SORLA/SORL1 Functionally Interacts with SPAK To Control Renal Activation of Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) Cotransporter 2*\(^{7}\)

Juliane Reiche,1 Franziska Theilig,2 Fatema H. Rafiqi,3 Anne-Sophie Carlo,1 Daniel Militz,1 Kerim Mutig,2 Mihail Todiras,1 Erik Ilso Christensen,4 David H. Ellison,6 Michael Bader,1 Anders Nykjaer,5 Sebastian Bachmann,2 Dario Alessi,3 and Thomas E. Willnow1*

Max Delbrück Center for Molecular Medicine1 and Institute for Anatomy,2 Charité, Universitätsmedizin, 13125 Berlin, Germany; School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom; Department of Anatomy3 and Lundbeck Foundation Research Center MIND,5 University of Aarhus, 8000 Aarhus, Denmark; and Oregon Health and Science University, Portland, Oregon 97239

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Proper control of NaCl excretion in the kidney is central to bodily functions, yet many mechanisms that regulate reabsorption of sodium and chloride in the kidney remain incompletely understood. Here, we identify an important role played by the intracellular sorting receptor SORLA (sorting protein-related receptor with A-type repeats) in functional activation of renal ion transporters. We demonstrate that SORLA is expressed in epithelial cells of the thick ascending limb (TAL) of Henle’s loop and that lack of receptor expression in this cell type in SORLA-deficient mice results in an inability to properly reabsorb sodium and chloride during osmotic stress. The underlying cellular defect was correlated with an inability of the TAL to phosphorylate Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) cotransporter 2 (NKCC2), the major sodium transporter in the distal nephron. SORLA functionally interacts with Ste-20-related proline-alanine-rich kinase (SPAK), an activator of NKCC2, and receptor deficiency is associated with missorting of SPAK. Our data suggest a novel regulatory pathway whereby intracellular trafficking of SPAK by the sorting receptor SORLA is crucial for proper NKCC2 activation and for maintenance of renal ion balance.

Sorting-protein-related receptor with A-type repeats (SORLA; also known as SORL1 or LR11) is a member of the VPS10P domain receptor gene family, a group of sorting receptors structurally related to the yeast vacuolar protein sorting 10 protein (VPS10P) (11, 30). VPS10P is a receptor that transports carboxypeptidase Y into the vacuole (the yeast lysosome) (12). VPS10P domain receptors, including SORLA, have been shown to shuttle between the cell surface, early endosomes, and the trans-Golgi network (TGN) in various cell types (16, 26). Based on this distinct trafficking pattern and on their homology to VPS10P, a role for SORLA and other family members as intracellular sorting receptors has been suggested (reviewed in reference 29).

Recently, a role for SORLA in protein trafficking was confirmed in neurons in the brain, where it acts as a receptor for the amyloid precursor protein (APP), the main etiological agent in Alzheimer’s disease. SORLA controls the sorting of APP between the TGN and endosomal compartments, thereby regulating proteolytic breakdown into amyloidogenic and nonamyloidogenic products (1, 17, 24).

In addition to the brain, expression of SORLA has also been reported in other tissues, including testis, pancreas, smooth muscle, and kidney (11, 15, 30). In the kidney, expression was mapped to the collecting duct epithelium (23). However, the relevance of the receptor for renal physiology remained enigmatic.

In this study, we addressed the physiological role of SORLA in the kidney. Contrary to previous reports, we detected significant expression of SORLA in epithelial cells of the thick ascending limb (TAL) of Henle’s loop, the distal convoluted tubule, and the connecting tubule. Lack of receptor expression in SORLA-deficient mice results in an inability to properly reabsorb sodium and chloride under osmotic stress induced by thirsting. The underlying cellular defect was linked to an inability of the TAL to activate Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) cotransporter 2 (NKCC2), the major sodium transporter in the distal nephron. Interaction of SORLA with Ste-20-related proline-alanine-rich kinase (SPAK), the kinase that activates NKCC2, and missorting of SPAK in SORLA-deficient cells suggest a novel regulatory pathway in which intracellular trafficking of SPAK by SORLA is essential for NKCC2 activation and for renal ion handling.

