Macula Densa Control of Renin Secretion and Preglomerular Resistance in Mice with Selective Deletion of the B Isoform of the Na,K,2Cl Co-Transporter

Mona Oppermann,* Diane Mizel,* George Huang,* Cuiling Li,* Chuxia Deng,* Franziska Theilig,† Sebastian Bachmann,† Josie Briggs,* Jurgen Schnermann,* and Hayo Castrop*‡

*National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; †Anatomy, Charite, Humboldt University, Berlin, Germany; and ‡Institute of Physiology, University of Regensburg, Regensburg, Germany

Na,K,2Cl co-transporter (NKCC2), the primary NaCl uptake pathway in the thick ascending limb of Henle, is expressed in three different full-length splice variants, called NKCC2F, NKCC2A, and NKCC2B. These variants, derived by differential splicing of the variable exon 4, show a distinct distribution pattern along the loop of Henle, but the functional significance of this organization is unclear. By introduction of premature stop codons into exon 4B, specific for the B isoform, mice with an exclusive NKCC2B deficiency were generated. Relative expression levels and distribution patterns of NKCC2A and NKCC2F were not altered in the NKCC2B-deficient mice. NKCC2B-deficient mice did not display a salt-losing phenotype; basal plasma renin and aldosterone levels were not different from those of wild-type mice. Ambient urine osmolarities, however, were slightly but significantly reduced. Distal Cl concentration was significantly elevated and loop of Henle Cl absorption was reduced in microperfused superficial loops of Henle of NKCC2B-deficient mice. Because of the presence of NKCC2A in the macula densa, maximum tubuloglomerular feedback responses were normal, but tubuloglomerular feedback function curves were right-shifted, indicating reduced sensitivity in the subnormal flow range. Plasma renin concentration in NKCC2B-deficient mice was reduced under conditions of salt loading compared with that in wild-type mice. This study shows the feasibility of generating mice with specific deletions of single splice variants. The mild phenotype of mice that are deficient in the B isoform of NKCC2 indicates a limited role for NKCC2B for overall salt retrieval. Nevertheless, the high-affinity NKCC2B contributes to salt absorption and macula densa function in the low NaCl concentration range.


In addition to being affected by numerous systemic factors, substantial evidence supports the concept that renal vascular resistance and renin secretion are the end points of an intrarenal regulatory pathway that resides in the juxtaglomerular apparatus. By connecting tubular epithelial and preglomerular vascular cells, this structure provides an anatomic route along which information about tubular fluid composition can be signaled to vascular effector cells of the same nephron. Specifically, renin secretion and preglomerular resistance have been shown to be strongly affected by changes of luminal NaCl concentration at the level of the epithelial sensor cells of the macula densa (1–6). Transport activity of the Na,K,2Cl co-transporter (NKCC2; BSC1) in the apical membrane of macula densa cells is thought to be the mechanism by which these cells detect tubular salt concentration (7–12).

In all mammalian species so far examined, NKCC2 is expressed along the thick ascending limb of Henle (TAL) in at least three different splice variants, called NKCC2F, NKCC2A, and NKCC2B (13–17). These variants are derived from one distinct gene by differential splicing of the variable exon 4, which encodes for the second transmembrane domain and parts of the adjacent intracellular loop of the transporter protein (14,15,18). NKCC2F seems to be the dominant isoform of NKCC2, with highest expression levels in the medullary portion of the TAL (13,19). NKCC2A and NKCC2B seem to be expressed in the more distal or cortical segments of the TAL (13,19). A recent attempt to localize NKCC2 isoforms by in situ hybridization in the mouse suggested that macula densa cells seem to express exclusively and selectively the B isoform of the co-transporter (13). Therefore, NKCC2B seemed to be the most likely candidate for serving as the salt sensor in macula densa control of renin secretion and preglomerular resistance.

Studies of the NKCC2 sensor function in macula densa control of renin secretion and preglomerular resistance in vivo traditionally have been performed by examination of the effects...
of loop diuretics such as furosemide or bumetanide (20, 21). However, these agents block the transport activity of all isoforms of NKCC2. Complete inactivation of NKCC2 by gene targeting results in severe electrolyte disturbances that lead to early postnatal death in most animals and therefore represents a model that cannot be studied easily physiologically (22).

