Signal Sequence Cleavage and Plasma Membrane Targeting of the Retinal Rod NCKX1 and Cone NCKX2 $Na^+/Ca^{2+}-K^+$ Exchangers†

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ABSTRACT: Retinal rod and cone photoreceptors express two distinct $Na^+/Ca^{2+}-K^+$ exchanger (NCKX) gene products. Both the rod NCKX1 and cone NCKX2 are polytopic membrane proteins thought to contain a putative cleavable signal peptide. A cleavable signal peptide is unusual for plasma membrane proteins; moreover, predictive algorithms suggest the presence of a cleavable signal peptide for all rod NCKX1 proteins and a noncleavable signal anchor for the cone NCKX2 proteins. In this study we have placed a peptide tag at different positions of the NCKX sequence to examine whether the putative signal sequence is indeed cleaved in either NCKX1 or NCKX2 proteins expressed in heterologous systems. The signal peptide was found to be, at least in part, cleaved in dolphin rod NCKX1 and in chicken and human cone NCKX2 expressed in HEK293 cells; no signal peptide cleavage was observed for chicken rod NCKX1 despite the fact that the SignalP predictive algorithm assigned this sequence to have the highest likelihood for a cleavable signal peptide among the three NCKX sequences tested here. For the two NCKX proteins that contained a cleavable signal peptide, only cleaved NCKX protein was found in the plasma membrane of HEK293 cells. Deletion of the signal sequence in both dolphin rod NCKX1 or cone NCKX2 did not affect NCKX protein synthesis but did disrupt plasma membrane targeting as judged from abolition of NCKX function and from lack of surface biotinylation. These results are consistent with delayed signal peptide cleavage for the rod and cone NCKX proteins.

The $Na^+/Ca^{2+}-K^+$ exchanger (NCKX)[1] is a polytopic membrane protein, thought to be composed of two sets of multiple transmembrane-spanning helices and two large hydrophilic loops, one at the N-terminus and one located in the cytosol (*1*). Predictive algorithms suggest that an additional hydrophobic segment at the N-terminus represents a cleavable signal peptide. The N-terminal signal sequence is known to be responsible for signal recognition particle (SRP) dependent translocation into the endoplasmic reticulum (ER) (*2*). The NCKX1 gene product is found in retinal rod photoreceptors (*3*−*5*) and is predicted to contain a signal peptide of about 38 residues. The NCKX2 gene product is found in retinal cones and in retinal ganglion cells (*4*, *5*) as well as in various parts of the brain (*6*); NCKX2 is predicted to contain an unusually long signal peptide of 58 residues. The physiological role of NCKX proteins in retinal rod and cone photoreceptors is to extrude Ca^{2+} that enters photoreceptors in the dark via the cGMP-gated and light-regulated channels; under illumination NCKX readily lowers cytosolic Ca^{2+} to increase guanylyl cyclase activity and initiate a negative feedback loop important for the process of light adaptation and recovery from previous illumination; for a recent review, see ref *7*.

Relatively few plasma membrane ion transporters have been described to contain a cleavable signal sequence. For example, some members of the Na^+/H^+ exchanger gene family have recently been suggested to contain a cleavable signal sequence, but this matter remains to be resolved unambiguously (*8*). The Na^+/Ca^{2+} (NCX) exchanger has an arrangement of transmembrane-spanning helices very similar to that of NCKX, and in this case it has been demonstrated that the protein expressed in heterologous systems is cleaved; moreover, the signal peptide could be deleted from the cDNA sequence without impacting on protein synthesis and protein targeting (*9*, *10*). However, it is controversial whether the signal sequence of bovine rod NCKX1 is cleaved. In one study, it was suggested that, like NCX1, bovine rod NCKX1 contained a cleavable N-terminal signal sequence, since N-terminal sequencing of the purified bovine rod NCKX1 led to a sequence starting at Asp66 (*3*). In contrast, analysis of bovine rod NCKX1 polypeptides obtained in a cell-free system showed no evidence for cleavage but suggested that the N-terminal sequence was important for a proper topology, inferred by glycosylation of the extracelluar loop (*11*).

In this study we have examined whether the first N-terminal hydrophobic segment of different NCKX proteins is cleaved when the corresponding NCKX cDNAs are expressed in heterologous cell systems. The importance of

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[1] Abbreviations: NCKX, $Na^+/Ca^{2+}-K^+$ exchanger; NCX, Na^+/Ca^{2+} exchanger; SRP, signal recognition particle; IP, immunoprecipitation; MW, molecular weight; EDTA, ethylenediaminetetraacetic acid; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; PNGase F, peptide:*N*-glycosidase F.

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this putative signal sequence for protein synthesis and protein targeting in cells was also examined. We have used the chicken rod NCKX1 and human cone NCKX2 clones to represent the short NCKX proteins (~660 residues) and the dolphin rod NCKX1 to represent the much larger (1000−1200 residues) NCKX1 proteins typical for those found only in mammalian rod photoreceptors (*12*). Our results show that in two of the NCKX proteins tested the signal peptide was cleaved, at least in part, when these NCKX proteins were expressed in cell lines. Moreover, the signal sequence was essential for correct targeting of the NCKX protein to the plasma membrane in a mammalian cell line.

METHODS

Myc-Tagged NCKX cDNAs. The human Myc tag (EQKLISEEDL) was inserted into the N-terminus of all NCKX cDNAs using appropriate restriction sites; correct insertion was verified by sequencing of all PCR- and oligonucleotide-generated fragments. The Myc tag was inserted after residue Thr97 of chicken rod NCKX1 (AAF25808), after residue Pro98 of chicken cone NCKX2 (AAF25810), after residue Gln81 of human cone NCKX2 (AAF25811), and after residue His215 of dolphin rod NCKX1 (AAC18119). Insertion of the Myc tag into the N-terminus had no deleterious effect on NCKX function (data not shown).

