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**Vasopressin-inducible ubiquitin-specific protease 10 increases ENaC cell surface expression by deubiquitylating and stabilizing sorting nexin 3**

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Boulkroun S, Ruffieux-Daidié D, Vitagliano J-J, Poirot O, Charles R-P, Lagnaz D, Firsov D, Kellenberger S, Staub O. Vasopressin-inducible ubiquitin-specific protease 10 increases ENaC cell surface expression by deubiquitylating and stabilizing sorting nexin 3. Am J Physiol Renal Physiol 295: F889–F900, 2008. First published July 16, 2008; doi:10.1152/ajprenal.00001.2008.—Adjustment of Na$^+$ balance in extracellular fluids is achieved by regulated Na$^+$ transport involving the amiloride-sensitive epithelial Na$^+$ channel (ENaC) in the distal nephron. In this context, ENaC is controlled by a number of hormones, including vasopressin, which promotes rapid translocation of water and Na$^+$ channels to the plasma membrane and long-term effects on transcription of vasopressin-induced and -reduced transcripts. We have identified a mRNA encoding the deubiquitylating enzyme ubiquitin-specific protease 10 (Usp10), whose expression is increased by vasopressin at both the mRNA and the protein level. Coexpression of Usp10 in ENaC-transfected HEK-293 cells causes a more than fivefold increase in amiloride-sensitive Na$^+$ currents, as measured by whole cell patch clamping. This is accompanied by a three- to fourfold increase in surface expression of α- and γ-ENaC, as shown by cell surface biotinylation experiments. Although ENaC is well known to be regulated by its direct ubiquitylation, Usp10 does not affect the ubiquitylation level of ENaC, suggesting an indirect effect. A two-hybrid screen identified sorting nexin 3 (SNX3) as a novel substrate of Usp10. We show that it is a ubiquitylated protein that is degraded by the proteasome; interaction with Usp10 leads to its deubiquitylation and stabilization. When coexpressed with ENaC, SNX3 increases the channel’s cell surface expression, similarly to Usp10. In mCCD-1a cells, vasopressin increases SNX3 protein but not mRNA, supporting the idea that the vasopressin-induced Usp10 deubiquitylates and stabilizes endogenous SNX3 and consequently promotes cell surface expression of ENaC.

**SORTING NexIN 3; epithelial sodium channel**

Vertebrates must regulate salt and water excretion to maintain extracellular fluid volume, Na$^+$ homeostasis, and blood pressure. In the kidney, the fine regulation of Na$^+$ balance takes place in the aldosterone-sensitive distal nephron (ASDN) (28), where Na$^+$ enters into the cell through the apical epithelial Na$^+$ channel (ENaC) and is extruded through the basolateral Na$^+$–K$^+$–ATPase, a process highly regulated by aldosterone and in synergism with vasopressin (25, 52). Arginine vasopressin (AVP) is a polypeptide hormone that acts on the V2 vasopressin receptor, a member of the G protein-coupled receptor superfamily, leading to both increased water and Na$^+$ reabsorption (31, 32, 36). This process includes both short-term nongenomic effects and long-term genomic effects. Short-term vasopressin binding to the V2 receptors increases intracellular cAMP via G$\alpha$ proteins and adenylyl cyclase, causing activation of protein kinase A (PKA) and rapid translocation of ENaC (3, 12, 23, 33, 43) (for a review, see Refs. 39, 44) and water channels (aquaporin 2, AQP2) (15, 24) from an intracellular pool to the cell surface. Whereas it is generally accepted that phosphorylation of AQP2 and possibly other proteins is involved in AQP2 translocation to the apical membrane (for a review, see Ref. 36), this is less clear for ENaC. Direct phosphorylation of either ENaC (41) or other proteins such as the ubiquitin-protein ligase Nedd4-2 may be involved (45). Long-term genomic effects of vasopressin have been well documented and involve the increase of water, Na$^+$, and Cl$^-$ transport (10, 11, 13, 21, 22, 26). These effects occur through a cAMP-dependant transcriptional activation of a gene network including AQP2 (9, 35), Na$^+$–K$^+$–ATPase, ENaC, and the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (10, 12, 13). The mechanism of transcription-dependant regulation of vasopressin is not yet very well understood. It was proposed that the rise in cAMP leads to PKA-dependant phosphorylation of the transcription factor CREB [cAMP responsive element (CRE) binding protein], but a recent study showed that vasopressin stimulates the synthesis of AQP2 independently of PKA (51).

In recent years, two studies have been carried out to identify new vasopressin regulated transcripts [vasopressin-induced (VITs) and -reduced transcripts (VRTs)]. In 2002, Courtin-Courty et al. (8) performed subtractive hybridization experiments in RCCD1 cortical collecting duct cells and identified calcyclin as an early AVP-induced gene. It was demonstrated that this early vasopressin-induced gene product participates in the late phase of the hormone response in transepithelial ion transport. In the other study, Robert-Nicoud et al. (37) performed a serial analysis of gene expression (SAGE) on untreated or vasopressin-induced renal epithelial mpkCCDCl4 cells. Forty-eight VITs and 11 VRTs were identified, and some of them validated, in the mpkCCDCl4 cell line by either Northern blot hybridization or RT-PCR. Of interest among the VITs was ubiquitin-specific protease 10 (Usp10), a deubiquitylating enzyme. We followed up on this protein because ubiquitylation (i.e., the covalent modification of a target pro-

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tein with ubiquitin polypeptides) plays an important role in
ENaC regulation (for review, see Refs. 30, 44, 49).