In these experiments, we assessed the effect of a more localized inactivation of NKCC2 on macula densa function. On the basis of the premise that NKCC2B is the dominant isoform of NKCC2 in macula densa cells, we generated a mouse strain that specifically is deficient in NKCC2B. NKCC2B-deficient mice showed an impairment of TAL diluting function accompanied by a slight decrease of osmotic urine concentration and a significant right shift of the tubuloglomerular feedback (TGF) function curve. Renin secretion was suppressed during salt loading. Partial maintenance of macula densa signaling indicates that another NKCC2 isoform in addition to NKCC2B must be involved in the macula densa sensing function.

Materials and Methods

Generation of NKCC2B-Deficient Mice
All animal studies were performed according to protocols that were examined and approved by the Animal Use and Care Committee of the National Institute of Diabetes and Digestive and Kidney Diseases and by the Animal Care Committee of the University of Regensburg. A targeting vector was generated to introduce premature stop codons into exon 4B of the NKCC2 gene (Sli12a1; Figure 1, A and C). This targeting vector consisted of a 5′ homologous arm that was introduced into the vector ploxP (23), followed by the coding sequence for the FLAG peptide, by stop codons in all reading frames, and by a neomycin-resistance (neo) cassette flanked by loxP sites. 3′-Adjacent to the neo cassette, a 3′ homologous arm was inserted (Figure 1B). Homologous arms were generated by long-distance PCR (Roche, Indianapolis, IN). Primers used were 5′-GGAGGTATTTGGGCTTGGTCCTG-3′ and 5′-ACACCGGACATCTCAGTGAGG-3′ for the upstream arm and 5′-GATATCATCGGCCTTAGCCGTGACAG-3′ and 5′-GGAGTTGCGCACACTACATGGGCTC-3′ for the downstream arm. The targeting vector was linearized with NotI and transfected into 129SvEv embryonic stem cells. Primers used for PCR screening of the clones were 5′-CGACGCCATGGCTTCTCTATGCCTC-3′ and 5′-CCACGGTGATGGAACCGATGATG-3′ (inside the neo cassette) and 5′-CCACGGTGATGGAACCGATGATG-3′ (3′ outside of the downstream homologous region). Embryonic stem cell clones that had undergone homologous recombination were injected into blastocysts of C57BL/6 mice and implanted into pseudopregnant foster female mice. Two male chimeras were crossed to C57BL/6 female mice to test for germline transmission. Genotyping was performed on tail biopsies by PCR using primers for the neo gene (5′-GCAGGGCAACAGAGCTCTGCACATTCC-3′ and 5′-TGCGCGCCTTGAGCCTGGCGAAC-3′). This yielded a 2.6-kb band for the targeting results in severe electrolyte disturbances that lead to early postnatal death in most animals and therefore represents a model that cannot be studied easily physiologically (22).

In these experiments, we assessed the effect of a more localized inactivation of NKCC2 on macula densa function. On the basis of the premise that NKCC2B is the dominant isoform of NKCC2 in macula densa cells, we generated a mouse strain that specifically is deficient in NKCC2B. NKCC2B-deficient mice showed an impairment of TAL diluting function accompanied by a slight decrease of osmotic urine concentration and a significant right shift of the tubuloglomerular feedback (TGF) function curve. Renin secretion was suppressed during salt loading. Partial maintenance of macula densa signaling indicates that another NKCC2 isoform in addition to NKCC2B must be involved in the macula densa sensing function.

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Removal of the Neo Cassette
The loxP-flanked neo cassette was excised by crossing homozygous NKCC2B+/−/neo+ mice with Ella-Cre mice (24). Offspring DNA was tested for the absence of neo, and progeny that lacked the neo gene were intercrossed to obtain homozygous NKCC2B−/− mice and wild-type littermates (Figure 1B).

