Characterization of the Sodium/Hydrogen Exchanger NHA2

Daniel G. Fuster,* Jianning Zhang,† Mingjun Shi,†‡ I. Alexandru Bobulescu,† Stefan Andersson,§ and Orson W. Moe†‡

*Division of Nephrology and Hypertension and Institute for Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland; and Departments of †Internal Medicine, §Gynecology and Obstetrics, and ‡Physiology and ‡Charles and Jane Pak Center for Mineral Metabolism, University of Texas Southwestern Medical Center, Dallas, Texas

ABSTRACT

Cation/proton exchange has been recognized for decades in mammalian mitochondria, but the exchanger proteins have eluded identification. In this study, a cDNA from a human brain library, previously designated NHA2 in the genome, was cloned and characterized. The NHA2 transcript bears more similarity to prokaryotic than known eukaryotic sodium/proton exchangers, but it was found to be expressed in multiple mammalian organs and cultured cells. A mAb to NHA2 was generated and found to label an approximately 55-kD native protein in multiple tissues and cell lines. The specificity of this antibody was confirmed by demonstrating the loss of the native NHA2 band on immunoblots when cultured cells were treated with NHA2-specific small interfering RNA. Although NHA2 protein was detected in multiple organs, within each, its expression was restricted to specific cell types. In the kidney, co-localization with calbindin 28k and reverse transcription–PCR of microdissected tubules revealed that NHA2 is limited to the distal convoluted tubule. In cell lines, native NHA2 was localized both to the plasma membrane and to the intracellular compartment; immunogold electron microscopy of rat distal convoluted tubule demonstrated NHA2 predominantly but not exclusively on the inner mitochondrial membrane. Furthermore, co-sedimentation of NHA2 antigen and mitochondrial membranes was observed with differential centrifugation, and two mitochondrial markers co-localized with NHA2 in cultured cells. Regarding function, human NHA2 reversed the sodium/hydrogen exchanger–null phenotype when expressed in sodium/hydrogen exchanger–deficient yeast and restored the ability to defend high salinity in the presence of acidic extracellular pH. In summary, NHA2 is a ubiquitous mammalian sodium proton/exchanger that is restricted to the distal convoluted tubule in the kidney.


Sodium/hydrogen exchangers (NHE) are ubiquitous ion transporters present in lipid bilayers in simple prokaryotes and eukaryotes, including plants, fungi, and animals, that harness the electrochemical gradient of one ion to energize the uphill transport of the other.1 More than 40 yr ago, Mitchell and Moyle2 first described a cation/proton antiport system allowing mitochondria to extrude sodium and potassium against a highly unfavorable inward cationic electrochemical gradient. Ten NHE isoforms have since been cloned in mammals, which include plasmalemmal NHE1 through 5 and intracellular NHE6 through 9. In addition, a sperm-specific plasmalemmal NHE was cloned.3 Although it was first described functionally, the molecular identity of the mitochondrial cation/proton antiport system remains elusive.

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Correspondence: Dr. Daniel G. Fuster, Division of Nephrology and Hypertension and Institute for Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland. Phone: +41-31-631-47-39; Fax: +41-31-631-37-37; E-mail: danielfuster@hotmail.com

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By controlling cell volume and maintaining cellular and organellar pH, NHE have been proposed to play a pivotal role in diverse physiologic processes, including control of cell cycle and cell proliferation, cell migration, transepithelial proton, bicarbonate and sodium transport, salt tolerance, and vesicle trafficking and biogenesis.1,4 At the whole-organism level, NHE are key players in extremely diverse areas such as cancer biology, central nervous system integrity, and cardiovascular pathophysiology.4,5

The superfamily of monovalent cation/proton antiporters (CPA) as proposed by Saier6 has two large clades, named CPA1 and CPA2. CPA1 includes the currently known mammalian NHE with the exception of the recently cloned sperm NHE. In a comprehensive phylogenetic analysis of CPA families, Brett et al.7 proposed two novel, previously unrecognized, putative NHE in the mammalian CPA2 clade. These putative NHE have higher homology to fungal and bacterial NHE, especially Escherichia coli NhaA, than the currently known mammalian NHE. On the basis of this homology, they have been named NHA1 and NHA2. NHA1, also referred to as NHEDC1, has been cloned and proposed to be expressed exclusively in testis.8 No functional characterization has been reported so far. One recent article described the ubiquitous distribution of NHA2,9 but another reported a highly specific distribution in osteoclasts only.10,11 Although there was evidence for cell surface expression in one study,9 an exclusively intracellular distribution was described in the other.11

In this study, we describe tissue distribution, intracellular localization, and functional characterization of NHA2. NHA2 is a new intracellular and plasmalemmal NHE with wide tissue distribution, and, in the kidney, it is specifically localized to the distal convoluted tubules (DCT).