METHODS

Cell culture and transfection procedure. mCCDcl1 cells (16) were
used between passages 10 and 23. Cells were cultured in complete
medium containing Dulbecco’s modified Eagle’s medium (DMEM)-
F12 medium (1:1), 5 μg/ml insulin, 5 μg/ml transferrin, 6 × 10⁻⁸ M
sodium selenate, 5 × 10⁻⁹ M dexamethasone, 1 nM triiodothyronine,
10 ng/ml epidermal growth factor, 10 mM HEPES, pH 7.4, and 2%
fetal calf serum. For experiments, cells were grown on transwell filters
coated with collagen, cultured the first 5 days in complete medium,
and then for 5 more days in filter medium containing DMEM-F12
medium (1:1), 5 μg/ml insulin, 6 × 10⁻⁸ M sodium selenate, 3 ×
10⁻⁹ M dexamethasone, 1 nM triiodothyronine and 10 mM HEPES,
pH 7.4. Before experiments, cells were incubated overnight in mini-
imal medium [DMEM-F12 (1:1) and 10 mM HEPES, pH 7.4] and then
treated or not with 10 nM AVP (Sigma) for 4 h. Collagen was
prepared as previously described (16). HEK-293 cells were used
between passages 6 and 40. Cells were cultured in medium containing

Fig. 1. Ubiquitin-specific protease 10 (Usp10) mRNA and protein are induced by vasopressin (AVP) in mCCDcl1 cells. mCCDcl1 cells grown on collagen-coated permeable filters were treated for 4 h with 10 nM AVP or vehicle. A: transepithelial currents were measured using the short-circuit (Isc) method (n = 9 filters from 3 independent experiments). B: quantitative real-time PCR analysis, normalized to hypoxanthine phosphoribosyltransferase 1 (Hprt1) and control conditions (n = 3). C: representative Western blots showing expression of Usp10 and actin. D: quantification of Usp10 expression in untreated or vasopressin-induced cells, normalized to actin. *P < 0.05; ***P < 0.001. E: Usp10 confocal staining of cells grown on filters with or without AVP treatment. The changes in localization of Usp10 are shown from a homogeneous distribution to a denser and granulated one.
DMEM and 10% fetal bovine serum (FBS). Cells were transiently transfected using the calcium phosphate method. HEK-293 cells stably transfected with the three ENaC subunits were raised and cultured as described by Ruffieux-Daidié et al. (38). These cells were transfected with Lipofectamine (Invitrogen).

**DNA constructs.** Rat hemagglutinin (HA)-tagged α-ENaC in pCMV4, rat β-ENaC in pCB6, and rat γ-ENaC, tagged either with VSV-G [in pcDNA3.1(+)Zeo] or flag (in pCB6) were described previously (5, 38, 47, 48). Mouse Usp10 was generated by PCR and subcloned into KpnI-XhoI-digested PET30a(+) (Novagen) to add S- and (His)6 tag and was then subcloned into XbaI-XhoI-digested pcDNA3.1(−) (Invitrogen). The Cys418Ala mutation was introduced into Usp10 by using a PCR approach. The 5'-GGAAACTGGGCCTACATTAATGC-3' and 5'-CCTTTGACCCGGATGTAATTACG-3' primers were used to mutate the Cys418 codon. Mouse HA- and S-tagged sorting nexin 3 (SNX3) were generated by PCR and subcloned into EcoRI-digested pcDNA3.1(+) (Invitrogen). Mouse HA-tagged SNX3 was also subcloned into XhoI-digested pGEX4-T1. All the constructs generated by PCR were verified by DNA sequencing.

**Electrophysiological measurements and analysis.** Electrophysiological measurements were done in HEK-293 cells stably transfected with the three ENaC subunits, with α-ENaC under the control of a dexamethasone-inducible promoter, as described previously (38). The measurements were done 24–48 h after induction of the ENaC α-subunit. Macroscopic amiloride-sensitive currents, defined as the difference between ionic currents obtained in the presence and ab-

![Fig. 2. Characterization of anti-Usp10 antibody. Lysates of HEK-293 cells (control or transfected with Usp10) or mCCDcl1 cells (control or stimulated with 10 nM AVP) were analyzed by SDS-PAGE/Western blotting using anti-Usp10 antibodies.](image)

