Early Aldosterone-Induced Gene Product Regulates the Epithelial Sodium Channel by Deubiquitylation

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The mineralocorticoid hormone aldosterone controls sodium reabsorption and BP largely by regulating the cell-surface expression and function of the epithelial sodium channel (ENaC) in target kidney tubules. Part of the stimulatory effect of aldosterone on ENaC is mediated by the induction of serum- and glucocorticoid-regulated kinase 1 (Sgk1), a kinase that interferes with the ubiquitylation of ENaC by ubiquitin-protein ligase Nedd4-2. In vivo early aldosterone-regulated mRNA now has been identified in microselected mouse distal nephron by microarray. From 22 mRNA that displayed a two-fold or more change, 13 were downregulated and nine were upregulated. Besides Sgk1, the induced mRNA include Grem2 (protein related to DAN and cerebrosphceric [PRDC]), activating transcription factor 3, cAMP responsive element modulator, and the ubiquitin-specific protease Usp2-45. The induction of this last enzyme isoform was verified in mouse distal nephron tubule at the protein level. With the use of Hek293 cells, Xenopus oocytes, and mpkCCD cell lines, it was shown that Usp2-45 deubiquitylates ENaC and stimulates ENaC-mediated sodium transport, an effect that is not additive to that of Sgk1. A deubiquitylating enzyme that targets ENaC in vitro and thus may play a role in sodium transport regulation was identified within a series of new in vivo early aldosterone-regulated gene products.


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The Sgk1–Nedd4-2 pathway, however, cannot be the only link between aldosterone and ENaC function. This has been indicated by the results of experiments that were performed with mpkCCD<sub>c14</sub> cells that expressed Liddle-type ENaC channels or with mice that expressed an ENaC-β subunit with Liddle mutation, thus displaying impaired ENaC/Nedd4-2 interaction (23,24). In these systems, aldosterone was still able to stimulate transepithelial sodium transport. Hence, the pathways that link the transcriptional activity of activated mineralocorticoid receptor to ENaC function independent of ENaC ubiquitylation still need to be clarified. In that respect, it is interesting to mention two observations that support the possibility of an additional aldosterone-induced regulatory mechanism that has an impact on ENaC function/surface expression, namely the demonstration of an unsuspected regulatory role of α-ENaC in collecting duct cells and of a stimulation of ENaC by Sgk1 that is mediated by the phosphorylation of the α subunit (25,26). It therefore is anticipated that other gene products are rapidly regulated by aldosterone in target cells that participate to the control of Na<sup>+</sup> reabsorption and K<sup>+</sup> secretion.

In this study, we describe the comparison by microarray of RNA that were extracted from epithelial cells of the aldosterone-sensitive distal nephron (ASDN) of control and aldosterone-treated mice. We thereby identified 22 significantly and more than two-fold regulated RNA. We anticipate that some of them encode proteins that participate in early regulatory effects of aldosterone that are independent of ENaC ubiquitylation. However, in this study, we focused on the induced deubiquitylating enzyme Usp2-45 and demonstrated in expression systems that it deubiquitylates ENaC and increases its function.

**Materials and Methods**

Animal studies were in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Science, Bethesda, MD).