Analysis of the Mutated Transcripts of NKCC2B−/− Mice
Total RNA from kidneys of NKCC2B+/+ and −/− mice was isolated using Trizol reagent (Life Technologies, Carlsbad, CA). NKCC2B mutated transcripts were analyzed by reverse transcription–PCR (RT-PCR) using primers that spanned from exon 4B to exon 5 and from exon 2 to exon 4B. To verify the integrity of the remaining isoforms, we also performed analogous RT-PCR for NKCC2A and NKCC2F transcripts. In addition, cDNA fragments that spanned from exon 2 to exon 5 were cloned into TOPO XL (Life Technologies), and all fragments were sequenced.
Localization of NKCC2B, NKCC2A, and NKCC2F Transcripts in Kidney Segments

A localization study for the three different isoforms of NKCC2 was performed by real-time RT-PCR on RNA that was derived from different kidney regions. Kidneys were dissected under a microscope, and cortex, outer medulla, and inner medulla tissue pieces were collected. Real-time PCR was performed using isoform-specific TaqMan probes in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA).

Localization of NKCC2B, NKCC2A, and NKCC2F Transcripts by In Situ Hybridization

For in situ hybridization, kidney sections were prepared as described previously (25). The mRNA expression of NKCC2A, -B, and -F was localized using digoxigenin-labeled riboprobes according to the manufacturer’s protocol (Roche). Sense and antisense probes were generated by in vitro transcription of a 350-bp cDNA of each isoform that spanned from exon 4 into exon 5. In addition, a 96-bp antisense probe of each isoform from exon 4 was used. Sense probes were applied in parallel with antisense probes to test for unspecific hybridization signals.

Immunohistochemistry

Immunolabeling was performed as described (25) using guinea pig anti-NKCC2 (directed against the C-terminal region; gift from Dr. Ellison, Oregon Health Sciences University, Portland, OR) followed by incubation with donkey anti-guinea pig cy3-lg (Dianova, Hamburg, Germany).

BP and Heart Rate

BP and heart rate in conscious mice were determined by tail-cuff manometry (Visitech Systems, Apex, NC) (26).

Blood Collection and Plasma Renin Determination

Plasma renin concentration (PRC) in blood samples from tail veins was measured with a commercial RIA kit (DiaSorin, Stillwater, MN), as described previously (27). For assessment of renin secretion after inhibition of macula densa salt transport activity, a single dose of furosemide (40 mg/kg) was injected intraperitoneally and blood samples were drawn 60 min later. For determination of macula densa–dependent inhibition of renin release, mice received a single intravenous (tail vein) injection of 1 ml of saline, and blood samples were collected 60 min later (27). For chronic modulation of the renin-angiotensin system, mice were subjected to the following treatments: (1) Control group: Mice were fed a standard rodent diet (0.3% NaCl [wt/wt]) for 1 wk; 2) salt-deficient group: After injection of a single dose of furosemide (40 mg/kg intraperitoneally), mice received a salt-deficient diet (0.03% NaCl [wt/wt]) for 1 wk; 3) and salt load group: Mice were fed a high-salt diet (4% NaCl [wt/wt]) for 1 wk.

Micropuncture Experiments

Measurements of stop flow pressure (P_{sf}) during loop of Henle perfusion were done as described (12). When P_{sf} had stabilized, loop of Henle perfusion rate was increased to 30 nl/min, and maximum P_{sf} responses were determined. Perfusion rates then were decreased to 20, 15, 10, 5, and 0 nl/min and maintained until steady states were achieved at each flow rate. Logistic curve parameters were determined at each flow rate. Logistic curve parameters were determined by real-time RT-PCR of RNA that was derived from different kidney regions. Kidneys were dissected under a microscope, and cortex, outer medulla, and inner medulla tissue pieces were collected. Real-time PCR was performed using isoform-specific TaqMan probes in an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA).

For determination of absorptive TAL function, loops of Henle were perfused as described above. Fluid samples were collected from distal tubular segments of the perfused segments for 1 to 3 min at set flow rates of 6 and 15 nl/min. After volume determinations, a 1-nl sample was removed for determination of Cl⁻ concentration using an electro-metric Cl⁻ microtitrator (28).

Statistical Analyses

Unpaired t test was used to compare two values between different animals. Multiple groups were analyzed with ANOVA followed by Bonferroni post test. P < 0.05 was considered significant.