![Fig. 3. Usp10 induces amiloride-sensitive Na⁺ currents in epithelial Na⁺ channel α,β,γ-subunit (α,β,γ-ENaC) stably transfected HEK 293 cells. Cells were transfected with either wild-type or mutant (CA) Usp10 or with an empty plasmid (pcDNA3). After 24 h, cells were diluted by trypsinization and induced with 1 μM dexamethasone for another 24 h. Amiloride-sensitive currents were then measured by whole cell patch clamp at a holding potential of −60 mV. A: amiloride-sensitive current densities (current amplitude normalized to cell capacitance) are shown for the 3 conditions (n = 26–36 per condition). The inhibition by amiloride (n = 5; B) and the current-voltage relationship for the amiloride-sensitive current (C; n = 5) are shown for the cells coexpressing ENaC subunits with Usp10. I_amiloride, amiloride-sensitive current.](image)
sence of 10 μM amiloride in the bath, were recorded using the
patch-clamp technique in the whole cell configuration at room
temperature. All macroscopic currents shown are amiloride-sensitive
currents as defined above. We used an EPC-10 amplifier (HEKA
Electronics, Lambrecht, Germany) and Pulse and PulseFit software
for data acquisition and analysis. The sampling interval was 5 ms for
all measurements except for current-voltage relationships, in which it
was 100 μs. Filtering was set to 5 kHz. For changes of extracellular
solutions, we used a micromanifold that brings eight tubes into one
outlet tube (MPRE8; Cell MicroControls, Norfolk, VA). The solution
flow was controlled by computer-driven solenoid valves. The standard
extracellular solution contained 140 mM NaCl, 3 mM KCl, 2 mM
CaCl₂, 1 mM MgCl₂, 10 mM MES, 10 mM HEPES, and 10 mM
glucose, and pH was adjusted to 7.4 with NaOH. For selectivity
measurements, we used solutions containing 10 mM MES, 10 mM
HEPES, and 10 mM glucose with either 140 mM NaCl or 140 mM
KCl, and pH was adjusted to 7.4 with Tris base. Pipettes were pulled
from borosilicate glass (World Precision Instruments, Sarasota, FL).
Pipettes had a resistance of 1–3 MΩ when filled with the pipette
solution. The pipette solution contained 90 mM potassium gluconate,
10 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 60 mM HEPES, and 10 mM
EGTA, with pH 7.3 adjusted using KOH. All chemicals were obtained
from Sigma or Fluka (Buchs, Switzerland). Except for current-voltage
curves, whole cell currents were measured at
60 mV.

Immunohistochemistry. Immunohistochemistry was performed on
cells grown on filters. After 1× PBS washes, cells were fixed for 10
min with 4% paraformaldehyde and permeabilized for 10 min with
Triton X-100 0.2%. Primary (anti-Usp10) and secondary antibodies
(Alexa 488-conjugated anti-rabbit IgG; Invitrogen Molecular Probes)
were incubated for 1 h at room temperature. Staining was visualized
using laser scanning microscopy (LSM) with a confocal microscope
(model LSMS10 Meta; Carl Zeiss MicroImaging). Nuclei were counter-
tastered with 0.2 μg/ml 4,6-diamidino-2-phenylindole in mounting
medium (glycergel; DAKO, Basel, Switzerland). No signal was
detected when the primary anti-Usp10 antibody was omitted.

Two-hybrid screen. The NH₂-terminal part of Usp10 (nt 25-925)
was amplified by PCR and cloned into pGBK7, a plasmid carrying
the TRP1 selection marker and a myc epitope (Clontech). These
constructs were used to screen a cDNA library derived from micro-
dissected mouse cortical collecting duct cells (in a pGADT7-rec
plasmid; Clontech). Fifty-five positive clones were identified, two of
which encoded SNX3.

Quantitative real-time PCR. Total RNA was extracted from
mCCD1a1 cells with Trizol (Invitrogen) according to the manufactur-
er’s instructions, and reverse transcription was carried out using
SuperScript II reverse transcriptase (Invitrogen). Quantitative real-
time PCR analysis of Usp10 was done on an ABI 7500 sequence
detector (Applied Biosystems). The primers were obtained from
Applied Biosystems (corresponding to the following order num-
bers: Usp10, Mm00494058_m1; SNX3, Mm00444384_m1; and
HPRT1, Mm01545399_m1). The reactions were done according to
the manufacturer’s instructions; each reaction was done in tripli-

Fig. 4. Usp10 increased the total pool and cell surface expression of α- and γ-ENaC. HEK-293 cells were transiently cotransfected with hemagglutinin
(HA)-tagged α-ENaC, β-ENaC, and Flag-tagged γ-ENaC with or without wild-type or inactive Usp10 (CA). After 24 h, biontination experiments
were performed. Total (A) and biontinated proteins (B) were analyzed by SDS-PAGE/Western blotting (WB) using anti-Usp10, anti-HA (α-ENaC), anti-VSV-G [VSV
(γ-ENaC)], anti-α-Na⁺-K⁺-ATPase (α-NaK), and anti-actin antibodies. The levels of α- and γ-ENaC and the α-NaK were quantified on a molecular imager FX.
Values were normalized to the control (ENaC alone) and are means ± SE (n = 3–6 experiments). *P < 0.05; ***P < 0.001.
cate. The data were analyzed using the software provided with the instrument, and results were normalized to hypoxanthine phosphoribosyltransferase 1 (Hprt1) and to the control condition.

