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# Aldosterone-Induced Serum and Glucocorticoid-Induced Kinase 1 Expression Is Accompanied by Nedd4-2 Phosphorylation and Increased $Na^+$ Transport in Cortical Collecting Duct Cells

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**Aldosterone plays a central role in $Na^+$ homeostasis by controlling $Na^+$ reabsorption in the aldosterone-sensitive distal nephron involving the epithelial $Na^+$ channel (ENaC). Part of the effects of aldosterone is mediated by serum and glucocorticoid-induced kinase 1 (Sgk1), a Ser/Thr kinase whose expression is rapidly induced by aldosterone and that increases in heterologous expression systems ENaC cell surface abundance and activity. Previous work in *Xenopus laevis* oocytes suggested that Sgk1 phosphorylates specific residues (Ser212 and Ser328) on the ubiquitin-protein ligase Nedd4-2, an enzyme that directly interacts with ENaC and negatively controls channel density at the plasma membrane. It further indicated that phosphorylation of Nedd4-2 led to impairment of ENaC/Nedd4-2 interaction and consequently to more channels at the cell surface. These data suggested a novel mode of aldosterone-dependent action, yet this was not demonstrated formally in epithelial cells that physiologically express ENaC. Here it is shown, with the use of an anti–phospho-Ser328-mNedd4-2 antibody, that 2 to 6 h of aldosterone treatment induces an increase in Nedd4-2 phosphorylation, both in a mouse cortical collecting duct cell line ($mpkCCD_{cl4}$) and in kidneys of adrenalectomized rats. This augmentation, which is accompanied by a raise in Sgk1 expression and transepithelial $Na^+$ transport, is sensitive to phosphatidylinositol-3 kinase inhibition, as is Sgk1 phosphorylation and $Na^+$ transport. Hence, these data provide evidence in cortical collecting duct cells *in vitro* and *in vivo* that Sgk1-dependent phosphorylation of Nedd4-2 is part of the aldosterone response.**



In the aldosterone-sensitive distal nephron (1), aldosterone controls transepithelial $Na^+$ and $K^+$ transport, consequently regulating whole-body $Na^+$ and $K^+$ balance, blood volume, and arterial BP (2). The hormone is known to interact with the mineralocorticoid receptor, which, upon binding, translocates to the nucleus, where it orchestrates transcriptional regulation of many different genes (2). The effect of aldosterone on transepithelial $Na^+$ transport has been well described. After approximately 45 min, one observes an early response, during which $Na^+$ transport increases and the transepithelial electrical resistance decreases. This is followed by a late response, in which $Na^+$ transport rate further increases, without change of the epithelial resistance. The late response shows an increase of the transcriptional/translational rate of the two principal membrane complexes involved in $Na^+$ transport, the apical epithelial $Na^+$ channel (ENaC) and the basolateral $Na^+$,$K^+$-ATPase. The early response depends on the action of early induced or repressed proteins (3). A number of such proteins have been described, including K-Ras2A (3), GILZ (4), and the serum and glucocorticoid-induced kinase 1 (Sgk1) (5,6). The last is a Ser/Thr kinase, originally identified as a glucocorticoid-induced mRNA in mammary epithelial cells (7). Cell volume control, regulation of apoptosis, stress responses, and activation of $K^+$ channels are among the cellular functions affected by Sgk1 (for a recent review see reference 8). It shares strong homology with protein kinase B/Akt kinases and is highly conserved in all eukaryotes. It is itself a phosphoprotein constituent of the phosphatidylinositol-3 kinase (PI-3K) signaling cascade positioned downstream of PDK-1 (9). Sgk1 fulfills a number of properties of an early aldosterone-induced regulator of transepithelial $Na^+$ transport: (*1*) Its expression increases shortly before the increase in $Na^+$ transport (6,5,10), (*2*) it stimulates transepithelial $Na^+$ current in epithelial cells (11–13), (*3*) its expression is elevated in adrenalectomized rats upon acute administration of aldosterone in the cortical collecting duct (CCD) with low circulating levels of aldosterone and correlates with a decrease in urinary $Na^+$ concentrations (10), and (*4*) an Sgk1 mouse knockout model displays a salt-dependent pseudohypoaldosteronism type I phenotype (14). The consensus motif for phosphorylation by Sgk1 is RXRXXS/T (15,16), a motif also present on the ubiquitin-protein ligase Nedd4-2.

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Nedd4-2, which is composed of a calcium-dependent lipid binding domains C2, three to four WW protein:protein interaction domains, and the catalytic HECT domain (17), negatively controls ENaC cell surface expression (18). We and others have shown in *Xenopus laevis* oocytes that Sgk1 regulates ENaC by phosphorylating Nedd4-2. Such phosphorylation decreases the interaction between ENaC and Nedd4-2 interaction and leads to the accumulation of functional ENaC channels at the cell surface (19,20). It was postulated that this mechanism contributes to the aldosterone response. According to this working model, aldosterone stimulates the expression of Sgk1, which will increase the phosphorylation level of Nedd4-2 primarily on Ser328 but also on Ser212, leading as in the oocytes to increased density of ENaC at the apical membrane. However, to date, the mode of Sgk1-dependent phosphorylation of Nedd4-2, as seen in the oocytes, has not been demonstrated in renal epithelial cells *in vitro* or *in vivo*.