MATERIALS AND METHODS

Mouse models. Generation of SORLA-deficient mice by targeted gene disruption has been described previously (1). This line was kept on hybrid (129SvEmTer × C57BL/6N) and (129SvEmTer × BALB/c) genetic backgrounds. A second receptor-null line on a (129/Ola × C57BL/6N) background was generated by insertional mutagenesis of Sorla by a lacZ reporter gene (24). All three lines were used in this study and gave identical results compared with age- and sex-matched control mice with an identical genetic background.

Antisera. Antibodies against SORLA were provided by C. M. Petersen (Aarhus University). Commercial antibodies were used to detect renal outer medullary potassium channel (ROMK) (anti-K\(_{\text{ir}}\)-1.1; Alomone Laboratories), actin (Sigma), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam), epithelial Na\(^{+}\) channel (ENaC) (AlphaDiagnosics), and Na\(^{+}\)/H\(^{+}\) exchanger (NHE3) (Chemicon).

* Corresponding author. Mailing address: Max Delbrück Center for Molecular Medicine, R.-Rössle-Strasse 10, D-13125 Berlin, Germany. Phone: 49-30-9406-2569. Fax: 49-30-9406-3382. E-mail: willnow@mdc-berlin.de.

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Precipitation of SPAK, a protocol of Richardson et al. was applied (22). For published previously (1) and subjected to standard immunoblotting. For immunoprecipitations were performed using anti-SORLA, anti-YFP, or nonimmune IgG and protein G-coupled Sepharose beads (1).

**Immunohistochemistry.** Immunohistochemical detections were performed on paraffin, Tissue Tek-, or LR-White-embedded specimens. Sections were blocked with 5% skim milk-phosphate-buffered saline (PBS) and incubated with primary antibody at 4°C overnight, followed by incubation with Cy-2-, Cy-3-, or horse-radish peroxidase (HRP)-coupled secondary antibody. Secondary antibodies conjugated with HRP were detected using diaminobenzidine. For electron microscopy (anti-NKCC2 T4 antibody, Developmental Studies Hybridoma Bank), gold staining was intensified using the IntenSE-silver enhancement system. Signals were quantified by counting gold particles over the luminal plasma membrane or cytoplasmic vesicular compartment. For colocalization studies of SPAK and NCC, regions of interest (ROI) were set on individual tubules. After the threshold was set, the number of colocalizing pixels compared to the total number of pixels in each ROI was determined across individual tubular epithelial cells. Signal intensities over distance were indicated as percentages of the maximal intensity and percentages of the maximal distance, set to 100%, respectively, using ImageJ software.

**Physiology.** Urine samples were collected from mice placed in metabolic cages for 18 h. Blood sampling was performed by retro-orbital puncture. Ion concentrations in urine and serum were measured using a standard clinical analyzer. Endocrine parameters were determined using competitive radioimmunoassay assays, and osmolality was determined by the freezing-point method. Blood pressure telemetry was performed as described previously using a pressure-sensing catheter in the abdominal aorta (10). Mice were allowed 10 days of recovery from surgery before baseline blood pressure was recorded for 3 days. The statistical significance of all data was determined by Student’s t-test.

**Immunoprecipitation and Western blot analyses.** Membrane preparations were generated from freshly isolated kidney tissues according to protocols published previously (1) and subjected to standard immunoblotting. For immunoprecipitation of SPAK, a protocol of Richardson et al. was applied (22). For

**RESULTS**

To study the renal expression pattern of SORLA, we made use of a mouse model with insertion of a β-galactosidase (lacZ) reporter gene into the murine Sorla locus (24). Expression of lacZ is controlled by the endogenous Sorla promoter and recapitulates the expression pattern of the receptor. In renal tissues, lacZ expression was seen in the inner stripe of the outer medulla, but also in cortical regions (Fig. 1A). Using an antibody directed against SORLA, cell types expressing the receptor were identified as epithelial cells in the TAL, the distal convoluted tubule, the connecting tubule, and the cortical collecting duct (Fig. 1B to D). In these cells, the receptor localized to vesicular structures in the perinuclear region, in line with the predominant intracellular localization of the receptor in other cell types (1) (arrowheads in Fig. 1B to D). This renal expression pattern was reproduced using two different anti-SORLA antisera, as well as in situ hybridization (not shown).