Results

Mutated Transcripts in NKCC2B−/− Mice

NKCC2-deficient mice were viable and showed no gross anatomic, behavioral, or fertility abnormalities (Table 1). Crossing of heterozygous mice yielded offspring of the different genotypes in near Mendelian ratio. In a first set of experiments, we addressed the nature of the mutated NKCC2B transcripts and those of the remaining isoforms. As can be seen in Figure 2, the targeting event led to the introduction of several premature stop codons in all reading frames, rendering NKCC2B transcripts nonfunctional. NKCC2A and NKCC2F transcripts, in contrast, were found to be unaltered. Before removal of the bulky neomycin-cassette, splicing of the mutated exon 4B was compromised, leading to two different transcripts with partial losses of neo (Figure 2A). In contrast, correct splicing was fully preserved after Cre-mediated removal of the floxed selection cassette (Figure 2B). For assessment of whether targeting NKCC2B would influence the quantity and the localization of the remaining NKCC2A and NKCC2F, their respective mRNA expression was determined by real-time RT-PCR of RNA that was derived from the inner medulla, outer medulla, and cortex. As can be seen in Figure 3, targeting NKCC2B did not alter the expression levels of the remaining NKCC2A and NKCC2F isoforms. NKCC2F was the dominant isoform in the medulla with highest expression levels in the inner stripe of the outer medulla. NKCC2B was expressed mainly in the cortex and to very low degrees in the outer medulla. NKCC2A was present in both cortex and medulla. NKCC2A mRNA levels in the outer medulla were roughly twice those in the cortex. No NKCC2 mRNA was detected in the renal inner medulla.

Urine Osmolarity and Concentrating Ability

To assess the effect of the loss of NKCC2B on urine-concentrating ability, we determined urine osmolality both under ambient conditions and after 48 h of water deprivation (Figure 4). Ambient urine osmolality averaged 1763 ± 121 in wild-type mice and 1192 ± 60 in NKCC2B-deficient mice (n = 30 and 34, respectively; P < 0.0001). Water restriction substantially increased urine osmolality in both wild-type (n = 19) and NKCC2B knockout (n = 20) mice to 3601 ± 115 and 3311 ± 97 mmol/L, respectively (P = 0.06).

Plasma Renin and Plasma Aldosterone Concentrations

To address the consequences of the loss of the macula densa NKCC2B for the regulation of the renin system, we determined baseline PRC in NKCC2B-deficient mice, lacking NKCC2B in
the macula densa and upstream parts of the TAL, and wild-type mice under control conditions and after acute inhibition of NKCC2 by administration of furosemide. Basal PRC levels were similar in wild-type (n = 30) and NKCC2B−/− mice (n = 22), averaging 1911 ± 150 and 1735 ± 160 ng angiotensin I (Ang I)/ml per h (P = 0.53). As can be seen in Figure 5, acute administration of furosemide to inhibit all isoforms of NKCC2 increased PRC to the same extent in wild-type and knockout mice (7451 ± 1016 [n = 15] and 6213 ± 449 ng Ang I/ml per h [n = 12], respectively; P = 0.315 between genotypes). To investigate the renin system under conditions of chronic modulation of oral salt intake, we determined PRC in mice that were fed either a high-salt diet or a salt-deficient diet for 1 wk (Figure 6). After the high-salt diet, PRC was slightly reduced in NKCC2B mice (7218 ± 111 to 1832 ± 174 ng Ang I/ml per h [n = 12; P = 0.038] versus control diet) and wild-type mice, respectively (P = 0.315 between genotypes). Absolute Cl absorption exceeded those in wild-type mice by 22.0 and 13.1 mEq/L, averaging 88.8 ± 4.3 versus 66.8 ± 6.1 mEq/L at 6 nl/min and 106 ± 6.2 versus 92.9 ± 4.0 mEq/L at 15 nl/min in NKCC2B−/− and wild-type mice, respectively (P = 0.008 and P = 0.042, between genotypes). Absolute Cl absorption along the loop of Henle was significantly higher in wild-type than in NKCC2B−/− mice (n = 10). PRC was significantly lower after saline injection in NKCC2B−/− compared with NKCC2B+/+ mice (P = 0.0015).