RESULTS

We have cloned the human NHA1 and NHA2 isoforms (HsNHA1 and HsNHA2, respectively) from a human brain cDNA library. The human NHA2 open reading frame (ORF) spans 1614 bp, giving rise to a protein of 537 amino acids with a predicted molecular weight of approximately 57 kD. All completely sequenced metazoan genomes contain orthologs of NHA2 with high conservation on the amino acid level (Figure 1).7 By hydrophathy-based analysis and depending on the algorithm used, the protein is predicted to contain 10 to 12 transmembrane domains with short intracellular N- and C-termini (www.expasy.org/tools). The E. coli Nha crystal structure shows 12 transmembrane spans.12 The predicted structure of

Figure 1. Sequence comparison of NHA2. Amino acid alignment of human (Hs), mouse (Mm), and rat (Rn) NHA2. Sequence identity is 81% between human and mouse or human and rat NHA2. Epitope of MAB45D12D is marked in yellow highlight.
the two termini are in contrast to the currently known mammalian NHE, which have long intracellular C-termini important in the functional regulation.

Human NHA2 is expressed in all human cell lines tested. Figure 2A shows a representative RNA blot in HeLa, HEK293, and Caco-2 cells. The corresponding mRNA is approximately 2.4 kb without evidence of alternate splice variants with differential mobility in these cell lines. Presence of NHA2 in these cell lines was also confirmed with reverse transcription–PCR (RT-PCR) of NHA2 ORF followed by sequencing of the PCR product (data not shown). We next performed RT-PCR using mouse-specific NHA2 primers from different mouse tissues. With the exception of heart and blood, NHA2 transcript was detected in all organs, including the kidney (Figure 2B).

To determine NHA2 expression at the protein level, we raised a mAb to a conserved epitope (DTARSHGEEKLEDYG; Figure 1, yellow highlight). We obtained a highly specific mAb, designated MAB45D12D of the IgG2a subclass. On immunoblot, this antibody recognizes a single band of approximately 55 kD, which is compatible with the predicted ORF (Figure 3A). For verification of the specificity of our antibody, endogenous NHA2 in NRK cells was knocked down using RNA interference. Compared with control cells, transfection of cells with small interference RNA (siRNA) targeting rat NHA2 led to almost complete disappearance of the approximately 55 kD band on immunoblot (Figure 3A). Global silencing was ruled out by the fact that protein levels of NHE1 or NHE8 in these cells were not affected (Figure 3A). To rule out cross-reactivity with the highly homologous NHA1 isoform, we tran...
siently transfected plasmalemmal NHE-deficient AP-1 cells with a C-terminally GFP-tagged human NHA1 construct. Although the construct was expressed as detected by a monoclonal anti-GFP antibody, MAB45D12D failed to label NHA1 (Figure 3B).

We next analyzed the tissue distribution of NHA2 at the protein level in the mouse using MAB45D12D. The protein expression pattern mirrors our RT-PCR findings with the exception of the heart (Figure 3C). NHA2 transcript in heart was also absent when two different PCR primer pairs were used and in another mouse strain (C57 Black/6 strain; data not shown). This discrepancy can be explained by either the presence of alternate splice variants in heart that failed to anneal to our primers or that, although the tissues were perfused with PBS before homogenization, residual red blood cells (RBC) in the capillary bed could be responsible for the signal. Also, the possibility of cross-reactivity of MAB45D12D in RBC and heart cannot be completely ruled out. Indeed, we and others have detected NHA2 in RBC (Figure 3D), despite the absence of mRNA detected by RT-PCR of whole blood (Figure 2B). The negative RT-PCR findings are supported by the absence of a signal in heart by immunohistochemistry (Figure 4).