Expression of SORLA in distal nephron segments argued for a possible contribution to the renal salt and/or water balance. Thus, we tested urine volume and salt excretion in wild-type mice and in animals genetically deficient for Sorla generated previously (Fig. 2A, inset) (1). The animals were studied with free access to drinking water and after thirsting for 18 h.

FIG. 1. Expression of SORLA in the mouse kidney. (A) Detection of SORLA gene expression in the cortex (⋆) and inner stripe of the outer medulla (IS) based on activity of a β-galactosidase reporter gene inserted into the Sorla locus. (B to D) Immunodetection of SORLA in cells of the TAL of Henle’s loop, distal convoluted tubule (DCT), connecting tubule (CNT), and cortical collecting duct (CCD). The receptor is seen in vesicular structures in the perinuclear region (arrowheads). Magnification, ×40 (A) and ×1,000 (B to D).
The urine volume was independent of the genotype under both experimental conditions, indicating normal water handling in mutants (Fig. 2A). There was a trend toward higher urine levels of anti-diuretic hormone vasopressin in Sorla\(^-/-\) mice under basal conditions (52.1 versus 35.1 pg/ml) (Table 1). Still, the animals reacted to thirsting with proper induction of the hormone in response to osmotic stress (Fig. 2B).

In contrast to water homeostasis, we observed a striking anomaly in salt handling in SORLA-deficient mice (Fig. 3). With access to water, excretion of sodium and chloride was normal but excretion of potassium was increased (Fig. 3A). With access to water, excretion of sodium and chloride was normal. The inability of SORLA-deficient mice to regulate salt homeostasis was confirmed by more detailed characterization of ion and endocrine parameters (Table 1). Under basal conditions, SORLA-deficient mice exhibited significant urinary loss of potassium and calcium compared to controls. Serum Na\(^+\), Cl\(^-\), and K\(^+\) levels were normal, but aldosterone levels were elevated 2-fold. Mean arterial blood pressure was decreased despite the hyperaldosteronemic phenotype.

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Immunohistological inspection confirmed upregulation of NHE3 both in the proximal tubules (Fig. 6C) and in the TAL (Fig. 6D) of mutants with access to water. Apparently, ENaC (connecting tubule and collecting duct) and NCC (distal convoluted tubule), as well as NHE3 (proximal tubules and TAL), are induced and/or hyperphosphorylated in mice lacking SORLA. Intriguingly, the opposite effect was seen for NKCC2, the quantitatively most important Na\(^+\)/H\(^+\) and Cl\(^-\)/H\(^+\) cotransporter in the TAL. As shown by Western blot analysis, receptor-null mice expressed normal levels of the cotransporter, but the protein lacked phosphorylation, a hallmark of functional activation (Fig. 7A and B). Lack of NKCC2 phosphorylation was confirmed by immunohistology using antisera directed against total or phosphorylated variants of the transporter (Fig. 7C). Loss of NKCC2 phosphorylation was seen in

![Fig. 3](image-url)  
**Fig. 3.** Salt handling in wild-type and SORLA-deficient mice. (A) Urinary sodium, chloride, and potassium concentrations in Sorla\(^{+/+}\) and Sorla\(^{-/-}\) mice with free access to water (water) or thirsted for 24 h (no water). (B) Total amounts of sodium and chloride excreted by Sorla\(^{+/+}\) and Sorla\(^{-/-}\) mice in 24 h. The mice had free access to water or were thirsted for 18 h (no water). The data are shown as percentages of the salt excreted by mice of the same genotype with access to water (set to 100%). Total excretion of sodium and chloride in 24 h in wild-type mice was reduced by approximately 50% upon thirsting (P < 0.01). Salt excretion in SORLA-deficient mice was not statistically different (n.s. when basal and thirsting conditions were compared. The data represent mean values plus standard errors of the mean. Statistical significance was determined using Student’s t test (n = 11 to 13 animals per genotype).

