

# Defective processing and expression of thiazide-sensitive Na-Cl cotransporter as a cause of Gitelman's syndrome

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**Kunchaparty, Shanti, Matthew Palcso, Jennifer Berkman, Heino Velázquez, Gary V. Desir, Paul Bernstein, Robert F. Reilly, and David H. Ellison.** Defective processing and expression of thiazide-sensitive Na-Cl cotransporter as a cause of Gitelman's syndrome. *Am. J. Physiol.* 277 (*Renal Physiol.* 46): F643–F649, 1999.—Gitelman's syndrome is an autosomal recessive disorder of salt wasting and hypokalemia caused by mutations in the thiazide-sensitive Na-Cl cotransporter. To investigate the pathogenesis of Gitelman's syndrome, eight disease mutations were introduced into the mouse thiazide-sensitive Na-Cl cotransporter and studied by functional expression in *Xenopus* oocytes. Sodium uptake into oocytes that expressed the wild-type clone was more than sevenfold greater than uptake into control oocytes. Uptake into oocytes that expressed the mutated transporters was not different from control. Hydrochlorothiazide reduced Na uptake by oocytes expressing the wild-type gene to control values but had no effect on oocytes expressing the mutant clones. Western blots of oocytes injected with the wild-type clone showed bands representing glycosylated (125 kDa) and unglycosylated (110 kDa) forms of the transport protein. Immunoblot of oocytes expressing the mutated clones showed only the unglycosylated protein, indicating that protein processing was disrupted. Immunocytochemistry with an antibody against the transport protein showed intense membrane staining of oocytes expressing the wild-type protein. Membrane staining was completely absent from oocytes expressing mNCC<sub>R948X</sub>; instead, diffuse cytoplasmic staining was evident. In summary, the results show that several mutations that cause Gitelman's syndrome are nonfunctional because the mutant thiazide-sensitive Na-Cl cotransporter is not processed normally, probably activating the "quality control" mechanism of the endoplasmic reticulum.

distal convoluted tubule; familial hypokalemia-hypomagnesemia; thiazide-sensitive sodium-chloride cotransporter; kidney tubules; distal; diuretics; thiazide; blood pressure

GITELMAN'S SYNDROME of inherited hypokalemic alkalosis, salt wasting, and normal to low blood pressure is distinguished from Bartter's syndrome by the presence of low rates of urinary calcium excretion and by hypomagnesemia (1). Both Gitelman's and Bartter's syndromes are inherited as autosomal recessive traits, but in contrast to Bartter's syndrome (22, 24–28), Gitelman's syndrome results from mutations in a single

transport protein, the thiazide-sensitive Na-Cl cotransporter (NCC) (11, 20, 28, 29). This transport protein is expressed at the apical membrane of mammalian distal convoluted tubule (DCT) cells (2, 18, 19). It is the predominant pathway by which Na and Cl are reabsorbed by DCT cells, transporting ~5% of the filtered NaCl from the lumen to the cell. The NCC was cloned first from flounder bladder and later from rat and rabbit kidney (6, 7, 30). It is a member of the cation-chloride cotransporter gene family, which also comprises the bumetanide-sensitive Na-K-2Cl cotransporters and the K-Cl cotransporters (8). On the basis of hydropathy analysis, the NCC is believed to contain 12 membrane-spanning domains and be oriented with the amino and carboxy termini within the cytoplasm (7).

Mutations throughout the NCC have been shown to cause Gitelman's syndrome. These mutations include nonsense mutations, splice site mutations, and missense mutations (28, 29). To date, correlations between phenotypic variability and specific inherited mutations have not been reported, in part because no single mutation is most common (23). A cluster of Gitelman's syndrome-causing mutations is found within the carboxy-terminal region of the protein (14). One such mutation (*R948X*)<sup>1</sup> is predicted to truncate the protein by 54 amino acids. Another (*G738R*), was reported to be the most common of 80 mutations studied and to account for ~10% of Gitelman's cases (23). The present experiments were designed to investigate the mechanisms by which Gitelman's mutations impair function of the thiazide-sensitive Na-Cl cotransporter. To this end, the wild-type NCC was cloned from mouse kidney cortex, and site-directed mutagenesis was used to generate eight discrete Gitelman's mutations. When these clones were expressed in *Xenopus* oocytes, each of the mutations was shown to be inactive and to impair protein processing.