Here we show, using a phospho-Nedd4-2–specific antibody, that aldosterone induces phosphorylation of Nedd4-2 *in vivo* and in an epithelial cell line derived from the mouse CCD ($mpkCCD_{cl4}$) (21). The increased phosphorylation level of Nedd4-2 correlates with elevated Sgk1 expression and an increase in transepithelial $Na^+$ transport.



## Materials and Methods

### *Plasmids and cDNA Constructs*

cDNA constructs, encoding mouse Nedd4-2 and *Xenopus laevis* Sgk1, the latter harboring a myc tag cDNA, both cloned into pSDeasy have been described previously (17,19). Wild-type mNedd4-2 and mNedd4-2-S328A (AF277232; 338 to 2905 bp) were cloned into the pAdTrack-CMV shuttle vector (22). Mouse Nedd4-2 cDNA, lacking the C2 domain and mutated on Ser328 or not, was cloned into the Epstein-Barr virus–based retroviral vector (LZRS) (23).

### *Materials*

Culture medium was from Invitrogen (San Diego, CA). Hormones and reagents were from Sigma (St. Louis, MO). Tissue culture–treated Transwell filters were from Corning Costar Corp. (Corning, NY).

### *Cell Culture*

Experiments were carried out in the $mpkCCD_{c14}$ cell line, a clone of principal cells that has been derived from microdissected CCD of an SV-PK/Tag transgenic mouse (21). The ecotropic retrovirus producer Phoenix ($\phi$nx) cell line was grown in DMEM that contained 10% FCS with 100 U/$\mu$l penicillin and 100 $\mu$g/$\mu$l streptomycin at 37°C/5% $CO_2$. Both cell lines were maintained and cultured as described previously (23).

### *Expression in* Xenopus *Oocyte and Phosphorylation Assay*

pSDeasy mNedd4-2 and *Xenopus laevis* Sgk1 constructs were transcribed; the cRNA was injected into *Xenopus* oocytes; and after overnight incubation, lysates were prepared as described previously (19). For characterizing the anti–p328S-Nedd4-2 antibody, lysates were treated with 1/10 vol of 2 M Tris/HCl (pH 9.0) with or without calf intestinal alkaline phosphatase (CIP; Roche, Rotkreuz, Switzerland) and incubated for 3 h at 37°C. The enzyme was inactivated by adding 5× SDS-PAGE sample buffer and heating for 5 min at 95°C. Immunoblots were prepared and analyzed with anti–p-S328-Nedd4-2, anti-myc, and anti–Nedd4-2 antibodies as described previously (19,24).

### *Retroviral Infection of $mpkCCD_{cl4}$ Cells*

Retroviruses encoding LZRS–mNedd4-2 that were prepared in Phoenix ($\phi$nx) retrovirus producer cells were used to infect $mpkCCD_{cl4}$ cells as described previously (23).

### *Adenovirus Production*

mNedd4-2 and mNedd4-2S328A were cloned into the pAdTrack-CMV shuttle vector. The constructs were linearized with PmeI and transformed into *Escherichia coli* BJ5183-AD-1 electroporation competent cells (Stratagene, La Jolla, CA). From transformation on, adenoviruses were produced according to the manufacturer's protocol (AdEasyXL Adenoviral Vector System; Stratagene).

### *Short-Circuit Current Measurements*

Transepithelial short circuit currents ($I_{sc}$) were recorded on $mpkCCD_{cl4}$ cells that were grown on collagen-coated Transwell filters mounted in a modified Ussing chamber (25) as described previously (23). Briefly, cell layers were bathed in symmetrical solutions (120 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM Na-pyruvate, 0.9 mM Na-phosphate, 10 mM D-glucose, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) and gassed continuously with $O_2/CO_2$ 95%/5% to keep the pH at 7.4 in the presence or absence of 10 $\mu$M amiloride. $I_{sc}$ was measured by clamping the open-circuit $P_D$ to 0 mV. The amiloride-sensitive $I_{sc}$ was calculated, and values were normalized against control amiloride-sensitive $I_{sc}$.

### *Antibodies*

The anti–Nedd4-2 phosphopeptide antibody was raised in rabbits using a keyhole limpet hemocyanin-coupled, synthetic phosphopeptide (KPRSL-$S^{Phos}$-SPTV), affinity-purified on the corresponding phospho- and non-phosphopeptide and used as described previously (24). For the peptide competition assay (Figure 1B), the antibodies first were incubated overnight at 4°C with 20 $\mu$g/ml of either phospho- or dephosphopeptide. An antibody against Nedd4-2 was raised in rabbits against a GST fusion protein that comprised mouse Nedd4-2 amino acids 201 to 260 (GEQFSSLIQREPSSRLRSCSVTDTVAEQAHLPPPSTPTGRARSSTVTGGEESTPSVAY). For immunofluorescence experiments, it was affinity-purified by passing it over a GST column, followed by binding and elution on a GST–mNedd4-2 (<201 to 260) column, according to standard techniques. Anti-Sgk and anti-actin antibodies were from Sigma. Anti–enhanced green fluorescent protein (JL-18 living colors) was from Clontech (Palo Alto, CA).