![Fig. 4](image-url)  
**Fig. 4.** Expression of ENaC in wild-type and Sorla\(^{-/-}\) mice. Western blot analysis of the α and γ subunits of ENaC in kidney extracts from four Sorla\(^{+/+}\) and four Sorla\(^{-/-}\) mice with (+) or without (−) access to drinking water. The amount of the α subunit is chronically increased in Sorla\(^{-/-}\) (lanes 5 to 8) compared with Sorla\(^{+/+}\) (lanes 1 to 4) mice. Expression of the γ subunit was strongly induced in thirsted Sorla\(^{-/-}\) mice (lanes 7 and 8) compared to all other conditions. Immunodetection of Na/K ATPase served as a loading control.

![Fig. 5](image-url)  
**Fig. 5.** Expression of Na\(^+\)/Cl\(^-\) cotransporter in kidney extracts from wild-type and Sorla\(^{-/-}\) mice. (A) Western blot analysis of total NCC and of the phosphorylated variants phospho-Thr\(^{55}\) (NCC T55-p) and phospho-Ser\(^{91}\) (S91-p) in renal extracts from wild-type and SORLA-deficient mice. The animals had free access to water (lanes 7 to 11) or were thirsted for 18 h (lanes 1 to 6). Immunodetection of GAPDH served as a loading control. (B) Densitometric scanning of the Western blots in panel A demonstrated a modest but statistically significant increase in total and NCC T55-p but a massive increase in NCC S91-p in SORLA-deficient mice compared to controls (set at 100%) (n = 5). The mean values plus standard errors of the mean are shown.
mutant mice under basal (Fig. 7) and under thirsting (data not shown) conditions.

Taking the data together, we concluded that loss of NKCC2 activity may be a primary consequence of SORLA deficiency and that upregulation of other sodium transport mechanisms represents secondary compensatory processes. The fact that alterations in all sodium transport pathways were seen in mice with free access to water supported our notion of a renal defect that already manifests itself under basal conditions.

The activity of NKCC2 in the TAL epithelium is intimately linked to the activities of other ion transport systems in this cell type. In line with low activity of NKCC2, the expression of the apical ROMK was reduced in SORLA-null kidneys, as documented by Western blotting (Fig. 8A and B) and immunohistochemistry (Fig. 8C). The expression levels of barttin, the \(\alpha/H\) subunit of the voltage-dependent chloride conductance channel \(\alpha/K\)-Cl cotransporter 4; and of the catalytic subunit of the \(\alpha/K\)-ATPase were unchanged (data not shown).

SPAK is a serine/threonine kinase implicated in functional activation of NKCC2. To test for SPAK expression, we immunoprecipitated the protein from renal extracts and performed Western blotting using antisera specific for total kinase or phosphorylated (active) variants of the kinase. Intriguingly, a massive increase in phosphorylated SPAK (pSPAK) but not in total SPAK was seen for receptor-deficient kidneys under normal and thirsting conditions despite the failure to phosphorylate NKCC2 (Fig. 9A). Densitometric scanning of the Western blot experiments showed 186.0 ± 19.1 relative intensity levels for pSPAK in \(\text{Sorla}^{-/-}\) versus 100.0 ± 15.5 in \(\text{Sorla}^{+/+}\) mice (\(P < 0.01\)) (Fig. 9B). Elevated levels of pSPAK in receptor-deficient animals were further substantiated by immunohistochemical analysis (Fig. 9C). Remarkably, strong apical signals for pSPAK in receptor-deficient kidneys were restricted to cells of the TAL, as shown by costaining with NKCC2. Less intense signals for pSPAK were seen in other renal cell types, such as the cells of the distal convoluted tubule (costained for NCC) (Fig. 9C). These data suggested that hyperphosphorylation of SPAK in the SORLA-deficient kidney is mainly relevant in the TAL, but not in other nephron segments.