Plasma aldosterone concentrations after exposure to high- or low-salt diet changed in parallel to PRC. As shown in Table 2, no significant differences between genotypes were observed under these conditions.

**BP and Heart Rate**

Systolic BP measured by the tail-cuff compression method in awake wild-type mice averaged 120 ± 2.8 mmHg, and heart rate was 610 ± 19 bpm (n = 10). In NKCC2B-deficient mice, systolic BP and heart rate were 119 ± 2.0 mmHg and 615 ± 15 bpm, respectively (n = 10; NS).

**Distal Tubular Chloride Concentration**

To determine the effect of an NKCC2B deficiency on overall loop of Henle diluting ability, we determined chloride concentrations in distal tubular fluid of superficial loops of Henle that were micropерfused in situ at normal and elevated flow rates (Figure 8A). At tubular perfusion rates of 6 or 15 nl/min, distal chloride concentrations in NKCC2B−/− exceeded those in wild-type mice by 22.0 and 13.1 mEq/L, averaging 88.8 ± 4.3 versus 66.8 ± 6.1 mEq/L at 6 nl/min and 106 ± 6.2 versus 92.9 ± 4.0 mEq/L at 15 nl/min in NKCC2B−/− and wild-type mice, respectively (P = 0.008 and P = 0.042, between genotypes). Absolute Cl absorption along the loop of Henle was significantly higher in wild-type than in NKCC2B−/− mice at the low flow rate (723 ± 19 versus 602 ± 26 pEq/min; P = 0.001) but not at the high flow rate (1420 ± 72 versus 1269 ± 100 pEq/min; P = 0.22). Similarly, fractional Cl

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**Table 1. Average concentrations of a number of plasma constituents in NKCC2B+/+ and NKCC2B−/− mice (n = 5 for each genotype)**

<table>
<thead>
<tr>
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<th>NKCC2B+/+</th>
<th>NKCC2B−/−</th>
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<tr>
<td></td>
<td>Mean [mg/dl]</td>
<td>Mean [mg/dl]</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>290.7</td>
<td>345.1</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>70.7</td>
<td>72.6</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>76.8</td>
<td>55.2</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>150.0</td>
<td>148.9</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>4.4</td>
<td>4.1</td>
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<tr>
<td>Cl (mmol/L)</td>
<td>114.3</td>
<td>114.0</td>
</tr>
<tr>
<td>Ca (mmol/L)</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>P (mg/dl)</td>
<td>7.9</td>
<td>7.7</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>21.8</td>
<td>17.8</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>59.0</td>
<td>41.1</td>
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<tr>
<td>ALT/GPT (U/L)</td>
<td>21.0</td>
<td>19.5</td>
</tr>
<tr>
<td>AST/GOT (U/L)</td>
<td>36.4</td>
<td>37.5</td>
</tr>
<tr>
<td>Creatinine kinase (U/L)</td>
<td>102.2</td>
<td>89.6</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>4.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*ALT/GPT, alanine aminotransferase/glutamate pyruvate transaminase; AST/GOT, aspartate aminotransferase/glutamate oxaloacetic transaminase; BUN, blood urea nitrogen.*
absorption was significantly higher in wild-type than in knockout mice at the low but not at the high flow rate (Figure 8B). Water absorption along the loop of Henle also was significantly higher in wild-type than knockout mice at the low flow rate (4.2 \pm 0.3 versus 3.3 \pm 0.3 nl/min; \( P = 0.032 \)), whereas differences were NS at the high flow rate (7.9 \pm 0.6 versus 7.5 \pm 0.7 nl/min).

**TGF Regulation of GFR**

TGF responses were determined by measurement of proximal PSF during loop perfusion rates of 0, 5, 7.5, 10, 15, 20, and 25 ml/min. Data are given as percentage of the zone with highest expression levels of the respective isoforms. IM, inner medulla; OMIS, outer medulla inner stripe; OMOS, outer medulla outer stripe.