### *Immunofluorescence in Kidneys of Rats*

Kidneys of adrenalectomized (ADX) male Wistar rats (body weights 180 to 200 g) were taken from a previous experiment (1) in which rats received either a single injection of vehicle or 50 $\mu$g/kg body wt aldosterone. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD). The kidneys had been fixed by vascular perfusion with 3% paraformaldehyde and 0.05% picric acid as described (1) and had been stored at −80°C until use. Cryosections (5 $\mu$m thick) of kidneys from three vehicle-treated rats and three rats with an aldosterone injection 2 h before being killed were incubated overnight at 4°C with either affinity-purified anti–Nedd4-2 phosphopeptide antibody (dilution 1:50) or affinity-purified anti–Nedd4-2 antibody (dilution 1:200). Binding sites of the primary antibodies were detected with a Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:1000. In some experiments, the primary anti–Nedd4-2 phosphopeptide antibody was preincubated overnight at 4°C with the immunogenic phosphopeptide (20 $\mu$g/ml) or its nonphosphorylated counterpart (20 $\mu$g/ml). For histologic analysis, the slides were studied

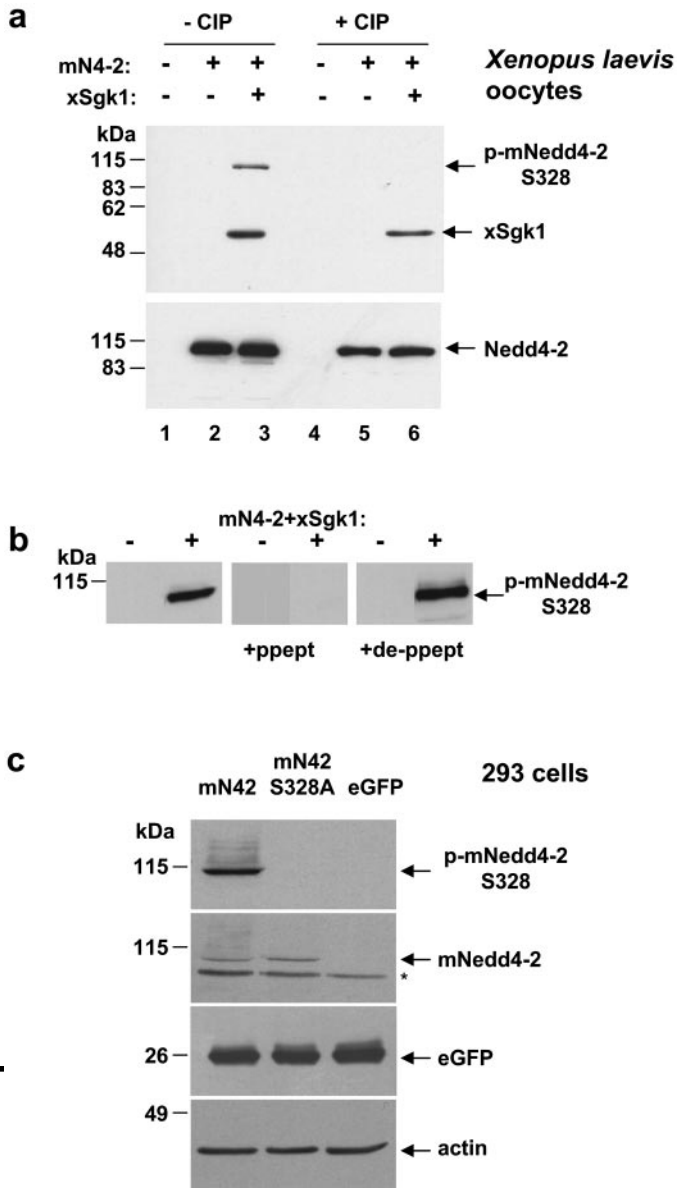


*Figure 1.* Characterization of an antibody against a phosphopeptide covering mNedd4-2 serine 328. (A) *Xenopus laevis* oocytes were injected with cRNA encoding mNedd4-2 or myc-xSgk1 as indicated. After 24 h, lysates were prepared, treated or not with calf intestinal phosphatase (− or + calf intestinal alkaline phosphatase), and analyzed by SDS-PAGE/Western blotting with anti–pSer328-Nedd4-2 and anti-myc (top) and anti–Nedd4-2 (bottom). (B) Oocytes expressing Sgk1 and Nedd4-2 (as indicated) were analyzed by Western blotting, using anti–pNedd4-2 antibodies that were incubated overnight with excess of phospho- (ppept) or dephosphopeptide (de-ppept). (C) Western blotting analysis of 293 cells that were infected for 24 h with adenovirus encoding mNedd4-2 and enhanced green fluorescence protein (eGFP), mNedd4-2S328A and eGFP, or eGFP alone. Lysates were blotted with anti–phospho-Nedd4-2, anti–Nedd4-2, anti-GFP (to monitor infection efficiency), and anti-actin antibodies. *Endogenous cross-reacting protein, likely endogenous Nedd4-2.

by two investigators who were blinded to the treatment of the rats. The staining intensities with the two Nedd4-2 antibodies were rated for each rat on a scale from 1 to 5, with 1 for the lowest and 5 for the highest staining intensities. According to these ratings, the kidney samples were ranked from weak to high staining intensities. These qualitative rankings were confirmed further by digital image analysis. For each rat (three vehicle-treated ADX rats and three aldosterone-treated rats), at least four micrographs of randomly selected CCD profiles were acquired by using a charge-coupled device camera. Camera settings were kept constant for each image. The mean pixel intensity for the collecting duct epithelia were determined with the NIH Image software (National Institutes of Health, Bethesda, MD).