**Mouse Treatment, Kidney Dissection, and Tubule Microcollection**

Female C57BL/6J Ola Hsd mice (Harlan, Horst, The Netherlands), approximately 8 wk of age, received drinking water that contained 0.3% NaCl for at least 5 d before the treatment. All mice were housed in a temperature- and humidity-controlled room with an automatic 12-h light/dark cycle (6 a.m./6 p.m.). Aldosterone (Acros Organics, Pittsburgh, PA; 10 μg/kg body wt in 0.33% ethanol and 0.9% NaCl) or canrenoate (Sigma, Sternheim, Germany; 10 mg/kg in 0.9% NaCl) was injected intraperitoneally, or mice were left untreated. Forty-two minutes later, mice were anesthetized with ketamine (Vetoquinol AG, Uznach, Switzerland) and xylazine (Streuli AG, Uznach, Switzerland; intraperitoneally 100 and 0.5 μg/g, respectively). They were then perfused through the left ventricle with Ringer solution that contained collagenase and protease (5 and 1 mg in 15 ml, respectively; Sigma) while the lower vena cava was opened. Kidneys were removed and cut transversally in 2-mm slices that were incubated for 10 min at 38°C in a collagenase solution (1 mg/ml Ringer solution). These tubule preparations were then transferred to ice-cold Ringer (1 h after aldosterone injection), washed, and resuspended three times. Segments (75 to 85) of cortical collecting duct/connecting tubule (CCD/CNT; approximately 1.1 mm of total length) were identified under a binocular microscope (SMZ1000; Nikon AG, Egg, Switzerland) at 4°C and transferred directly into RLT lysis buffer of the Qiagen RNeasy Micro Kit (Qiagen AG, Hombrechtikon, Switzerland) that contained 1% β-mercaptoethanol (Sigma-Aldrich, Buchs, Switzerland) using a pair of sharp forceps (F.S.T. GmbH, Heidelberg, Germany). For plasma aldosterone and corticosterone measurements, mice were anesthetized as described, and blood was collected by puncture of the vena cava. Plasma aldosterone and corticosterone levels were measured using an ELISA and a 125I-RIA kit from Immuno-Biologic Laboratories (Hamburg, Germany), respectively.

RNA Purification and Quality Control, Microarray Target cRNA Preparation, Target cRNA Hybridization and GeneChip Scanning, Real-Time Reverse Transcriptase–PCR, Quality Control of Microarrays, and Statistical Analyses

Please refer to online supplementary materials for detailed methods.

Prediction of Mineralocorticoid Hormone Response Elements in the Promoter Region of the Mouse Usp2-45 Gene and Western Blotting of Usp2-45 in Microcollected Mouse Kidney CNT and CCD

Please refer to online supplementary materials for detailed methods.

Cloning of Mouse Usp2-45, Usp2-69, and Usp15; Site-Directed Mutagenesis and cRNA Preparation; and Coexpression of mUsp2-45, Usp2-69, mUsp15, αβγXENaC, and XSgk in Xenopus laevis Oocytes

Please refer to online supplementary materials for detailed methods.

Two-Electrode Voltage Clamp on Xenopus Oocytes, Ubiquitylation of ENaC in Hek293 Cells, and Culture and Retroviral Transduction of mpkCCD Cells

Please refer to online supplementary materials for detailed methods.

Western Blotting and Real-Time PCR of Usp2-45 in mpkCCD<sub>c14</sub> Cells and Transepithelial Electrophysiologic Measurements

Please refer to online supplementary materials for detailed methods.

**Results**

Microarray to Identify Early Aldosterone-Regulated mRNA in Mouse ASDN

To identify other early aldosterone-regulated gene products in kidney target cells in vivo, we first established a short-term aldosterone treatment scheme in mice. Female C57BL/6J mice that received 0.3% NaCl in their water to repress partially their endogenous aldosterone production were administered an injection at 10:00 a.m. (4 h after beginning of daytime) with aldosterone (10 μg/kg body wt; Figure 1, A and B) or were left untreated. The aldosterone injection led to a transient increase in plasma aldosterone that 15 min after injection was at a level of approximately 10 nM, a value within the range expected for mice on a low-salt diet (27). The plasma aldosterone concentration was nearly back to the initial value of approximately 0.5 nM after 60 min.