**Antibodies.** Polyclonal anti-Usp10 antibodies were generated by Cocalico Biologicals (Reamstown, PA). Rabbits were injected with the purified NH2-terminal part of Usp10 (amino acids 1-300) fused to glutathione S-transferase (GST). Antibodies against the α-subunit of the Na+–K+-ATPase were described previously (17). Rabbit anti-actin, mouse anti-VSV-G, and anti-flag (M2) were purchased from Sigma. Mouse anti-HA and goat anti-SNX3 were obtained from Santa Cruz Biotechnology, mouse anti-ubiquitin (FK2) was obtained from Biomol, and biotinylated S-protein was obtained from Novagen. ENaC antibodies were described previously (27).

**Cell surface biotinylation.** Twenty-four hours after transfection of HEK-293 cells with cDNA encoding ENaC, Usp10, and/or SNX3, cells were washed twice with 1× cold PBS and then incubated for 30 min at 4°C with 1.2 mg of biotin (EZ-link sulfo-NHS-SS-biotin). Cells were washed twice with 1× cold Tris-buffered saline, and the protein extracts were prepared by scraping the cells directly in lysis buffer: 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, pH 8.0, 10% glycerol, Triton X-100, 1.2 mg/ml N-ethylmaleimide (NEM), and Complete protease inhibitor cocktail (1 tablet/20 ml; Roche). Proteins were solubilized for 3 h at 4°C, under end-over-end rotation, and then centrifuged at 20,000 g for 30 min at 4°C. Supernatants were assayed for total protein using the Bradford method (Brunschwig) according to the instructions provided. For immunoprecipitation of biotinylated proteins, 1–3 mg of cell lysates were diluted to a final volume of 1 ml with lysis buffer and incubated in the presence of 30 μl of streptavidin Sepharose overnight at 4°C on a gentle rotating mixer. Beads were washed five times with lysis buffer containing 1 mM PMSF, resuspended in 100 mM DTT, and incubated for 1 h at 37°C with agitation. After centrifugation for 1 min at 5,000 g, the supernatant was removed and the beads were washed five times with 1× cold PBS. Beads were then resuspended in 2× SDS-PAGE buffer and boiled for 2 min. After centrifugation, the supernatant was removed and the protein concentration was determined using the Bradford method. After SDS-PAGE/WB using anti-α-ENaC, anti-γ-ENaC, or anti-ubiquitin (Ub) antibodies, the blots were stained with anti-Usp10 antibodies. A: representative blots for α- and γ-ENaC. B: quantification of 3 experiments. Results are expressed as the ratio between ubiquitylated material (A: top) and immunoprecipitated ENaC (A; bottom). IP, immunoprecipitate.

**Fig. 5.** Usp10 does not affect the ubiquitylation level of ENaC. HEK-293 cells were transfected with HA-tagged α-ENaC, β-ENaC, and Flag-tagged γ-ENaC with or without Usp10. After 24 h, cells were lysed and α-ENaC was immunoprecipitated using HA- and γ-ENaC with Flag antibodies, and the immunoprecipitated material was analyzed by SDS-PAGE/WB using anti-α-ENaC, anti-γ-ENaC, or anti-ubiquitin (Ub) antibodies.

**Fig. 6.** Usp10 interacts with sorting nexin 3 (SNX3) in vitro. Pull-down experiments were performed using glutathione S-transferase (GST) or GST-SNX3 fusion proteins. HEK-293 cells were transfected (or not; nt) with Usp10 or with a control plasmid (mock). Lysates were collected after 24 h, and pull-down experiments were carried out using immobilized GST or GST-SNX3 fusion protein. Precipitated proteins were analyzed by SDS-PAGE/WB using Usp10 antibodies.
rpm and 4°C, supernatant was collected and protein sample buffer added. Proteins were analyzed by SDS-PAGE/Western blotting using antibodies directed against tagged ENaC and anti-actin antibodies (negative control for biotinylated fraction).

**Immunoprecipitation.** For immunoprecipitation experiments, HEK-293 cells were used 24 h after transfection. Cells were washed once in 1× PBS and then scraped in 1 ml of cell dissociation buffer: 5% glycerol, 1 mM EDTA, pH 8.0, and 1 mM EGTA, pH 8.0, in 1× PBS. After centrifugation for 5 min at 3,000 rpm and 4°C, the pellet was frozen at −70°C for at least 15 min and then resuspended in 1 ml of lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, pH 8.0, 10% glycerol, 1% Triton X-100, 1.2 mg/ml NEM, and Complete protease inhibitor cocktail (1 tablet/20 ml; Roche). Prior use 1 mM DTT, 100 mM sodium fluoride, and 10 mM disodium pyro-