## METHODS

**RNA isolation and DNA synthesis.** Total RNA was extracted from BALB/c mice, and first-strand cDNA was generated. Degenerate primers were designed based on the flounder (7) or rat (6) amino acid sequences. Each was ~100-fold

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<sup>1</sup> The amino acid numbers employed in this study are those of the mouse NCC. The following list shows the human Gitelman's mutations that were investigated, together with the corresponding mouse amino acid numbers in parenthesis: R209W (206), P349L (346), C421R (418), L738R (735), G741R (738), R968X (948), R1018X (989), and T1026I (997).

degenerate and 20 nucleotides in length. Three hundred nanograms of each of was used to amplify the sequences using the Perkin-Elmer GeneAmp PCR kit. Six individual PCR products were obtained, cloned into pBluescript, and sequenced.

Rapid amplification of cDNA ends (5'-RACE) was carried out using the 5'-RACE System from GIBCO BRL. 3'-RACE was carried out as described (5). The products of 5' and 3'-RACE were cloned into pBluescript and sequenced. After the entire sequence including 5' and 3' untranslated regions were obtained, primers were designed to generate a full-length cDNA. These primers included a Kozak sequence (12) upstream of the start methionine. A full-length clone was obtained by long range PCR using the PCR-XL kit and mouse kidney cDNA. The full-length product was cloned into pCR II and sequenced (by the University of Colorado Cancer Center DNA Sequencing Facility). Because this product was completely independent of the products derived by individual PCR reactions (except for the region of the two PCR primers that lay outside the coding region), it provided a check on the accuracy of the sequence. The predicted amino acid sequences of the mouse NCC (mNCC) obtained using the two approaches are identical. The product was then excised using *EcoR* I and cloned into the *EcoR* I site of pgh 19 (derived from pGEM-HE; Ref. 15), a gift of John Orloff.

Site-directed mutagenesis was performed using the Quick Change kit from Stratagene, according to the manufacturer's instructions. Eight mutations reported to cause Gitelman's syndrome were generated. These were R206W, P346L, C418R, L735R, G738R, R948X, R989X, and T997I. Each product of site-directed mutagenesis was sequenced to confirm that unexpected changes did not occur during mutagenesis.

**cRNA synthesis.** DNA template was linearized downstream from the 3' untranslated region with *Not* I (NEB), treated with proteinase K (mCAP mRNA Capping Kit, Stratagene) and precipitated in 0.1 vol of 3 M sodium acetate and 2.5 vol of 100% ethanol on dry ice. The DNA was resuspended in RNase-free buffer, and size and amount were checked by agarose gel electrophoresis. The cRNA was transcribed via the mCAP Kit protocol with the addition of RNase inhibitor (PRIME RNase Inhibitor, 5 Prime → 3 Prime, Inc.). Transcript size and quantity were checked by RNA agarose gel electrophoresis.

**Oocyte harvesting and sorting.** Oocytes were removed from *Xenopus laevis* and placed in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.5, sterile filtered, stored at 4°C). Ovarian lobes were cut into flat sheets and nutated in OR2 for 1–2 h with several solution changes to loosen the connective tissue. The oocytes were then added to OR2 with 1 mg/ml collagenase (type A, Boehringer-Mannheim) and nutated for the minimum time needed to remove the follicular membrane from each oocyte (usually ~1 h). The oocytes were washed with Barth's wash solution [88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, and 10 mM HEPES, pH 7.4] and then added to Barth's storage solution [Barth's wash solution, with 2.5 mM sodium pyruvate, and 5 mg/dl gentamicin (Sigma, 10 mg/ml)]. Healthy stage V and VI oocytes were selected from this group for injection.

***Xenopus* oocyte injection.** Micropipettes (Microcaps, Drummond) were heated and pulled (INJECT+MATIC puller), and the tips were ground to 20–35 μm. RNA was heated at 65°C for 2 min, quick spun, and aspirated into the pipette. Sorted *Xenopus* oocytes were injected with 50 nl of water or fluid containing RNA (total injected 12.5 ng/oocyte). Oocytes were injected at the transitional zone between the animal and vegetal poles using the INJECT+MATIC injection system

(INJECT+MATIC, Geneva, Switzerland). After injection, oocytes were stored in Barth's storage solution 3–5 days at 18°C, with fresh solution changes daily.