To obtain kidney aldosterone-target tissue RNA 1 h after hormone injection, mice were anesthetized 42 min after aldosterone injection and rapidly perfused with collagenase solution. Kidneys were removed, sliced, and further digested in
ANOVA. vehicle at 10 a.m. (means (cort) at 10 a.m. and at 10:30 a.m. with or without injection of
Comparison of plasma aldosterone (aldo) and corticosterone
ENaC), Sgk1 and Na/H11001
slightly (Figure 1, A and B) but did not have an impact on Sgk1
manipulation of the mice increased plasma corticosterone
five- to six-fold by aldosterone. It is interesting that the mere
the total kidney preparation and specifically further increased
mRNA was measured by real-time reverse transcriptase–PCR
expression (Figure 1, C and D). More than two thirds of the
prepared RNA samples fulfilled the selection criteria of quantity, integrity, enrichment, lack of contamination, and Sgk1
induction and could be used as template for cDNA amplification
and labeled target RNA synthesis. Target cRNA made
from six control and from six aldosterone-injected mice were
separately hybridized to mouse full genome GeneChip mi-
croarrays (MOE 430 v2; Affymetrix, Santa Clara, CA). Raw
array data were processed using the GCRMA and the MA55
algorithms (Microarray data are available at Array Express
with the accession number E-TABM-229.) The data were fil-
tered using a t test with a cutoff P ≤ 0.05 and using a fold-
change limit of 2.0. The cumulative list of genes that fulfilled
these criteria after processing with one of the algorithms com-
prised 90 genes (73 genes with GCRMA and 39 with MA55). As
detailed in Table 1, 22 genes fulfilled these criteria with both
algorithms, nine that are upregulated and 13 that are down-
regulated. A scatter plot and the hierarchical clustering of the
results that were calculated by the GCRMA method are shown
in Supplementary Figure 1B. We retested the differential ex-
pression level of eight mRNA that were identified in the mi-
croarray screen by real-time RT-PCR (see primers and probes
sequences in the online supplementary material) on RNA from
from three other mice per condition. As shown in Table 1, the regu-
lation was verified in six cases.

**Aldosterone-Induced Usp2-45 Deubiquitylates ENaC**

Ubiquitylation of ENaC had previously been shown to play
an important role in the control of Na\(^+\) reabsorption (12) and
our screening had confirmed that Sgk1, a kinase that is known
to inhibit the action of the ubiquitin-protein ligase Nedd4-2 on
ENaC, was rapidly induced by aldosterone. We focused now
on Usp2, a ubiquitin-specific protease that was also induced
and belongs to a large protein family that is involved in reversing
the ubiquitylation of target proteins (28–30). We specifically
investigated the potential role of the isoform Usp2-45 that we
detected in kidney by reverse transcriptase–PCR (RT-PCR),
unlike the other isoform Usp2-69 that is expressed only at low
levels in the kidney (31) (P.F. and F.V., unpublished observa-
tion) and in mpkCCD\(_{14}\) cell (10-fold difference; discussed fur-
ther). The analysis of the promoter region around the TATA
box of the Usp2-45 isoform identified at least four potential
hormone response elements that support the possibility of a
direct regulation by aldosterone (see Supplementary Figure 2).
An induction by aldosterone of the shorter Usp2-45 isoform but
not of the Usp2-69 isoform was also detected in mpkCCD cells
discussed further).

To test whether the aldosterone-induced Usp2-45 mRNA
induction translates in vivo into an increase in Usp2-45 protein,
we performed Western blotting of proteins that were prepared
from microselected segments of CNT/CCD that were obtained
from mice that were subjected to the same hormone treatment
as previously performed for the mRNA studies. As shown in
Figure 2, the polyclonal rabbit anti-mouse Usp2-45 antibody
recognized a 45-kD band that was blocked by competing im-
munizing peptide. This band was selectively increased 1 h after
an aldosterone injection by a factor of approximately 2, as
previously shown for the mRNA. That the Usp2-45 mRNA indeed encodes an active ubiquitin-specific protease was confirmed by the fact that its core domain that is produced in *Escherichia coli* very efficiently cleaves ubiquitin off from a test substrate *in vitro* (B. Oberfeld *et al.*, unpublished observations) (32).

To test the possible impact of Usp2-45 on ENaC function, we coexpressed it with ENaC in *Xenopus laevis* oocytes and compared the effect of Usp2-45 with that of Sgk1, the kinase that inhibits endogenous Nedd4-2. Coexpression of Usp2-45 with ENaC increased the level of amiloride-sensitive Na/H current nearly four-fold, an effect similar to that of Sgk1 (Figure 3A and Supplementary Figure 3, A and B). It is interesting that coexpression of Sgk1 with Usp2-45 did not further increase ENaC function, suggesting that these two enzymes have an impact on the same regulatory mechanism (Figure 3A and Supplementary Figure 3B).