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Fig. 7. Usp10 stabilized the expression of SNX3 by regulation of its ubiquitylation levels. A: HEK-293 cells were transfected with HA-SNX3, Usp10, or Usp10-CA (as indicated). Twenty-four hours after transfection, cells were lysed and SNX3 was immunoprecipitated with anti-HA antibodies and analyzed with either anti-SNX3 or anti-ubiquitin antibodies. The proteins were quantified, and the ratio between ubiquitylated material (Ub) and precipitated HA-SNX3 was calculated (n = 3 experiments). Asterisk indicates nonspecific band. B: HEK-293 cells were transfected with HA-tagged SNX3, Usp10, and Usp10-CA (as indicated). After 24 h, cells were lysed, and lysates were analyzed by SDS-PAGE/WB using anti-Usp10, HA-SNX3, and actin antibodies. The HA bands were quantified and normalized against actin and SNX3 control (transfected with SNX; n = 3 for each condition). *P < 0.05. C: HEK-293 cells were transfected or not with HA-tagged SNX3. After 24 h, cells were treated with 10 μM lactacystin for the indicated times and then lysed. SDS-PAGE/WB was done using anti-HA and -actin antibodies.
phosphate were added to the lysis buffer. Proteins were solubilized for 30 min at 4°C under rotation. After centrifugation for 15 min at 14,000 rpm and 4°C, supernatants were collected and assayed for total protein. Proteins (0.2–1 mg) were incubated for 2.5 h at 4°C under rotation with the antibody directed against Usp10 (1:250), HA for α-ENaC (Santa Cruz; 1:250), or Flag-M2 agarose (Sigma; 1:250) for γ-ENaC. In the last case, incubation time was 4 h. Immunoprecipitates were then incubated with protein A-Sepharose beads (Amersham) for Usp10 or protein G agarose (Amersham) for HA at 4°C for 1.5 h. Beads were washed three times with 1 ml of wash buffer: 50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EGTA, pH 8.0, 10% glycerol, 1% Triton X-100, and 1.5 mM MgCl2 and resuspended in 50 μl of 2X sample buffer. Samples of eluted immunoprecipitates were submitted to 8% SDS-PAGE and transferred onto nitrocellulose membrane.

**Pull-down experiments.** SNX3 (nt 1-486) was amplified by PCR using Pfu polymerase (Promega) and subcloned into pGEX4-T1 (Pharmacia). The constructed plasmid was transformed into BL21, and protein expression was induced for 4 h with 1 mM isopropylthiogalactoside. Proteins were then affinity purified with glutathione-agarose beads and used directly for pull-down experiments. The concentration of the protein was estimated by Coomassie blue staining on SDS-PAGE. For the pull-down experiments, HEK cells were transfected with Usp10 or empty vector. After 24 h of transfection, cells were scraped directly in 1 ml of lysis buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton, 1.2 mg/ml NEM, and EDTA-free Complete protease inhibitor cocktail (1 tablet/20 ml; Roche). Each lysate (1 mg) was incubated with 3 μg of GST-SNX3 coupled with glutathione-agarose beads for 2 h at 4°C under rotation. Beads were then washed three times with wash buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton, and 1 mM PMSF and resuspended in 35 μl of 2X sample buffer. Samples were submitted to 10% SDS-PAGE and transferred onto nitrocellulose membrane.

**Ubiquitylation of α-ENaC or SNX3.** HEK-293 cells grown on 75-cm² dishes were transfected using the calcium phosphate technique with 4.5 μg of the plasmids encoding rat ENaC subunits (HA-tagged α-ENaC, β-ENaC, and Flag-tagged γ-ENaC) or HA-tagged SNX3 and 13.5 μg of Usp10. After 24 h, cells were washed with 10 ml of PBS and dissociated in 1 ml of dissociation buffer (5% glycerol, 1 mM EDTA, and 1 mM EGTA, pH 8.0). Cells were recovered with 10 ml of PBS, centrifuged for 2 min at 4°C and 1,500 g, and pellets were frozen at −70°C. Cells were lysed in 1 ml of buffer containing 50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 0.2% Triton X-100, 1 mM dithiothreitol, 100 mM NaF, 10 mM disodium pyrophosphate, 1 mM PMSF, and 1X protease cocktail (Roche) and then centrifuged for 15 min at 21,500 g (4°C). Supernatants were recovered, and immunoprecipitation was carried out with anti-HA antibodies (recognizing HA-tagged rat α-ENaC or SNX3; Santa Cruz) or anti-Flag antibodies (recognizing Flag-tagged rat γ-ENaC; M2, Sigma) for 2.5 h. Immunocomplexes were recovered with protein G-Sepharose during 1.25 h at 4°C on a rotating wheel. Protein G-Sepharose was washed with washing buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 0.2% Triton X-100, 1.5 mM MgCl2, and 1 mM PMSF), and the immune complexes were recovered in SDS-PAGE sample buffer. The samples were analyzed on 8 or 5–15% SDS-PAGE and Western blotting using rat anti-α-ENaC, rat γ-ENaC antibodies, anti-HA antibodies (SNX3), or anti-ubiquitin antibodies (FK2).