**Uptakes.** Injected oocytes were transferred to ND96 Cl-free solution (96 mM sodium isethionate, 2 mM potassium gluconate, 1.8 mM calcium gluconate, 1 mM magnesium gluconate, 5 mM HEPES, 2.5 mM sodium pyruvate, and 5 mg/dl gentamicin, pH 7.4) 24 h prior to the uptake assay. Thirty minutes prior to the addition of uptake medium, the oocytes were added to ND96 Cl-free solution with inhibitors (1 mM ouabain, 100 μM amiloride, and 100 μM bumetanide), according to the protocol of Gamba et al. (6). The oocytes were then transferred to isotonic uptake medium (58 mM NaCl, 38 mM *N*-methyl-D-glucamine, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, with inhibitors, pH 7.4), with 10 μCi/ml final concentration of <sup>22</sup>Na, and incubated at room temperature for 1 h. The oocytes were washed five times with 5 ml each of ice cold isotonic uptake medium, lysed in 0.1% SDS, added to 5 ml of liquid scintillant (Hionic-Fluor, Packard), and counted for 2 min in a liquid scintillation counter.

**Oocyte membrane preparation.** Groups of five oocytes were transferred to Eppendorf tubes and chilled on ice for 5 min. Barth's buffer was then replaced with 500 ml of homogenization buffer (7.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, 1 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin), and the oocytes were lysed by repeated vortexing and pipetting. The yolk and cellular debris were pelleted at 750 *g* for 5 min, and the supernatant was centrifuged at 16,000 *g* for 30 min to pellet the membranes. All centrifugation steps were performed at 4°C. The additional floating yolk was then removed with a cotton swab, and the supernatant was discarded. The pellet was then washed gently with ice-cold homogenization buffer and resuspended in 10 μl of 1.25% SDS per oocyte. Protein samples were stored at –20°C.

**SDS-PAGE electrophoresis and transfer of proteins to PVDF paper.** Oocyte membrane proteins (0.1–5 μg) were then separated on a 3–8% NuPAGE Tris-acetate gel (Novex no. EA0378) by electrophoresis at 150 V for 1 h in NuPAGE SDS Running Buffer (50 mM Tris base, 50 mM tricine, 3.5 mM SDS). Proteins were then transferred to polyvinylidene difluoride (PVDF) paper (Novex no. LC2002) using the X-Cell II blotting apparatus (Novex no. EI9002) in NuPAGE transfer buffer [25 mM bicine, 25 mM bis-Tris (free base), 1 mM EDTA, 0.05 mM chlorobutanol, 20% methanol] at 30 V for 1 h.

**Western blot and ECL detection.** PVDF paper was dipped in methanol, immersed in water for 5 min, and then rinsed in PBS-T (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween-20). The blot was blocked with Blotto-T (5% nonfat dried milk in PBS-T), incubated with mNCCnAb1 diluted 1:1,000 in Blotto-T. The blot was then washed, incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit, Zymed no. 62-6120, diluted 1:1,000), and then washed. All steps were performed at room temperature. Detection was carried out using the enhanced chemiluminescence kit (ECL+, Amersham Life Science no. RPN2132) according to the manufacturer's instructions. Deglycosylations were performed using the N-glycosidase F deglycosylation kit (Boehringer-Mannheim no. 1-836-552) according to the manufacturer's instructions.

**Oocyte immunocytochemistry.** Four days after injection, oocytes were fixed with 3% paraformaldehyde in phosphate-buffered saline for 4 h at 4°C. Oocytes were frozen rapidly, and 6-μm thick cryosections were placed on chrom-alum gelatin-coated glass slides. Nonspecific antibody binding was blocked by incubation (30 min at room temperature) of the sections in TSA blocking buffer (TSA-Direct kit; NEN, Boston,

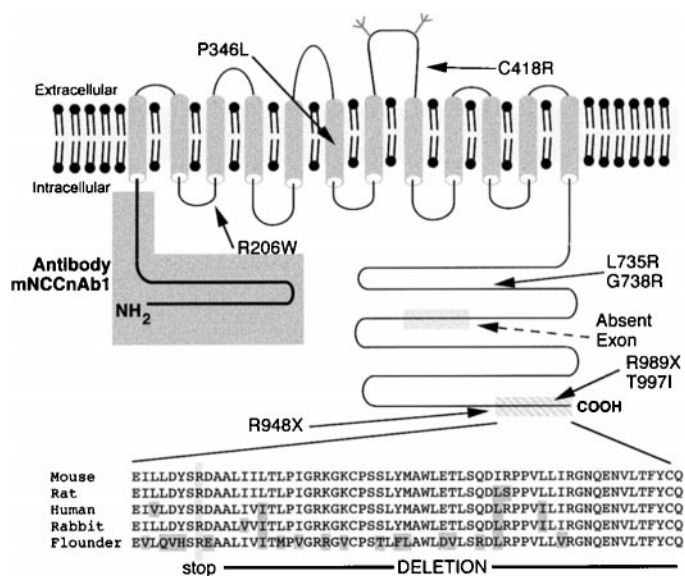


Fig. 1. Model of the thiazide-sensitive Na-Cl cotransporter, based on hydropathy analysis. Model shows the region used to generate a polyclonal antibody against, as described (2). Model also shows the region in which an additional exon is present in the human transport protein (light gray box, "absent exon"). Region deleted by the R948X mutation is indicated by the hatched box. Note that this region is far removed from the sites of glycosylation (shown by the forks) which are extracellular. The amino acid sequence of the region deleted by this mutation is given in single letter code; the sequences from other species are shown for comparison. Site of the Gitelman's mutations (studied in the current experiments) are indicated by arrows. Note that these are distributed throughout the protein.