Construction of FLAG-Inserted NCKX Clones and Deletion of N-Termini. FLAG epitopes were inserted either upstream or downstream of the putative signal peptide (labeled H0 in previous studies on NCKX) in both human cone NCKX2 and dolphin rod NCKX1 clones. Briefly, oligonucleotide primers were prepared with FLAG epitope sequences included in them. The FLAG tags were introduced into the exchanger sequences by standard, two-step, overlapping high-fidelity PCR reactions (Qiagen, Proofstart DNA polymerase). PCR-generated fragments were digested with appropriate restriction endonucleases and cloned as replacements of native fragments in the clones. In the case of the human cone NCKX2 exchanger, the FLAG epitopes were inserted after amino acid residue 15 or 62 by replacement of an *Apa*I and *Xho* restriction fragment in the wild-type pBluescript (pBS) human cone clone. For dolphin rod NCKX1, FLAG tags were introduced after amino acid residue 15 or 53 by replacement of an *Apa*I and *Acc*I restriction fragment in a pBS-based dolphin clone. For chicken rod NCKX1, the FLAG epitope was inserted after residue 2. All constructs were excised from pBS clones using *Apa*I and *Not*I restriction digestion and recloned into similarly digested pEIA4+ and pCDNA3.1+.

To delete the first 75 amino acid residues from wild-type human cone NCKX, a pair of complementary oligonucleotide primers, containing an in-frame ATG codon between *Apa*I and *Avr*II restriction sites, was annealed, digested with those two restriction endonucleases, and cloned into similarly digested human cone NCKX in pBluescript (pBS). The modified human cone NCKX sequence, with the introduced methionine codon, was excised from pBS by digestion with *Apa*I and *Not*I and then recloned into pEIA4+ and pCDNA3.1+ (Invitrogen) using those same restriction sites in those vectors. To delete the N-terminal 84 residues of the dolphin rod NCKX, a primer was synthesized to introduce an *Apa*I restriction site just upstream of the methionine residue at position 85. High-fidelity PCR (Qiagen, Proofstart) was performed using the above primer and one prepared to sequences downstream of the native *Acc*I restriction site. This fragment was digested with those two enzymes and cloned into the pBS-based dolphin clone digested with those same restriction endonucleases. The entire modified dolphin clone was excised with *Apa*I and *Not*I and then recloned into pEIA4+ and pCDNA3.1. The regions of all clones modified by either PCR fragment replacement or cloning of annealed nucleotides were thoroughly sequenced to ensure correct nucleotide sequence using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (USB).

Surface Biotinylation. Surface biotinylation was carried out with the impermeant reagent Sulfo NHS-LC-biotin (Pierce) which targets lysine residues. Transiently transfected HEK293 cells were washed two times with PBS on 10 cm plates. Immediately before biotinylation, the reactive biotin was diluted to 0.1 mg/mL. After 2.5 mL of 0.1 mg/mL Sulfo NHS-LC-biotin was applied on plates for 10 min at room temperature, the residual reactive biotin was inactivated by 1 mL of 1 M Tris-HCl, pH 7.5, for 15 min. After the biotinylated cells were harvested and washed with PBS, proteins were extracted with 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). To pull down NCKX molecules, 0.2 mg of protein extract was volumed up to 1 mL with TBS containing 0.05% Tween 20 (TBST) and was incubated with either Myc monoclonal antibody (9E10, Roche) or FLAG M2 monoclonal antibody (Kodak), and protein G−agarose (Santa Cruz Biotech) overnight, rotating end to end. The immunoprecipitates were washed three to five times with TBST. Proteins were separated by SDS−PAGE, transferred to nitrocellulose filter, and probed with avidin−HRP (Pierce). Subsequently, membranes were stripped and reprobed with anti-Myc-HRP (Roche).

Deglycosylation of Different NCKX1 and NCKX2 Proteins. High Five cells transiently transfected with the various NCKX cDNAs were collected 48 h posttransfection and washed two times with 150 mM NaCl, 20 mM Hepes, 80 mM sucrose, and 200 μM EDTA, pH 7.4. The cells were then incubated for 20 min in ice-cold RIPA buffer. The suspension was spun at 20000*g* for 5 min, and the supernatant was collected. The protein concentration of the supernatant was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The extract was then denatured by adding 0.5% SDS and 1% β-mercaptoethanol and incubated at 37 °C for 10 min. NP-40 and Na_2HPO_4 were added to each sample to a final concentration of 1% and 50 mM, respectively. PNGase F was then added to the treated samples to a final concentration of 2 units/μL. All samples were then incubated at 37 °C for 1.5 h. Sample buffer was added, and samples were subjected to gel electrophoresis in an 8% Laemmli gel. Myc-tagged NCKX protein was detected by Western blotting with the anti-Myc antibody.

Fluorometric Assay for $Na^+/Ca^{2+}-K^+$ Exchange Function. HEK293 cells, transiently transfected with NCKX cDNA, were loaded with the Ca^{2+}-indicating dye fluo-3 as described previously (*13*). After being loaded, 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 sulfinpyra-

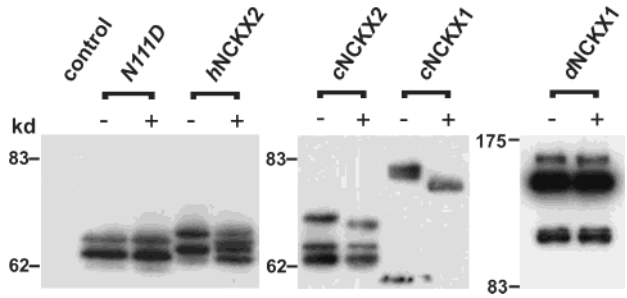

FIGURE 1: Effect of *N*-glycosidase on the apparent MW of heterologously expressed NCKX proteins. Human cone *h*NCKX2, chicken rod *c*NCKX1 and cone *c*NCKX2, and dolphin rod *d*NCKX1 were transiently expressed in High Five cells. All NCKX cDNAs contained the Myc tag inserted in the N-terminal extracellular loop for protein detection. Samples were untreated (−) or treated with PNGase F (+) as indicated. Left panel: mock-transfected cells, the human *h*NCKX2 N111D mutant, and wild-type human *h*NCKX2. Middle panel: chicken cone *c*NCKX2 and chicken rod *c*NCKX1. Right panel: dolphin rod *d*NCKX1. Each lane contains 10 μg of protein.

zone, and 20 mM Hepes (pH 7.4) and stored at room temperature until use. Transfected HEK293 cells were diluted 20-fold into a cuvette in a medium containing 75 mM NaCl, 75 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 with a stirrer. Fluo-3 fluorescence was monitored after the addition of various agents to the cuvette under constant stirring; further details on instrumentation and procedures have been published elsewhere (*14*).