**Statistical analysis.** Data are means ± SE. Statistical analysis was performed using Student’s t-test.

**RESULTS**

Usp10 expression is increased by vasopressin treatment in mCCDcl1 cells. Usp10 was previously identified in a SAGE screen as a VIT in the mouse cortical collecting duct cell line nppCCDCl4 (2, 37). To confirm the effect on Usp10 expression in another, novel cell line derived from the murine cortical collecting duct (mCCDcl1; Ref. 16), we treated these cells for 4 h with 10 nM vasopressin. Figure 1A shows that this induced a non-significant increase of the short-circuit current (Isc): 9.33 ±

![AJP-Renal Physiol • VOL 295 • OCTOBER 2008 • www.ajprenal.org](image-url)
0.93 μA/cm² for control vs. 35.30 ± 2.79 μA/cm². This was accompanied by a 2.3-fold increase in expression of Usp10 mRNA (relative to Hprt1) as determined by quantitative real-time PCR (Fig. 1B). To follow Usp10 protein expression, we generated an anti-Usp10 antibody directed against the NH₂-terminal part of Usp10. This antibody recognizes specifically Usp10, as shown in Fig. 2. Using this antibody, we observed a 4.8-fold increase of the Usp10 protein when mCCD₁₁ cells were stimulated with vasopressin (Fig. 1B). Moreover, immunofluorescence studies showed that the cytoplasmic distribution of Usp10 in mCCD₁₁ cells changed to a more punctuate distribution when cells were treated with vasopressin (Fig. 1E).

Usp10 stimulates ENaC currents and cell surface expression but not its ubiquitylation level. Because ENaC density has been shown to be negatively regulated by Nedd4-2-dependent ubiquitylation, we wondered whether Usp10 increases channel activity and surface expression via deubiquitylation of ENaC. We used stably transfected HEK-293 cells that express all three subunits of ENaC (38) and monitored amiloride-sensitive Na⁺ currents by using the whole cell patch-clamp technique (Fig. 3). As observed previously, control cells expressing only the ENaC subunits displayed relatively small currents with a current density of 2.6 ± 0.9 pA/pF. When wild-type Usp10 was cotransfected, these currents increased fourfold to 11.0 ± 3.1 pA/pF, whereas coexpression of the inactive Usp10-C418A mutant did not increase the current density and even had a slight, but not significant, inhibitory effect, consistent with a role of Usp10 in ENaC regulation (Fig. 3A). The current measured in cells coexpressing ENaC with Usp10 was inhibited by amiloride with an IC₅₀ value of 128 ± 9 nM (Fig. 3B), and the current-voltage relationship had a reversal potential of +55 mV, consistent with a Na⁺-selective current (Fig. 3C). We determined the Na⁺/K⁺ current ratio at −100 mV as 50 ± 25 (n = 9) by replacing the extracellular monovalent cation from Na⁺ to K⁺. These properties of the Usp10-induced currents correspond to the known properties of ENaC.

To investigate whether the effect was due to changes in channel density, we carried out cell surface biotinylation experiments. We coexpressed in HEK-293 cells all three ENaC subunits and either wild-type or catalytically inactive Usp10 (Usp10-CA). Twenty-four hours after transfection, cells were surface biotinylated and lysed, and the biotinylated proteins were precipitated with streptavidin Sepharose. Total lysates and biotinylated proteins were analyzed by SDS-PAGE/Western blotting using antibodies against tagged ENaC (Fig. 4). We found that expression of wild-type Usp10, but not its inactive mutant form (Usp10-CA), increased the steady-state level of α- and γ-ENaC subunits in total cell lysates twofold (Fig. 4A). This was accompanied by a simultaneous three- to fourfold rise of α- and γ-ENaC at cell surface expression of ENaC as evidenced by the biotinylation experiments (Fig. 4B), corresponding well with the observed augmentation in ENaC currents (Fig. 3). On the other hand, the α-subunit of the Na⁺-K⁺-ATPase was not affected either in the lysate or at the cell surface. Because ENaC is a protein that is regulated by direct ubiquitylation of its subunits (49), we wondered whether Usp10 increased ENaC expression by deubiquitylating the ENaC subunits. Therefore, we coexpressed Usp10 with ENaC and immunoprecipitated either the α- or the γ-subunit. The precipitated proteins were analyzed by SDS-PAGE/Western blotting using either anti-ENaC or anti-ubiquitin antibodies.
(Fig. 5A). We found that the relative ENaC ubiquitylation (ratio of ubiquitylated ENaC to total ENaC) level was not affected by Usp10 (Fig. 5B), suggesting that it affects ENaC expression indirectly, possibly via deubiquitylation of another protein.

