Mutations in the Human Na-K-2Cl Cotransporter (NKCC2) Identified in Bartter Syndrome Type I Consistently Result in Nonfunctional Transporters

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Abstract. Bartter syndrome (BS) is a heterogeneous renal tubular disorder affecting Na-K-Cl reabsorption in the thick ascending limb of Henle’s loop. BS type I patients typically present with profound hypokalemia and metabolic alkalosis. The main goal of the present study was to elucidate the functional implications of six homozygous mutations (G193R, A267S, G319R, A508T, del526N, and Y998X) in the bumetanide-sensitive Na-K-2Cl cotransporter (hNKCC2) identified in patients diagnosed with BS type I. To this end, capped RNA (cRNA) of FLAG-tagged hNKCC2 and the corresponding mutants was injected in *Xenopus laevis* oocytes and transporter activity was measured after 72 h by means of a bumetanide-sensitive $^{22}\text{Na}^+$ uptake assay at 30°C. Injection of 25 ng of hNKCC2 cRNA resulted in bumetanide-sensitive $^{22}\text{Na}^+$ uptake of 2.5 ± 0.5 nmol/oocyte per 30 min. Injection of 25 ng of mutant cRNA yielded no significant bumetanide-sensitive $^{22}\text{Na}^+$ uptake. Expression of wild-type and mutant transporters was confirmed by immunoblotting, showing significantly less mutant protein compared with wild-type at the same cRNA injection levels. However, when the wild-type cRNA injection level was reduced to obtain a protein expression level equal to that of the mutants, the wild-type still exhibited a significant bumetanide-sensitive $^{22}\text{Na}^+$ uptake. Immunocytochemical analysis showed immunopositive staining of hNKCC2 at the plasma membrane for wild-type and all studied mutants. In conclusion, mutations in hNKCC2 identified in type I BS patients, when expressed in *Xenopus* oocytes, result in a low expression of normally routed but functionally impaired transporters. These results are in line with the hypothesis that the mutations in hNKCC2 are the underlying cause of the clinical abnormalities seen in patients with type I BS.

Barter syndrome (BS) is an autosomal recessive heterogeneous renal tubular disorder in which one of the key transport proteins involved in transeellular Na-K-Cl transport in the thick ascending limb of Henle’s loop (TAL) is impaired (1–4). Approximately 23% of the body’s total NaCl reabsorption takes place through active transport pathways located in this nephron segment, emphasizing its physiologic importance. The primary mediator for Na-K-Cl uptake in the apical membrane of the TAL is the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2), of which currently six different isoforms have been identified (5–7). In rat, NKCC2a is present in both the medullary (mTAL) and cortical (cTAL) part of the thick ascending limb, whereas NKCC2b is mainly present in the inner part of the mTAL and NKCC2f in the outer part of the cTAL and macula densa. Besides being present in a larger physical region of the TAL, NKCC2a also appeared to have the largest Na$^+$ transport capacity and would, therefore, be the predominant contributor to Na-K-Cl reabsorption in TAL (8). These cotransporters, in interplay with the basolaterally located chloride channel complex (CLC-Kb/Barttin) and the Na/K-ATPase, transport NaCl from the lumen back to the blood compartment. Another key component in this system is the apical inwardly rectifying ATP-sensitive K$^+$ channel, designated ROMK, which ensures adequate presence of luminal K$^+$ critical for continuous Na-K-Cl uptake by NKCC2 and generates the luminal-positive electrical gradient driving paracellular Ca$^{2+}$ and Mg$^{2+}$ reabsorption (9,10).

So far, mutations in NKCC2, ROMK, ClC-Kb, and Barttin have been linked to the four types of BS that are currently distinguished. Types I and II BS correspond to mutations in NKCC2 (2) and ROMK (3,11), respectively. These two variants are life-threatening disorders in which both the hypokalemic alkalosis as well as profound systemic symptoms are present at birth (12–14). Some of these symptoms already arise in utero, where fetal polyuria can cause polyhydramnios between 24 and 30 wk of gestation followed typically by premature delivery. Affected neonates have severe salt wasting and hypostenuria as well as hyperprostaglandinuria and failure to
thrive. An essential feature is marked hypercalciuria, which may lead to nephrocalcinosis and osteopenia (15,16). Type III BS is coupled to mutations in the basolateral CLC-Kb (4,17) and has a more heterogeneous phenotype. Usually, the clinical features formerly associated with the “Classical Bartter” type are seen. However, mutations in the gene encoding CLC-Kb can also cause an antenatal onset of BS or even display a more Gitelman-like phenotype with hypocalciuria and hypomagnesaemia (18). Type IV BS is composed of a rare subset of patients with sensorineural deafness and has recently been linked to an essential chloride channel β-subunit, called Barttin, which is present not only in the kidney, but also in the inner ear (19,20). There are still BS cases in which the four genes encoding the known ion transport proteins have been excluded as being the underlying disease genes, suggesting the presence of at least one other causative gene.