To study plasmalemmal expression of endogenous NHA2, we used a surface biotinylation assay. Figure 3D shows absence of endogenous NHA2 from the plasma membrane in HEK293 cells as determined by this method. Simultaneous presence of NHA2 in lysate suggests an intracellular localization in these cells. Identical results were obtained from other cell lines (HeLa, NRK; results not shown). In contrast, plasmalemmal NHA2 could be detected in IN5-1E cells, a rat insulinoma cell line, and in HEK293 cells transiently transfected with a C-terminally HA-tagged human NHA2 construct and in RBC. NHE1 was used as a biotinylation control (Figure 3D) to show that absence of biotinylatable NHA2 was not due to inaccessibility of the reagent in these cells.

To study further native NHA2 expression, we performed confocal microscopy studies in NRK cells as shown in Figure 5A. By fluorescence immunocytochemistry, native NHA2 was not found on the plasma membrane of NRK cells. Identical staining patterns were observed in other cell lines tested for native NHA2 localization (HeLa, HEK293, MDCK; data not shown). To delineate further the intracellular localization of NHA2, we performed co-staining with the mitochondrial marker Mitotracker. There is partial co-localization of endogenous NHA2 with mitochondria in NRK cells. Although there is considerable variance from cell to cell, 39 ± 17% (mean ± SD; n = 40 cells) of the intracellular signal co-localizes with mitochondria (Figure 5A). Because there are some nonoverlapping signals of NHA2 and mitochondria, we performed additional co-localization of NHA2 with anti-CPS1 as a second mitochondrial marker, anti-GOLPH4 antibody for Golgi, and anti-PDI for endoplasmic reticulum (ER). The overlap of NHA2 with CPS1 was 30 ± 19% (mean ± SD; n = 52 cells; Figure 5A). Although there is no overlap with Golgi, there is some co-localization with ER (Figure 5A). We next isolated mitochondria from HEK293 and NRK cells by differential centrifugation. Native NHA2 is enriched in the mitochondrial fraction (3000 × g pellets; Figure 5B) as are the mitochondrial proteins Tom40 and CPS1. Although there is some co-purification of lysosomes as evidenced by the presence of the lysosomal membrane protein LAMP1 in the pellet, the majority of LAMP1 is present in the 3000 × g supernatant (Figure 5B). Knockdown of endogenous NHA2 leads to disappearance of the co-staining in NRK cells (Figure 5C). The intracellular localization as determined by imaging thus fits the biochemical evidence obtained with fractionation studies.

To study intracellular localization of NHA2 in native tissue, we performed immunogold electron microscopy of rat DCT cells in the kidney using MAB45D12D, which demonstrates presence of NHA2 on the inner membrane of mitochondria (Figure 5, D and E). In addition to mitochondria, NHA2 is seen in other intracellular organelles. Most of these are vesicular structures. A small amount of NHA2 is also seen in other locations. Figure 5F shows quantitative gold particle density of NHA2 in the DCT. The most abundant sites were mitochondria and intracellular vesicles. A few or no gold particles were present in the plasma membrane (Figure 5F).
seen in the proximal tubule, which was very variable and much lower than that of the DCT (data not shown).

The quasi-ubiquitous expression of NHA2 is striking, and the question arises in which cell types NHA2 expression is present in different tissues. By immunohistochemistry analysis of mouse tissues, NHA2 is present in epithelial cells of the small intestine, lung, mammary gland, and renal tubules. In addition, NHA2 is strongly expressed in neurons of the brain (Figure 4). Although quite ubiquitous in organ distribution, within each organ NHA2 is not universally expressed in all cells. In the kidney, NHA2 seems to be exclusively expressed in distal tubular cells (Figure 4) by light microscopy. This is further supported by co-labeling of NHA2 with calbindin 28k, a DCT marker (Figure 6A), and exclusive presence of the transcript in DCT by RT-PCR of microdissected rat tubules (Figure 6B).

This is comparable to the immunogold electron microscopy described in Figure 5.