MA). Sections were then incubated with mNCCnAb1 (2) diluted in the TSA blocking buffer (1:100). Sections were rinsed in PBS containing 0.05% Tween. Sections were incubated with a horseradish peroxidase-conjugated secondary antibody (1:200), rinsed, and revealed by incubating with the provided FITC-tyramide conjugates (TSA-Direct kit, NEN).

## RESULTS

**Cloning.** The thiazide-sensitive Na-Cl cotransporter was cloned from mouse kidney cortex.<sup>2</sup> The predicted amino acid sequence is 97% identical to the amino acid sequence of the rat NCC (6). The mouse sequence is also similar to the sequence of the transporter cloned from rabbit (30) and from human (28). Like the rat sequence, the mouse clone lacks a 26-amino-acid stretch (between mouse amino acid residues 654–655) that is present in the rabbit, human, and flounder clones. This region is predicted to be within the carboxy terminal cytoplasmic domain; it comprises one exon in the human clone (exon 24). Gitelman's mutations have not been described within this region.

Figure 1 shows a hydropathy model of the thiazide-sensitive Na-Cl cotransporter. It comprises 12 putative membrane-spanning domains and cytoplasmic amino and carboxy termini. Figure 1 shows the locations of the mutations introduced in the clone by site-directed mutagenesis. Two are predicted to truncate the protein

by 54 and 4 amino acids. The others are missense mutations predicted to change only a single amino acid residue. Figure 1 also shows the region of the protein that was used to generate a polyclonal antibody (2). This region lies within the putative cytoplasmic amino terminus. Mutation within the carboxy-terminal domain would not be predicted to alter the ability of the antibody to recognize the transport protein.

**Expression in oocytes.** In a first series of experiments, the effects of the mNCC<sub>R948X</sub> mutation were examined. Figure 2 shows that sodium uptake into oocytes injected with the mNCC transcript was significantly more rapid than sodium uptake into oocytes that were uninjected (mNCC,  $28 \pm 5.1$ ; uninjected,  $5 \pm 0.8$  nmol·oocyte<sup>-1</sup>·h<sup>-1</sup>;  $P < 0.005$  by unpaired *t*-test). Oocytes injected with an antisense mNCC had uptakes that were similar to uninjected oocytes ( $4 \pm 0.3$  nmol·oocyte<sup>-1</sup>·h<sup>-1</sup>). Hydrochlorothiazide ( $10^{-4}$  M) incubation completely prevented the stimulation of Na uptake by the mNCC clone (mNCC + hydrochlorothiazide,  $5 \pm 1.1$  nmol·oocyte<sup>-1</sup>·h<sup>-1</sup>). In contrast, Na uptake into oocytes that were injected with the mNCC<sub>R948X</sub> clone (mNCC<sub>R948X</sub>:  $5 \pm 1.6$  nmol·oocyte<sup>-1</sup>·h<sup>-1</sup>) was not significantly different from uptake into uninjected oocytes or oocytes injected with antisense clone. Hydrochlorothiazide had no effect on uptake by uninjected oocytes or oocytes that were injected with the antisense clone or mNCC<sub>R948X</sub>.

One mechanism by which a mutation could alter transport function is by altering the abundance of the transport protein. To determine whether NCC protein expression was similar in oocytes expressing the wild-type and mNCC<sub>R948X</sub> clones, Western blotting was performed. For these experiments, mNCCnAb1, a fusion-protein antibody directed against the amino

