Scanning Mutagenesis of the Alpha Repeats and of the Transmembrane Acidic Residues of the Human Retinal Cone Na/Ca-K Exchanger†

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ABSTRACT: The Na/Ca-K exchanger (NCKX) utilizes the inward sodium gradient and outward potassium gradient for Ca^{2+} extrusion; two distinct NCKX isoforms are expressed in the outer segments of retinal rod (NCKX1) and cone (NCKX2) photoreceptors, respectively, where NCKX extrudes Ca^{2+} that enters photoreceptors via the cGMP-gated channels. We carried out the first systematic NCKX mutagenesis study in which 96 residues were mutated in the human cone NCKX2 cDNA, and functional consequences of these mutations were measured; the residues selected for mutagenesis are conserved between rod and cone NCKX, the large majority are also conserved in NCKX paralogs found in lower organisms, and finally, they include the few residues conserved between members of the NCKX and members of the NCX (potassium-independent Na/Ca exchange) gene families. Twenty-five residues were identified for which mutagenesis reduced NCKX function to <20% of wild-type cone NCKX2 activity, while protein expression and plasma membrane targeting were not affected. Three classes of residues were found to be most sensitive toward mutagenesis: acidic (glutamate/aspartate) residues, polar (serines/threonine) residues, and glycine residues. These results are discussed with respect to residues that may contribute to the NCKX cation binding site(s).

Na/Ca-K (NCKX)[1] exchange was first described in the outer segments of bovine retinal rod photoreceptors and was shown to operate at a stoichiometry of $4Na^+$:($1Ca^{2+} + 1K^+$) (*1*, *2*). In rod and cone photoreceptors, the Na/Ca-K exchangers are responsible for extruding calcium that enters the rod and cone outer segments via the cGMP-gated channels in darkness or under nonsaturating illumination (*3*). Two distinct gene products, NCKX1 and NCKX2, have been shown to code for the rod and cone Na/Ca-K exchangers, respectively (*4*−*7*). Analysis of genomic sequencing projects, molecular cloning, and transcript analysis by means of Northern blots has indicated the presence of several NCKX-related proteins expressed in other tissues (*8*−*10*) as well as in lower organisms that lack vertebrate-type photoreceptors, such as *Caenorhabditis elegans* (*11*) and *Drosophila* (*12*). However, nothing has been reported yet on NCKX physiology in cells other than rod and cone photoreceptors. Hydropathy analysis suggests that all NCKX-related proteins consist of two clusters of highly conserved hydrophobic transmembrane spanning segments (TM) and two large hydrophilic loops that vary greatly in length and are not conserved between different NCKX paralogs (*13*). In our lab, we have demonstrated that both potassium-dependent Na/Ca exchange and calcium-dependent ^{86}Rb (used as a potassium congener) transport were observed when cDNAs coding for either rod NCKX1, cone NCKX2, a rod NCKX1 deletion mutant (from which the two large hydrophilic loops accounting for close to two-thirds of the sequence were removed), or a distantly related *C. elegans* NCKX were expressed in cultured insect cells (*7*, *11*, *14*); the stoichiometry of the heterologously expressed chicken and human cone NCKX2 proteins was shown to be $4Na^+$:($1Ca^{2+} + 1K^+$), identical to previous measurements on rod NCKX1 in situ (*14*). Moreover, the kinetic parameters of Na/Ca-K exchange (apparent Na^+, Ca^{2+}, and K^+ dissociation constants) were found to be very similar for heterologously expressed NCKX1, NCKX2, and *C. elegans* NCKX cDNAs (*7*, *11*, *14*, *15*). This demonstrates that residues involved in cation binding and cation transport are located in the conserved TM's, although no information on specific residues important to Na/Ca-K exchange is yet available. In this study we have carried out scanning mutagenesis of the core of the two sets of TM's that make up the so-called alpha1 and alpha2 repeats, sequence elements that are thought to have resulted from an ancient gene duplication event and contain the only sequence elements conserved between the Na/Ca and Na/Ca-K gene families, respectively (*16*). In addition, we have carried out mutagenesis of all 20 acidic residues found in the two TM's as Ca^{2+} transport is likely to be critically dependent on such residues.

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[1] Abbreviations: NCKX, Na/Ca-K exchange; NCX, Na/Ca exchange; PBS, phosphate buffered saline; RIPA, radioimmune precipitation buffer; GFP, green fluorescent protein; EDTA, ethylenediaminetetracetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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EXPERIMENTAL PROCEDURES

Mutagenesis of the Myc-Tagged Human NCKX2 Clone. The short splice variant of the human retinal cone NCKX2 cDNA (AAF25811) was used in all our mutant constructs; the short splice variant lacks a stretch of 17 amino acids located in the middle of the large cytosolic loop (*7*). The human c-Myc tag (EQKLISEEDL) was inserted at the BstE II site between bases 241−242 (at amino acid residue 81) of the human cone NCKX2 sequence. Mutations in the Myc-tagged human cone NCXK2 cDNA were prepared by generating mutated cassettes and reintroducing the cassette into the wild-type sequence. Mutations were prepared by synthesizing oligonucleotide primers containing the desired mutation as well as upstream and downstream primers (just outside of the restriction sites used to clone the cassette) and generating two overlapping fragments (mutation upstream and mutation downstream) by PCR. The two fragments were isolated, combined, and reamplified by PCR again using only the upstream and downstream primers and digested with the appropriate restriction endonucleases before cloning the cassette back into the wild-type clone. All PCR reactions were performed using ProofStart DNA polymerase (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's directions. All fragments generated by PCR were thoroughly sequenced after cloning to ensure that no unwanted mutations were generated by PCR errors. Plasmid DNAs to be transfected into insect cells were prepared using the EndoFree Plasmid Maxi Kit system from Qiagen (Mississauga, Ontario, Canada).

Transient Expression of Human Mutant and Myc-Tagged Cone NCKX2 cDNA's in Insect Cells. A lepidopteran insect cell expression system was used for transient transfection of BTI-TN-5B1-4 (High Five; Invitrogen) insect cells with the various mutant human cone NCKX2 cDNAs as described before (*11*). High Five cells were subcultured at 28 °C in IPL-41 insect medium (Life Technologies, Burlington, Ontario, Canada) 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 $AIK(SO_4)_2 \cdot 12H_2O$, 10% heat-inactivated fetal bovine serum (GibcoBRL), and penicillin-streptomycin-fungizone (GibcoBRL).