Obviously, hyperactive SPAK in SORLA-deficient kidneys fails to activate NKCC2. Because activation of NKCC2 involves trafficking of the transporter between intracellular compartments and the cell surface, we explored a possible role for SORLA as a sorting protein for NKCC2 and/or its kinase, SPAK. Using confocal immunofluorescence microscopy, SORLA was localized to intracellular vesicles in the subapical region of TAL cells (Fig. 10, middle column). Likewise, staining for SPAK, pSPAK, and NKCC2 showed staining of the subapical region in this cell type. The pattern for pNKCC2 was different, with more restricted labeling of the apical cell membranes (Fig. 10, left column). This observation is in line with translocation of the activated transporter to the luminal cell surface of the TAL. In merged micrographs (Fig. 10, right column), partial colocalization of SORLA was seen with SPAK and pSPAK and to a minor extent with NKCC2 in the subapical space. No apparent colocalization of SORLA was seen with pNKCC2 at the apical plasma membrane.

Partial colocalization of SORLA with SPAK, pSPAK, and

**FIG. 6.** Expression of NHE3 in kidneys from wild-type and \(\text{Sorla}^{-/-}\) mice. (A) Western blot analysis of NHE3 and \(\alpha/K\)-ATPase (loading control) in total kidney extracts of \(\text{Sorla}^{+/+}\) (lanes 1 and 2) and \(\text{Sorla}^{-/-}\) (lanes 3 and 4) mice with free access to drinking water. (B) Densitometric scanning of Western blot experiments exemplified in panel A demonstrated a statistically significant increase in expression of NHE3 in SORLA-deficient mice under basal conditions (signal intensity in the wild type was set at 100%) (\(n = 4\)). The mean values plus standard errors of the mean are shown. (C and D) Immunohistological detection of NHE3 in sections from mouse kidneys confirmed upregulation of NHE3 expression in cells of the proximal tubules (asterisks in panel C) and the TAL (arrowheads in panel D) of SORLA-deficient compared with wild-type mice. Magnification, \(\times600\).
NKCC2 is consistent with the transient interaction of the receptor with confirmed ligands, such as APP (26). Accordingly, we tested whether lack of SORLA may affect the subcellular localization of these proteins in the TAL. The localization of total NKCC2 was not affected by absence of the receptor, as shown by immunofluorescence microscopy of wild-type and Sorla−/− kidneys (Fig. 11A, middle). This assumption was confirmed by immunoelectron microscopy, which demonstrated equal distribution of the transporter molecules between intracellular compartments and the apical cell surface (Fig. 12).

However, the situation was different for SPAK and pSPAK. In wild-type TAL cells, SPAK and pSPAK mainly colocalized with NKCC2 to vesicles in the immediate subapical compartment (Fig. 11A, right). In contrast, in TAL cells lacking SORLA, the signals for SPAK and pSPAK were partially mislocalized, as both proteins also appeared in vesicles more distant from the apical plasma membrane. These vesicular structures were devoid of NKCC2 signal (Fig. 11A, right column).

To further substantiate impaired colocalization of the kinase, the immunostaining patterns were evaluated using image analysis software (see Materials and Methods for details). In these analyses, the percentage of SPAK immunosignals that colocalized with NKCC2 dropped from 39.8% to 17.9% in Sorla−/− kidneys (Fig. 11A, right column). Accordingly, the overlap coefficient (after Manders) was reduced from 0.001.