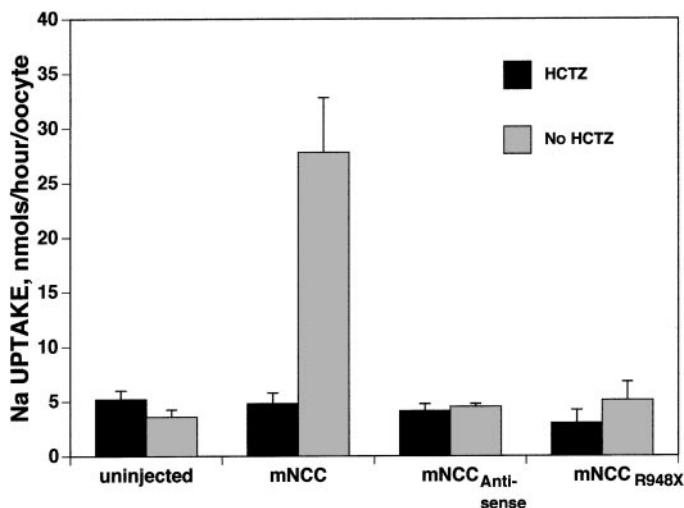


Fig. 2. Uptake of Na by oocytes (in nmol·oocyte<sup>-1</sup>·h<sup>-1</sup>; values are means + SE) in presence and absence of  $10^{-4}$  M hydrochlorothiazide (HCTZ). Rates of Na uptake by oocytes injected with mNCC<sub>R948X</sub> were similar to rates of uptake by uninjected oocytes or oocytes injected with an antisense mNCC. Twenty to 30 oocytes were used for each condition in each experiment. Number of experiments per number of frogs was 10/10 for uninjected, 13/8 for mNCC, 3/3 for mNCC antisense, and 7/7 for mNCC<sub>R948X</sub>. mNCC, mouse thiazide-sensitive Na-Cl cotransporter.

<sup>2</sup> The sequence of the mouse thiazide-sensitive Na-Cl cotransporter has been deposited in GenBank with accession number U61085.

terminal cytoplasmic domain of the mouse thiazide-sensitive Na-Cl cotransporter, was used (2). The specificity of this antibody in kidney cortex has been documented previously (2). Figure 3A shows that mNCCnAb1 recognizes a protein in mouse kidney cortex that runs as a broad band between 125 and 180 kDa (*band C*). A second band, present but very faint, migrates at 110 kDa, the size predicted for the core unglycosylated mNCC (*band A*). *Xenopus* oocytes (Fig. 3A) expressing the wild-type mNCC show predominantly the 110-kDa protein band (*band A*). A second band (*band B*) that is more diffuse and runs at a higher molecular weight is also present in oocytes that express the wild-type mNCC. This band migrates at an apparent molecular size of 120–140 kDa, a size that is clearly distinct from both the fully glycosylated, mature mouse kidney protein and the core unglycosylated protein. To confirm that the larger product is a result of glycosylation, membranes from oocytes injected with the wild-type mNCC clone were treated with N-glycosidase F and then immunoblotted. Figure 3B shows that treatment with N-glycosidase F converts all of *band B* to *band A*, confirming that *band B* is a glycosylated form of *band A*.

Figure 4 shows an immunoblot comparing oocytes injected with the wild-type mNCC and mNCC<sub>R948X</sub> and with water-injected control. Although these blots are not quantitative, they show that the wild-type and mutant clones generate similar levels of protein expression. Both clones produce primarily proteins that run in the range predicted for the core unglycosylated protein, i.e., 110 kDa for the wild type and 105 kDa for mNCC<sub>R948X</sub>. In contrast, although the glycosylated form (*band B*) is clearly evident in oocytes injected with the wild-type clone, it is greatly reduced or absent in oocytes injected with mNCC<sub>R948X</sub>. The immunoblot pattern from oocytes injected with mNCC<sub>R948X</sub> (Fig. 4) resembles that observed following treatment of the

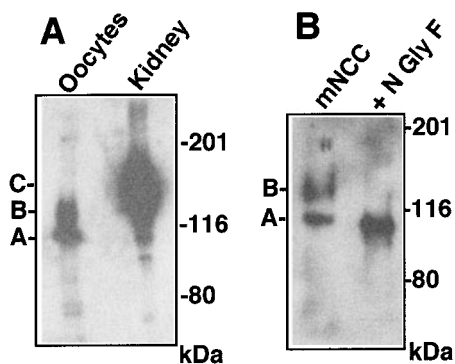


Fig. 3. Immunoblots probed with mNCCnAb1. *A*: compares kidney cortical membranes and *Xenopus* oocytes injected with mNCC. In kidney cortical membranes, the antibody recognizes predominantly a broad band between 120 and 180 kDa (*band C*). A narrow band of ~110 kDa (*band A*) is also present. In *Xenopus* oocytes, the antibody recognizes primarily *band A*, but a second, more diffuse band is visible at ~120 kDa (*band B*). *B*: compares blots of oocytes expressing the wild-type clone before and after treatment with N-glycosidase F (N Gly F). Note that *bands A* and *B* are visible before N-glycosidase F treatment. *Band B* collapses into *band A*, which is more abundant, following treatment.