NCKX Expression in High Five Cells. In addition to HEK293 cells, we also used a lepidopteran insect cell expression system for transient transfection of NCKX cDNAs in High Five insect cells (BTI-TN-5B1-4, High Five; Invitrogen). This cell system results in very consistent expression of NCKX protein and NCKX function as described before (*15*, *16*).

RESULTS

Effect of N-Glycosidase on the Banding Pattern of NCKX Proteins on SDS−PAGE. We introduced a Myc tag downstream of putative signal peptide cleavage sites into the N-terminus of all the NCKX clones studied in our laboratory (see Methods); the Myc tag at this position did not affect NCKX function and permits uniform MW determination of the different expressed NCKX proteins. Figure 1 illustrates the effect of the glycosidase PNGase F on the MW of different Myc-tagged NCKX proteins as determined by SDS−PAGE. The left panel shows that human cone *h*NCKX2 ran as a doublet around 62 kDa with both bands showing a significant increase in mobility after PNGase F treatment (occasionally a triplet was observed with the additional band at slightly higher mobility, e.g., Figure 8). The human cone *h*NCKX2 has a single N-glycosylation site (N111), and mutating this residue to aspartate resulted in NCKX protein that ran as a doublet of lower MW compared with wild type, but in this case PNGase F treatment had no effect on mobility. The middle panel shows that chicken cone *c*NCKX2 ran as a triplet, consisting of a doublet similar to that observed with human *h*NCKX2 plus an additional band of higher apparent MW. PNGase F treatment showed a significant increase in mobility for the upper band but had much less effect on the doublet at ~65 kDa unlike what was observed for human *h*NCKX2. Chicken rod *c*NCKX1 ran as a single band on SDS−PAGE, and this band underwent a significant reduction in MW upon PNGase F treatment. Finally, the right panel of Figure 1 shows that dolphin rod *d*NCKX1 ran as a single major band with often, as illustrated here, a few minor bands; PNGase F treatement caused a very slight lowering of the MW, in particular of the highest MW band observed. The multiple banding pattern observed with dolphin *d*NCKX1 is often seen with the bovine *b*NCKX1 in situ or expressed in cell lines (*13*) and may be typical for the large mammalian NCKX1 proteins. The results illustrated in Figure 1 show that deglycosylation can cause significant MW shifts for some heterologously expressed NCKX proteins, but this did not affect the different banding patterns observed. The small Myc-tagged NCKX proteins showed a remarkable range of MW's despite the fact that the calculated MW (inclusive the Myc tag) is very similar. The calculated nonglycosylated MW's of the different NCKX proteins are as follows (between parentheses the MW after cleavage at the predicated site; see below): chicken rod *c*NCKX1, 75.0 kDa (71.1) runs at ~80 kDa; chicken cone *c*NCKX2, 71.9 kDa (65.5) runs at ~73, 67, and 64 kDa; human cone *h*NCKX2, 72.9 kDa (66.4) runs at ~68 and 65 kDa; dolphin rod *d*NCKX1, 112.6 kDa (107.0) runs at ~146 kDa. It is clear from this survey that analysis of apparent MW on SDS−PAGE alone is unlikely to provide clear evidence for processing of a putative signal sequence.

N-Terminal Sequences of both Dolphin Rod NCKX1 and Human Cone NCKX2 Are Partially Cleaved in Heterologous Expression Systems. Next, we examined whether the banding patterns observed for the different NCKX proteins represented cleavage of putative signal peptides. SignalP, a web-based server to predict signal peptide cleavage sites (http://www.cbs.dtu.dk/services/SignalP-2.0/), predicts a putative cleavage site between residues 58 and 59 of the cone NCKX2 proteins and predicts a putative cleavage site between residues 38 and 39 of the dolphin rod *d*NCKX1 protein. On the basis of these predictions we inserted the FLAG epitope either before or after the predicted cleavage site at positions 15 and 62 of the human cone *h*NCKX2 sequence (F15-*h*NCKX2 and F62-*h*NCKX2) and at positions 15 and 53 of the dolphin rod *d*NCKX1 sequence (F15-*d*NCKX1 and F53-*d*NCKX1). Figure 2 illustrates the positions of the different FLAG inserts relative to the position of the Myc tag. All of the FLAG constructs were tested for NCKX function after transfection in High Five cells by measuring ^{45}Ca uptake associated with reverse $Na^+/Ca^{2+}-K^+$ exchange, a simple and quantitative assay for NCKX function established in our laboratory (*15*, *16*). No effect on NCKX function was observed (data not shown), suggesting that insertion of the FLAG epitope at these positions did not alter NCKX topology.