FIG. 7. Expression of Na⁺-K⁺-Cl⁻ transporter 2 in wild-type and Sorla−/− mice. (A) Western blot analysis of total (NKCC2) and phosphorylated (p) variants of NKCC2 in kidney extracts from two Sorla+/+ (WT; lanes 1 and 2) and two Sorla−/− (KO; lanes 3 and 4) mice with free access to drinking water. Detection of actin served as a loading control. (B) Densitometric scanning of Western blot experiments as exemplified in panel A (n = 9) demonstrated a significant decrease in pNKCC2, but not in total NKCC2, signal in mutant mice (the wild-type signal was set at 100%). Identical findings were obtained in thirsted mice (data not shown). n.s., not significant. The mean values plus standard errors of the mean are shown. (C) Immunohistological detection of NKCC2 and pNKCC2 in Sorla+/+ and Sorla−/− kidneys. Magnification, ×400.

FIG. 8. Expression of ROMK in wild-type and Sorla−/− mice. (A) Western blot analysis of ROMK and actin (loading control) in kidney extracts from Sorla+/+ (WT) and Sorla−/− (KO) mice with free access to drinking water. Detection of actin served as a loading control. (B) Densitometric scanning of Western blot experiments as in panel A (n = 4) demonstrated a statistically significant decrease in expression of ROMK in Sorla−/− kidneys (Fig. 11A, middle). This assumption was confirmed by immunoelectron microscopy, which demonstrated equal distribution of the transporter molecules between intracellular compartments and the apical cell surface (Fig. 12). However, the situation was different for SPAK and pSPAK. In wild-type TAL cells, SPAK and pSPAK mainly colocalized with NKCC2 to vesicles in the immediate subapical compartment (Fig. 11A, right). In contrast, in TAL cells lacking SORLA, the signals for SPAK and pSPAK were partially mislocalized, as both proteins also appeared in vesicles more distant from the apical plasma membrane. These vesicular structures were devoid of NKCC2 signal (Fig. 11A, right column).
Reduced colocalization of NKCC2 and total SPAK was not caused by altered levels of expression but by displacement of total SPAK signals to more distal cell compartments devoid of NKCC2. This fact was demonstrated by cross-sectional quantification of the NKCC2 and SPAK immunoreactivity in TAL tubules (Fig. 11B).

All of the above-mentioned data suggested a potential role for SORLA in proper trafficking of SPAK/pSAPK in the TAL. To test the ability of SORLA to physically interact with SPAK in cells, HEK 293 cells that either expressed or lacked SORLA were transiently transfected with an expression construct for HA-tagged SPAK. Forty-eight hours later, proteins were immunoprecipitated with anti-SORLA IgG (Fig. 13A). Coimmunoprecipitation of HA-SPAK was seen in cells expressing SORLA (Fig. 13A, lane 4), but not in cells devoid of the receptor (Fig. 13A, lane 3). Coimmunoprecipitation of SPAK was also seen with a truncated polypeptide encompassing the intracellular domain (ICD) of SORLA only (Fig. 13B, lane 4). These findings confirmed the ability of the receptor to interact with SPAK through its cytosolic tail, substantiating its role as an intracellular sorting factor for the kinase.

**DISCUSSION**

Here, we have identified a potential role for the sorting receptor SORLA as a trafficking factor for SPAK that sorts the kinase into intracellular compartments where NKCC2 is local-
The absence of SORLA in gene-targeted mice results in missorting of SPAK and in an inability to activate NKCC2 by phosphorylation.

NKCC2 is a member of the cation-chloride cotransporter family, a group of electroneutral ion transporters (reviewed in reference 25). In contrast to other family members that are more widely expressed, expression of NKCC2 is restricted to cells in the TAL. NKCC2 is a central player in transepithelial transport of NaCl in this tissue. It is expressed on the apical surface of the epithelium and uses electrochemical gradients to take up Na\(^+/\)H\(^+\), Cl\(^-/\)H\(^+\), and K\(^+/\)H\(^+\) (25). Other components of the NaCl transport machinery in the TAL include ROMK, which circulates K\(^+/\)H\(^+\) back into the tubule fluid. Sodium is transported across the basolateral membrane by the Na\(^+/\)H\(^+\)/K\(^+/\)H\(^+\)-ATPase, whereas chloride exits via the chloride channels CLCNKA and CLCNKB (of which barttin is an essential subunit). A defect in salt handling in the TAL of SORLA-deficient mice is suggested not only by lack of phosphorylation of NKCC2, but also by the modest but significant reduction in ROMK expression, likely a consequence of reduced potassium intake into this cell type (Fig. 8). Also, compensatory upregulation of other renal sodium transport mechanisms, such as NHE3 (Fig. 6), NCC (Fig. 5), and ENaC (Fig. 4), support the notion that impaired NaCl transport by NKCC2 underlies the renal phenotypes seen in this model.