To study function of NHA2, we took advantage of the fact that overexpressed NHA2 can be detected on the plasma membrane of mammalian cells. For this purpose, we used cells devoid of plasmalemmal cation/proton exchange activity to express our constructs. This included AP-1 cells that have been derived from CHO cells by means of random chemical mutagenesis and skin fibroblasts derived from NHE1 knockout mice. First, we used a well-documented NHE transport assay using the intracellularly trapped pH-sensitive dye BCECF and were unable to detected cation/proton exchange (data not shown). We then resorted to a recently developed electrophysiologic approach that allows determination with high sensitivity of simultaneously ion fluxes and currents elicited by

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**Figure 5.** Intracellular localization of endogenous NHA2. (A) Fluorescence immunocytochemistry. Bar = 10 μm. NRK cells were treated with the specific markers (mitochondria: Mitotracker [B] and α-CPS1 [E]; Golgi: α-GOLPH4 [H]; ER: α-PDI [K]) and then with MAB45D12D (α-NHA2 [A, D, G, and J]). Merged images (C, F, I, and L) show overlap of the organellar markers with NHA2. Note the absence of plasmalemmal staining for NHA2. (B) Mitochondrial isolation of HEK293 and NRK cells. Native NHA2 is present in the 3000 × g pellets as the mitochondrial proteins Tom40 and CPS1. LAMP1, a lysosomal membrane protein, partially co-purifies with the mitochondrial pellet but is also present in the 3000 × g supernatant. (C) Fluorescence immunocytochemical images of NRK cells treated with Mitotracker and stained with MAB45D12D. Disappearance of the MAB45D12D signal after siRNA transfection. (D and E) Immunogold electron microscopy of rat DCT cells using MAB45D12D. NHA2 is present on the inner mitochondrial membrane. Bar = 1 μm. (F) Quantitative gold particle density of rat DCT. Bars and error bars are means ± SD (n = 56). Background particle density (gold-conjugated secondary antibody only) was <0.5/μm².
NHE16; however, even with the latter highly sensitive method combined with using highly favorable pH and cation gradients, we were unable to demonstrate NHA2-induced cation (Na⁺, K⁺, or H⁺) fluxes or currents in a mammalian system using untagged expression constructs and NHA2 from different species (human and mouse; data not shown).

Because NHA2 has high similarity to bacterial and fungal NHE, we examined the ability of heterologously expressed human NHA2 to rescue the salt-sensitive growth phenotype of Saccharomyces cerevisiae strains lacking two B31 2/6c (ena1–4/nha1Δ) or three AXT3 (ena1–4/nha1Δ/nhx1Δ) major salt-handling ion transporters.17–19 As expected, the host strain AXT3 transformed with the empty vector is unable to grow at 100 mM extracellular Na⁺, at both extracellular pH of 4.0 and 7.4 (Figure 7A). Expression of prevacuolar (Nhx1) and plasma-membral (Nha1) S. cerevisiae NHE and human NHA2 all rescue the salt-sensitive phenotype at pH 4 but not at pH 7.4. This finding is compatible with NHA2-induced Na⁺/H⁺ exchange activity. Expression of C-terminally HA-tagged NHA2 constructs in yeast are shown in Figure 7B. Findings were identical for the yeast strain B31 2/6c (data not shown). At mildly acidic extracellular pH of 6.0, no rescue of the salt-sensitive phenotype could be observed. Also, no rescue could be observed when Na⁺ was replaced by K⁺ (data not shown). We also determined K⁺ transport in a K⁺ uptake–deficient S. cerevisiae strain WΔ3 (trk1Δtrk2Δ) that grows only on high external K⁺ medium.20 At different pH, no rescue could be observed with heterologous expression of NHA2 (data not shown). All of these observations are in agreement with the functional characterization by Xiang et al.9 and suggest that NHA2 indeed is a functional Na⁺-selective NHE.