### *Biochemical Analysis in CCD Cell Lines*

Differentiated mpkCCD$_{cl4}$ monolayers or 293 cells were lysed in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, and 1% Triton X-100) that contained protease inhibitors (1 mM PMSF, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin A, and 10 $\mu$g/ml aprotinin) and phosphatase inhibitors (100 mM NaF, 10 mM Na-pyrophosphate, and 10 mM Na-orthovanadate). This lysate was centrifuged (20,000 × *g*, 4°C, 15 min), and the supernatant was recovered. Samples were analyzed by SDS-PAGE/Western blotting as indicated. Briefly, samples were run on 8% SDS-PAGE gels and transferred on nitrocellulose. Blots were blocked in blocking buffer (1× PBS, 0.05% Triton X-100, 0.02% Na-azide, and 5% nonfat dry milk) for 30 min and incubated with primary antibody for 1 h in the same solution. Excess primary antibody was removed by four washes with Wash buffer (1× PBS and 0.05% Triton X-100) plus 3% skim milk and exposed to secondary antibody coupled to horseradish peroxidase (Amersham) during 30 min. After four washes with Wash buffer, blots were developed either with the ECL Western Blotting Detection (Amersham, Otelfingen, Switzerland) or with the SuperSignal West Dura Kit (Pierce, Rockford, IL) and exposed to Biomax XAR films (Kodak, Rochester, NY). Quantification of recognized levels was performed on these fluorograms using a molecular imager FX (Biorad, Hercules, CA), and the results were normalized to the controls and expressed as mean ± SEM. Statistical analyses were performed using the unpaired two-tailed *t* test. For Figure 2D, lysates were mixed with one tenth of their volume with shrimp alkaline phosphatase (Roche; 10× reaction buffer and 28 $\mu$l of shrimp alkaline phosphatase) and incubated for 30 min at 37°C. SDS-PAGE sample buffer (5×) was added, and samples were boiled at 95°C for 5 min. Immunoblots were as described above.

### *Analysis of Sgk1 Phosphorylation by Phosphatase Treatment*

Cells that were treated or not with 1 $\mu$M aldosterone for 3 h were lysed in lysis buffer that contained phosphatase inhibitors or not (100 mM NaF, 10 mM Na-pyrophosphate, and 10 mM Na-orthovanadate; as indicated in the Figure 3). Samples were treated with Lambda protein phosphatase (10 U/$\mu$l; New England Biolabs, Beverly, MA) during 30 min at 30°C. Reactions were stopped by adding SDS-PAGE sample buffer and boiling at 95°C for 5 min. Samples were analyzed by SDS-PAGE/Western blotting using anti-*Sgk* antibody.

### *Statistical Analyses*

Data are presented as means of normalized values ± SEM. The difference between means was analyzed by the two-tailed *t* test for unpaired data.

## Results

### *Specificity of Anti–pSer328-Nedd4-2 Antibody*

Recently we showed in *Xenopus laevis* oocytes that Sgk1 phosphorylates *Xenopus laevis* Nedd4-2 primarily on Ser444