To test whether the effect of Usp2-45 was mediated by its protease activity, we mutated its cysteine 67 that corresponds to one of the catalytically active amino acids of its cysteine protease motif (31,33). Consistent with a requirement for an enzymatically active Usp2-45, this mutant did not affect ENaC activity at the membrane (Figure 3B and Supplementary Figure 3C). To control whether this effect of Usp2-45 was specific for this ubiquitin-specific protease, we carried out the same experiment with another randomly chosen ubiquitin-specific protease, Usp15. The results indicated that the effect of Usp2-45 is specific, because coexpression of Usp15 in contrast rather decreased ENaC function (Figure 3C and Supplementary Figure 3D). It is probable that this latter effect is due to the deubiquitylation of other proteins that interfere with the expression of ENaC. In addition, we coexpressed the longer Usp2-69 isoform that turned out not to have an impact on ENaC function (Figure

### Table 1. Kidney CNT/CCD RNA that changed two-fold or more by 1 ho faldosterone

<table>
<thead>
<tr>
<th>RNA</th>
<th>Genebank Accession No.</th>
<th>Fold Change</th>
<th>Range</th>
<th>P</th>
<th>Fold Change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein related to DAN and cerberus</td>
<td>NM_011825</td>
<td>7.2</td>
<td>2.1 to 17.7</td>
<td>0.045</td>
<td>5.6</td>
<td>0.029</td>
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<tr>
<td>serum/glucocorticoid regulated kinase</td>
<td>NM_011361</td>
<td>4.0</td>
<td>3.0 to 5.1</td>
<td>&lt;0.0001</td>
<td>8.1</td>
<td>0.001</td>
</tr>
<tr>
<td>activating transcription factor 3</td>
<td>BC019946</td>
<td>3.5</td>
<td>2.5 to 4.9</td>
<td>0.001</td>
<td>8.6</td>
<td>0.018</td>
</tr>
<tr>
<td>cAMP responsive element modulator</td>
<td>NM_013498</td>
<td>3.0</td>
<td>1.3 to 4.5</td>
<td>0.004</td>
<td>1.1</td>
<td>0.061</td>
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<tr>
<td>mus musculus transcribed sequences</td>
<td>AI098139</td>
<td>2.2</td>
<td>1.5 to 2.9</td>
<td>&lt;0.0001</td>
<td>1.6</td>
<td>0.001</td>
</tr>
<tr>
<td>ubiquitin specific protease 2</td>
<td>BG069663</td>
<td>2.8</td>
<td>1.7 to 4.4</td>
<td>0.004</td>
<td>1.6</td>
<td>0.001</td>
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<tr>
<td>growth arrest and DNA-damage-inducible 45 gamma</td>
<td>AK007410</td>
<td>2.2</td>
<td>1.5 to 3.2</td>
<td>0.001</td>
<td>1.6</td>
<td>0.001</td>
</tr>
<tr>
<td>RIKEN cDNA clone 6430570G24</td>
<td>BG069663</td>
<td>2.2</td>
<td>1.5 to 2.9</td>
<td>&lt;0.0001</td>
<td>1.6</td>
<td>0.001</td>
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<tr>
<td>Downregulated genes</td>
<td></td>
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<td></td>
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<tr>
<td>RIKEN cDNA 4833417L20 gene</td>
<td>AK017914</td>
<td>0.5</td>
<td>0.3 to 0.9</td>
<td>0.014</td>
<td>1.0</td>
<td>0.946</td>
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<tr>
<td>similarity to pir:A45973 (H.sapiens)</td>
<td>AI997808</td>
<td>0.5</td>
<td>0.3 to 0.6</td>
<td>0.009</td>
<td>0.1</td>
<td>0.024</td>
</tr>
<tr>
<td>argininosuccinate synthetase 1</td>
<td>BM213298</td>
<td>0.4</td>
<td>0.3 to 0.5</td>
<td>0.007</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>RIKEN cDNA clone D330044A17</td>
<td>BB480395</td>
<td>0.4</td>
<td>0.2 to 0.6</td>
<td>0.006</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>partial mRNA for hypothetical prot.</td>
<td>BC181292</td>
<td>0.4</td>
<td>0.3 to 0.6</td>
<td>0.006</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>fatty acid binding protein 1, liver</td>
<td>NM_017399</td>
<td>0.4</td>
<td>0.2 to 0.8</td>