**DISCUSSION**

In this study, we describe the detailed characterization of a novel, almost ubiquitously expressed sodium/hydrogen exchanger NHA2. Mitchell and Moyle2 were the first to propose a mitochondrial cation/proton antiport system. Because of the highly favorable electrochemical gradient, mitochondria rep-
resent a sink for cations. High mitochondrial sodium concentrations can lead to mitochondrial swelling and, in parallel with sodium/calcium exchange, can lead to calcium accumulation in the mitochondria. The finding of mitochondrial localization of NHA2 may at first glance contrast with the striking cell-specific expression of NHA2 in the organs examined. There is, however, both functional and biochemical evidence for a heterogeneity of mitochondrial cation/proton antiporters. Also, there is ample evidence for tissue-specific differences in mitochondria, even along the nephron. NHA2 does not have the classical N-terminal motif for mitochondrial import but may use internal recognition signals for translocation into mitochondria. The sodium-selective NHE of mitochondria has been partially purified and reconstituted in proteoliposomes with a predicted molecular weight of approximately 59 kD. This is very close to the predicted and detected molecular weight of approximately 57 kD of human and rodent NHA2. In S. cerevisiae, NHE activity can be demonstrated for NHA2 under highly favorable conditions, including a 3-log-unit difference in proton concentrations. The physiologic implications are unclear because this surrogate system is considerably divergent from the native habitat of NHA2. High proton gradients may be found in gastric epithelia, medullary collecting duct of the kidney under maximal urinary acidification, or possibly microdomains between organelles and cytoplasm. It is also conceivable that the overexpression system S. cerevisiae is lacking accessory proteins or the right lipid environment for proper function of NHA2, mandating the requirement of a high pH gradient.

During the preparation of this manuscript, Battaglino and Xiang and co-workers published initial reports on NHA2. Our data partially support and extend these initial findings. The one major discrepancy between the findings of Battaglino and those of Xiang and ours is that Battaglino found bone-restricted expression of NHA2. The reason for this incongruity is unclear.

Although NHA2 is present on the plasma membrane of certain specialized cells, in many cell types it seems to be a completely intracellular protein. It has been documented for several “intracellular” NHE that they can also reside and function on the plasma membrane under certain circumstances, and dual mitochondrial and plasmalemmal localization has also been reported for the voltage-dependent anion channel VDAC. For the intracellular NHE isoform NHE6, the scaffolding protein RACK1 was recently shown to regulate the balance between plasmalemmal and intracellular localization. Clearly, further studies are needed to elucidate the regulatory pathways underlying this dual localization.

One salient finding is that renal NHA2 is specific to the DCT. Because only 1% of mitochondrial proteins are encoded by the mitochondrial genome, protein import from nucleus-originated transcripts is not uncommon and in fact is expected to exhibit cell specificity. One unique feature of the mammalian DCT is that it mediates the highest transcellular flux of calcium. The mitochondria act in concert with many other mechanisms to provide intracellular calcium buffering in many cell types, including the DCT. Mitochondrial sodium/calcium exchange may be deployed by the DCT to unload the mitochondria of calcium as previously shown in cardiac cells. This forward sodium/calcium exchange may burden the mitochondria with increased sodium load. This can be extruded by sodium/proton exchange by expending part of the proton gradient that is usually used for ATP generation. These and other speculations for the DCT-specific expression remain to be proved.

In summary, our data indicate that NHA2 is both an intracellular and a plasmalemmal sodium/proton exchanger expressed in multiple tissues but in a cell type–specific manner. Whether this is indeed the cation/proton exchanger of Mitchell and what the physiologic function for NHA2 is remains to be determined.

CONCISE METHODS

Chemicals

Unless otherwise specified, all chemicals and reagents were obtained from Sigma (St. Louis, MO).

DNA Constructs

Human brain cDNA library in pADSL-Nx vector was a gift from I. Stagljar (University of Toronto, Toronto, ON, Canada). Human NHA2 (accession no. NM_178833) was cloned into pmH vector (Roche, Indianapolis, IN) containing a C-terminal HA-tag. Human NHA1 (accession no. BC132712) was cloned into pEGFP-N3 (Clontech, Mountain View, CA). Monoclonal anti-GFP antibody was obtained from Clontech. S. cerevisiae Nha1 and Nhx1 cDNA were obtained from Open Biosystems (Huntsville, AL) and subcloned into p426TEF expression vector. C-terminal HA tags were introduced by PCR. All constructs were verified by sequencing.

Generation of MAB

Mouse mAb were raised against the peptide H,N-CDTARSH-GEKQLEDYG-COOH linked to KLH as described previously. The anti-NHA2 mAb designated MAB45D12D was determined to belong to the IgG2a subclass as revealed by an ELISA-based mouse typer subisotyping kit (Bio-Rad, Hercules, CA).