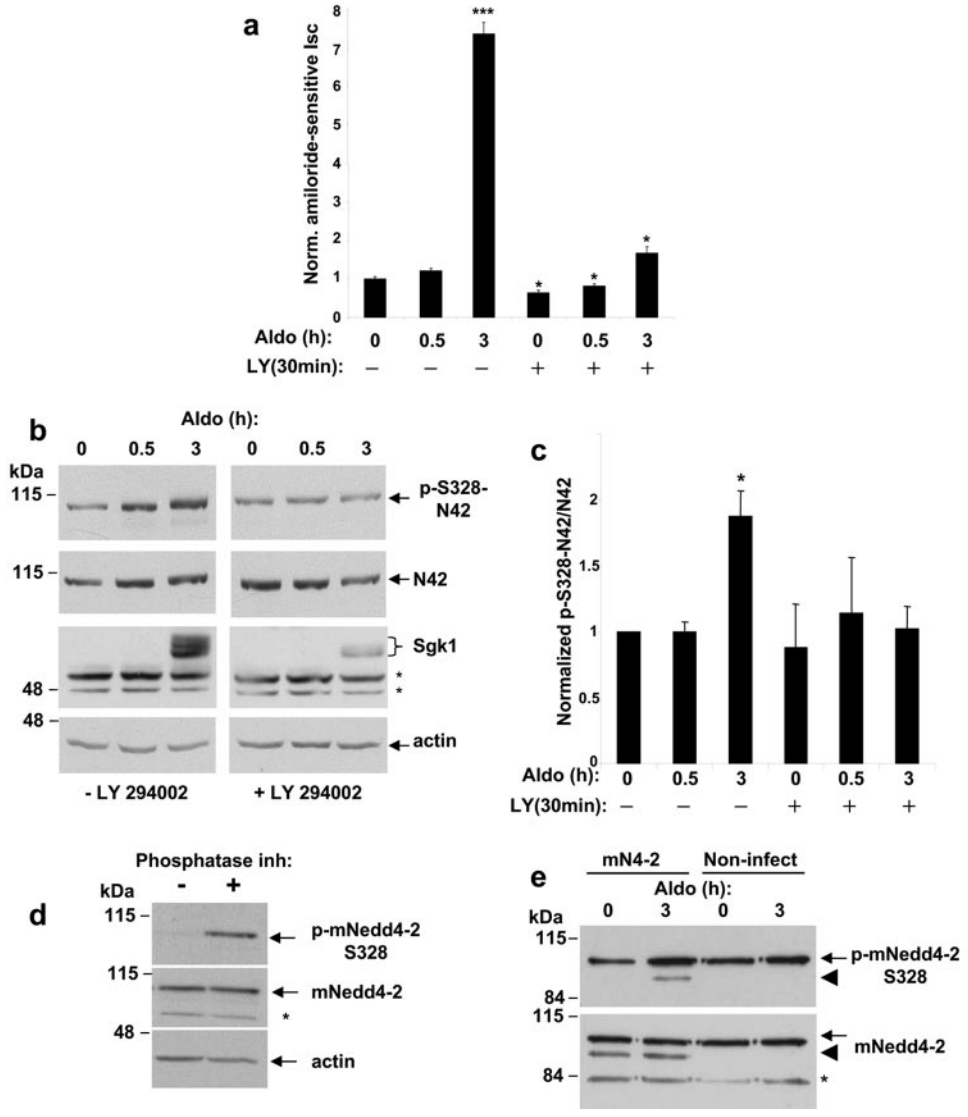


*Figure 2.* Effect of aldosterone and phosphatidylinositol-3 kinase (PI-3K) inhibition on transepithelial $Na^+$ transport, Sgk1 expression, and phosphorylation of S328–Nedd4-2. (A) Mouse cortical collecting duct (CCD) cells ($mpkCCD_{cl4}$) grown on collagen-coated filters were stimulated for the indicated times with 1 $\mu$M aldosterone (aldo). When indicated, 50 $\mu$M of LY-294002 (LY) was added to inhibit PI-3K activity 30 min before electrophysiologic measurements of the cells. Amiloride-sensitive short-circuit currents were measured and normalized to the currents of nonstimulated cells ($n$ = 9 to 12 filters from three independent experiments). *$P < 0.05$ *versus* control; ***$P < 0.005$ *versus* nonstimulated cells. (B) $mpkCCD_{cl4}$ cells were treated with 1 $\mu$M aldosterone (aldo) for the indicated times and/or 30 min of 50 $\mu$M LY-294002 before lysis. Lysates then were analyzed by SDS-PAGE/Western blot, using anti–pS328-mNedd4-2, mNedd4-2, Sgk, and actin antibodies. (C) p-mNedd4-2Ser328 and mNedd4-2 bands on fluorograms (of B) were quantified, and the ratio of pS328–mNedd4-2 to mNedd4-2 was calculated and normalized to the control condition ($n$ = 4 to 7 independent experiments). *$P <$ 0.05 *versus* control. (D) Western blotting of lysates of $mpkCCD_{cl4}$ monolayers prepared in lysis buffer that contained or did not contain phosphatase inhibitors, using the indicated antibodies. (E) $mpkCCD_{cl4}$ cells transduced with a retrovirus encoding mNedd4-2 or noninfected control cells were treated or not treated for 3 h with 1 $\mu$M aldosterone. Lysates were analyzed as described in B. Endogenous Nedd4-2 is indicated by arrows; exogenous Nedd4-2 is shown by arrowheads. * in B, D, and E denotes a cross-reacting, endogenous protein, likely an alternatively spliced Nedd4-2 isoform.

(corresponding to Ser328 in mouse Nedd4-2) (19). To study the phosphorylation of mouse Nedd4-2 (mNedd4-2) on Ser328 in epithelial cells, we raised and purified a phosphopeptide antibody against this region (anti–pS328-Nedd4-2) as described previously (24). Although the antibody was characterized toward human Nedd4-2, we confirmed its specificity for phosphorylated mouse Nedd4-2 by three means. First, we expressed Nedd4-2 with or without Sgk1 in *Xenopus laevis* oocytes and carried out Western blot analysis using anti–pSer328-Nedd4-2 (Figure 1A, top). Consistent with our previous observation that in oocytes Nedd4-2 is phosphorylated in the presence of Sgk1 (19), we detected mNedd4-2 only when mNedd4-2 and Sgk1

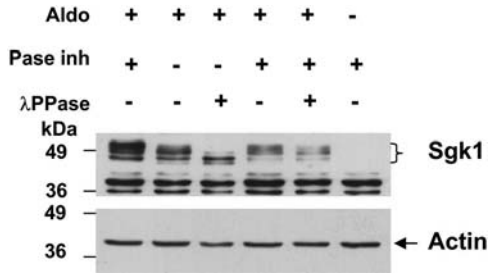


*Figure 3.* Dephosphorylation of Sgk1 by phosphatase treatment in mpkCCD$_{cl4}$ lysate. Cells that treated or not with 1 $\mu$M aldosterone for 3 h were lysed in lysis buffer that contained phosphatase inhibitors or not (as indicated). Samples were treated with Lambda protein phosphatase during 30 min at 30°C, and reactions were stopped by adding SDS-PAGE sample buffer and boiling at 95°C for 5 min. Samples were analyzed by SDS-PAGE/Western blotting using anti-Sgk antibody.