Measurement of Surface Expression by Surface Biotinylation. Cells were transfected with the various mutants cone NCKX2 cDNAs or with green fluorescent protein (GFP). Biotinylation was carried out in PBS medium with 80 mM added sucrose and 1 mM EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 7 min. The reaction was terminated by addition of 10 mM glycine. Cells were washed two times in the above PBS medium; incubated for 20 min with ice-cold RIPA buffer containing 140 mM NaCl, 25 mM Tris (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 mM EDTA, and a protease inhibitor tablet (Roche Molecular Biochemicals); sedimented (5 min at 20 000*g*); and the supernatant was used. Protein concentration was determined with the Bradford reagent. Samples containing 170 μg of total protein were incubated for 2 h at 4 °C with 1 μL of monoclonal Myc antibody (NEB), or 1 μL of polyclonal GFP antibody (Clontech). Subsequently, the samples incubated with the Myc or GFP antibodies were mixed with protein A/G Plus-agarose beads (Santa Cruz Biotechnology), while another sample of 170 μg of total protein was mixed with 30 μL of immoblized NeutrAvidin agarose beads. The beads were mixed overnight at 4 °C, and then washed five times with RIPA-TBS (1:6.7) cocktail. Sample buffer was added and heated for 2 min at 95 °C, the beads were spun down, and samples were subjected to gel electrophoresis in an 8% Laemmli gel.

Measurement of $^{45}Ca^{2+}$ Uptake via Reverse Na/Ca-K Exchange. Potassium-dependent Ca^{2+} uptake was measured in sodium-loaded High Five cells after transient transfection with the various mutant human cone NCKX2 cDNA's. Control cells were either untransfected High Five cells or High Five cells transfected with empty vector, both of which resulted in very similar background $^{45}Ca^{2+}$ uptake (*11*). Transfected High Five cells expressing the various cone NCKX2 mutants were loaded with high internal sodium with the aid of the ionophore monensin as described before (*11*). The final cell pellet was resuspended in 150 mM choline chloride, 80 mM sucrose, 20 mM Hepes, pH 7.4, and 0.05 mM EDTA, and was stored at 25 °C. $^{45}Ca^{2+}$ (0.5−1.0:Ci per experiment) (Amersham Pharmacia Biotech) uptake experiments were carried out in a medium containing 140 mM KCl, 80 mM sucrose, 20 mM Hepes, pH 7.4, and 0.036 mM $CaCl_2$ in addition to $^{45}Ca^{2+}$. Nonspecific $^{45}Ca^{2+}$ uptake was measured when the uptake medium contained NaCl instead of KCl, which results in complete inhibition of $^{45}Ca^{2+}$ uptake via reverse Na/Ca-K exchange (*11*). External $^{45}Ca^{2+}$ was removed from $^{45}Ca^{2+}$ taken up by cells by a rapid filtration and washing procedure with the use of borosilicate glass fiber filters as described previously; the washing medium contained 140 mM KCl, 80 mM sucrose, 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, and 1 mM EGTA. NaCl, KCl, LiCl, and choline chloride were all SigmaUltra grade (Sigma-Aldrich, Oakville, Ontario, Canada). Protein content of cell samples was determined with the Bio-Rad protein assay (Biorad Laboratories, Mississauga, Ontario, Canada). Sample preparation, SDS gel electrophoresis, and Western blotting with the human c-Myc antibody were carried out as described (*11*).

RESULTS

In an earlier study we examined in a heterologous system the functional consequences of six missense mutations found in either rod NCKX1 or cone NCKX2 of patients with retinal disease (*17*). Two of these mutations are located as near neighbors in the highly conserved alpha2 repeat, found in the sequences of both human rod NCKX1 and cone NCKX2, as well as in the sequences of NCKX paralogs from *Drosophila* and *C. elegans*; Ile554Thr showed a >80% reduction in reverse Na/Ca-K exchange activity, whereas Ala556Thr showed wild-type activity. To get a more complete picture of residues that are important for Na/Ca-K exchange function, we have now systematically mutated 80 residues comprising most of the alpha1 and alpha2 repeats, and examined the functional consequences of these mutations after expression of the mutated proteins in High Five cells. In addition, we examined functional consequences of mutating all the acidic residues found in the two sets of transmembrane spanning segments (TMs) (the location of these residues is illustrated in Figure 6). In these experiments, we measured the maximal velocity of reverse Na/Ca-K exchange under conditions that resulted in close to optimal occupancy of the cation transport sites (*11*, *14*). Reverse Na/Ca-K

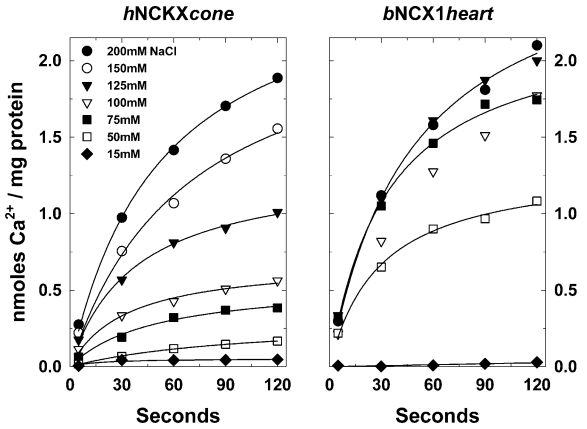

FIGURE 1: Internal Na^+ dependence of reverse Na/Ca-K exchange mediated by NCKX2 and NCX1. High Five cells, stably transfected with either human cone NCKX2 or bovine heart NCX1, were loaded with the indicated Na^+ concentrations as described under Methods. Tonicity was maintained by the use of NaCl−LiCl mixtures. ^{45}Ca uptake was measured as described under Methods. Temperature: 25 °C.

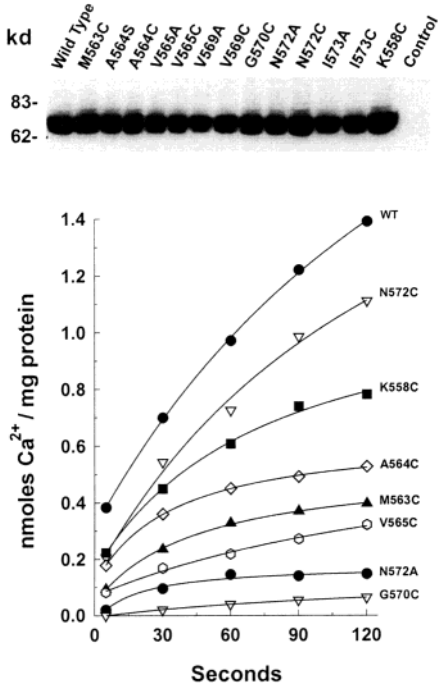

FIGURE 2: Protein expression and Na/Ca-K exchange activity in various cone NCKX2 mutants. High Five cells were transiently transfected with the indicated human cone NCKX2 mutants. Expressed NCKX2 protein was determined by Western blotting (top panel), while NCKX2 function was measured as ^{45}Ca uptake via reverse Na/Ca-K exchange (bottom panel). Temperature: 25 °C.

exchange is measured as ^{45}Ca uptake in sodium-loaded cells suspended in a sodium-free medium. However, we first examined the internal Na^+ dependence of reverse Na/Ca-K exchange as this had not been determined before for heterologously expressed NCKX proteins.