<td>0.006</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>transcribed sequences</td>
<td>BE991175</td>
<td>0.4</td>
<td>0.2 to 0.6</td>
<td>0.006</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>RIKEN cDNA clone C230084M03</td>
<td>BM123508</td>
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<td>0.2 to 0.8</td>
<td>0.005</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>RIKEN cDNA 4833417L20 gene</td>
<td>BM217861</td>
<td>0.4</td>
<td>0.3 to 0.4</td>
<td>0.004</td>
<td>0.2</td>
<td>0.015</td>
</tr>
<tr>
<td>H+/K+-ATPase alpha, nongastric</td>
<td>NM_138652</td>
<td>0.4</td>
<td>0.1 to 0.8</td>
<td>0.004</td>
<td>1.0</td>
<td>0.946</td>
</tr>
<tr>
<td>Slc22a8 (Oat3)</td>
<td>AV330315</td>
<td>0.3</td>
<td>0.1 to 0.6</td>
<td>0.004</td>
<td>0.1</td>
<td>0.024</td>
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<td>G0/G1 switch gene 2</td>
<td>NM_008059</td>
<td>0.3</td>
<td>0.2 to 0.6</td>
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<td>0.2</td>
<td>0.015</td>
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<tr>
<td>RIKEN cDNA clone E030031K24</td>
<td>BB553736</td>
<td>0.3</td>
<td>0.2 to 0.5</td>
<td>0.003</td>
<td>0.2</td>
<td>0.015</td>
</tr>
</tbody>
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aRNA in italics were also tested by real-time reverse transcriptase–PCR.
bRNA with fold-change ≥2 and t test *P* ≤ 0.05 using GCRMA and MAS5 algorithms.
cValues were obtained using GCRMA.
dReal-time PCR data were obtained with specific intron spanning primer/probe sets, *n* = 3 mice.
Figure 3. Usp2-45 increases amiloride-sensitive Na\(^+\) current (I\(_{\text{amil}}\)) carried by coexpressed αβγ-ENaC. Original tracings are shown in Supplementary Figure 3. (A) Summary of amiloride-sensitive whole-cell currents at a holding potential of \(-100\) mV. Coexpression of Usp2-45 or of XSgk1 increased the ENaC-mediated amiloride-sensitive I\(_{\text{amil}}\) to a similar extent. These effects were not additive. Data are means ± SEM; \(n = 23\) to \(27\). ***\(P < 0.001\); ****\(P < 0.0001\). (B) Through (D) Characterization of Usp2-45 stimulatory effect on I\(_{\text{amil}}\) in Xenopus oocytes. (B) Cys67Ala protease-dead Usp2-45 mutant (Usp2-45mut) does not stimulate XENaC current I\(_{\text{amil}}\) at \(-100\) mV. Data are means ± SEM; \(n = 12\) to \(15\). **\(P < 0.01\). (C) Another Usp (Usp15) does not stimulate XENaC-mediated I\(_{\text{amil}}\) at \(-100\) mV. Data are means ± SEM; \(n = 20\) to \(24\). *\(P < 0.05\); ****\(P < 0.0001\). (D) The Usp2-45 isoform does not stimulate XENaC-mediated I\(_{\text{amil}}\) at \(-100\) mV. Data are means ± SEM; \(n = 20\) to \(22\). ****\(P < 0.0001\). (E) Western blot made with lysate of control and Usp2-45- and Usp2-69-expressing Xenopus oocytes and incubated with a pan-Usp2 antibody shows specific bands that correspond to each isoform, confirming that both were similarly expressed and that the lack of ENaC stimulation by Usp2-69 was not due to a lack of expression of this isoform. (F) Effect of Usp2-45 on ENaC-mediated I\(_{\text{amil}}\) is prevented by mutation of rENaC PY motif (rENaC-PY\(_{\text{mut}}\)). I\(_{\text{amil}}\) at \(-100\) mV is shown. Data are means ± SEM; \(n = 4\) independent experiments with a total of \(20\) oocytes. *\(P < 0.05\) using paired \(t\) test. (G) Effect of Usp2-45 on ENaC-mediated I\(_{\text{amil}}\) is prevented by mutation of rENaC ubiquitylation site (lysine-mutant rENaC-K\(_{\text{mut}}\)). I\(_{\text{amil}}\) at \(-100\) mV is shown. Data are means ± SEM; \(n = 4\) to \(5\) independent experiments with a total of \(20\) and \(25\) oocytes, respectively. *\(P < 0.05\); **\(P < 0.01\); ****\(P < 0.001\).