were coexpressed (Figure 1A, lane 3, top). When the lysate was treated with calf-intestinal phosphatase (calf intestinal alkaline phosphatase, lanes 4 to 6) or when Sgk1 was omitted (lanes 2 and 5), no band was observed despite the presence of Nedd4-2, as shown with another non–phosphoform-specific anti–Nedd4-2 antibody (Figure 1A, bottom). Second, in a competition assay, the anti–pSer328-Nedd4-2 antibody was incubated overnight with excess mNedd4-2 phospho- (ppept) or dephosphopeptide (de-ppept), and Western blot analysis of oocytes that were injected or not with mNedd4-2+xSgk1 was performed. The signal disappears only in the presence of the phosphopeptide, further corroborating the specificity of the anti–pSer328-Nedd4-2 antibody (Figure 1B). Finally, we infected 293 cells with an adenovirus encoding either mNedd4-2 or mNedd4-2-S328A (Ser328 mutated to alanine). As shown in Figure 1C, anti–pSer328-Nedd4-2 recognized only mNedd4-2 but not mNedd4-2-S328A, further demonstrating the specificity for p-S328–mNedd4-2.

### *Aldosterone Stimulates Transepithelial $Na^+$ Currents, Sgk1 Expression, and Nedd4-2 Phosphorylation in a PI-3K–Dependent Manner*

Having established the specificity of anti–p-mNedd4-2S328, we tested whether aldosterone augmented Nedd4-2 phosphorylation on Ser328 in renal epithelial cells. To carry out these studies, we took advantage of the mpkCCD$_{cl4}$ cell line, a highly differentiated cell model, derived from the mouse CCD and displaying aldosterone-dependent transepithelial $Na^+$ transport (21). mpkCCD$_{cl4}$ monolayers that were grown on collagen-coated filters were treated with 1 $\mu$M aldosterone for 30 min and 3 h. Because it was shown previously in A6 cells that the PI-3K inhibitor LY-294002 inhibits the aldosterone response (26), we tested the effect of 50 $\mu$M of LY-294002 on a subset of filters 30 min before the measurements. Amiloride-sensitive short-circuit current, representing $Na^+$ currents *via* ENaC, was measured in a modified Ussing chamber and normalized to control currents (*i.e.*, to the mean currents of untreated cells of the same day; Figure 2A). In agreement with previous reports (27), a 3-h aldosterone treatment significantly stimulated $Na^+$ currents. When LY-294002 was added, the basal and 30-min aldosterone currents were reduced by approximately 30%. After 3 h of aldosterone treatment, LY-294002 inhibited most of the current increase, indicating that the aldosterone early response depends on PI-3K activity (as reported previously for A6 cells [26]).

To evaluate the effect of aldosterone and LY-294002 on Sgk1 expression and Nedd4-2 phosphorylation, we analyzed cell lysates by SDS-PAGE/Western blotting, using antibodies against Sgk, pS328–Nedd4-2, Nedd4-2, or actin (Figure 2B). As reported previously (27), 3 h of aldosterone treatment stimulated the expression of Sgk1 kinase, displaying several bands on the Western blot (Figure 2B, third panel). To corroborate that the slower migrating protein bands represented differentially phosphorylated Sgk1, we treated the lysate with λ-phosphatase and found that these bands were disappearing, whereas treatment with phosphatase inhibitors increased the intensity of these bands. Moreover, without aldosterone treatment, none of the Sgk1 forms was detectable (Figure 3). Concomitant with the expression of Sgk1, the level of pS328–Nedd4-2 also increased (Figure 2B, top), whereas the total amount of Nedd4-2 remained unchanged. Quantification of the protein bands of four to seven independent experiments and calculation of the p-Nedd4-2 to Nedd4-2 ratio revealed a 1.8-fold increase of phospho Nedd4-2 as compared with the control (Figure 2C). Importantly, in contrast to the situation in *Xenopus* oocytes (19), Nedd4-2 seemed to be phosphorylated on Ser328 even in the absence of any detectable Sgk1 (*i.e.*, under control condition or 30 min of aldosterone stimulation). The notion that this band at basal conditions indeed represents phosphorylated Nedd4-2 is supported by the finding that incubation of cell lysates without phosphatase inhibitors for 30 min at 30°C leads to its near-complete disappearance when probed with anti–pS328-Nedd4-2 antibodies (Figure 2D, top).

When cells were treated with LY-294002, the slower migrating bands of Sgk1 became weaker (consistent with PI-3K–dependent phosphorylation of Sgk1), and the global expression levels of Sgk1 decreased (Figure 2B, third right panel). In parallel, the increase of phosphorylated Nedd4-2 with 3 h of aldosterone treatment was completely prevented by the PI-3K inhibitor (Figure 2B, top right panel; quantification in Figure 2C). Notably, the basal phosphorylation level of Nedd4-2 observed in the control and with 30 min of aldosterone treatment persisted with the PI-3K inhibitor.

To confirm further that aldosterone induces the phosphorylation of Nedd4-2, we expressed an exogenous mNedd4-2 protein in mpkCCD$_{cl4}$ cells, using a retroviral vector. This protein, which lacks the C2 domain, migrates faster than endogenous Nedd4-2 and therefore can be identified simultaneously by anti–Nedd4-2 antibodies. Nedd4-2ΔC2 was found to be expressed properly, although at lower levels than the endogenous proteins (Figure 2E, bottom). Under basal conditions, the endogenous Nedd4-2 was phosphorylated, whereas the exogenously expressed Nedd4-2ΔC2 protein was not detectable with the anti–pS328-Nedd4-2 antibody (Figure 2E, top). When cells were stimulated with aldosterone, phosphorylation levels of both endogenous and exogenous Nedd4-2 increased, sup-

porting the notion that aldosterone controls phosphorylation levels of Nedd4-2.