Internal Sodium Dependence of Reverse Na/Ca-K Exchange. High Five cells expressing the Myc-tagged human cone NCKX2 were loaded with different concentrations of Na^+ with the use of the alkali cation ionophore monensin as described under Methods; tonicity was maintained by using LiCl−NaCl mixtures to a final concentration of 200 mM since Li^+ cannot replace Na^+ in Na/Ca-K exchange transport (*11*, *18*). Ca^{2+} uptake via reverse Na/Ca-K exchange was

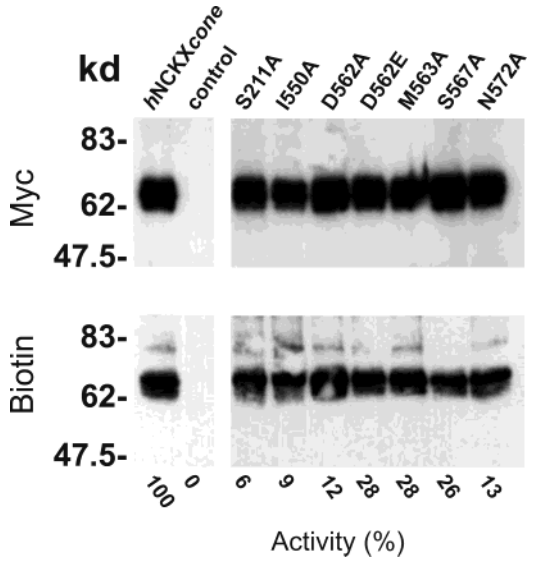

FIGURE 3: Surface labeling of different NCKX2 mutants. High Five cells were transiently transfected with the indicated cone NCKX2 mutants. Surface biotinylation (probed with avidin-HRP, bottom panel) was compared with total protein expression (probed with Myc antibody, top panel).

obtained by measuring the difference between ^{45}Ca uptake into sodium-loaded cells suspended in a medium containing 150 mM KCl (which optimizes reverse Na/Ca-K exchange) and ^{45}Ca uptake into cells suspended in a medium containing 150 mM NaCl (which completely inhibits reverse Na/Ca-K exchange) (*11*). The left panel of Figure 1 shows that reverse Na/Ca-K exchange required surprisingly high internal Na^+ concentrations of >100 mM, perhaps unexpected when considering external Na^+ dissociation constants of 35−50 mM, reported for forward Na/Ca-K exchange in situ in bovine rod outer segments (*19*) and for heterologously expressed rod NCKX1 and cone NCKX2 (*15*). A Hill plot of the averaged results of five experiments suggested a K_d of 150 mM for Na^+ and a Hill coefficient of 3.1. To ensure that Na^+ loading was not limited in some way by our monensin loading protocol, we measured the internal Na^+ dependence of the heart Na/Ca exchanger in High Five cells expressing bovine NCX1; the right panel of Figure 1 shows that considerably lower internal Na^+ concentrations were sufficient for reverse Na/Ca exchange, consistent with reported values of 15−25 mM for the internal Na^+ dissociation constant of NCX1 measured with the giant-excised patch technique (*20*). A practical consequence of the low internal Na^+ sensitivity of reverse Na/Ca-K exchange is that consistent measurements of NCKX activity require high Na^+ loading concentrations (150 mM in our experiments) to avoid the highly nonlinear part of the Na^+ dependence curve at Na^+ concentrations below 75 mM; also, subsequent loss of Na^+ due to passive leakage should be carefully controlled (i.e., the time elapsed between the Na^+ loading procedure and the subsequent Ca^{2+} uptake experiment should be minimized).

Protein Expression and Targeting of Human Cone NCKX2 Mutants. To monitor NCKX2 protein expression levels, we inserted a Myc tag in the N-terminal extracellular loop of our wild-type human cone NCKX2 cDNA and used this construct for all our single residue mutants. Through many experiments, our protocol for transient transfection of High Five cells with the wild-type Myc-tagged human cone

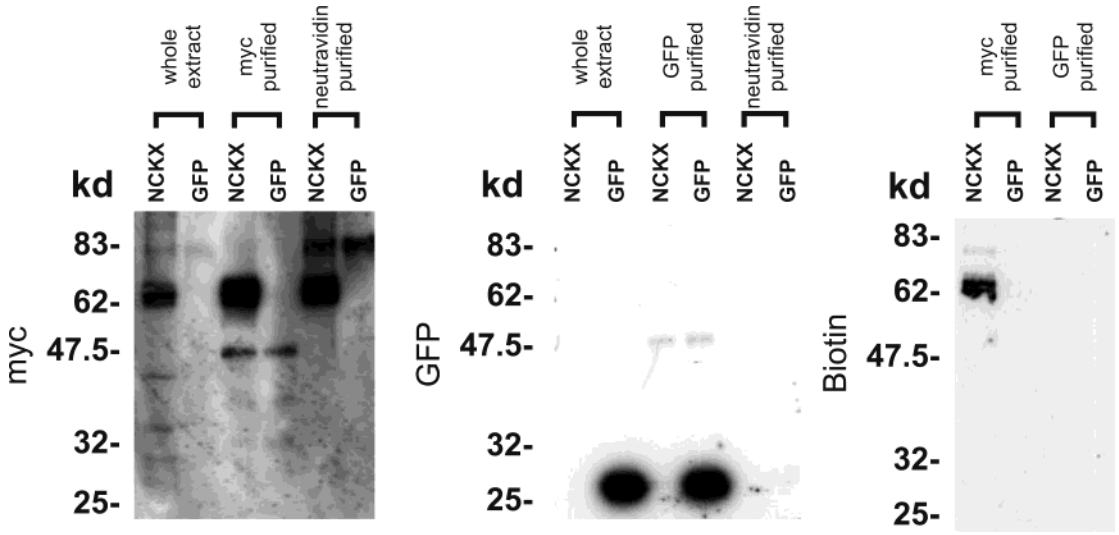

FIGURE 4: Surface labeling of cone NCKX2 as compared with labeling of GFP. High Five cells were transiently transfected with Myc-tagged human cone NCKX2 or with GFP. Blots were probed with monoclonal Myc antibody to detect NCKX2 (left panel), GFP antibody to detect GFP (middle panel), or avidin-HRP to detect biotinylated protein (right panel). Samples represented whole protein extract (10 μg of total protein/lane for the Myc blot and 2 μg of total protein/lane for the GFP blot), Myc affinity purified proteins (starting with 16 μg of total protein), GFP affinity purified proteins (starting with 2.6 μg of total protein), or NeutrAvidin affinity purified proteins (starting with 16 μg of total protein for the Myc blot and 2.6 μg of total protein for the GFP blot) as indicated.

