form homo-oligomers and also that both NCKX1 and NCKX2 can form hetero-oligomers with the CNGA subunit of cGMP-gated channels when coexpressed (*10*). The presence of such complexes had previously been demonstrated in bovine rod outer segment membranes (*8, 9*), and thiol-specific cross-linking reagents have been reported to affect the bovine rod $Na^+/Ca^{2+}-K^+$ exchanger by modulating the NCKX−NCKX interaction (*25*). Here, we examined the possible role of cysteine residues in both the NCKX−NCKX and NCKX−CNG interactions by means of coimmunoprecipitation experiments. Immunoprecipitation was carried out with the monoclonal 6H2 antibody, the epitope for which is contained in a 40 amino acid stretch of the N-terminal extracellular loop of the cardiac NCX1 Na^+/Ca^{2+}exchanger (SHVDHI-SAETEMEGEGNETGECTGSYYCKKGVILPIWEDEP). We inserted this 40 residue oligopeptide containing the 6H2 epitope tag into the N-terminus of the chicken rod CNGA1 and chicken cone CNGA3 proteins and also within the cytosolic loop of the chicken NCKX2 sequence (in the latter case replacing the entire cytosolic loop of the NCKX2). These CNGA and NCKX constructs are described in more detail elsewhere (*10*). The above 6H2-tagged CNGA or NCKX2 constructs were each individually coexpressed either with wild-type NCKX2 or with Cys-less NCKX2, both of

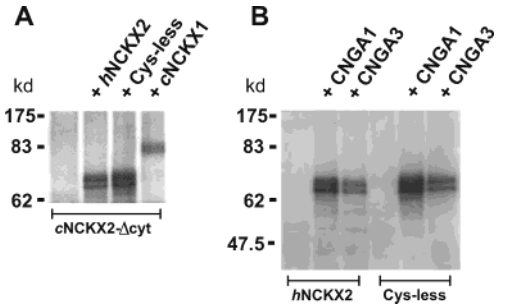

FIGURE 5: Cysteine residues and the NCKX−NCKX and NCKX−CNG interactions. A: High five cells were transfected with *c*NCKX2-Δcyt and no further cDNA (lane 1), wild-type NCKX2 (lane 2), Cys-less NCKX2 (lane 3), or chicken rod NCKX1 (lane 4). The *c*NCKX2-Δcyt construct contains a 40 residue spacer (SHVDHISAETEMEGEGNETGECTGSYYCKKGVILPIWEDEP) in place of the large cytosolic loop (10). This spacer contains the epitope of the monoclonal 6H2 antibody, which was used for immunoprecipitation as described in Methods. Immunoprecipitates were separated on SDS-PAGE and blotted with the anti-[c-Myc]-peroxidase monoclonal antibody. B: High Five cells were transfected with wild-type NCKX2 or the Cys-less NCKX2 and no further cDNA (lanes 1 and 4), chicken rod CNGA1 (lanes 2 and 5), or chicken cone CNGA3 (lanes 3 and 6). Immunoprecipitates were separated on SDS-PAGE and blotted with the anti-[c-Myc]-peroxidase monoclonal antibody, which recognizes the NCXK proteins but the not the CNGA proteins.

which contain the Myc tag but no 6H2 tag. Immunoprecipitation was carried out with the monoclonal 6H2 antibody, while detection was carried out with the monoclonal Myc antibody. In the experiment illustrated in Figure 5A, the 6H2-tagged NCKX2 construct (*c*NCKX2-Δcyt) could pull down wild-type NCKX2, the Cys-less NCKX2, and also the chicken rod NCKX1. The experiment illustrated in Figure 5B illustrates that both the chicken rod CNGA1 and cone CNGA3 cGMP-gated channel subunits (containing the 6H2 tag) could pull down wild-type NCKX2 as well as the Cys-less NCKX2. These results show that the cysteine residues of NCKX2 are not critically important to either the NCKX−NCKX or the NCKX−CNGA interaction.

DISCUSSION

Cysteine Residues of NCKX2: Generation of a Functional Cys-Less NCKX2. Cysteine residues play critical roles in many proteins, as a key residue in the active site of many enzymes or in the formation of either intramolecular or intermolecular disulfide bonds. The retinal rod NCKX1 and retinal cone/brain NCKX2 $Na^+/Ca^{2+}-K^+$ exchangers contain five conserved cysteine residues (Figure 1). Both NCKX1 and NCKX2 have been shown to form NCKX homo-oligomers and also hetero-oligomers with the CNGA subunit of cGMP-gated channels (*8*, *10*). Thus, NCKX cysteine residues could possibly mediate these associations. In view of the unique reactivity of the thiol group of the cysteine residue, many tools are available for studying protein structure based on scanning cysteine mutagenesis. Ideally, endogenous cysteine residues are removed before inserting cysteine residues at various controlled positions. In this study, we have examined whether we could replace all endogenous cysteine residues in the human NCKX2 protein with serine residues without generating major defects in NCKX2 transport function, in NCKX2 protein expression and targeting, and in NCKX2 interactions with other proteins. As is illustrated in Figures 2 and 3, individual replacement of any of the five conserved cysteine residues (highlighted in Figure 1) by serine resulted in a significant decrease in NCKX2 transport in most cases but had no effect on NCKX2 protein synthesis and processing. Still, significant NCKX2 function was retained by each of these five NCKX2 mutant proteins. Of the five cysteines conserved between NCKX1 and NCKX2, Cys153 and Cys223 are conserved in a broader group of NCKX proteins, including NCKX3, NCKX4, two NCKX isoforms from *Drosophila*, and one isoform from *C. elegans*, while Cys605 and Cys624 are not found in NCKX3, NCKX4, or *Drosophila* NCKX-X (*26*). Various multiple-cysteine mutations (e.g., the Cys605/Cys657 or the Cys-less) were analyzed, and the deleterious effect on the NCKX2 transport function found in individual cysteine mutants proved not to be additive in multiple-cysteine mutants. Thus, it proved to be possible to generate a Cys-less NCKX2 that showed unaltered protein expression and ~50% of the wild-type transport activity in both HEK293 cells and High Five cells (Figures 2 and 4). We conclude that cysteine residues are not critically important for NCKX2 protein synthesis, folding, and targeting and also not essential for NCKX2 transport function. Similarly, formation of NCKX2 oligomers and the interaction between NCKX2 and the CNGA cGMP-gated channel subunit do not appear to involve a disulfide bridge, as these interactions could be observed with the Cys-less NCKX2 (Figure 5). The 6H2-tagged NCKX2 construct used in this experiment (Figure 5) lacks the large cytosolic loop, suggesting that the NCKX−NCKX and NCKX−CNG interactions are mediated by the transmembrane domains of NCKX2, as observed before (*10*).