### *Sgk1 Expression Precedes Increase in Nedd4-2 Phosphorylation*

Because it has been suggested that Sgk1 is involved in S328–Nedd4-2 phosphorylation (19), we were interested to know whether such phosphorylation follows Sgk1 expression. We carried out a time course of aldosterone treatment over 6 h (Figure 4). mpkCCD$_{cl4}$ lysates were collected and analyzed by Western blotting for Sgk1 and Nedd4-2 expression, as well as phosphorylation level of Nedd4-2 Ser238. Sgk1 became detectable after 1 h of aldosterone induction and reached a peak at 3 h (Figure 4A, third panel). This was followed by an increase in S328–Nedd4-2 phosphorylation (Figure 4A, top, and quantification in Figure 4B), compatible with the notion that Sgk1 is involved in Nedd4-2 phosphorylation.

### *Aldosterone Increases Phosphorylation of mNedd4-2 in the CCD*

Having established that aldosterone is able to stimulate Sgk1 expression and Nedd4-2 phosphorylation in mpkCCD$_{cl4}$ cells, we wished to know whether aldosterone increases the level of phosphorylation in the CCD *in vivo*, as well. To address this question, we carried out immunohistochemistry on kidneys from adrenalectomized rats with and without aldosterone treatment. First, we tested the usefulness and the specificity of the anti–p328-Nedd4-2 antibody for immunohistochemical studies. On kidney sections from intact rats, the anti–p328-Nedd4-2 antibody (Figure 5A) strongly labeled the cytoplasm of epithelial cells lining the collecting ducts as shown for medullary collecting duct profiles in Figure 5A. Weak staining was also seen in other tubules such as thick ascending limbs (Figure 5A) and proximal tubules in the renal cortex (data not shown). Preincubation of the anti–p328-Nedd4-2 antibody with the immunogenic phosphopeptide completely abolished the immunostaining, whereas preincubation with the nonphosphopeptide was without any effect on the staining qualities of the antibody, indicating that the antibody recognizes only Nedd4-2 phosphorylated at position 328. Previous studies on ADX rats

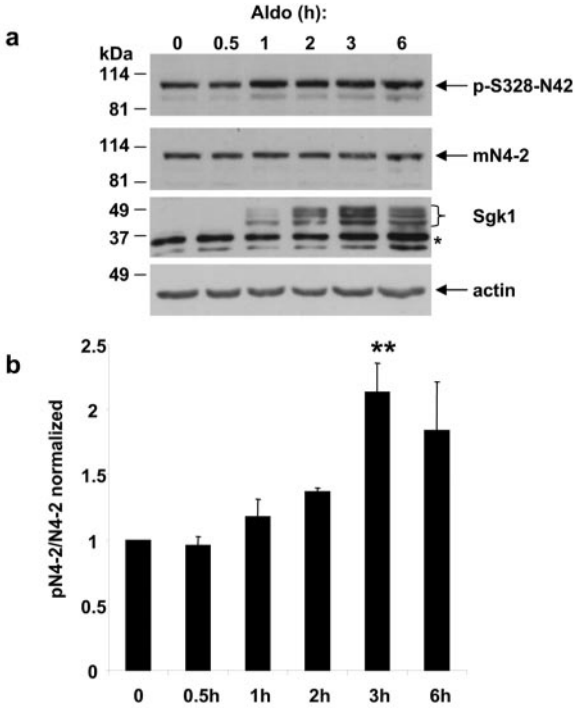


*Figure 4.* Time-dependent effect of aldosterone effect on S328–mNedd4-2 phosphorylation in mpkCCD$_{cl4}$. (A) Cells were treated with 1 $\mu$M aldosterone for the indicated times. Lysates were prepared and analyzed by Western blotting with anti–p-mNedd4-2Ser328, mNedd4-2, Sgk1, or actin antibodies. *Endogenous cross-reacting band. (B) pS328–mNedd4 and mNedd4-2 bands on fluorograms (A) were quantified using a phosphoimager, and the ratio of p-mNedd4-2Ser328 to mNedd4-2 was calculated and normalized to control condition. **$P < 0.01$ *versus* nonstimulated cells ($t = 0$).