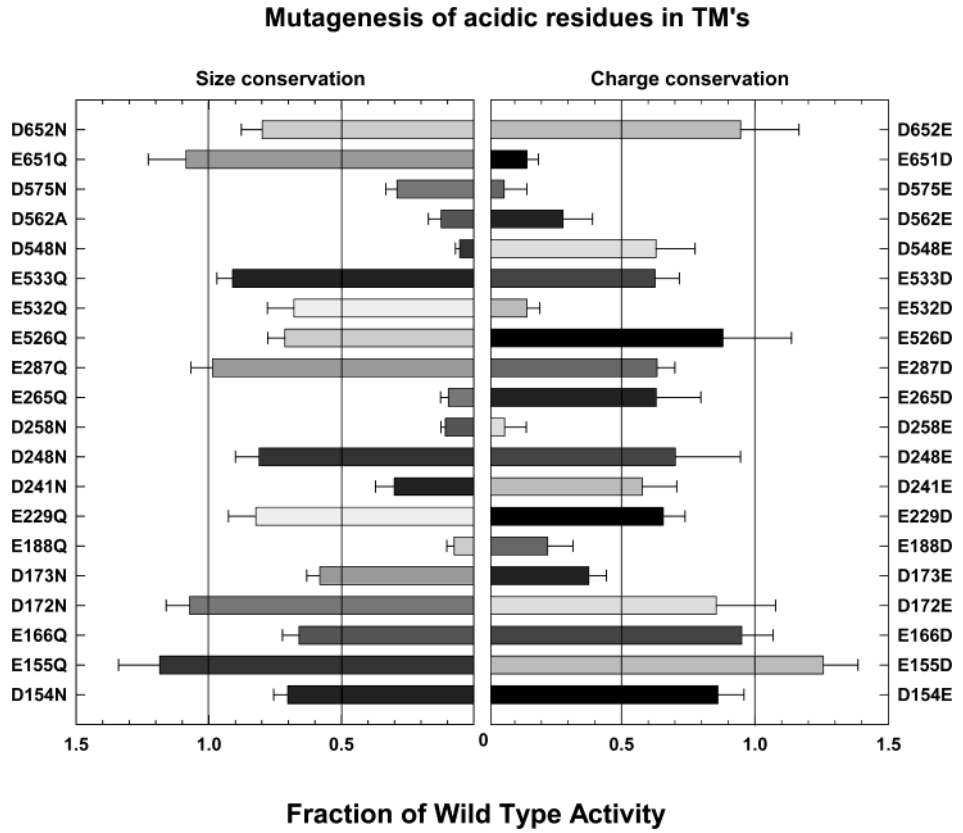

FIGURE 5: Functional consequences of mutagenesis of acidic residues. High Five cells were transiently transfected with the indicated human cone NCKX2 mutants. NCKX activity was measured as described under Methods and illustrated in Figure 2; activity was normalized with respect to that observed for wild-type human cone NCKX2. Average values ($\pm$ standard error of the mean) is shown representing 4−7 separate transfection experiments for each mutant tested. Temperature: 25 °C.

NCKX2 gave very consistent levels of NCKX2 function and NCKX2 protein expression. NCKX2 protein expression levels were measured for every mutant in each experiment and compared with expression levels of wild-type NCKX2 protein in the same transfection experiment; a representative experiment is illustrated in Figure 2. For a series of cone NCKX2 mutant proteins representing a wide range of functional activities from almost a complete loss of activity (G570C) to near wild-type activity (N572C), expression levels were very similar as judged by Western blotting. For all experiments, the blots were scanned to assess differences in mutant NCKX2 protein expression, and functional activity was corrected for changes in NCKX2 protein expression. In general, mutant NCKX2 protein expression levels were

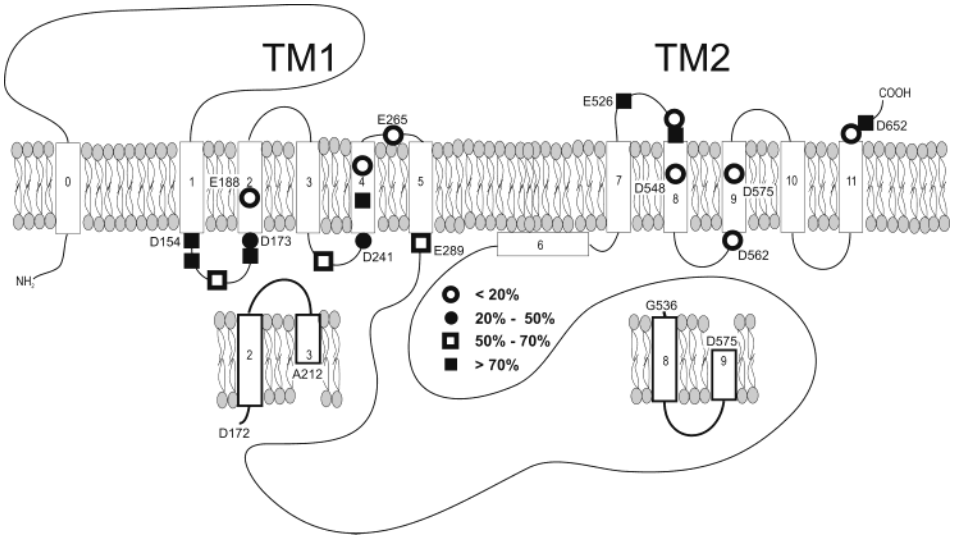

FIGURE 6: Localization and sensitivity to mutagenesis of acidic residues within the two TM's of cone NCKX2. The predicted NCKX topology and the position of the acidic residues in the two sets of TM's is illustrated. Sensitivity toward mutagenesis (i.e., percent activity with respect to wild-type NCKX2 activity) represented either charge-conservative or size-conservative substitutions, whichever had the greatest effect on mutant NCKX2 function. The insets indicate the position of the stretches within the alpha1 and alpha2 repeat, respectively, that were systematically mutated in this study.

within 30% of that of wild-type cone NCKX2. We obtained somewhat anomalous results in only two mutants when functional activity was corrected for differences in NCKX2 protein expression as judged from the Western blot. Both the F574A and the F574C mutations gave rather high and quite variable activity as compared with wild-type activity when this correction was applied, while much smaller error bars were observed without correction (as presented here).