Figure 3. Quantification and localization of NKCC2B, NKCC2A, and NKCC2F mRNA in kidney regions of NKCC2B+/- and -/- mice; real-time PCR was performed using isoform-specific TaqMan probes and 18s RNA as a reference. Data are given as percentage of the zone with highest expression levels of the respective isoforms. IM, inner medulla; OMIS, outer medulla inner stripe; OMOS, outer medulla outer stripe.

Figure 2. Sequencing of NKCC2B transcripts before and after removal of the neomycin resistance (neo) cassette. For both strains, the targeting event led to the introduction of several premature stop codons in all reading frames (underlined italic base triplets), rendering NKCC2B transcripts nonfunctional. (A) Presence of the neo cassette affected splicing of the mutated exon 4B and led to two different transcripts with partial losses of neo (* in the annotated sequence). (B) Correct splicing was observed after Cre-mediated removal of the floxed selection cassette; gaps in the annotated sequence refer to remaining vector sequences. Arrows in the schematic drawing of the transcripts (A and B) indicate PCR primers used for cloning and sequencing.
30 nl/min. Experiments were done in seven male NKCC2B wild-type and four male NKCC2B-deficient mice, with the number of nephrons examined being 13 and 11, respectively. Body weight averaged 33.1 ± 1.0 g in wild-type and 36.1 ± 1.2 g in knockout mice (NS). Mean arterial BP during the period of micropuncture experiments was 95.1 ± 2.4 and 94.1 ± 3.0 mmHg for wild-type and knockout mice, respectively (NS). Mean PSF at zero perfusion was not significantly different between genotypes (43.1 ± 2.1 versus 45.5 ± 1.9 mmHg; P = 0.42). PSF at a saturating perfusion rate (30 nl/min) tended to be lower in nephrons from wild-type compared with knockout mice (31.9 ± 2.3 and 37.1 ± 2.6 mmHg; P = 0.14). At intermediate perfusion rates of 5, 7.5, 10, 15, and 20 nl/min, PSF decreased from 43.1 ± 2.1 (zero perfusion) to 41.3 ± 2.1, 43.6 ± 3.4, 39.5 ± 2.0, 34.5 ± 2.4, and 32.5 ± 2.5 mmHg in wild-type mice, whereas it changed from 45.5 ± 1.9 (zero perfusion) to 47.1 ± 3.2, 46.1 ± 2.6, 45.7 ± 1.7, 41.5 ± 1.9, and 38.8 ± 2.1 mmHg in NKCC2B−/− mice (P = 0.42, 0.14, 0.59, 0.04, 0.036, and 0.07 for genotype comparison). Half-maximum TGF responses were calculated to be reached at a perfusion flow of 12.3 ± 0.6 in wild-type and of 15.2 ± 1.2 nl/min in knockout mice (P = 0.033). Therefore, TGF response curves were right-shifted in NKCC2B-deficient mice (Figure 9).

Localization of the NKCC2 Isoforms along the TAL
In view of the largely preserved macula densa function in NKCC2B-deficient mice, we performed isoform-specific in situ hybridizations to determine whether an additional isoform of NKCC2 is present in macula densa cells. Using antisense riboprobes that were specific for NKCC2B, NKCC2A, and NKCC2F mRNA, the macula densa segment of the TAL showed hybrid-
ization signals for both NKCC2B and NKCC2A but not NKCC2F (Figure 10). No signal was detected using respective sense probes (data not shown). Consistent with the presence of the mRNA of an NKCC2 isoform other than NKCC2B, NKCC2 protein was detected in the macula densa of NKCC2B-deficient mice (Figure 11). In line with our localization study on kidney segments using RT-PCR, NKCC2B and NKCC2A also were detected in parts of the TAL upstream of the macula densa segment (data not shown).

**Discussion**

Movement of NaCl mediated by NKCC2 is the major uptake mechanism in the TAL of the loop of Henle and macula densa cells, and adequate NKCC2 operation is directly required for loop salt absorption and body Na balance, urine-concentrating ability, renin secretion, and TGF. By alternative splicing, a number of different NKCC2 isoforms that show distinct spatial distribution along the loop of Henle and that differ on their affinity for NaCl are generated (13–17).