The mutant human *h*NCKX2 FLAG-tagged proteins were expressed in either High Five cells (Figure 3A) or HEK293 cells (Figure 3B). The expressed proteins were separated on SDS−PAGE and analyzed by Western blotting. Figure 3A (Myc blot) illustrates that the upper bands of both F15-*h*NCKX2 and F62-*h*NCKX2 as well as the lower band of F62-*h*NCKX2 showed a slightly lower migration rate compared with the FLAG-free *h*NCKX2 protein, consistent

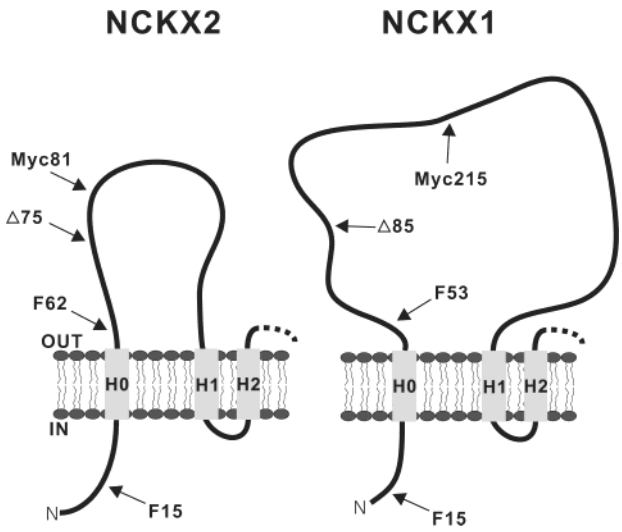

FIGURE 2: Diagram indicating the different FLAG insertion constructs. The N-terminal parts of human *h*NCKX2 (left) and dolphin *d*NCKX1 (right) are illustrated with the positions at which various modifications were made. F indicates the different positions at which the FLAG epitope was inserted. Myc indicates the position of the Myc tag, present in all constructs. △ indicates the position of the N-terminal deletion constructs.

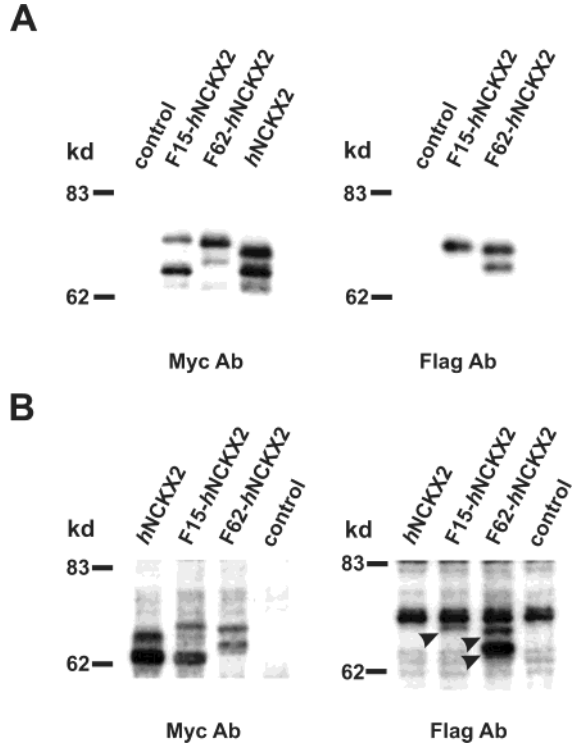

FIGURE 3: Detection of double-tagged *h*NCKX2 proteins in High Five cells and in HEK293 cells. The indicated NCKX proteins were transiently expressed in High Five cells (A) or HEK293 cells (B). The FLAG blot shows a nonspecific band at ~75 kDa in HEK293 cells; the arrowheads indicate unprocessed (upper) and processed (lower) FLAG-tagged NCKX protein positioned just below this nonspecific band. Control indicates mock-transfected cells. Blots were probed with Myc or FLAG monoclonal antibodies as indicated. Each lane contains 10 μg of protein.

with the addition of the mass of the FLAG epitope. In contrast, the lower band of F15-*h*NCKX2 was very similar to that of FLAG-free *h*NCKX2 protein, suggesting the lower band represented the *h*NCKX2 protein from which extra mass of the FLAG15 epitope was removed. Judged by the

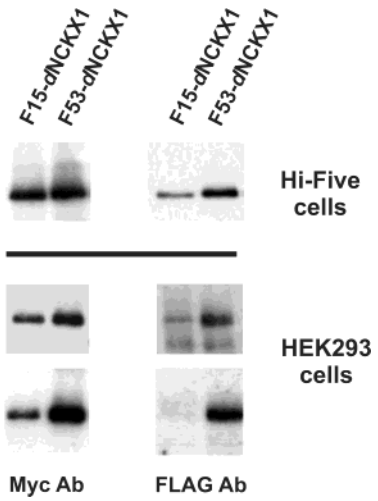

FIGURE 4: Detection of double-tagged *d*NCKX1 proteins in High Five and in HEK293 cells. The dolphin rod NCKX1-based FLAG inserts F15*d*NCKX1 and F53*d*NCKX1 were transiently expressed in High Five or HEK293 cells as indicated. Blots were probed with Myc or FLAG monoclonal antibodies as indicated. Each lane contains 20 μg of protein.

relative intensities of the upper and lower bands, F62-*h*NCKX2 appeared to be cleaved to a lesser degree, perhaps because the close proximity of the FLAG epitope (inserted at residue 62) to the predicted cleavage site at residue 58 might interfere with proper cleavage. The FLAG immunoblot (Figure 3A) showed a single upper band for the F15-*h*NCKX2 construct and two bands for the F62-*h*NCKX2 construct, confirming that the FLAG at position 15 was cleaved but not the FLAG at position 62. When the same *h*NCKX2 constructs were expressed in HEK293 cells, very similar results were obtained (Figure 3B; note the nonspecific band at ~75 kDa in the FLAG blot of protein extracts from HEK293 cells; the arrowheads indicate the unprocessed and processed NCKX bands positioned just below this nonspecific band). Results very similar to those illustrated in Figure 3 were obtained in two other experiments.

We repeated these experiments with the dolphin rod *d*NCKX1 protein. In this case, due to the larger size of the dolphin rod *d*NCKX1 protein and the smaller size of the predicted cleaved fragment, separation on SDS−PAGE did not result in a doublet as observed for the cone NCKX2 proteins. Therefore, we compared the intensity of the bands on the FLAG blot, measured with densitometry using Sigmagel, with the intensity of the bands on the Myc blots. In a total of eight different transfection experiments, in six cases >90% of *d*NCKX1 appeared to be cleaved, while in two experiments a significant amount (55% and 35%, respectively) of uncleaved *d*NCKX1 remained. The bottom panel of Figure 4 illustrates two of these experiments. Less processing of *d*NCKX1 was obtained when expressed in High Five cells: in four experiments, the percentage of uncleaved *d*NCKX1 amounted to 95%, 19%, 37%, and 36%, respectively; the 36% case is illustrated in the top panel of Figure 4.