3D). In this case, we also verified by Western blotting that this ubiquitin-specific protease isoform was indeed expressed as expected (Figure 3E).

We then asked whether Usp2-45 interferes with Nedd4-2-dependent regulation of ENaC, a mechanism that involves the interaction of Nedd4-2 with PY motifs that are present on the C-termini of the ENaC subunits and is defective in Liddle syndrome (14). ENaC that was devoid of PY motifs (\(α\) Y673A, \(β\) Y618H, γ Y628A) is resistant to Nedd4-2 and thus displayed a higher level of amiloride-sensitive Na\(^+\) current than did wild-type ENaC. This current was indeed resistant to the stimulatory action of Usp2-45 (Figure 3F). This observation supports the hypothesis that Usp2-45 cleaves off ubiquitin moieties that Nedd4-2 ligates onto ENaC. The involvement of direct ENaC ubiquitylation was then verified by coexpressing Usp2-45 with ENaC that was devoid of potential ubiquitylation sites (lysine residues of the intracellular NH\(_2\)-terminal domains of all three subunits mutated to arginine) in Xenopus oocytes. As expected for the case that Usp2-45 deubiquitylates ENaC, this mutant channel was not activated in the presence of Usp2-45 (Figure 3G).

To verify that the effect of Usp2-45 on ENaC function was not an artifact that was due to the coexpression of two proteins in Xenopus oocytes but also takes place with endogenous ENaC in CCD cells, we addressed this question using the mouse CCD cell line mpkCCD\(_{\text{cl4}}\). We actually first verified whether Usp2-45 is expressed and regulated by aldosterone in these cells. The relative expression of the two isoforms in mpkCCD\(_{\text{cl4}}\) cells was similar to the observation made in microdissected CNT/CCD with a 10-fold higher expression of the Usp2-45 isoform-specific real-time RT-PCR primers and probes are shown in online supplementary material. (B) Baseline trans-epithelial I\(_{\text{amil}}\) across mpkCCD\(_{\text{cl4}}\) cell monolayers that were cultured on permeable supports is significantly increased in cells that overexpressed Usp2-45 compared with vector-transduced negative control cells (NC) and cells that were transduced with protease-dead Usp2-45mut (\(●\)). *\(P < 0.05\); \(n = 9\) to 12. Aldosterone (300 nM) given for 3 h increased I\(_{\text{amil}}\) similarly in the monolayers that were made of the different mpkCCD\(_{\text{cl4}}\) cell populations (\(■\)).

Figure 4. Expression of endogenous and exogenous Usp2-45 in mpkCCD\(_{\text{cl4}}\) cells. (A) Regulation of endogenous Usp2-45, Usp2-69, and Sgk1 mRNA by aldosterone (10\(^{-5}\) M; see Materials and Methods in online supplementary material and reference [35]) in mpkCCD\(_{\text{cl4}}\) cells. Usp2-45 upregulation is slightly more delayed than that of Sgk1. Unlike its isoform Usp2-45, Usp2-69 is not induced by aldosterone. Baseline mRNA abundance of Usp2-45 was 10-fold higher than that of Usp2-69. Fold changes of normalized means relative to β-actin ± SEM are given (\(n = 3\)). The isoform-specific real-time RT-PCR primers and probes are shown in online supplementary material. (B) Baseline trans-epithelial I\(_{\text{amil}}\) across mpkCCD\(_{\text{cl4}}\) cell monolayers that were cultured on permeable supports is significantly increased in cells that overexpressed Usp2-45 compared with vector-transduced negative control cells (NC) and cells that were transduced with protease-dead Usp2-45mut (\(●\)). *\(P < 0.05\); \(n = 9\) to 12. Aldosterone (300 nM) given for 3 h increased I\(_{\text{amil}}\) similarly in the monolayers that were made of the different mpkCCD\(_{\text{cl4}}\) cell populations (\(■\)).
mRNA. The time course of the regulation by aldosterone revealed that only the Usp2-45 isoform was regulated (Figure 4A). To investigate the effect of Usp2-45 on the transepithelial Na\(^+\) transport function, we expressed exogenous Usp2-45 as well as mutant protease-dead Usp2-45 in mpkCCD cells by retroviral transduction and verified by Western blot that the expression levels were equal (Supplementary Figure 4). As expected, when ENaC expression and activity were limited by its ubiquitylation, expression of functional Usp2-45 increased the baseline transepithelial amiloride-sensitive Na\(^+\)/H\(^+\) transport (Figure 4B). In contrast, the expression of the protease-dead mutant had no effect. The increase in Na\(^+\) transport that was induced by aldosterone (300 \(\mu\)M; see Materials and Methods in the online supplementary material and reference [35]) that was given for 3 h was not modified significantly by the expression of wild-type and mutant Usp2-45, as expected considering that short-term aldosterone prevents the Nedd4-2-mediated ubiquitylation of ENaC by inducing Sgk1. These results that were obtained in CCD cells confirm that Usp2-45, a ubiquitin-specific protease that was identified because of its \textit{in vivo} regulation by aldosterone, also acts on endogenous ENaC in kidney tubule cells.