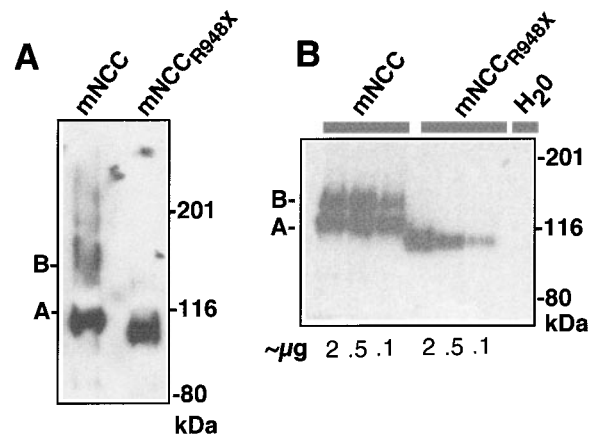


Fig. 4. *A*: immunoblots of oocytes expressing wild-type mNCC and mutant clone mNCC<sub>R948X</sub>. Note that *band B* is visible only in oocytes expressing the wild-type clone. *B*: even when increasing amounts of oocyte membrane protein are loaded on the gel (approximate amounts loaded are shown at bottom), *band B* is visible in oocytes injected with the wild-type clone, but absent from oocytes injected with mNCC<sub>R948X</sub>. Note that *band A* is smaller in oocytes injected with the mutant clone, because of truncation of the protein.

wild-type clone with N-glycosidase F (compare Fig. 3B). To determine whether the defect in glycosylation of NCC is incomplete, increasing amounts of protein were loaded on the gel. Figure 4 shows that, despite increasing amounts of *band A* in both groups, *band B* is evident only in oocytes injected with the wild-type clone.

To determine whether the mutant clone is inserted normally into the plasma membrane, uninjected oocytes and oocytes injected with the wild-type mNCC and mNCC<sub>R948X</sub> were fixed, frozen, sectioned, and stained with mNCCnAb1. Figure 5A shows that mNCCnAb1 recognizes an antigen in the plasma membrane of oocytes injected with mNCC. These oocytes also show cytosolic fluorescence that is significantly greater than background (compare an uninjected oocyte in Fig. 5C). The plasma membrane of oocytes injected with mNCC<sub>R948X</sub> is devoid of any fluorescence (Fig. 5B) and resembles the plasma membrane of uninjected oocytes. In contrast to uninjected oocytes, however, cytosolic fluorescence is evident in the oocytes injected with mNCC<sub>R948X</sub>.

Figure 6 compares Na uptake into oocytes expressing seven additional Gitelman's mutations with uptake by oocytes expressing the wild-type clone. As was the case for mNCC<sub>R948X</sub>, rates of Na uptake by oocytes expressing these mutated clones are not different from rates of uptake by uninjected oocytes. Figure 6 also shows Western blot of membranes from aliquots of oocytes used for uptake experiments. The results confirm that, in each case, the mutant NCC protein was synthesized normally. In each case, however, the mutant clone generated only a single band on Western blot, in the range predicted for the core, unglycosylated protein. A second protein band, representing the mature glycosylated protein, was evident only in oocytes that expressed the wild-type clone. No consistent differences in abundance of the mutant proteins were detected in

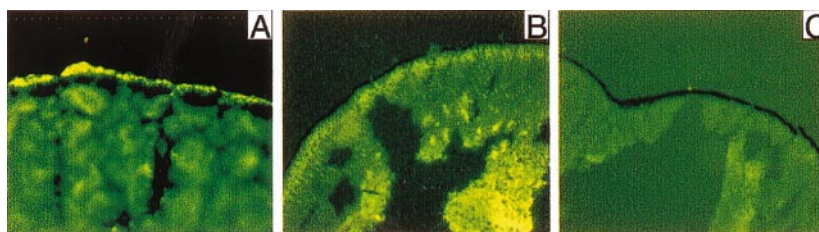


Fig. 5. Cryosections of oocytes stained with mNCCnAb1. *A*: an oocyte injected with mNCC. Note the linear staining along the plasma membrane as well as more diffuse fluorescence in the cytosolic region. *B*: an oocyte injected with mNCC<sub>R948X</sub>. Note that staining of the plasma membrane is completely absent, but that diffuse cytosolic staining is evident. *C*: an uninjected oocyte. There is no staining of either the plasma membrane or the cytosol. Note that the time of exposure of *C* was longer than that of *A* or *B* because of the complete absence of cellular staining; this makes background staining more apparent.

different batches of oocytes. In each case, the total amount of expressed protein was slightly greater in oocytes that expressed the wild-type clone.