The aim of the present study was to assess the functional consequences of type I BS mutations selected from different regions in the hNKCC2 sequence. To this end, human wild-type NKCC2 and six mutants identified in unrelated patients diagnosed with type I BS were expressed in Xenopus laevis oocytes. hNKCC2 transporter activity was determined, and mutant and wild-type expression levels were analyzed. Subsequently, their subcellular localization was visualized using immunocytochemical techniques.

Materials and Methods

Synthesis of hNKCC2 Constructs

hNKCC2a cDNA was obtained from a human kidney cDNA library (Clontech Laboratories Inc, Palo Alto, CA) by means of PCR and cloned into a pGEM-Teasy Vector (Promega Corp, Madison, WI). The coding sequence was subcloned into a custom oocyte expression vector, pTLN (21). A FLAG-epitope (“DYKDDDDK”, IBI; Kodak, New Haven, CT) and Kozak sequence (22) were cloned into the vector, pTLN (21). A FLAG-epitope (DYKDDDDK, IBI; Kodak, New Haven, CT) and Kozak sequence (22) were cloned into the construct replacing the original ATG. Selected mutations in hNKCC2 were introduced by using the Quikchange Site-directed mutagenesis kit (Stratagene, La Jolla, CA), and all constructs were checked by double-stranded sequence analysis.

Preparation and Injection of Oocytes

Oocytes were obtained from Xenopus laevis and defolliculated by incubation for 2 h in modified Barth’s solution (MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM HEPES-Tris [N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid], pH 7.4, 0.8 mM MgSO4, 0.3 mM Ca(NO3)2, 0.4 mM CaCl2, and 25 μg/ml gentamycin) containing 0.2% (vol/vol) Tween-20 and 5 μg/ml gentamycin. The dissolved membrane samples were subjected to electrophoresis on 7% (wt/vol) SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (PVDF; Millipore Corp, Bedford, MA) by standard procedures. Blots were incubated with mouse anti-FLAG-PO antibodies (Sigma Chemical Co., St. Louis, MO) diluted 1:2000 in Tris-buffered saline, pH 7.4, containing 0.2% (vol/vol) Tween-20 and supplemented with 5% (wt/vol) nonfat dried milk. Finally, blots were washed and immunopositive bands were visualized using an enhanced chemoluminescence system (Pierce, Rockford, IL). Relative protein amounts were determined by analysis of immunopositive signals of the films using a model GS-690 imaging densitometer (Bio-Rad, Richmond, VA) operated by Molecular Analyst software. The density of the wild-type protein was set at 100% after correction for background.

Immunocytochemistry

After removal of the follicle membranes, oocytes were fixated in 1% (wt/vol) paraformaldehyde solution for 1 h (24,25), washed twice in 80% (vol/vol) ethanol, and embedded in paraffin using a Citadel Tissue Processor and Histocenter 2 (Shandon Southern Products Ltd, Cheshire, UK). Seven-micron sections were cut, deparaffinized, and incubated for 30 min in TN (100 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 0.2% (wt/vol) SDS and subsequently blocked in TNB (TN containing 0.5% [wt/vol] blocking reagent from Renais-
sance TSA-direct kit; NEN Lifescience Products Inc, Boston, MA) for 1 h at room temperature. Sections were subsequently incubated overnight at 4°C with 1:1000 diluted mouse M2 anti-FLAG monoclonal (Sigma-Aldrich, St. Louis, MO) in TNB. After three washes in TNT (TN containing 0.05% [wt/vol] Tween-20) sections were stained for 1 h at room temperature with a 1:1000 diluted Alexa 594 conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) in TNB. Finally sections were washed three times in TNT, dehydrated in subsequently 50% (vol/vol) and 100% (vol/vol) methanol and mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA). Digital images were made using a MRC-1000 confocal laser scanning microscope (Bio-Rad, Richmond, VA).