Is an Intramolecular Disulfide Bond Important for NCKX2 Synthesis and Function? Generation of functional Cys-less human NCKX2 reported here is inconsistent with a recent report that concluded that a disulfide linkage is critically important for the stability of the rat NCKX2 exchanger expressed in HEK293 cells; this conclusion was based on the observation that no heterologously expressed NCKX2 protein was observed when each of two endogenous cysteine residues was mutated individually (the equivalent of Cys605 and Cys657 shown in Figure 1) (*20*). The human NCKX2 shares over 89% conservation in amino acid sequence with that of the rat NCKX2. Sequence variation between the two types of NCKX is found mainly in two large hydophilic loops with nearly complete conservation within the TMs containing the five highly conserved cysteine residues, including Cys605 and Cys657. Cys605 and Cys657 are among the five cysteine residues found in all known NCKX1 and NCKX2 sequences, but Cys605 is not conserved in mammalian NCKX3, NCKX4, or *Drosophila* NCKX-X. A functional Cys-less NCKX2 precludes any of the nine cysteine residues in NCKX2 from being of critical importance for either NCKX2 protein synthesis, protein folding, or plasma membrane targeting. When analyzed on SDS-PAGE, NCKX2 shows a doublet representing full-length NCKX2 and NCKX2 from which an N-terminal signal peptide of about 50 residues is cleaved (*12*). As shown in Figures 2 and 4, signal peptide cleavage, which is associated with plasma membrane targeting, was very similar in all cysteine mutant studies here, including the Cys-less, again suggesting that cysteine residues were not essential for protein folding and correct targeting to the plasma membrane. The reasons for the discrepancies

between the results reported here and those reported by Cai et al. (*20*) are unclear. In the latter study, cysteine residues were replaced by alanine residues, and, therefore, we made a Cys605Ala/Cys657Ala double mutant. This double mutant showed normal NCKX2 protein expression and ~50% transport activity compared to wild-type NCKX2 (Figure 2), and, hence, the discrepancy cannot be explained by the nature of the cysteine substituents. It is interesting to note the lack of a critical importance of strongly conserved residues such as Cys153 and Cys223. In a previous study, we observed that many residues in the so-called α1 and α2 repeats are not very sensitive toward mutagenesis despite the fact that most are conserved among the first four human NCKX isoforms and conserved between mammalian NCKX and NCKX from lower organisms (*15*). The availability of a functionally competent Cys-less NCKX2 exchanger will be useful for future structure and topology studies that take advantage of the unique reactivity of the thiol group of cysteine residues reintroduced in the Cys-less background.

Functional Importance of NCKX2 Cysteine Residues? Another way to address the potential importance of cysteine residues in NCKX is to measure the effect of sulfhydryl reagents on NCKX function. A recent study described activation of rat NCKX2 currents by high concentrations of β-mercaptoethanol (*20*). In the NCKX assays used in our study, reducing agents such as β-mercaptoethanol or DTT had no effect on NCKX2 function measured as either ^{45}Ca uptake or changes in fluo-3 fluorescence (data not shown). The impermeant sulfhydryl modifying reagent MTSET had no effect on NCKX2 function (*11*), whereas membrane-permeant reagents such as NEM or MTSEA had strong effects on intracellular Ca^{2+} homeostasis and interfered with our NCKX assays in ways that made it difficult to assess their effect on NCKX2 function (data not illustrated). In the case of the NCX gene family of Na^+/Ca^{2+} exchangers, modulation of NCX1 activity in cardiac sarcolemmal vesicles had been ascribed to cyclic oxidation−reduction of an internal disulfide linkage (*27*). Later work identified the cysteine residues of the NCX1 exchanger involved in this intramolecular disulfide bond that alters the mobility of the NCX1 protein when analyzed by SDS-PAGE, but it was shown that cysteine residues were not involved in redox stimulation of NCX1 activity (*28*). Finally, the creation of a functional Cys-less NCX1 exchanger showed that disulfide bonds were not critically important for NCX1 function (*24*), as observed in our study for NCKX2 function.

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BI049538Y