## DISCUSSION

Gitelman's syndrome is an autosomal recessive disorder of renal salt wasting, hypokalemia, and alkalosis. It is distinguished from classic Bartter's syndrome by the presence of hypocalciuria and hypomagnesemia (1). Simon et al. (28) showed complete linkage of Gitelman's syndrome to the locus encoding the thiazide-sensitive Na-Cl cotransporter. Nineteen nonconservative mutations in this transport protein were detected (28). Other mutations in the same gene have been reported subsequently by others (11, 14, 20, 29). To date, mutations in the thiazide-sensitive Na-Cl cotransporter have been detected in most cases of Gitelman's syndrome that have been screened; mutations in no other gene have been detected. On the basis of the autosomal recessive inheritance and the phenotypic features of the syndrome, Simon and colleagues (28) postulated that Gitel-

man's syndrome results from defective Na and Cl transport by the mutant gene products. Recently, Schultheis and colleagues (21) reported that the phenotype of an NCC knockout mouse resembles Gitelman's syndrome, confirming that dysfunction of the transport protein underlies this disease.

The current experiments utilized a mouse thiazide-sensitive Na-Cl cotransporter clone. This clone is 97% identical, at the amino acid level, to the rat clone that has been described previously (6). It is 92% identical to the human clone and is similar to the rabbit clone (30) as well. One difference between the rodent forms of the transport protein and the rabbit and human forms is the presence in rabbit and human of one exon within the carboxy-terminal region that has not been described in rat or mouse (see Fig. 1) (28, 30). Gitelman's mutations have not been described in this region, and it is far removed from the mutation site in the current experiments, thus this difference is unlikely to affect results of the mutational experiments described herein. Each of the mutations that have been reported to cause Gitelman's syndrome alters an amino acid that has been conserved through evolution and is identical in human and mouse. Thus mutations in the mouse NCC should affect protein function by mechanisms that are similar to those resulting from mutations of the human protein.

The current results confirm that several mutations that cause Gitelman's syndrome in humans are not functional, when expressed in oocytes. At least six possible mechanisms by which mutations might reduce or abolish transporter activity were considered. A mutation could 1) impair protein synthesis, 2) impair protein processing, 3) impair protein insertion into the plasma membrane, 4) impair the intrinsic transporter activity, 5) accelerate protein removal or degradation, or 6) alter functional regulation. As shown by immunoblot analysis of oocytes, the mutations tested in these experiments do not substantially alter the abundance of the expressed protein. Although the abundance of the mutant protein was slightly less than the abundance of the wild-type protein in each experiment, it seems unlikely that differences in protein abundance account for the nearly complete absence of functional activity of the mutant clones. Levels of exogenous protein expression by oocytes are very high. Instead, the mutations appear to affect protein processing. Unlike the wild-

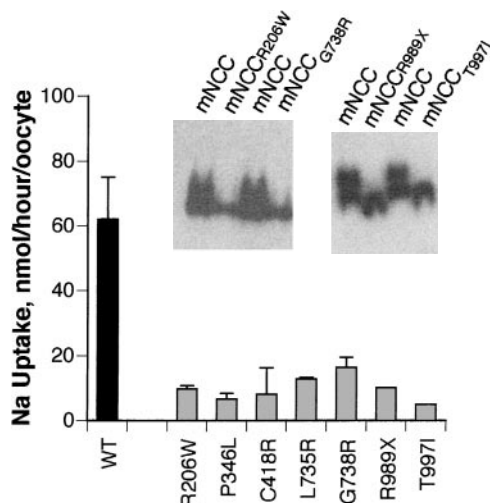


Fig. 6. Effects of Gitelman's mutations on Na uptake (values are means  $\pm$  SE) and protein processing. Na uptake by oocytes expressing the wild-type (WT) or mutant NCC clones is shown in  $\text{nmol} \cdot \text{oocyte}^{-1} \cdot \text{h}^{-1}$ . *Insets*: results of Western blot of membrane proteins from aliquots of oocytes, used for uptake experiments. Note that in each case, the glycosylated protein is present in oocytes expressing the wild-type clone (mNCC), but not in oocytes expressing the mutant clones. Results of Western blot from mutations C418R, P346L, and L735R and are similar but are not shown.

type protein, the mutant proteins are not glycosylated normally and do not appear to be expressed at the plasma membrane. Thus at least some Gitelman's mutations impair the function of the transport protein by interfering with protein processing and insertion into the plasma membrane.