**Usp10 interacts with SNX3 and regulates its steady-state level through deubiquitylation.** To identify Usp10 targets, we carried out a two-hybrid screen using the NH2-terminal region of Usp10 (amino acids 1-300) and screened a cDNA library derived from microdissected cortical collecting duct cells. With this bait, we identified two clones containing cDNA encoding SNX3. SNX3 is part of a larger protein family, the sorting nexins, that are characterized by the presence of a phosphatidylinositol-3-phosphate [PtdIns(3)P] binding Phox or PX-domain (40, 53). Interestingly, many sorting nexins play a role in intracellular protein traffic (40, 53). SNX3 is the smallest member of the family, just composed of the Phox domain and short NH2-terminal and COOH-terminal extensions. It is localized to the sorting endosomes and vesicular intermediates that are involved in transport to the recycling endosome (54). We biochemically confirmed the interaction between Usp10 and SNX3 by performing a GST pull-down experiment as shown in Fig. 6. In these experiments, Usp10 was transfected into HEK-293 cells, the cells were lysed, and the proteins were pulled down with immobilized GST-SNX3 or GST alone. Usp10 bound only to GST-SNX3, confirming the interaction between the two proteins.

We wondered whether SNX3 is a substrate of Usp10, i.e., is deubiquitylated by Usp10. Therefore, we expressed HA-tagged SNX3 together with wild-type or mutant Usp10 in HEK-293 cells and immunoprecipitated SNX3 with anti-HA antibodies (Fig. 7A). Immunoprecipitates were immunoblotted with either an anti-SNX3 antibody (bottom) or an anti-ubiquitin antibody (top). Overexpression of Usp10 (but not its inactive mutant Usp10-CA) led to an increase in SNX3 protein (Fig. 7A, bottom), as expected for a protein that becomes deubiquitylated and consequently stabilized. In parallel, relative SNX3 ubiquitylation levels (ratio of ubiquitylated SNX3 to total SNX3) decreased when Usp10 (but not its mutant) was coexpressed (Fig. 7A, top), compatible with the idea that Usp10 deubiquitylates SNX3. Ubiquitylation of cytosolic proteins is generally associated with rapid degradation of these proteins by the proteasome (18). We therefore determined whether Usp10 has an effect on the total pool of SNX3. Comparable with the observed elevated quantity of immunoprecipitable HA-SNX3 in Fig. 7A, Usp10 caused a more than fourfold increase in SNX3 levels, whereas its catalytic inactive mutant had no significant effect (Fig. 7B). We also studied the effect of the protease inhibitor lactacystin on steady-state levels of HA-SNX3 and found that 10 μM lactacystin increased SNX3-HA levels in a time-dependent fashion (Fig. 7C). Hence, SNX3 is a ubiquitylated protein that interacts with and is deubiquitylated by Usp10. Deubiquitylation of SNX3 stabilizes the protein, which is a substrate of the proteasome.

**SNX3 increases ENaC steady-state levels and cell surface density.** We wanted to know whether SNX3 modifies the total pool and cell surface expression of ENaC, similarly to Usp10. Thus we overexpressed SNX3 in the presence of ENaC and monitored ENaC steady-state levels and the biotinylated surface pool as we did previously with Usp10 (Fig. 8). As with Usp10, SNX3 increased the total pool of α- and, to a more variable extent, that of γ-ENaC (Fig. 8A). Moreover, the expression levels of both ENaC subunits at the cell surface increased three- to fourfold (Fig. 8B). The observation that Usp10 and SNX3 have similar effects on ENaC is compatible with the idea that Usp10 acts on ENaC via stabilization of SNX3.

**Usp10 and SNX3 interact with ENaC.** We wondered whether Usp10 and SNX3 interact with ENaC to regulate the channel. We transfected cells with all three ENaC subunits and with S-tagged Usp10, Usp10-CA (Fig. 9A), or SNX3 (Fig. 9B). Cells were lysed, Usp10 or SNX3 was precipitated with biotinylated S-protein (recognizing the S-tag) and streptavidin agarose, and the precipitated material was analyzed by SDS-PAGE/Western blotting using anti-HA (recognizing HA-tagged α-ENaC), anti-Usp10, or anti-SNX3 antibody. α-ENaC coprecipitated with both Usp10 (wild type and mutant) and SNX3, demonstrating that these proteins interact directly or indirectly with ENaC.

**Vasopressin induces SNX3 protein but not SNX3 mRNA.** In the SAGE study performed in mpkCCDcl4 cells (37), SNX3 was not identified has a VIT. The finding that Usp10 stabilizes SNX3 by deubiquitylation may suggest that SNX3 is regulated by vasopressin at the posttranslational level. We carried out real-time PCR analysis on both mpkCCDcl4 (not shown) and mCCDcl1 cells (Fig. 10A) and confirmed that SNX3 mRNA was not upregulated by vasopressin. On the other hand, Western blots on lysates of control and vasopressin-stimulated mCCD cells revealed that vasopressin promoted a 1.65-fold increase in SNX3 protein but not mRNA. mCCDcl1 cells grown on collagen-coated filters were treated 4 h with 10 nM AVP. A: RNA was extracted and used for real-time PCR analysis of SNX3 mRNA. Data are normalized to Hprt1 and control conditions. **P < 0.005.
increase in SNX3 protein levels compared with nonstimulated cells (Fig. 10B). Altogether, these results support the idea that increased expression of Usp10 leads to elevated SNX3 protein levels, independently of SNX3 transcription. The increase in SNX3 then causes an upregulation of ENaC at the total pool and at the plasma membrane.