Results

hNKCC2 Wild-Type and Mutant Constructs

In Figure 1, the predicted topology of hNKCC2 is shown (26). On the basis of the results of a recent NKCC2 study in mouse by Plata et al. (8), NKCC2a was selected as representative NKCC2 isoform. We investigated six mutations located in various regions of the hNKCC2 sequence, which were previously described, but not functionally characterized (27). All patients presented a similar clinical phenotype characteristic for antenatal BS (28). The studied mutations included missense, deletion, and nonsense mutations. Missense mutations G193R, A267S, and G319R are situated in or near putative transmembrane regions (TM1–4), while A508T is located in an intracellular loop between TM8 and 9. The del526N mutation is also located in this region; here the in-frame deletion of a nucleotide triplet causes the loss of an asparagine at position 526. Finally, the Y998X mutation introduces a premature stop-codon at this position causing the formation of a truncated protein, missing the last 101 AA of the C-terminal tail.

Bumetanide-Sensitive $^{22}$Na$^+$ Uptake Studies

Because polyclonal antibodies directed against the C-terminal part (AA921–1055) of human NKCC2 gave rise to non-specific background on oocyte paraffin sections, a FLAG-epitope was incorporated N-terminally to allow immunocytochemical detection. Both FLAG-tagged and untagged human NKCC2a were expressed in oocytes, and their function was compared by means of $^{22}$Na$^+$ uptake experiments. Upon injection of 25 ng of cRNA, a bumetanide-sensitive $^{22}$Na$^+$ uptake of 2.5 $\pm$ 0.5 nmol/oocyte per 30 min was observed for both FLAG-tagged and untagged hNKCC2a as is demonstrated in Figure 2A. On the basis of these results, FLAG-tagged hNKCC2a was used in all further experiments. To optimize protein expression for $^{22}$Na$^+$ uptake studies the cRNA injection levels were varied between 0.4 to 25 ng of hNKCC2a cRNA. At injection levels above 1.5 ng of hNKCC2a cRNA, the $^{22}$Na$^+$ uptake reached a plateau (Figure 2B). Furthermore, as shown in Figure 2C, at an injection level of 25 ng $^{22}$Na$^+$ uptake was linear up to 30 min of incubation.

Figure 1. Proposed topology of hNKCC2. The mutations investigated in the present study as well as potential glycosylation sites have been highlighted, and the location of the exon 4 cassettes and their relative sequence homology has been outlined as described by Gamba et al. (26) and updated in Swiss-Prot entry Q13621.
Oocytes were then injected with 3 ng of either wild-type or mutant hNKCC2a cRNA, and the batches were divided for functional analyses and immunoblotting. None of the six tested mutants exhibited a significant bumetanide-sensitive $^{22}\text{Na}^+$ uptake when compared with non-injected controls, and no immunopositive signal for these mutants could be detected on immunoblot (data not shown). Injection levels were then raised to 25 ng of either wild-type or mutant hNKCC2a cRNA. Now, an immunopositive signal could be detected for both wild-type and mutants, but the mutants still did not exhibit a significant bumetanide-sensitive $^{22}\text{Na}^+$ uptake when compared with non-injected controls (Figure 3).

**Immunoblot Analysis of hNKCC2a Mutants**

To determine the expression levels of the hNKCC2a mutants, total membranes isolated from oocytes expressing either wild-type or mutant hNKCC2a were subjected to immunoblotting (Figure 4). The lane loaded with wild-type hNKCC2a showed a 125-kD and approximately 170-kD band that were not present in the control lane. In the lanes loaded with mutants G193R, A267S, and G319R, the same two bands were detected. Interestingly, for mutants A508T and del526N, only the lower 125-kD band was observed. Finally, the lane loaded with the Y998X mutant contained a specific band at approximately 110 kD, corresponding to the size of the truncated protein, but also a band was observed at 125 kD. Densitometric analysis showed that the protein abundance compared with wild-type was 3.7% for G193R, 2.1% for A267S, 7.9% for G319R, 0.2% for A508T, 0.8% for del526N, and 0.2% for Y998X. Thus, all mutants exhibited a significantly lower expression level than wild-type when an equivalent amount of oocyte membranes was loaded.