Cell Culture, Transfection, and siRNA

NRK and HEK293 cells were obtained from ATCC and cultured in high-glucose (450 mg/dl) DMEM supplemented with 10% (vol/vol) FBS. INS-1E cells were obtained from P. Maechler (University of Geneva, Geneva, Switzerland) and grown in RPMI-1640 medium supplemented with 10% (vol/vol) FBS. NHE1-deficient AP-1 cells, derived from CHO cells (gift from S. Grinstein, Hospital for Sick Children, Toronto, ON, Canada), were maintained in α-modified minimal essential medium. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cells were used 24 to 36 h after transfection for further experiments.
Two independent stealth siRNA targeting rat NHA2 (1, forward 5’-AGGAACGCUCCAGCUCAUGCUGAU-3’; 2, 5’-CAUCCCU-CUAUCCGCUCUUCUAU-3’) were obtained from Invitrogen and transfected into NRK cells according to the manufacturer’s instructions.

**Yeast Transformation and Growth Selection**

Expression constructs were transformed into *S. cerevisiae* strains AXT3 (MATα, ade2, his3, leu2, trpl, ura3, ena1::HIS3::ena4, nha1::LEU2, nx1::TRP1), WΔ3 (MATα, ade2, his3, leu2, trpl, ura3, trk1::LEU2, trk2::HIS3), or B31 2/6c (MATα, ade2, can1, his3, leu2, trpl, ura3, nha10 ena1::HIS3::ena4, nha1::LEU2). Transformation of yeast was performed as described previously. Yeast were grown at 30°C on AP medium agar plates containing 10 mM arginine, 8 mM glutamine, 2% glucose, 2 mM MgSO₄, 1 mM KCl, 0.2 mM CaCl₂, trace minerals, and vitamins. pH was adjusted with phosphoric acid or Tris base. Salt-sensitive growth was assayed after 4 d.

For immunoblot, 10 OD units of yeast were washed once with 1 mM EDTA/H₂O and lysed in 2 M NaOH for 10 min on ice. Proteins were isolated by TCA/acetone precipitation, resuspended in SDS sample buffer (25 mM Tris-Cl [pH 6.8], 9 M urea, 1 mM KCl, 0.2 M CaCl₂, trace minerals, and vitamins). pH was adjusted with phosphoric acid or Tris base. Salt-sensitive growth was assayed after 4 d.

Mitochondria were isolated using the Pierce (Rockford, IL) mitochondrial isolation kit based on differential centrifugation. As mitochondrial fraction, the 3000 × g pellet was used. Polyclonal anti-Tom20 and anti-Tom40 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-Lamp1 antibody was obtained from Abcam (Cambridge, MA).

RBC isolation

RBC ghosts were isolated from freshly drawn human whole blood as described previously. Briefly, sedimented RBC were washed three times with saline, and RBC ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate/1 mM EDTA (pH 8.0) in the presence of protease inhibitors (Roche). The lysate was centrifuged at 25,000 × g for 20 min, and the supernatant was removed. The membrane pellets were resuspended and washed five additional times in lysis buffer before further use.

**Immunoprecipitation, Cell-Surface Biotinylation, and Immunoblotting**

Immunoprecipitation and cell-surface biotinylation were performed as described previously. For immunoblotting, undiluted hybridoma supernatant containing 40 mM Tris-HCl (pH 7.4) and 0.2% Tween 20 or monochlonal or polyclonal anti-HA antibodies (1:1000 dilution) were used followed by secondary horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit IgG antibodies (1:10,000 and 1:20,000 dilution, respectively; Bio-Rad). Signals were detected using enhanced chemiluminescence. Monoclonal anti-NHE1 antibody (clone 4E9) was obtained from Calbiochem (Temecula, CA); monoclonal anti-NHE8 antibody has been described before.

**Immunocytochemistry and Immunohistochemistry**

Preparation of samples for immunocytochemistry and immunohistochemistry was performed as described previously. Confuent quiescent NRK cells were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 3 min, and blocked by 1.5% BSA and 10% goat serum in PBS for 1 h. Fixed monolayers were incubated with anti-NHA2 mAb (1:3), followed by Alexa 488–conjugated goat anti-mouse antibody (1:800; Invitrogen), and then incubated with anti-GOLPH4 antibody (1:400; Abcam) or anti-PDI antibody (ER marker, 1:50; Abcam) or anti-CPS1 antibody (mitochondrial marker, 1:5000; Abcam) followed by Alexa 568–conjugated goat anti-rabbit antibody (1:800; Invitrogen). Some samples were stained with Mitotracker Red 580 (Invitrogen). Images were visualized with a Zeiss LSM-510 laser scanning confocal microscope. Degree of overlap between NHA2 and the organelar markers were quantified by ImageJ as fluorescence area yellow/yellow + green integrated over approximately 50 randomly selected cells.