NKCC2 is subject to short-term regulation in response to cell volume and humoral stimulators. Two major regulatory steps involve phosphorylation and membrane translocation of the transporter (reviewed in reference 8). Activation by phosphorylation of Na\(^+/\)H\(^+\)-Cl\(^-/\)H\(^+\) cotransporters was first shown for shark NKCC1 (3, 6). Three threonine residues (T184, T189, T192) are targets for phosphorylation. These residues are conserved in mouse NKCC2. Phosphorylation of NKCC2 in response to volume expansion and chronic NaCl loading is reduced in SORLA-deficient mice (7, 9, 11). This is consistent with the reduced ROMK expression and NKCC2 phosphorylation in SORLA-deficient kidneys (Fig. 8). Given the close association of SPAK and pSPAK with NKCC2 in wild-type kidneys (Fig. 11) and the significant reduction in SPAK expression in SORLA-deficient kidneys (Fig. 8), a deficit in SPAK, pSPAK, or both is a likely explanation for the reduced level of NKCC2 phosphorylation in SORLA-deficient mice. The altered distribution of SPAK and pSPAK in SORLA-deficient kidneys (Fig. 11) suggests that SORLA may play a role in membrane sorting or transport of SPAK and pSPAK to the TAL in wild-type kidneys, consistent with the observed misrouting of SPAK in gene-targeted mice. However, the precise role of SORLA in transport of SPAK and pSPAK remains to be determined.

**FIG. 11.** Colocalization of NKCC2 with SPAK and pSPAK in kidneys from wild-type and SORLA-deficient mice. (A) Immunohistological detection of total or phosphorylated variants of SPAK (left column, red) and NKCC2 (middle column, green) in kidneys of the indicated genotypes. Colocalization of both proteins in cells of the TAL is indicated by yellow in the right column. Significant colocalization of NKCC2 with both SPAK and pSPAK is seen in wild-type cells. In contrast, SPAK- and pSPAK-positive vesicles that lack NKCC2 are apparent in the more distal cytoplasm of Sorla\(^/-\) cells (arrowheads). Magnification, ×1,000. (B) Cross-sectional distribution of immunofluorescence signals corresponding to total SPAK (red) and NKCC2 (green) in TAL segments from Sorla\(^+/+\) and Sorla\(^/-\) kidneys. Immunofluorescence signal intensities for both proteins were scored across individual TAL segments, as indicated in the inset, from the basolateral to the apical sides of the tubules. In wild-type tubules, peak intensity signals for SPAK and NKCC2 perfectly overlap at the apical tubular surface (indicated by the vertical lines). In contrast, in SORLA-deficient tubules, the main intensity peaks for SPAK are partially displaced and shifted more toward the subapical compartment. The data represent mean values from 8 to 14 tubules per mouse (3 mice per genotype).