Single residue mutations did not give rise to much variation in NCKX2 protein expression levels that facilitates the interpretation of measurements of NCKX2 function for the various mutants. Next, we addressed the issue of whether mutations could affect correct targeting to the plasma membrane. Functional activity was assessed by measuring ^{45}Ca uptake into sodium-loaded cells in samples typically containing $\sim 10^4$ cells. Surface labeling of mutant NCKX2 proteins was carried out in a cell suspension as well by biotinylation with the impermeant reagent EZ-Link Sulfo-NHS-LC-Biotin. Figure 3 illustrates a representative experiment: the top panel shows the Western blot (probed for Myc) illustrating total (mutant) cone NCKX2 protein expression, while the lower panel shows surface labeling by biotin (probed with avidin-HRP). For cone NCKX2 mutants representing a wide range of functional activities, the amount of surface labeling was found to be proportional to the amount of protein expressed, indicating that targeting to the plasma membrane was not affected in these mutant NCKX2 proteins. All NCKX2 mutants with functional activity levels of $<50\%$ of wild-type human cone NCKX2 were tested this way, and targeting to the plasma membrane was found to be very similar to that of wild-type cone NCKX2 in all cases. To demonstrate that biotinylation was restricted to plasma membrane proteins, we compared biotinylation of cone NCKX2 with biotinylation of GFP expressed in insect cells (Figure 4). Proteins were extracted either by binding to the Myc antibody, by binding to the GFP antibody, or by binding to the biotin binding protein NeutrAvidin. Samples representing similar starting amounts of total protein (see Figure 4 legend) were run on SDS gels and transferred, and blots were then probed for either Myc (left panel), GFP (middle panel), or biotin (right panel). The left panel shows that the Myc-tagged cone NCKX2 protein levels observed in the whole cell extract, in the Myc-affinity purified protein fraction, and in the NeutrAvidin-affinity purified protein fraction were very similar; the middle panel shows the GFP blot of whole cell extract, the GFP-antibody purified faction, and the NeutrAvidin-affinity purified protein fraction, respectively (similar results were obtained in three other experiments). The results show that most of the cone NCKX2 protein was immunoprecipitated by the Myc antibody and specifically bound to NeutrAvidin, indicating plasma membrane localization; in contrast and as expected, the GFP protein was only immunoprecipitated by the GFP antibody, but none was bound by NeutrAvidin, indicating that a cytosolic protein such as GFP was not accessible for biotinylation. This result shows that biotinylation under our experimental conditions was a proper indicator of plasma membrane targeting. The biotinylation procedure did not affect cell integrity as judged by Trypan blue exclusion (data not illustrated). From these results we conclude that changes in functional activity measured in our ^{45}Ca uptake assay reflect true changes in functional activity of the mutated NCKX2 proteins and were not caused by changes in either protein expression or protein targeting to the plasma membrane.

Functional Consequences of Mutagenesis of Acidic Residues in TMs. Ca^{2+} transport proteins are expected to contain critical glutamate and aspartate residues within the hydrophobic membrane spanning helices to bind and neutralize the charge of the Ca^{2+} ion. We mutated each of 20 acidic residues within the two TM's, in each case to two different residues: in one case a charge-conservative change was made (e.g., Glu to Asp), and in the other case a size-conservative change was made (e.g., Glu to Gln). The residue numbering used here represents residues of the full-length human cone NCKX2 sequence (accession no. AAF21810). Na/Ca-K exchange function was measured for each of the mutant NCKX2 proteins and compared with that obtained for wild-type NCKX2 protein (Figure 5). Figure 6 illustrates the

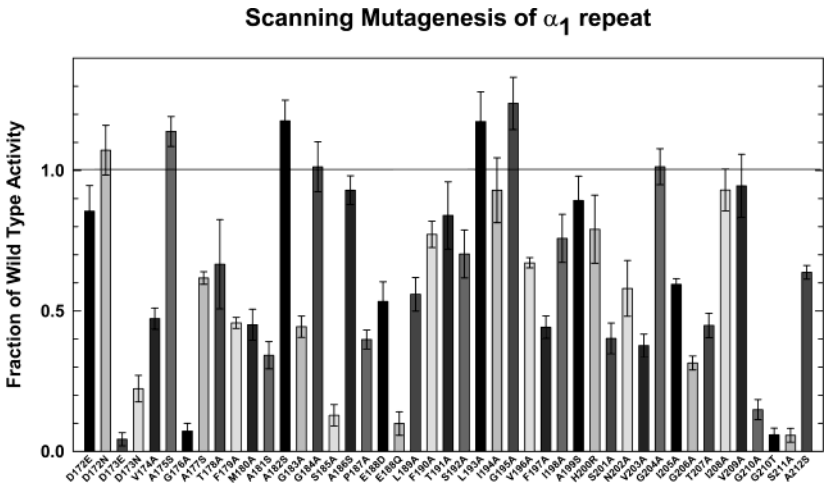

FIGURE 7: Alanine scanning mutagenesis of alpha1 repeat. High Five cells were transiently transfected with the indicated human cone NCKX2 mutants. NCKX activity was measured as described under Methods and illustrated in Figure 2; activity was normalized with respect to that observed for wild-type human cone NCKX2. Average values (± standard error of the mean) are shown representing 4−7 separate transfection experiments for each mutant tested. Temperature: 25 °C.

location of these residues in a model of NCKX topology on the basis of the TmHMM predictive algorithm (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Five of the acidic residues are thought to be located well within membrane spanning helices, whereas the remaining 15 are thought to be located in short loops connecting membrane spanning helices, often (in four cases) as a pair of adjacent acidic residues. Substitution of four out of the five residues thought to be located within the membrane resulted in a significant loss of NCKX2 function (E188, D258, D548, and D575), in three cases even when charge was conserved (E188, D258, and D575); one residue thought to be located within a transmembrane spanning helix was not important for NCKX2 function (D248). Mutation of acidic residues thought to be in the middle of short hydrophilic loops connecting the transmembrane helices led to >50% retention of function (E166, E229, and E526). The remaining 12 acidic residues are all thought to be located either in a very short connecting loop (E265) or at the membrane/water interface. In four cases, an adjacent pair of acidic residues is encountered at the membrane/water interface, and such a pair of charged residues may in fact demarcate such boundaries. One pair thought to be located at the H1/intracellular surface (D154 and E155) was unaffected by mutation; for the other three pairs (D172 and D173, E532 and E533, E651 and D652), mutation of one of the two acidic residues led to significant loss of transport function consequences, while mutation of its neighbor had little effect. Curiously, in two cases (E532 and E651), the size-conservative substitution had little effect on transport function, whereas in the case of the charge-conservative substitution transport function was reduced to <20% of wild-type human cone NCKX2.

Functional Consequences of Mutagenesis of Residues in the Two Alpha Repeats. We carried out scanning mutagenesis of 40 amino acid residues in each of the two alpha repeats of the short splice variant of human cone NCKX2. Except for acidic residues (see above), amino acids were replaced by alanine or by cysteine (a set made for the purpose of carrying out topology studies), while alanine residues were mutated to serine and cysteine residues. The two alpha repeats represent the most conserved elements between different NCKX sequences, and their location is illustrated in Figure 6. Figures 7, 8, 9, and 10 illustrate the results obtained. On average, residues in the alpha2 repeat were found to be considerably more sensitive toward mutagenesis as compared with residues in the alpha1 repeat. Figure 11 illustrates for each of the two alpha repeats a breakdown of the number of NCKX2 mutants as a function of percent of transport activity as compared with wild-type NCKX2 activity. Table 1 illustrates a breakdown of residual transport activity for different classes of amino acid residues. Not surprisingly, charged residues were most affected by mutagenesis, followed by polar residues (mostly serine and threonine) and glycine residues. In the latter case, substitution to the small alanine was often tolerated but not replacement by the bulkier cysteine. Interestingly, we replaced alanine in a not very conservative fashion with serine, but yet alanine proved to be among the least sensitive toward mutagenesis.