**N-Glycosidase Treatment of hNKCC2a and Mutants**

To gain more insight in the posttranslational modification of hNKCC2a, N-glycosidase treatment of total membranes from

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**Figure 2.** Functional characterization of wild-type hNKCC2a by $^{22}\text{Na}^+$ uptake studies. (A) Assessment of the influence of a FLAG-tag on the $^{22}\text{Na}^+$ uptake of hNKCC2a. Oocytes were injected with 25 ng of either FLAG-tagged or untagged hNKCC2a cRNA. Displayed are the bumetanide-sensitive $^{22}\text{Na}^+$ uptake rates of tagged and untagged hNKCC2a versus non-injected controls (Ni) both with (open bars) and without (filled bars) $10^{-5}$ M bumetanide. $n = 15$ oocytes per condition. (B) Analysis of the $^{22}\text{Na}^+$ uptake of FLAG-tagged hNKCC2a as a function of the cRNA injection level. Shown is the bumetanide-sensitive $^{22}\text{Na}^+$ uptake of oocytes injected with different cRNA amounts, measured after 30 min of incubation at 30°C. $n = 15$ oocytes per injected cRNA concentration. (C) Time-dependent $^{22}\text{Na}^+$ uptake of FLAG-tagged hNKCC2a. Oocytes were injected with 25 ng FLAG-tagged hNKCC2a cRNA, and bumetanide-sensitive $^{22}\text{Na}^+$ uptakes are displayed after 30, 60, 120, and 240 min of incubation at 30°C. $n = 15$ oocytes per time point.

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**Figure 3.** Functional analysis of mutations in hNKCC2a. Oocytes were injected with 25 ng cRNA, and the bumetanide-sensitive $^{22}\text{Na}^+$ uptake was subsequently measured. $^{22}\text{Na}^+$ uptake rates that are significantly different from the non-injected controls are marked with an asterisk. The data are the mean of two independent experiments with at least 15 oocytes per injected mutant.
oocytes expressing hNKCC2a was performed. Upon incubation of the wild-type protein with PNGase F, which cleaves high-mannose glycosylations, only the 125-kD band of wild-type was reduced, whereas the approximately 170-kD band was unaffected (Figure 4B, right). By a similar treatment of the Y998X mutant, the 125-kD band shifted to 110 kD, which corresponds to the predicted unglycosylated size of the truncated protein (Figure 4C).

Immunocytochemical Analyses of hNKCC2a Mutants

To further determine whether absence of transport activity was due to misrouting or to functional impairment, paraffin sections of oocytes expressing the various mutants were stained with an anti-FLAG antibody and the subcellular localization of hNKCC2a was determined. In sections expressing wild-type hNKCC2a, injected at 3 and 25 ng (Figure 5, A and B), immunopositive staining was observed at the plasma membrane, which was absent in non-injected controls (Figure 5I). The sections expressing the mutants consistently showed a significant immunopositive staining at the plasma membrane and in the cytoplasm (Figure 5, C through H).

Discussion

In the present study, the functional consequences of mutations in hNKCC2 identified in type I BS were characterized. Wild-type hNKCC2a, heterologously expressed in Xenopus...
laevis oocytes, exhibited a significant bumetanide-sensitive $^{22}\text{Na}^+$ uptake when compared with non-injected controls, which is consistent with earlier studies on mouse and rabbit NKCC2 (7,29,30). hNKCC2a yielded two bands on immunoblot corresponding to a high-mannose glycosylated form (125 kDa) and a larger complex glycosylated form of approximately 170 kDa. Immunocytochemical analysis showed that the immunopositive signal for hNKCC2 was predominantly present at the plasma membrane. All studied mutants, although dispersed over the hNKCC2 sequence, resulted in a low expression of normally routed, but functionally impaired, transporters.

The studied mutants, including the truncated Y998X, were consistently processed into high-mannose glycosylated forms. Mutants A508T, del526N, and Y998X were not further processed, but G193R, A267S, and G319R appeared to be modified into complex glycosylated forms. Interestingly, all mutants were routed predominantly to the plasma membrane, irrespective of their posttranslational modification. Similar observations have been made for other mutated transport proteins, including the thiazide-sensitive NaCl cotransporter, aquaporin-2, and several KC1 cotransporter mutants, where mutant transporters located at the plasma membrane were at least high-mannose glycosylated (35–37).