**FIG. 12.** Subcellular localization of NKCC2 in kidneys from wild-type and SORLA-deficient mice. Total NKCC2 was detected in cells of the TAL in wild-type (A) and SORLA-deficient (B) kidneys using immunoelectron microscopy. NKCC2 was seen both at the apical cell surface (arrowheads) and in intracellular compartments in this cell type. Scale bar, 1 μm. (C) Ratio of immunogold particles representing total NKCC2 molecules at the cell surface to intracellular compartments. The ratio for wild-type cells was set at 100%. No difference in the ratios was seen between wild-type and Sorla\(^/-\) cells. A total of 802 (+/+) and 1,282 (−/−) silver grains of 24 tubules from 3 animals per genotype were counted. The mean values plus standard errors of the mean are shown.
in the TAL requires the activity of SORLA, a novel sorting receptor for SPAK, identifying a hitherto unknown regulatory mechanism to control kinase activity. As well as activation by phosphorylation, translocation of NKCC2 to the apical cell surface is equally important for transporter activity. Thus, application of vasopressin has been shown to increase the membrane-bound fraction of NKCC2 both in cells and in vivo (9, 13, 18). Where exactly in the cell NKCC2 is localized and what mechanisms mediate trafficking from intracellular compartments to the plasma membrane are incompletely understood. Several factors, including vesicle-associated membrane proteins (VAMPs) (18) and aldolase B (2), have been implicated in this process. Given the role of SORLA in cell surface trafficking of APP in neurons (1, 26), a similar function for this receptor in pNKCC2 translocation may be envisioned. Because SORLA-deficient TAL cells show little, if any, immunoreactivity for pNKCC2, it is impossible to test whether the phosphorylated transporter requires SORLA for sorting to the apical cell membrane. Typically, levels of total NKCC2 at the cell surface are only marginally increased by activation (2.6% versus 5.6% of the total NKCC2 fraction [18]).

In contrast to NKCC2, an obvious alteration in subcellular localization could be appreciated for SPAK in SORLA-deficient TAL cells (Fig. 11). Colocalization of SPAK with NKCC2 is seen in receptor-deficient TAL cells to some extent (17.9% ± 1% compared to 39.8% ± 4% in wild-type cells). This partial colocalization likely accounts for the residual phosphorylation of NKCC2 seen in our model (34%) (Fig. 7B). Still, a major fraction of the SPAK signals in SORLA-deficient cells of the TAL localizes to intracellular vesicles more distally from the subsapical space that lacks NKCC2 (Fig. 11B). These vesicles are not seen to the same extent in wild-type cells. A possible role for SORLA as a sorting receptor for SPAK is also supported by the ability of the two proteins to physically interact in cells (Fig. 13).

The physiological relevance of NKCC2 activity for renal ion homeostasis is documented by mutations in the transporter gene causing autosomal recessive Bartter syndrome type I (27). Patients suffering from this disease are characterized by disturbances in handling of renal salt and volume, including polyuria and enhanced excretion of potassium and calcium, as well as high aldosterone. Blood pressure is below normal. Similar defects are seen in mice with targeted Nkcc2 disruption that die from perinatal fluid wasting and dehydration (28). Clearly, mice lacking expression of SORLA share some (e.g., potassium wasting, hypercalciuria, hyperaldosteronemia, and hypertension), but not all, phenotypes seen in mouse models and in patients with NKCC2 deficiency (e.g., hypokalemia and polyuria), in agreement with impairment rather than complete absence of transporter activity in our mouse line. Strong support for SPAK deficiency as the underlying defect in renal abnormalities in SORLA-deficient mice comes from a recent description of a mouse model expressing a mutant variant of SPAK that cannot be activated by phosphorylation (20). Hypophosphorylation of NKCC2 as a consequence of impaired SPAK activity coincides with mild hypotension. Urine and serum electrolytes are normal under basal conditions, but distinct sodium and potassium wasting is seen under certain stress conditions, such as switching between high- and low-salt diets. All subunits of ENaC are induced (20).
In conclusion, the data presented in this study suggest a novel regulatory pathway in control of renal ion homeostasis and blood pressure. According to our model, SORLA mediates the transport of SPAK to intracellular compartments that contain NKCC2 (Fig. 14a). Upon stimulation (e.g., by osmotic stress), activated SPAK (pSPAK) phosphorylates NKCC2, which in turn translocates to the apical cell surface (Fig. 14b). In SORLA-deficient cells, mislocalization of SPAK results in failure to properly activate NKCC2 and in renal salt loss (Fig. 14c). As a consequence of imbalanced salt homeostasis, compensatory mechanisms take effect and alternate sodium transporters become hyperactive.

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