Table 1

activity (%)	charged	polar	hydrophobic	aromatic	glycine		proline	alanine
					Ala	Cys		
>70	1	5	11	1	6	2		5
40−70	2	4	15	3	3	4	1	4
20−40		4	7	1	1	1		1
<20	5	6	3		2	5	1	

When residues were mutated to cysteine residues instead of alanine residues, functional consequences were very similar for the majority of substitutions. A difference in functional consequences for alanine versus cysteine substitutions was most often found in the case of glycine residues (five of 12 glycine residues examined): for residues G183, G204, G570, and G536, substitution to a cysteine led to a much greater loss of activity when compared to substitution to alanine, whereas the reverse was true in only one case (residue G206).

Hydroxyl containing residues located within the membrane may contribute to the binding of cations within cation transport proteins and may contribute to the formation of a hydrophilic surface of an amphipatic alpha helix. Consistent with this, mutation of several serine and threonine residues led to functionally compromised NCKX2 proteins (function <20% of wild-type NCKX2); the orientation of these resi-

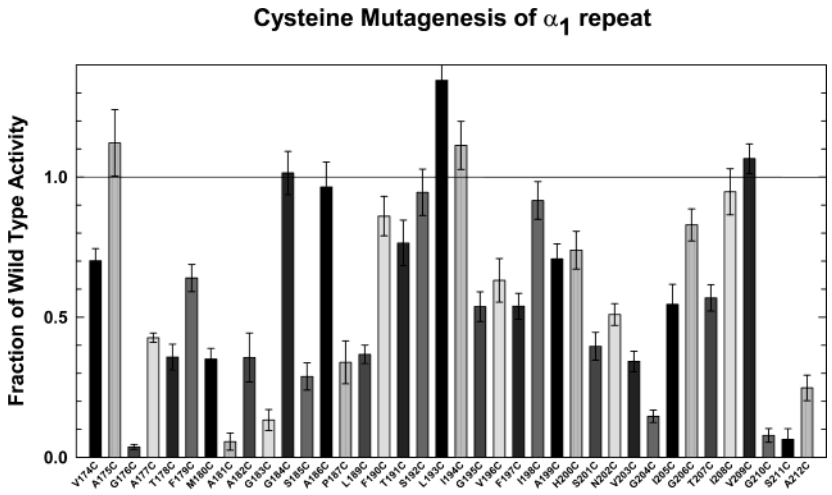

FIGURE 8: Cysteine scanning mutagenesis of alpha1 repeat. High Five cells were transiently transfected with the indicated human cone NCKX2 mutants. NCKX activity was measured as described under Methods and illustrated in Figure 2; activity was normalized with respect to that observed for wild-type human cone NCKX2. Average values (± standard error of the mean) are shown representing 4−7 separate transfection experiments for each mutant tested. Temperature: 25 °C.

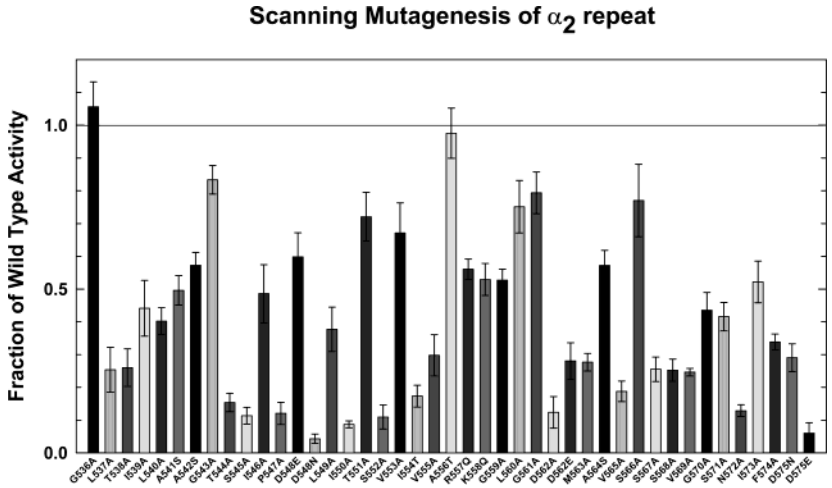

FIGURE 9: Alanine scanning mutagenesis of alpha2 repeat. High Five cells were transiently transfected with the indicated human cone NCKX2 mutants. NCKX activity was measured as described under Methods and illustrated in Figure 2; activity was normalized with respect to that observed for wild-type human cone NCKX2. Average values (± standard error of the mean) are shown representing 4−7 separate transfection experiments for each mutant tested. Temperature: 25 °C.

dues is illustrated in the helical wheel plot of Figure 12: S185 is located one helical turn away from E188 in helix 2; T544 and S545 are located one turn away from D548, while S552 is located one turn away from D548 in the other direction. Glycine and proline are known as helix breakers, and some residues may serve a critical function in this respect. In the first alpha repeat we found two glycine residues (G176 and G210) that could not be replaced by either an alanine or a cysteine residue without causing a near complete loss of NCKX2 function, whereas mutation of other glycine residues led to mutant NCKX2 proteins with >50% function. Mutation of the two proline residues assessed in our study resulted in moderate (P187) to strong (P547) impairment of NCKX function. These proline residues are found to occupy the identical position in the alpha1 and alpha2 repeats, respectively, immediately preceding the functionally important acidic residues E188 and D548 (Figure 12).

DISCUSSION

In this paper we present the first systematic mutagenesis study of a member of the Na/Ca-K exchanger gene family. We selected for mutagenesis 80 residues that make up the two so-called alpha repeats as well as all the acidic residues within the two sets of putative transmembrane spanning segments (Figure 6). These residues represent the most conserved sequence elements among different members of the NCKX gene family. We have used the Myc-tagged human retinal cone NCKX2 clone to introduce point mutations in view of the fact that transfection with this clone results in consistent high functional expression levels as compared with most other NCKX clones and in view if the fact that we have used this clone before to examine functional consequences of NCKX mutations observed in patients with retinal disease (*17*). Twenty-seven novel sequence changes were found in the human rod NCKX1 gene, six of which were considered to be likely pathogenic changes; 14 novel sequence changes were found in the human cone NCKX2 gene, three of which led to missense changes but were unlikely to be pathogenic (*17*). Two of the above sequence changes (I554T and A556T) were included in our scanning mutagenesis; they were found in rod NCKX1, but both residues are conserved between rod NCKX1 and cone