The expression level of the mutants was significantly lower than wild-type at similar cRNA injection levels. A decreased protein expression of mutants has also been observed for other proteins such as thrombomodulin and protein S mutants (31,32). The exact mechanism responsible for this diminished protein expression remains to be elucidated, but we hypothesize that the hNKCC2 mutants are subject to early degradation by the cellular ER quality control mechanism (33). This mechanism involves activation of the ubiquitin-proteasome proteolytic pathway by misfolded proteins in the endoplasmic reticulum and rerouting from the Golgi complex and downstream vesicle systems to lysosomes (34). Future research is needed to identify the specific interacting components responsible for this degradation.

Essentially, there are several basic levels at which mutations can affect protein function. For instance, based on the analysis of cystic fibrosis transmembrane conductance regulator (CFTR) mutations observed in cystic fibrosis (CF) patients, five distinct categories could be distinguished for which specific treatments are being developed (38). Class I (synthesis) mutations typically result from premature stop codons or nonsense mutations. The resulting mRNA is unstable and degraded. Class II (processing) mutations are normally synthesized, but retained in the endoplasmic reticulum because of protein folding defects and targeted for rapid degradation. This defect is often referred to as a “trafficking” defect because the protein is not transported to the plasma membrane. Most mutations in this class are either missense or deletion mutants. Class III (regulatory) mutations are defective in the activation of transport, although they are present in the proper location in the apical membrane. This class mainly consists of missense mutations in regulatory domains. Class IV (function) mutations are characterized by a normal intracellular localization, but they have a reduced function. These class IV mutations are generally associated with milder phenotypes. Finally, class V mutations affect the structure of the gene and the efficiency of mRNA transcription and processing, markedly reducing the levels of functional protein, and are mainly localized in intronic or promoter regions (39). Applying the same classification used for CFTR mutations on previous studies on BS type II (ROMK2) and Gitelman syndrome (NCC) would implicate that the three groups of BS type II mutations belong to classes II, III, and IV, whereas the Gitelman mutations group into classes II and IV (37,40,41).

All hNKCC2 mutations studied here can be categorized into a single group, namely class III, because all proteins are able to reach their appropriate location on the plasma membrane, but are not able to transport ions. These findings are rather unexpected, considering that different types of mutations (missense, deletion, and nonsense) have been selected randomly over the hNKCC2 sequence. However, in recent studies on closely related KC1 cotransporters, which are also part of the SLC12A solute carrier family, a similar mechanism for KCC1 and KCC3 mutants was shown (36,42). These mutants were able to reach the plasma membrane but were nonfunctional. Therefore, on the basis of the similarities in the observations, we hypothesize that the studied BS type I mutations apparently do not influence protein folding, routing, or processing in a significant way. Alternatively, these mutations may affect transporter affinity for one of its ions or negatively influence the proper regulation or activation of the transporter. The present study on hNKCC2 mutants as well as the aforementioned studies on KCC1 and KCC3 mutants are based on the Xenopus laevis oocyte expression system only, and the existence of another mechanism in native TAL cells can, therefore, not be excluded.

To date, several approaches have been suggested for the clinical treatment of diverse genetic disorders like the use of amino-glycosides for class I mutants to skip aberrant stop-codons during translation, (chemical) chaperones to induce proper protein folding for class II mutants, and the use of butyrate-derivatives to increase the plasma membrane presence of (partly) functional proteins by overexpressing them for class IV and V. However, no functional approach is available yet for class III mutations, like the presently studied hNKCC2 mutants. All therapies presently undergoing clinical trials are focused on routing a (partly) functional protein to its proper place in the plasma membrane in sufficient quantities and not in restoring function in an impaired, but correctly routed, protein.

In conclusion, human NKCC2a has been functionally expressed for the first time, and six mutations identified in patients with BS type I have been characterized. Expression levels for all studied mutants were significantly lower when compared with wild-type. Additionally, all mutants were correctly routed to the plasma membrane, but remained nonfunctional. As such, they can be grouped into a single category, equivalent to CFTR class III mutants. This study functionally supports the hypothesis that mutations in hNKCC2 are pathogenic and cause the phenotype associated with type I BS.
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References