DISCUSSION

In the present work, we report that long-term stimulation with vasopressin (4 h) of cells derived from the cortical collecting duct leads to a hitherto unrecognized mode of regulation of ENaC whereby vasopressin stimulates expression of Usp10 mRNA and protein, the latter interacting with both ENaC and the trafficking protein SNX3. These interactions influence not the ubiquitylation levels of ENaC but those of SNX3, leading to stabilization of the protein. The raise in SNX3 levels provokes an increase of both the total and the cell surface pool of ENaC.

It is well established that ENaC is regulated by the ubiquitin system, involving the ubiquitin-protein ligase Nedd4-2, which interacts via its WW domains with the PY motifs of ENaC and ubiquitylates the channel (1, 20, 46, 47, 55). This ubiquitylation leads to the internalization and subsequent degradation of ENaC (1, 29, 48, 55). Importantly, this regulation is defective in Liddle’s syndrome, an inherited form of salt-sensitive hypertension caused by mutations in the genes encoding β- or γ-ENaC that inactivate the PY-motifs in ENaC (19, 42). In recent years it has become obvious that ubiquitylation is a reversible process, involving deubiquitylation enzymes (DUBs; for a review, see Ref. 34). Indeed, two groups have recently reported that ENaC is regulated via deubiquitylation. Pakitasas et al. (14) found that the aldosterone-induced Usp2-45 is able to stimulate ENaC activity when coexpressed in Xenopus laevis oocytes or in mpkCCD14 cells and that it directly deubiquitylates ENaC, thereby likely interfering with internalization of ENaC. Moreover, we showed that Usp2-45 regulates both ENaC internalization and proteolytic cleavage (38). On the other hand, Butterworth et al. (4) identified another deubiquitylating enzyme, UCH-L3, using a chemical probe for active DUBs. Blocking UCH-L3 activity was shown to reduce channel numbers at the plasma membrane, to interfere with transepithelial Na⁺ transport in mpkCCD14 cells, and to increase ubiquitylation levels of α- and γ-ENaC. Hence, both these enzymes appear to directly modify the ubiquitylation status of ENaC.

The vasopressin-induced Usp10 enzyme identified in this study regulates ENaC via a different, indirect mechanism. We observe that the ubiquitylation signal of ENaC is proportionally increased to the total level of α-ENaC. This elevated polyubiquitylation of ENaC may be due to increased endoplasmic reticulum (ER)-associated degradation (ERAD) activity with respect to ENaC (38). Instead of deubiquitylating ENaC, Usp10 does so with SNX3. It increases the expression levels of SNX3 via deubiquitylation and by reducing proteasomal degradation (as evidenced in Fig. 7). Because ubiquitylation levels of SNX3 do not disappear completely with Usp10, we cannot exclude that Usp10 may affect SNX3 expression also via other mechanisms, for example, via regulation of a modulatory protein influencing SNX3. The finding that overexpression of both Usp10 and SNX3 similarly leads to an increase in ENaC levels, both in the total lysate and at the cell surface, strongly supports the idea that Usp10 acts via stabilization of SNX3. How does SNX3 function? The protein, which essentially consists of a PtdIns(3)P binding PX domain and just a few additional amino acids on both sides, has been shown to be localized to a subset of the early endosome and recycling endosome and appears to be involved in early to recycling endosome transport (54). Overexpression of SNX3 has been demonstrated to interfere with EGF degradation. On the other hand, the yeast homolog of SNX3, Grd19p, has been found to be involved as an adaptor protein in retromer-dependent recycling of the yeast reductive ion transporter to the plasma membrane (50). There is also a role for Usp10 in protein traffic in yeast. Its homolog, Ubp3, has been shown to deubiquitylate and stabilize Sec23p, which is part of the COPII coat complex involved in ER-to-Golgi transport (6) and of the β’-COP subunit of the COPI coat (Golgi-to-ER transport) (7). It is therefore conceivable that Usp10 together with SNX3 promotes export of ENaC via the secretory pathway to the plasma membrane. Our unpublished data did not provide any evidence for a change of the Sec23p or the β’-COP expression or ubiquitylation levels when Usp10 was overexpressed. Alternatively, SNX3 may act as an adaptor for ENaC and stimulate, via a retromer complex, recycling of ENaC from an endosomal compartment back to the cell surface. The finding that SNX3 (and Usp10) interacts with ENaC is compatible with such a model. The elevated total pool of ENaC (observed with Usp10 or SNX3) may be the result of increased recycling of channels, which would otherwise be degraded by the lysosomal system. A possible role of Usp10 and SNX3 for AQP2 regulation also may be envisioned. Clearly, further investigation is required to decipher the precise role of Usp10 and SNX3 in ENaC (and AQP2) trafficking.

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