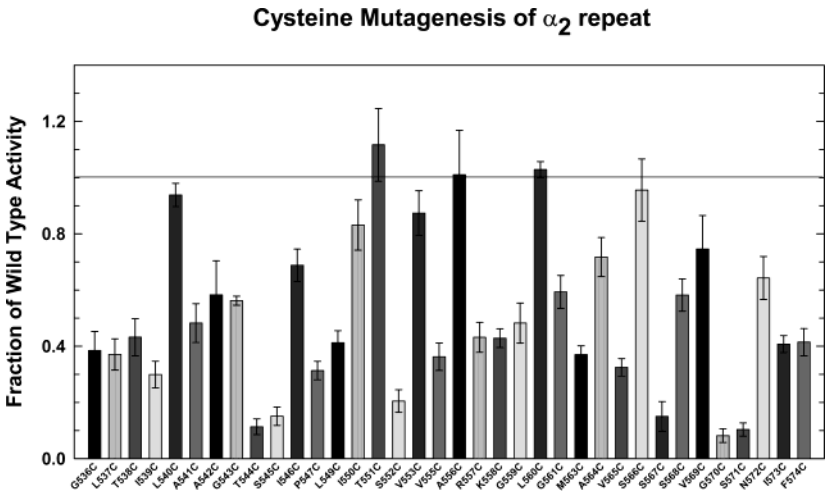

FIGURE 10: Cysteine scanning mutagenesis of alpha2 repeat. High Five cells were transiently transfected with the indicated human cone NCKX2 mutants. NCKX activity was measured as described under Methods and illustrated in Figure 2; activity was normalized with respect to that observed for wild-type human cone NCKX2. Average values (± standard error of the mean) are shown representing 4−7 separate transfection experiments for each mutant tested. Temperature: 25 °C.

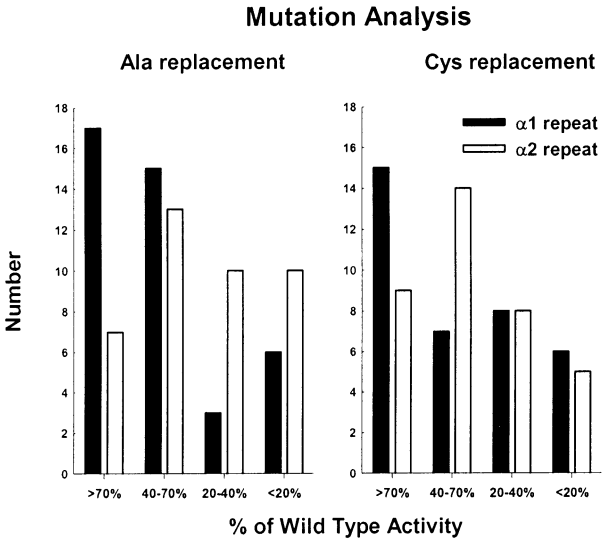

FIGURE 11: Sensitivity toward mutagenesis of residues within the alpha1 and alpha2 repeats, respectively. Solid bars represent the alpha1 repeat; open bars represent the alpha2 repeat.

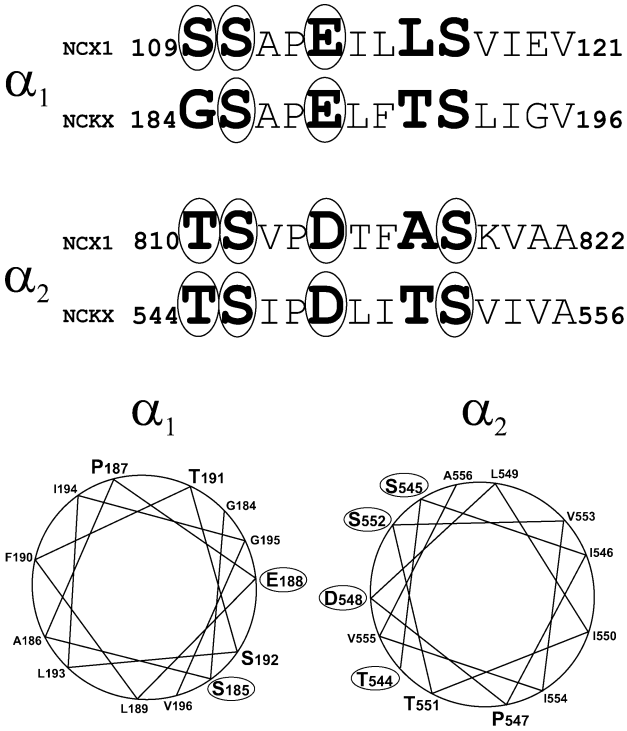

FIGURE 12: Alignment of critical acidic and hydroxyl-containing residues of NCX1 and NCKX. The sequences of the C-terminal parts of the alpha1 (two upper rows) and alpha2 (bottom two rows) repeats from canine heart NCX1 and human cone NCKX2 are compared. Circled residues indicate residues which show <20% of wild-type activity when mutated (activity levels are only indicated for the residues in large type-face). Bottom: helical wheel representations of the above stretches of NCKX sequence.

NCKX2. I554T reduced NCKX2 function to <20%, whereas A556T had no effect on NCKX2 function (Figure 9).

Validation of Procedures. Screening functional consequences of about 190 different point mutations requires efficient and simple methods to quantify and correlate NCKX function, protein expressing, and protein targeting in heterologous expression systems. We earlier developed a heterologous expression system based on transient (or stable) transfection of NCKX cDNAs in cultured insect cells. Insect cells readily grow in suspension, which permits efficient and quantitative analysis of NCKX function by measuring reverse Na/Ca-K exchange in sodium-loaded cells by means of ^{45}-Ca uptake sampling $\sim 10^4$ cells for each time point (*11*). It also permits simple assessment of transport function, protein expression, and surface targeting to be carried out with the same suspension of NCKX expressing cells. With the possible exception of Phe574, NCKX2 mutant protein expression levels as judged from Western blots (Figure 2) and mutant NCKX2 protein targeting to the plasma membrane as judged by surface biotinylation (Figures 3 and 4) were not affected for the mutations studied here; mutant NCKX2 proteins representing a wide range of functional activity with respect to wild-type cone NCKX2 showed very similar protein expression levels and were similarly targeted to the plasma membrane.

Intracellular Na^+ Dependence of Heterogously Expressed NCKX2. Cone NCKX2 expressed in insect cells showed a remarkably low affinity to intracellular Na^+ concentration, especially when compared with the intracellular Na^+ dependence of NCX1 (Figure 1). For a consistent and quantitative

analysis of NCKX function, cells required Na^+ loading to concentrations >100 mM to avoid the highly sigmoid and nonlinear part of the relationship that represents NCKX function versus internal Na^+ concentration. In High Five cells, this was readily accomplished with the shuttle ionophore monensin in a way that has proven to be not feasible in a number of mammalian cultured cell lines (*21*). Nevertheless, even at this high level of Na^+ loading care needs to be taken to control for rundown of activity because of Na^+ leakage from the cells.