This report summarizes experiments in which we applied a strategy to target NKCC2B without interfering with the expression of the remaining isoforms, NKCC2A and NKCC2F. Several mouse models with specific removal of single exons have been reported (23,29–32), but to our knowledge, this is the first report of the inactivation of one of several isoforms derived from differential splicing without affecting the mechanism of differential splicing of the remaining isoforms. In our approach, we introduced stop codons into the variable exon 4 (Figure 1), leading to premature termination of translation of the B isoform of NKCC2. Our experiments prove the feasibility of a specific inactivation of a splice variant of a gene by means of knock-in of premature stop codons. We assume that this strategy can be used as a general approach to obtain mouse models with inactivation of specific splice variants of proteins, although the generation of a nonfunctional protein may be more uncertain if the variable exon is located close to the C-terminal end of the protein.

Whereas inactivation of the entire NKCC2 protein by gene targeting leads to a severe salt-losing nephropathy with most animals dying within the first 2 wk of postnatal life (22), specific inactivation of the B isoform of NKCC2 was not associated with gross structural or functional abnormalities. This is consonant with this and previous studies indicating that NKCC2B transcripts account for only approximately 10% of total NKCC2 mRNA expression (19). Ambient urine osmolarity and concentrating ability after water restriction was only slightly compromised in NKCC2B-deficient mice. Therefore, NKCC2B does not seem to contribute importantly to the accumulation of medullary interstitial solute concentrations that is the basis of the urine-concentrating ability. In line with this finding are observations in a single Bartter patient with a missense mutation in exon 4B who displayed an unusually mild type I Bartter phenotype (33). Nevertheless, a significant increase in distal Cl concentration was observed in distal tubular fluid of micropерfused loops of Henle, suggesting an impairment of loop CI absorption, presumably along the cortical TAL. It is of note that the effect of NKCC2B deficiency on diluting ability of individual loops of Henle was more pronounced at the low than at the high flow rate.

**Table 2. Plasma aldosterone concentrations (pg/ml) in NKCC2B+/+ and −/− mice after 1 wk on control (0.3% NaCl), high-salt (4% NaCl), or low-salt diet (0.03% NaCl after a single dose of 40 mg/kg furosemide, intraperitoneally)**

<table>
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<tr>
<th>Aldosterone (pg/ml)</th>
<th>Normal Salt</th>
<th>n</th>
<th>High Salt</th>
<th>n</th>
<th>Low Salt</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKCC2B+/+</td>
<td>790 ± 56</td>
<td>9</td>
<td>199 ± 26</td>
<td>10</td>
<td>1327 ± 130</td>
<td>9</td>
</tr>
<tr>
<td>NKCC2B−/−</td>
<td>672 ± 43</td>
<td>10</td>
<td>159 ± 23</td>
<td>10</td>
<td>1737 ± 191</td>
<td>10</td>
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**Figure 7. Effect of a single intravenous injection of 1 ml of saline on PRC in NKCC2B+/+ (n = 9) and −/− mice (n = 10). Blood samples were taken 60 min after the injection. Lines connect values from the same animals; percentages indicate relative decrements of PRC.**
suggesting that NKCC2B may be more active in a low Cl concentration range.

In situ hybridization in the mouse kidney has identified a punctate pattern of NKCC2B expression in the glomerular vicinity consistent with predominant expression of the B isoform in macula densa cells (13). NKCC2B has been suggested to represent the transporter isoform that primarily is responsible for detecting variations of tubular chloride concentration by the macula densa. We therefore examined whether specific NKCC2B deficiency affects TGF regulation of afferent arteriolar tone and renin secretion the two end points that are known to be altered by macula densa input. Our data show that maximum TGF responses to increases in flow rate were not significantly different between NKCC2B-deficient and wild-type mice. However, TGF responses in the mutant mice were reduced in the subnormal flow range, causing a right shift of the TGF curve. The requirement of NKCC2B to maintain a normal TGF responsiveness to subnormal flow changes indicates that NKCC2B seems to operate most efficiently in the range of low NaCl concentrations. This observation is in general agreement with the recent demonstration that NKCC2B has a markedly

Figure 8. (A) Chloride concentration in individual samples of distal tubular fluid of microperfused superficial loops of Henle in NKCC2B+/+ (○) and NKCC2B−/− (○) mice; data are shown for perfusion rates of 6 and 15 nl/min. (B) Mean fractional Cl absorption along the loop of Henle perfused at 6 and 15 nl/min. *P < 0.05.