To test directly the hypothesis that Usp2-45 counteracts the effect of Nedd4-2 by deubiquitylating ENaC, we used Hek293 cells as an expression system. Coexpression of Nedd4-2 with ENaC in Hek293 cells massively increased the ubiquitylated form of the channel that was visualized with anti-ubiquitin antibody on immunoprecipitates of \(\alpha\)- and \(\gamma\)-ENaC, thereby confirming that ENaC was itself ubiquitylated (Figure 5A). The impact of ubiquitylation on the total amount of ENaC was demonstrated by the decreased amount of ENaC that was visualized in the same samples with anti-ENaC antibody. Importantly, Usp2-45 coexpression decreased the amount of ubiquitylated ENaC and increased total ENaC, demonstrating that Usp2-45 antagonizes the effect of Nedd4-2 by directly deubiquitylating ENaC. The low level of ubiquitylated ENaC and the substantial level of total ENaC that was observed with Usp2-45 coexpression were similar to those that were observed with Nedd4-2cs, a catalytically inactive Nedd4-2 (Figure 5A).

**Discussion**

**Early Aldosterone-Regulated Gene Products**

The number of mRNA the level of which was changed two-fold or more in mouse ASDN within 1 h after an aldosterone injection is relatively low (22 mRNA). It is noteworthy that the signal intensity of these regulated mRNA on control mice arrays differs by more than two orders of magnitude, indicating large differences in their absolute baseline expression levels (see Supplementary Figure 1A). Not unexpected, the gene product with the highest absolute expression level and the second highest relative level of induction is Sgk1, the only early aldosterone-regulated gene product of mammalian kidney that has been identified earlier and shown to activate ENaC.

Our list of early aldosterone-regulated mRNA also included 13 mRNA that are downregulated (Table 1). The known mRNA with the highest fractional decrease (0.3-fold in Affymetrix screen, 0.2-fold by real-time RT-PCR) encodes G0s2, a putative G0/G1 switch gene that regulates the cell cycle by acting on the cyclin-dependent kinases (see Discussion).

**Figure 5.** Inverse regulation of ENaC ubiquitylation and ENaC amount. (A) The ubiquitylated and the total amount of ENaC expressed in Hek293 cells are visualized by Western blotting (IB) of immunoprecipitated (IP) HA- and Flag-tagged \(\alpha\)- and \(\gamma\)-rENaC subunits, respectively. The molecular weight of marker proteins is indicated in kD. Coexpression of Nedd4-2 and not of ligase-inactive Nedd4-2 (Nedd4-2cs) increases the ubiquitylation of both tested ENaC subunits, whereas it decreases their total amount. In the presence of Usp2-45, ENaC ubiquitylation is decreased and the total subunit amount is increased. (B) Schematic representation of the convergent effect of Sgk1 and Usp2-45 that both decrease the amount of ubiquitylated ENaC and thus increase the amount of intact channel. The subcellular localization of these ubiquitylation/deubiquitylation reactions has not yet been identified.

The second upregulated gene product on the list is the already mentioned Sgk1 that is followed by two members of the cAMP response element binding (CREB) family of transcription factors, activating transcription factor 3 and cAMP responsive element modulator.

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that there are also two transport proteins among the downregulated gene products, namely the polyspecific organic anion transporter 3 (Slc22a8) and the colonic H\(^+\),K\(^+\)-ATPase.