Residues Important to NCKX Function. Three classes of amino acids within the transmembrane spanning segments proved to be most sensitive toward mutagenesis (Table 1): acidic residues (Glu and Asp), polar residues (serine and threonine), and glycine. Ca^{2+} transport through membranes is likely to involve negatively charged residues to ligand Ca^{2+} and to reduce the Born energy of placing a divalent cation into the low dielectric environment of the membrane interior. Figure 6 illustrates the location of the acidic residues found in two sets of transmembrane spanning segments of human cone NCKX2; the membrane/aqueous phase boundaries are drawn in such a way as to minimize the number of charged residues located within the membrane. The loops connecting individual TM helices are very short and contain strategically placed pairs of acidic residues or basic residues, unlikely to be located within the membrane. We believe that four of the five residues located within the membrane, E188, D258, D548, and D575, constitute the key residues involved in Ca^{2+} binding and transport based on the following: (1) these residues are localized within transmembrane spanning helices; (2) transport activity was greatly reduced when these residues were individually replaced by either charge-conservative or size-conservative substitutions (Figure 5); (3) these residues are conserved in all rod and cone NCKX sequences and are also conserved in two more distantly related NCKX sequences from *C. elegans* (*11*) and *Drosophila* (*12*), respectively. The fifth residue, D248, does not appear to play an important role since no loss in transport activity was observed upon mutagenesis, and it is not conserved or subject to conservative substitution in either the *C. elegans* or *Drosophila* NCKX.

The highly conserved nature of the short connecting loop sequences is reflected by the fact that mutagenesis of six out of 15 acidic residues, thought to be located in the connecting loops, lowered transport activity to <50% of wild-type and in four cases to <20% (Figure 5). Curiously, for three of these residues (D173, E532, and E651), activity was retained with a size-conservative substitution but not with a charge-conservative substitution; all three residues are thought to be located at the membrane/water interface, often as one of a pair of adjacent acidic residues. Location of acidic residues in the short connecting loops may suggest they play a role as topogenic markers. Alternatively, acidic residues may contribute to a negatively charged surface of the NCKX protein that would attract cations via surface charge effects.

Residues Important to the Putative Na^+/Ca^{2+} Binding Site of NCKX: Comparison with NCX1. Both NCX and NCKX appear to share the feature of a common Na^+/Ca^{2+} binding site(s) (*18*, *22*). Ca^{2+} binding sites in proteins often involve seven oxygen ligands (*23*). Two of the acidic residues located in the alpha1 (E188) and alpha2 (D548), respectively, have hydrophilic serine or threonine residues or pairs of such residues located one helical turn away in both directions (Figure 12); this could contribute to a hydrophilic helix surface lining the Ca^{2+} binding cavity/transport pathway and contribute oxygen ligands for coordinating Ca^{2+} binding. Consistent with this idea, several of these serine or threonine residues appear critical for transport and can be replaced neither with alanine nor with cysteine: E188 is bracketed by S185 (<20% activity) and T191/S192 (both >70% activity), while D548 is bracketed by T544/S545 (both <20% activity) and T551/S552 (T551 >70% activity; S552 <20% activity). As illustrated in Figure 12, most of the above residues are conserved between NCX1 and NCKX1/2 as part of the alpha1 (S185, E188, and S192) and alpha2 (T544, S545, D548, and S552) repeats, respectively. With the exception of S192 (and its counterpart in NCX1, S117), these serine/threonine residues were found to be important for both NCKX2 and NCX1 function (this study, ref *24*). In contrast, the residues that are not conserved between NCKX2 and NCX1 (T191 and T551) have hydrophobic residues in the corresponding position in NCX1 and were not important for NCKX function. Proline residues are often found in alpha helices of membrane proteins where they may contribute to the curvature of helices. Two proline residues (P187 and P547) are found in the sequences illustrated in Figure 12; they are positioned to the N-terminal side of the two acidic residues E188 and D548 (note NCX1 has similarly placed proline residues). These proline residues could position these acidic residues on the convex side of the helix at the narrowest part of a funnel-like structure (*25*).

SERCA and plasma membrane Ca^{2+} pumps are other proteins that carry out high-affinity Ca^{2+} transport through membranes. SERCA-type Ca^{2+} pumps contain four acidic residues critical for the binding of two Ca^{2+} ions, while the plasma membrane Ca^{2+} pump binds only a single Ca^{2+} and contains two critical acidic residues for Ca^{2+} binding (*26*). E188 and D548 are the likely candidates for residues critical to Ca^{2+} binding in NCKX (see above). NCKX has two additional acidic residues thought to be located within transmembrane spanning helices and shown to be critical for transport function (D258 and D575) (Figure 5). Interestingly, NCX1 does not appear to be similarly placed acidic residues.

Glycine Residues and NCKX Function. Twelve glycine residues are among the 80 residues that were mutated in this study. Eight out of 12 glycine residues were sensitive to mutagenesis, six of them located in the alpha1 repeat (Table 1). Mutating two of these residues, G176 and G210, to either alanine or cysteine resulted in <20% of wild-type NCKX activity (Figures 7 and 8). Strategically placed glycine residues allow flexibility, sharp turns, and may provide for contact points with other helices. These attributes could be important for conformational changes in the NCKX protein that are likely to be associated with each transport cycle that expose cation binding sites alternately to the extracellular and intracellular milieu. G210 is thought to be in the middle of a very hydrophobic surface of helix 3, while G176 is at the beginning of helix 2 with neighboring residues that were not particularly sensitive to mutagenesis. This may suggest that these residues provide contact points with another helix. Five glycine residues (G183, G195, G204, G536, and G570) tolerated a change to alanine but not to the larger cysteine, while in one case (G206) the reverse was true. G176 and

G183 are both located on the hydrophobic surface of helix 2, opposite of the hydrophilic surface containing the essential residues E188 and S185 discussed above. Identification and position of these glycine residues will be helpful to guide further topology and helix packing experiments underway in our laboratory.

The alpha2 repeat proved to be significantly more sensitive to mutagenesis as compared with the alpha1 repeat as is illustrated in Figure 11. A similar trend is also seen in a comparison of human NCKX2 with the more distantly related NCKX sequences from *Drosophila* and *C. elegans* cDNAs we have cloned (*11*, *12*): the alpha2 repeat is more conserved than the alpha1 repeat. The alpha1 and alpha2 repeats are the only parts of the NCKX sequence that show significant sequence identity with the heart NCX1 Na/Ca exchanger (*16*), and 10 residues of the alpha1 repeat are internally conserved in the alpha2 repeat of cone NCKX2. It might be expected that residues conserved between the NCX and the NCKX gene families or residues conserved internally between the alpha1 and alpha2 repeats would be particularly sensitive to mutagenesis. Surprisingly, the percentage of these conserved residues sensitive to mutagenesis was not much greater when compared with residues in the alpha repeats that are not conserved between the NCX and NCKX gene families. Similarly, about half of the 10 residues conserved internally between the two alpha repeats of cone NCKX2 were not very sensitive to mutagenesis.

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