Figure 9. Change of stop flow pressure (P_{max}) expressed as percentage reduction from P_{max} (P_{max} at zero loop flow) in response to stepwise increases of loop of Henle flow rates in wild-type (○) and NKCC2B−/− (○) mice (○). Flow rates that caused half-maximum responses (V_{1/2}) are indicated by vertical lines. *P < 0.05.

Figure 10. In situ hybridization of NKCC2 mRNA isoforms in the macula densa segment of the thick ascending limb (TAL; highlighted by arrows) in wild-type mice showing positive signals for NKCC2B and NKCC2A but not for the medullary F isoform of NKCC2.
higher Cl affinity than the other two isoforms (13,17). It remains to be investigated whether a constant exposure of the macula densa cells to higher Cl concentrations in NKCC2B-deficient mice, as suggested by our distal Cl concentration measurements, leads to some desensitization of the macula densa cells, supporting a right shift of the TGF response curve.

Maintenance of maximum TGF responses in NKCC2B knockout mice clearly indicates that NKCC2B is not the only isoform present in macula densa cells. In fact, the present in situ hybridization evidence shows the coexpression of both NKCC2B and NKCC2A in the macula densa segment and upstream parts of the TAL. In addition, NKCC2B protein was found immunohistochemically in the macula densa of NKCC2B-deficient mice with an antibody directed against the C-terminal part of the protein. Considering the different Cl affinities of the co-transporter isoforms, with a $K_m$ for Cl$^-$ of 9 to 12 and 22 to 45 mM for NKCC2B and NKCC2A, respectively (13,17,34), the A isoform seems to be better suited to serve as a Cl sensor, with tubular Cl concentrations at the macula densa segment being approximately 35 mM (35).

To explore the effect of NKCC2B deficiency on renin secretion, the second end point of macula densa signaling, we determined PRC in conscious knockout and wild-type mice. Interference with NKCC2 function by loop diuretics is consistently associated with a marked increase in renin secretion, which is mediated in part by inhibition of macula densa salt transport capacity (36). Consistent with the notion that macula densa function was largely intact in NKCC2B-deficient mice as a result of the coexpression of NKCC2A, PRC values were not significantly elevated in the knockout animals. In fact, PRC was found to be significantly reduced in NKCC2B-deficient mice during both chronic and acute conditions of salt loading. It is conceivable that the increase of macula densa NaCl concentration caused by a combination of NKCC2B deficiency in the cortical TAL upstream of the macula densa segment and salt loading is large enough to increase macula densa NaCl transport despite NKCC2B absence and that this inhibits renin release during conditions of salt loading. Micropuncture studies have shown that intravenous injections of isotonic saline indeed are accompanied with increased distal NaCl concentrations (2,37).

Overall, NKCC2B-deficient mice showed a mild phenotype compared with the massive salt-losing nephropathy observed in mice with inactivation of all NKCC2 isoforms (22). Further investigations will be necessary to distinguish clearly between a phenotype that is specifically related to loss of the B isoform of NKCC2 and a phenotype derived from the reduction of total NKCC2 expression as a result of NKCC2B deficiency.

Conclusion
We generated mice that lack the B isoform of NKCC2 by introducing premature stop codons into exon 4B. NKCC2B-deficient mice did not display a salt-losing phenotype, although ambient urine osmolarities were reduced. Distal Cl concentration was elevated and loop of Henle Cl absorption was reduced in microperfused superficial loops of Henle of NKCC2B-deficient mice. PRC in NKCC2B-deficient mice was reduced during salt loading. Maximum TGF responses were not significantly reduced in NKCC2B-deficient mice, indicating the operation of another isoform in the macula densa, presumably NKCC2A. However, NKCC2B absence caused a right shift of the TGF function curve consonant with dependence of TGF responsiveness on NKCC2B in the low NaCl concentration range.

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