N-Terminally Truncated NCKX Proteins Do Not Result in NCKX Function in the Plasma Membrane When Expressed in HEK293 Cells. To examine the role of the cleaved N-terminus in heterologous expression, the N-terminus was deleted from both human cone NCKX2 (Δ75N-*h*NCKX2) and dolphin rod NCKX1 (Δ85N-*d*NCKX1), and a new start

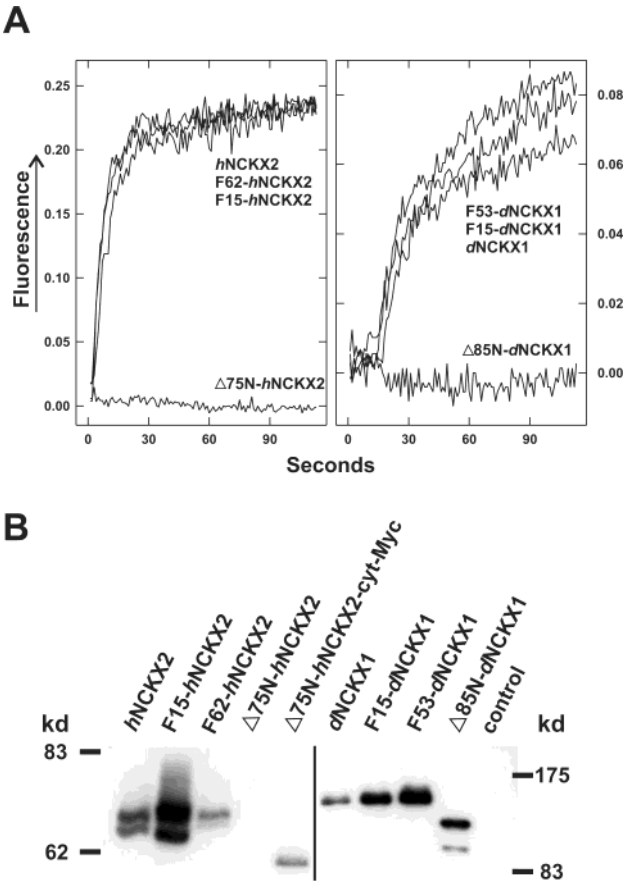

FIGURE 5: Functional consequences of N-terminal truncation of both rod *d*NCKX1 and cone *h*NCKX2. (A) Reverse $Na^+/Ca^{2+}-K^+$ exchange was measured in HEK293 cells as K^+-dependent (added at time zero) rise in free cytosolic Ca^{2+} detected by the Ca^{2+}-indicating dye fluo-3 as described in Methods. HEK293 cells were transfected with the indicated NCKX constructs. Mock-transfected cells or cells transfected with empty vector did not show any K^+-dependent rise in free cytosolic Ca^{2+}, similar to what is observed here for cells transfected with the truncated forms of *d*NCKX1 and *h*NCKX2. The Δ75N-*h*NCKX2 construct contained the Myc tag in the cytosolic loop. Temperature: 25 °C. (B) Protein levels of the cells tested in panel A were determined in Western blots with the monoclonal Myc antibody. Control indicates protein extracted from mock-transfected HEK293 cells. Each lane contains 10 μg of protein.

methionine was inserted. The human cone Δ75N-*h*NCKX2 construct did not result in protein that could be detected with the Myc antibody, perhaps because the Myc epitope (at position 81) was cleaved off. We inserted the Myc tag in the large cytosolic loop, and this rectified the detection problem. We expressed the various rod and cone NCKX constructs in HEK293 cells and assayed for NCKX function using the intracellular Ca^{2+}-indicating dye fluo-3 as described before (*14*). In these experiments we assayed for K^+-dependent Ca^{2+} influx (reverse $Na^+/Ca^{2+}-K^+$ exchange) in Na^+-loaded HEK293 cells. Cells were loaded with high intracellular Na^+ by collapsing the plasma membrane Na^+ gradient with the alkali cation-selective channel gramicidin. Reverse $Na^+/Ca^{2+}-K^+$ exchange was initiated by addition of 20 mM external KCl in the presence of Ca^{2+}. A rapid rise in fluo-3 fluorescence was observed, indicating Ca^{2+} influx into the cytosol when HEK293 cells expressed either wild-type human cone *h*NCKX2, the F15-*h*NCKX2 construct, or the F62-*h*NCKX2 construct (Figure 5A, left panel). In contrast, the Δ75N-*h*NCKX2 construct did not result in any detectable potassium-dependent Ca^{2+} influx (as observed in control mock-transfected cells; not shown), although protein expression was readily observed and was comparable to protein levels obtained with the other three NCKX2 constructs (Figure 5B, left panel).

We repeated this experiment with the four dolphin rod *d*NCKX1 constructs and obtained essentially the same result. Dolphin rod *d*NCKX1 and the F15-*d*NCKX1 and F53-*d*NCKX1 constructs derived from it showed clear K^+-dependent Ca^{2+} influx, whereas the truncated Δ85N-*d*NCKX1 did not show any NCKX function (Figure 5A, right panel), and this was not due to lack of protein expression (Figure 5B). Apart from the truncated NCKX clones, which showed no NCKX function, insertion of the FLAG epitope had little effect on either *h*NCKX2 or *d*NCKX1 function, but protein expression levels for the various constructs (as judged from the Western blot) appeared to be quite different as illustrated in Figure 5. Figure 5 illustrates results from a single experiment; very similar results were obtained in three other experiments, and the relative amounts of NCKX protein expressed for the different NCKX constructs were quite consistent as judged by the Western blots. The reason for this discrepancy between NCKX function and apparent NCKX protein expression level for the different Flag-tagged NCKX constructs is unclear. It could be noted that a similar discrepancy between NCKX function and NCKX protein expression was noted when various single residue mutants of *h*NCKX2 were expressed in HEK293 cells (K.-J. Kang, T. G. Kinjo, and P. P. M. Schnetkamp, unpublished results), in contrast to the uniform protein expression levels of the same *h*NCKX2 mutants observed in High Five cells (*16*).