**Role of Deubiquitylation in Aldosterone Action**

The regulation of the gene products discussed in the previous section seems to favor a decrease in proliferation of aldosterone target cells and to foster their differentiation. In addition, our screen identified with Sgk1 and Usp2-45 two aldosterone-induced gene products that interfere with protein ubiquitylation. As mentioned at the beginning of this article, Sgk1 was identified earlier as aldosterone-induced mRNA and protein, and its effect on ENaC function has been shown to be mediated to a large extent by its inhibitory action on the ubiquitylating enzyme Nedd4-2.

We have now indeed shown that the newly identified aldosterone-induced Usp2-45 directly deubiquitylates ENaC in the Hep273 cell expression system (Figure 5A). As expected, if the ubiquitylation of ENaC would lead to ENaC degradation, then the presence of Usp2-45 not only decreases the amount of ubiquitylated ENaC but also increases the total amount of ENaC. This biochemical evidence of ENaC deubiquitylation by Usp2-45 is supported by functional experiments made in *Xenopus* oocytes that show an increase in ENaC function that specifically is mediated by Usp2-45 and that depends on the integrity of its protease motif. Furthermore, we show both that the disruption of ENaC PY motifs and the disruption of ENaC lysine residues that potentially are targeted by Usp2-45 prevent the action of Usp2-45 on ENaC function. These results also strongly support the hypothesis that Usp2-45 acts on ubiquitin moieties that are ligated onto ENaC by the ubiquitin ligase Nedd4-2 (Figure 5B).

That a deubiquitylating enzyme that acts on ENaC is induced by aldosterone strengthens the notion that ubiquitylation plays a central role in the short-term regulation of ENaC. Although deubiquitylation of target proteins has been described since the early days of the ubiquitin system discovery (37), it has only recently gained considerable interest as a regulatory mechanism (for a review on deubiquitylation, see reference [29]). The mouse as the human genome encodes approximately 100 putative deubiquitylating enzymes, the function of most of which is not known. The Affymetrix screen that was presented in this article revealed that approximately 26 of these Usp are expressed in the aldosterone target cells of the mouse kidney tubule (present calls), but only Usp2-45 appeared in the list of regulated RNA (Table 1). On the basis of our results, the deubiquitylation of ENaC by Usp2-45 seems to be specific for Usp2-45, because none of the four other Usp that we tested in expression systems activated and/or deubiquitylated this channel (Figure 3, C and D; unpublished results). As yet, actually only a few examples of plasma membrane protein deubiquitylation have been published. One such example is the deubiquitylation of yeast membrane permeases that leads to their targeting to the vacuole (equivalent to the mammalian lysosome) by the ubiquitin-specific protease Dna4 (38). Another is the deubiquitylation and stabilization of the EGF receptor by the endosomal ubiquitin isopeptidases AMSH (associated mol-

cule with the SHB domain of STAM) and UBPY (ubiquitin-specific protease Y) (39,40).

The identification of ENaC as a target of Usp2-45 represents the first report of a hormonally controlled channel-deubiquitylating mechanism. It is noteworthy to mention that this effect is not selective for kidney tubule, because Usp2-45 is also expressed and regulated in distal colon, yet another target epithelium of aldosterone (P.F. and F.V., unpublished observations). Previously published data on Usp2 suggest that its isoform Usp2-69 is regulated by androgens in liver and prostate, where it deubiquitylates and stabilizes fatty acid synthase (33). Furthermore, Usp2 was shown to be regulated in bone by parathyroid hormone (41), indicating that the Usp2 gene products mediate various important hormonally regulated functions.

That the stimulatory action of Usp2-45 on ENaC depends on previous ENaC ubiquitylation indicates, however, that Usp2-45 induction cannot explain the effect of aldosterone on Liddle-type mutant ENaC (see the beginning of this article) (23,24). Therefore, it is expected that still other aldosterone-induced gene products, possibly also identified in our screen, act via different pathways on ENaC expression and function.

**Conclusion**

Our list of early aldosterone-regulated gene products contains two induced elements that interfere with the ubiquitylation of ENaC. Besides Sgk1, we identified Usp2-45 that we show to deubiquitylate ENaC in expression systems and thus represents a first example of a deubiquitylating enzyme acting on a channel.

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**Disclosures**

None.

**References**


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