Kidney sections, after blocking with 1.5% BSA/10% goat serum, were incubated with undiluted hybridoma supernatant (MAB45D12D) or rabbit anti-calbindin 28k (Swant, Bellinzona, Switzerland) antibody diluted 1:100 in 1.5% BSA/5% goat serum, followed by Alexa 488–or Alexa 568–conjugated goat anti-mouse or goat anti-rabbit antibody diluted 1:500 in 1.5% BSA/5% goat serum. Confocal images were obtained using a Zeiss LSM-510 laser-scanning confocal microscope.

**Immunogold Electron Microscopy and Gold Particle Quantification**

Anesthetized rats were laparatomized, and the aorta was ligated proximal to the renal arteries. Kidneys were fixed *in situ* by perfusion with 2.5% paraformaldehyde in PBS (pH 7.4), delivered via the distal aorta. Kidneys were then removed and postfixed in 4% paraformaldehyde in PBS at 4°C for 4 h. Immunogold labeling of ultrathin frozen tissue sections was performed according to the method of Tokuyasu. Kidney cortex was infiltrated with 2.3 M sucrose overnight and frozen in liquid nitrogen, and 70- to 80-nm sections were cut on a ultramicrotome (Leica ultracut UCT; Leica Mikrosysteme Gmbh, Vienna, Austria) and mounted on Formvar-coated nickel grids. Sections were processed using the following steps: Rehydration with PBS containing 20 mM glycine for 10 min followed by PBS (5 min × 2); blocking with 1.5% BSA in PBS for 15 min; incubation with anti-NHA2 mAb (1:3) for 60 min; washing with 1.5% BSA in PBS for 5 min and PBS (5 min × 3); incubation with gold-conjugated goat anti-mouse IgG antibody (10-nm gold particles, 1:5 dilution) for 60 min; washing with PBS (5 min × 3); fixation with 1% glutaraldehyde for 5 min; washing in water (5 min × 3); staining with a mixture (8:5:1) of 3% methylcellulose-water-3% uranyl acetate. Samples were visualized with a JEOL 1200 EX transmission electron microscope at a magnification of ×30,000. Random sampling was used at every stage (section of renal cortical tissue blocks, tissue sections, and transmission electron microscope fields) to yield a minimum of 60 randomly selected electron micrographs for each analysis. Label quantification was performed according to the methods of Mayhew and Kaur. Gold particles were counted on each electron micrograph, and the areas represented by mitochondria, intracellular vesicles, nucleus, and other cellular parts were measured using the ImageJ software. Labeling density was performed according to the methods of Mayhew and Kaur. Gold particles were counted on each electron micrograph, and the areas represented by mitochondria, intracellular vesicles, nucleus, and other cellular parts were measured using the ImageJ software.
(number of gold particles/μm²) for each cellular compartment was calculated, and results were expressed as relative labeling index.45

**Mouse Tissue Isolation and Rat Single-Tubule Microdissection**

Mice were perfused with ice-cold PBS, and individual organs were then harvested for RNA or protein isolation. For protein isolation, organ tissue was homogenized in ice-cold RIPA buffer (in mM: 150 NaCl, 50 Tris-HCl [pH 8.0], 5 EDTA, 1 EGTA, Triton X-100 1% [vol/vol], deoxycholate 0.5% [wt/vol], SDS 0.1% [wt/vol], and protease inhibitor cocktail from Roche), lysed for 1 h at 4°C, cleared twice at 10,000 × g for 15 min and immediately used for SDS-PAGE. Rat tubules were dissected as described previously.47 Total RNA from microdissected tubules was prepared using the Mircoprep kit (Stratagene, La Jolla, CA). Total RNA from cells and rat or mouse tissue was calculated, and results were expressed as relative labeling index.45

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**DISCLOSURES**

None.