Is Only Processed NCKX Present on the Surface of HEK293 Cells? The simplest interpretation of the above results is that truncation of the signal sequence prevents correct targeting to the plasma membrane of HEK293 cells. To confirm this, we carried out surface biotinylation with the impermeant reagent Sulfo NHS-LC-biotin. Expressed *h*NCKX2 and *d*NCKX1 proteins were immunoprecipitated with the monoclonal Myc antibody, separated by SDS−PAGE, and probed with avidin−HRP. Surface biotinylation was observed for the dolphin *d*NCKX1 and human *h*NCKX2 proteins tested but not for the truncated constructs, Δ85N-*d*NCKX1 and Δ75N-*h*NCKX2 (Figure 6, left panel); when the blot was reprobed with the Myc antibody, the amount of immunoprecipitated protein found for the truncated rod and cone NCKX constructs was not very different from that of the nontruncated NCKX proteins (Figure 6, right panel).

The above results illustrated in Figure 6 show that truncation of the cleavable signal peptide from human cone *h*NCKX2 and dolphin rod *d*NCKX1 prevented correct targeting to the plasma membrane, consistent with the lack of functional expression observed in Figure 5. Does this imply that proper cleavage of the signal sequence is required for or associated with plasma membrane targeting? To address this question, we carried out surface biotinylation on HEK293 cells expressing either human cone *h*NCKX2, the F15-*h*NCKX2 construct, or the F62-*h*NCKX2 construct. Immunoprecipitation was carried out with either the Myc or the FLAG antibody, and the precipitates were analyzed for biotinylation with avidin−HRP. The avidin−HRP blot predominantly showed single bands for all three NCKX2 constructs when immunoprecipitation was carried out with

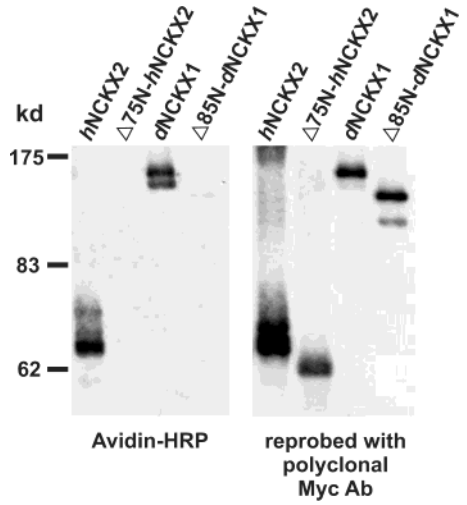

FIGURE 6: Surface biotinylation of full-length and truncated *d*NCKX1 and *h*NCKX2. Left panel: Surface biotinylation was carried in HEK293 cells expressing the indicated NCKX constructs as described under Methods. NCKX protein was immunoprecipitated with the monoclonal Myc antibody, separated on SDS−PAGE, and probed with avidin−HRP. Right panel: The membrane probed with avidin−HRP was stripped and reprobed with anti-Myc-HRP. The Δ75N-*h*NCKX2 construct contained the Myc tag in the cytosolic loop.

the Myc antibody (Figure 7A, top panel). In contrast, only the F62-*h*NCKX2 but not the F15-*h*NCKX2 was detected when immunoprecipitation was carried out with the FLAG antibody (Figure 7A, top panel). The latter implies that the noncleaved protein made by the F15-*h*NCKX2 construct is not present in the plasma membrane (the cleaved protein lost the FLAG epitope). To complement this observation, the membrane was stripped and reprobed with anti-Myc-HRP. In all cases two bands were present except for the single band observed for the F15-*h*NCKX2 construct immunoprecipitated with the FLAG antibody (Figure 7A, bottom panel) (in this case the lower band represents protein from which the FLAG epitope was cleaved and, hence, not immunoprecipitated with the FLAG antibody). These results are consistent with the model in which only NCKX2 protein represented by the lower band can be biotinylated with a membrane impermeant reagent. Similar results were obtained with the dolphin rod-based F15-*d*NCKX1 and F53-*d*NCKX1 constructs; i.e., immunoprecipipitation with the FLAG antibody yielded biotinylated *d*NCKX1 protein for the F53-*d*NCKX1 construct but not for the F15-*d*NCKX1 construct (Figure 7B). A high MW band was observed in the avidin−HRP blot of *d*NCKX1 in some (as shown here) but not all experiments. Otherwise, the results shown in Figure 7 are representative for three additional experiments not illustrated.

Processing and Function of Chicken Rod NCKX1. Chicken rod *c*NCKX1 codes for a much smaller protein compared with dolphin NCKX1. SignalP predicts a putative cleavage site between residues 31 and 32 of the chicken rod *c*NCKX1 protein. Of the three NCKX sequences examined here, *c*NCKX1 is predicted with the highest degree of confidence to have a cleaved signal peptide as opposed to a signal anchor. We introduced the FLAG tag after the second residue of the full-length *c*NCKX1 sequence. Unfortunately, the FLAG-tagged chicken rod *c*NCKX1 yielded much lower protein expression levels compared with *c*NCKX1 when expressed in either HEK293 or High Five cells. Nevertheless, chicken rod *c*NCKX1 containing both Myc and FLAG tags consistently showed a slightly lower mobility on SDS−PAGE when compared with the *c*NCKX1 containing only the Myc tag (Figure 8A). This is consistent with the added mass of the FLAG epitope and implies that neither the FLAG nor the rest of the signal peptide sequence was cleaved when expressed in either High Five cells or in HEK293 cells. From this we conclude that *c*NCKX1 is not cleaved in either HEK293 or High Five cells. Next, we examined both surface biotinylation and functional expression of *c*NCKX1 in HEK293 cells. Surface biotinylation was readily observed in four experiments (one such experiment illustrated in Figure 8C), while functional expression was variable. In two experiments good *c*NCKX1 function was observed, while in four other experiments no function was observed; two extreme cases are illustrated in Figure 8B and compared with the consistently strong NCKX function observed for *h*NCKX2.