The thiazide-sensitive Na-Cl cotransporter is a glycoprotein that runs as a broad band between 125 and 165 kDa on Western blot. Nishio et al. (17) showed recently that two sites in the 4th extracellular loop of the rat NCC exhibit N-linked glycosylation. In those experiments, removal by site-directed mutagenesis of either of the two putative glycosylation sites reduced transport activity significantly. Removal of both glycosylation sites led to a nonfunctional protein. Similar consensus N-linked glycosylation sites (NXT) are present in the mouse NCC (residues 403 and 423), and the results of digestion with N-glycosidase F confirm that the mouse protein is N-glycosylated. Glycosylation of asparagine moieties is a multistep procedure that begins in the rough endoplasmic reticulum. The first step involves core glycosylation and then partial deglycosylation while the protein is still in the endoplasmic reticulum. After this step, the partially processed protein moves to the Golgi, where further glycosylation occurs, leading to the mature structure.

Expression of the mutant NCCs led to production of proteins that were not glycosylated normally. Yet, except for one mutation, the amino acids affected by the mutations are distant from sites of glycosylation and would not be expected to influence glycosylation directly. Thus the current results suggest that these Gitelman's mutations generate proteins that are not glycosylated normally because of misfolding during synthesis, activating the "quality control" system of the protein secretory pathway. The quality control system (10, 16) monitors the structural integrity of proteins during synthesis on ribosomes. By incompletely understood mechanisms, this system recognizes incompletely folded, misfolded, or partially assembled proteins and prevents their release from the endoplasmic reticulum. Proteins that fail to fold normally are then degraded. This process serves several functions. It prevents the premature release of incompletely folded proteins from the endoplasmic reticulum. Because the endoplasmic reticulum is the only compartment in the secretory pathway that maintains a large concentration of molecular chaperones, it is important for protein folding to be complete before export to the Golgi. Thus the quality control system also prevents the deployment of misfolded or incompletely folded proteins into the cytoplasm and to plasma membrane. It is also used by some cells as a mechanism for posttranslational control of protein expression.

Although the present experiments show that mutation of mNCC disrupts glycosylation, they do not define which step in protein processing is disrupted. Yet the nearly complete absence of glycosylation of the mutant protein argues that the protein does not leave the rough endoplasmic reticulum. Western blots of oocytes expressing the wild-type mNCC show a broad band that runs

above 115–120 kDa. This band was sensitive to digestion with N-glycosidase F, confirming that it represents glycosylated product. This molecular size, however, is not identical to that of the protein expressed by native renal tissue, indicating that the glycosylation machinery of oocytes is not identical to that of DCT cells.

Defects in protein folding and processing have been shown to participate in the pathogenesis of several inherited disorders (13). One of the best understood examples is cystic fibrosis resulting from the  $\Delta F508$  mutation (3, 4). In contrast to the fully mature form, the mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR) is misfolded, achieves only partial glycosylation, and is retained and degraded within the endoplasmic reticulum (3, 4, 9). For this reason, it does not reach the cell surface. Thus the mechanism of transporter dysfunction identified by the current experiments appears to be similar to the mechanism responsible for most cases of cystic fibrosis. The mutant protein is not processed normally, does not become fully glycosylated, and does not reach the cell surface. In the case of cystic fibrosis, however, the  $\Delta F508$  mutation leads to temperature-dependent defects in protein processing. When expressed in cells grown at 18°C, CFTR $\Delta F508$  matures normally and is expressed at the plasma membrane. In contrast, when expressed in mammalian cells or in oocytes grown at higher temperatures, incomplete processing occurs (4). This effect has permitted experiments showing that the mutant CFTR is functional when it does move into the plasma membrane. There is no evidence that the defects in mNCC<sub>R948X</sub> protein processing are temperature sensitive, as the oocytes were grown at 18°C in the present experiments. Thus it is not possible to determine whether the mutant NCC would be functional if it did reach the plasma membrane.

The current results examine 8 of ~80 mutations that have been reported to cause Gitelman's syndrome. This group includes mNCC<sub>G738R</sub>, the most common mutation reported by Simon and colleagues (23), which is responsible for ~10% of Gitelman's cases. In each case, the mutant protein was synthesized normally, but was not glycosylated, when expressed in oocytes. This confirms that protein processing defects account for a significant fraction of Gitelman's syndrome. Although these experiments do not reveal the site within the cell at which malformed proteins are retained, they suggest that several Gitelman's mutations activate the quality control system, leading to retention of the mutant protein in the endoplasmic reticulum. Whether other Gitelman's mutations inactivate the transporter via different mechanisms remains to be determined.

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