DISCUSSION

Distinct $Na^+/Ca^{2+}-K^+$ exchanger gene products are expressed in the outer segments of retinal rod and cone photoreceptors, respectively. In the outer segment plasma membrane the $Na^+/Ca^{2+}-K^+$ exchanger extrudes Ca^{2+} that enters the outer segment via the cGMP-gated channels. The web-based SignalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/) predicts that both rod NCKX1 and cone NCKX2 contain either a cleavable signal sequence or a noncleaved signal anchor. In this study we have examined signal sequence processing for a number of different NCKX1 and NCKX2 proteins expressed in a mammalian cell line (HEK293 cells) or in an insect cell line (High Five cells). We have shown previously that High Five cells constitute a very consistent expression system for the quantitative analysis of NCKX protein expression and NCKX function (*4*, *16*). We examined the relatively small NCKX proteins (~660 residues) found in human and chicken cone photoreceptors (NCKX2) and in chicken rod photoreceptors (NCKX1) and the much larger dolphin rod NCKX1 (1016 residues) representative for NCKX1 proteins found in mammalian rod photoreceptor. Our results suggest that analysis of the MW of NCKX proteins on SDS−PAGE by itself was unlikely to provide unambiguous evidence for signal peptide cleavage or lack thereof due to the complicated banding patterns observed (Figures 1, 5, 7, and 8). Clear evidence for signal peptide processing or lack thereof was obtained by monitoring the presence of FLAG epitope tags inserted at different positions of the N-terminal part of the NCKX sequence. Curiously, different results were obtained for different NCKX proteins. A consistent pattern was observed for the human and chicken cone NCKX2 proteins, in which case a fraction of the expressed protein was cleaved (Figures 1 and 3) (similar results were obtained for bovine NCKX2 as well; data not shown). Lack of complete signal peptide cleavage may reflect the fact that the size of the predicted signal peptide for the cone NCKX2 proteins is unusually large (58 residues) (*17*, *18*). Clear evidence for signal peptide cleavage was also obtained for the dolphin rod NCKX1 protein expressed in HEK293 cells, but curiously, no signal peptide cleavage was observed for the chicken rod NCKX1. Oddly, the observed cleavage pattern runs contrary to the predictions of the SignalP algorithm, which attributes the highest probability of a cleaved signal peptide as opposed to

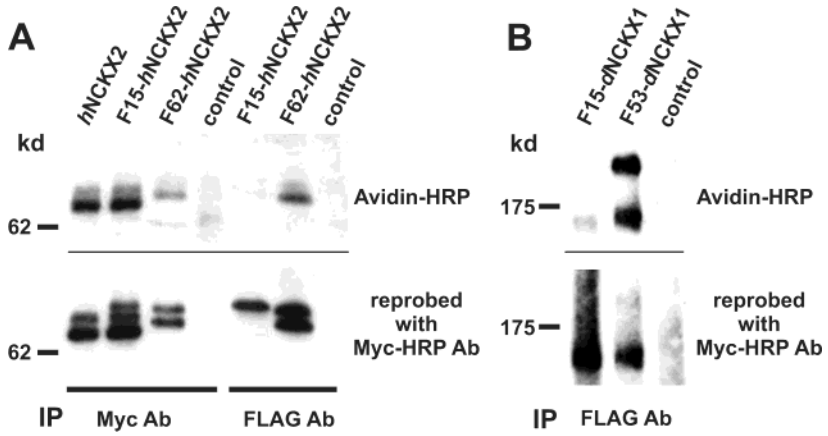

FIGURE 7: Surface biotinylation of the various FLAG-tagged NCKX. Surface biotinylation was carried in HEK293 cells expressing the indicated NCKX constructs as described under Methods. NCKX protein was immunoprecipitated with either monoclonal Myc or FLAG antibody (as indicated), separated on SDS−PAGE, and probed with avidin−HRP (labeled avidin−HRP). The membranes probed with avidin−HRP were stripped and reprobed with anti-Myc-HRP (labeled reprobed). Panel A: *h*NCKX2 constructs. Panel B: *d*NCKX1 constructs. Control represents a control lane containing material from mock-transfected HEK293 cells.

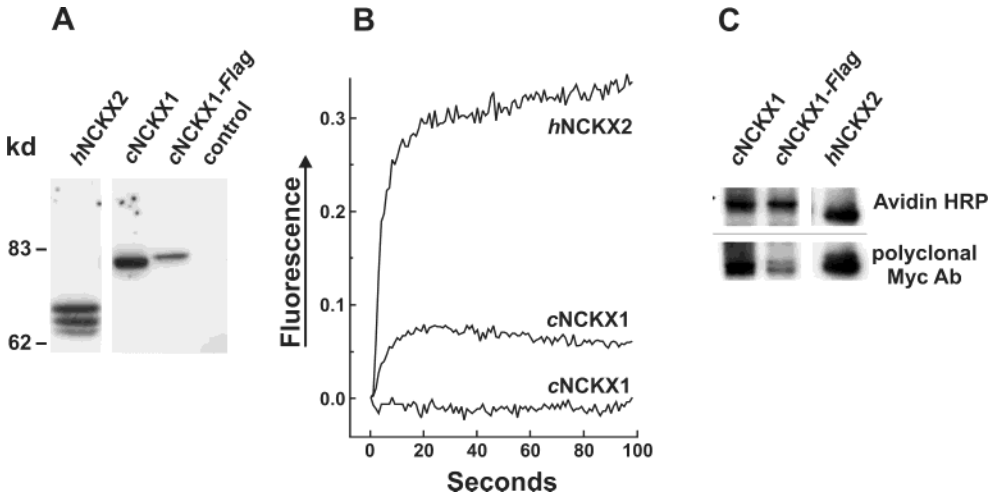

FIGURE 8: Plasma membrane localization of the chicken rod *c*NCKX1. *h*NCKX2, *c*NCKX1, and FLAG-tagged *c*NCKX1 were expressed in HEK293 cells. (A) Western blot probed with monoclonal Myc antibody. (B) Reverse $Na^+/Ca^{2+}-K^+$ exchange was measured as K^+-dependent (added at time zero) rise in free cytosolic Ca^{2+} detected by the Ca^{2+}-indicating dye fluo-3 as described in Methods. HEK293 cells were transfected with the indicated NCKX constructs. Mock-transfected cells or cells transfected with empty vector did not show any K^+-dependent rise in free cytosolic Ca^{2+}. Functional activity observed in two different experiments with cells expressing *c*NCKX1 is compared with that observed in cells expressing *h*NCKX2. Temperature: 25 °C. (C) Surface biotinylation of *c*NCKX1 compared with *h*NCKX2. Surface biotinylation was carried in HEK293 cells expressing the indicated NCKX constructs as described under Methods. NCKX protein was immunoprecipitated with the monoclonal Myc antibody, separated on SDS−PAGE, and probed with avidin−HRP. The blot was stripped and reprobed with polyclonal Myc antibody.

noncleaved signal anchor to chicken rod NCKX1 and attributes the lowest probability to the cone NCKX2 proteins. In general, all of the rod NCKX1 proteins (human, bovine, dolphin, chicken) are predicted to have a cleaved signal peptide, while all the cone NCKX2 proteins (human, bovine, chicken) are predicted to have a noncleaved signal anchor. The unusual NCKX signal peptides may be related to the unusual targeting issues faced by NCKX1 and NCKX2 in rod and cone photoreceptors, respectively. It has been shown that rod NCKX1 is only present in the outer segment organelle of the rod photoreceptor (*19*, *20*) and, within the outer segment, is only found in the plasma membrane, not in the intracellular disk membranes (*21*). Although the distribution of NCKX2 within cone photoreceptors is not known, it is reasonable to assume a similar distribution as observed in rod photoreceptors in view of the similarity in overall physiology between rod and cone photoreceptors.

Is Cleavage of Signal Peptide Associated with Targeting to Plasma Membrane? Signal sequences are often used to allow for specific targeting, although it is not common for plasma membrane proteins to have a cleavable signal sequence (*18*). In an earlier study it was reported that in vitro translation of bovine rod *b*NCKX1 did not result in signal sequence cleavage, implying that the latter did not occur cotranslationally in the endoplasmic reticulum (*11*). Truncation of the N-terminal signal sequence and insertion of a start methionine at residue 75 of human cone NCKX2 (Δ75N-*h*NCKX2) or at residue 85 of dolphin rod NCKX1 (Δ85N-*d*NCKX1) did not affect NCKX protein expression in HEK293 cells per se, but appeared to disrupt correct NCKX targeting to the plasma membrane as judged by (1) lack of NCKX function observed for the truncated human cone NCKX2 and dolphin rod NCKX1 (Figure 5) and (2) lack of surface biotinylation of the truncated human cone

NCKX2 and dolphin rod NCKX1 (Figure 6). This could suggest that delayed cleavage of the signal peptide is important for correct targeting to the plasma membrane. Delayed signal peptide cleavage on route to plasma membrane delivery is also suggested by the observation that in HEK293 cells only cleaved human NCKX2 or cleaved dolphin NCKX1 was present in the plasma membrane as determined by surface biotinylation (Figure 7). Chicken rod NCKX1 did not follow this pattern as no signal peptide processing was observed, but NCKX function was clearly observed in some but not all experiments in HEK293 cells (Figure 8). The sequence around the signal cleavage site and the sequence of the polar N-terminal region are both highly conserved among mammalian rod NCKX1 proteins but much less so with chicken NCKX1. The above discussion applies to NCKX expressed in the mammalian HEK293 cells. When expressed in insect High Five cells, the signal peptide cleavage patterns were qualitatively similar to those observed in HEK293 cells: partial cleavage of NCKX2 proteins was always observed as judged by the stereotypical banding pattern on SDS−PAGE, some signal peptide cleavage was observed for dolphin *d*NCKX1, and no signal peptide cleavage was observed for chicken rod NCKX1.

Significance of Signal Peptide Cleavage in Mammalian Rod NCKX1. When DNA of patients with retinal disease was analyzed for sequence changes in rod NCKX1, one of the changes observed was predicted to result in a novel acceptor splice site of high probability; if this splice site would be used "in vivo", it would remove most of the signal peptide (*22*). On the basis of our results with the related dolphin rod NCKX1, it seems likely that this truncation would result in incorrect targeting and, hence, affect NCKX function in the outer segment plasma membrane. Truncation of the signal sequence has also been reported to lead to incorrect membrane topology in "in vitro" translation studies (*11*). The latter study also suggests that no signal peptide cleavage occurs "in situ" in the bovine retina, based on the fact that Western blotting with an antibody against a peptide representing the far N-terminus of bovine NCKX1 detects unprocessed *b*NCKX1 in a retinal extract. However, this does not exclude the possibility that the majority of the NCKX1 protein is cleaved as suggested by N-terminal sequencing of the purified bovine NCKX1 protein (*3*). The high degree of sequence similarity at the far N-terminus and the predicted splice site of the signal peptide of different mammalian NCKX1 proteins (*13*) suggests that the signal peptide processing observed here for dolphin NCKX1 is also likely to occur with bovine NCKX1.

The results of this study point to a significant difference between members of the NCKX and NCX gene families, respectively. In the case of members of the NCX gene family, the signal sequence is cleaved cotranslationally and the cleaved N-terminal signal peptide is not required for functional integration into the plasma membrane (*9*, *10*). NCKX2 transcripts have been found in many parts of the brain (*6*), and it is likely to be coexpressed with NCX in many neurons in the brain. Perhaps the unusual signal peptide found in NCKX2 will allow for targeting to specific parts of the cell as opposed to a more general distribution expected from NCX.

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