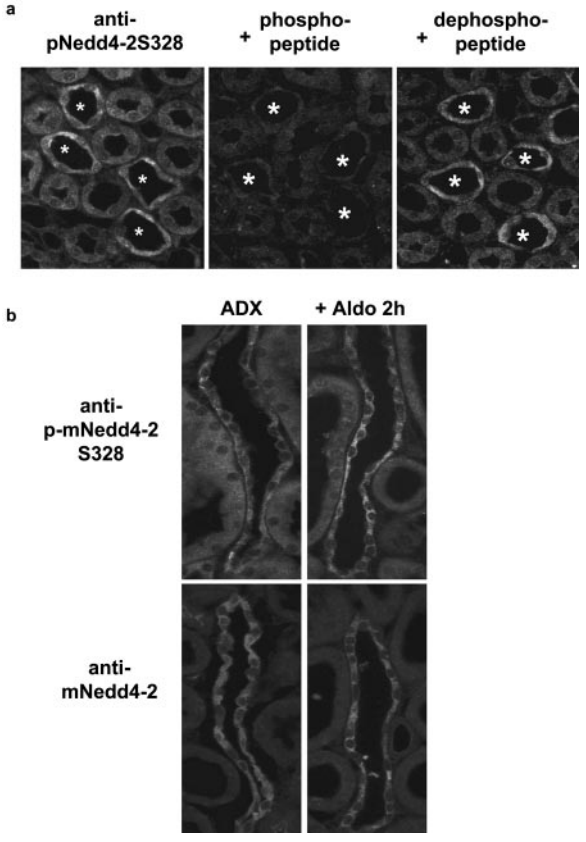


*Figure 5. In vivo* phosphorylation of S328–mNedd4-2 in cryosections of rat kidneys. (A) Inner stripe of outer kidney medulla immunostained with anti–pS328-mNedd4-2 antibodies (left) and with anti–p-mNedd4-2Ser328 antibodies that were preincubated with the immunogenic phosphopeptide (middle) or the corresponding dephosphopeptide (right). *Collecting ducts. (B) Kidney cortex immunostained with anti–p-mNedd4-2-Ser328 or mNedd4-2 antibodies. Two hours after a single aldosterone injection, p-mNedd4-2–Ser328 immunostaining is increased in the cytoplasm of CCD cells, whereas mNedd4-2–related immunostaining is unaffected by the aldosterone application.

showed that aldosterone increases Sgk1 expression from very low levels to high levels within 2 h and causes a redistribution of ENaC from the cytoplasm to the apical plasma membrane within 4 h (1). Now, we used the same tissues (from the experiments done for reference 1) to assess the phosphorylation status of Nedd4-2 in collecting ducts of vehicle- and aldosterone-treated ADX rats (Figure 5B). We found that pS328–Nedd4-2 was easily detectable in the collecting ducts of vehicle-treated ADX rats (in the absence of any aldosterone). However, 2 h after aldosterone injection, the p328–Nedd4-2–related immunostaining was clearly enhanced (Figure 5B, top). In contrast, the signal, obtained with an antibody that recognized both phosphorylated and unphosphorylated Nedd4-2, did not differ between collecting ducts from vehicle- and aldosterone-treated ADX rats (Figure 5B, bottom). Qualitative rating and digital image analysis of the staining intensities for each antibody and each rat (see the Materials and Methods section) confirmed these observations. For pNedd4-2 but not for Nedd4-2, all control rats were ranked below the aldosterone-treated rats. Measured mean pixel intensities for pNedd4-2 were consistently lower in the three vehicle-treated ADX rats (12.9, 13.8, and 19.6 arbitrary units; mean values per rat) than in the three aldosterone-treated ADX rats (21.2, 25.9, and 33.9 arbitrary units; mean values per rat). In contrast, measured values for Nedd4-2 did not clearly differ between vehicle-treated ADX rats (15.5, 19.0, and 26.7 arbitrary units) and aldosterone-treated rats (16.9, 19.5, and 37.3 arbitrary units). Taken together, these data indicate that aldosterone rapidly increases the level of phosphorylation on Ser328 of Nedd4-2 in the CCD *in vivo*, whereas it does not change the total pool of Nedd4-2, at least within the time frame of 2 h.

## Discussion

In this study, using the mpkCCD$_{cl4}$ cell line and ADX rats, we studied Sgk1 expression and correlated it with transepithelial $Na^+$ transport and S328–Nedd4-2 phosphorylation levels. We took advantage of a specific antibody raised against a phosphopeptide covering mNedd4-2 Ser328 (24), which we further characterized with respect to its specificity for phosphorylated mouse Nedd4-2.

Our data demonstrate that in mpkCCD$_{cl4}$ cells and *in vivo* in ADX rats, aldosterone stimulates the expression of Sgk1 and the phosphorylation of Ser328 of Nedd4-2 by approximately 1.8-fold. This increase in Nedd4-2–S328 phosphorylation is accompanied by the appearance of Sgk1 and by an increase of transepithelial, amiloride-sensitive $Na^+$ transport (Figure 2A). Moreover, when a time course of aldosterone stimulation is carried out, Sgk1 expression precedes slightly Nedd4-2 phosphorylation, but both peak at approximately 3 h of stimulation. When cells were treated with the PI-3K inhibitor LY-294002, the aldosterone-dependent increase of $Na^+$ transport and Nedd4-2 phosphorylation were inhibited, consistent with previous reports showing that PI-3K activity is required for mineralocorticoid-dependent $Na^+$ response (12,26,28) and for activation of Sgk1 (9,16). However, PI-3K inhibition reduces basal $Na^+$ transport but not basal phosphorylation levels of Nedd4-2. This suggests that a kinase different from Sgk1, Sgk2, Sgk3, or protein kinase B/Akt (all PI-3K dependant) is responsible for basal phosphorylation. One possible candidate is protein kinase A, as proposed recently by Snyder *et al.* (29), who have shown that the same residue can be phosphorylated by protein kinase A. Inhibition of PI-3K activity consistently resulted in a weakening of slower migrating Sgk1 bands (most likely phosphorylated forms of Sgk1) and also in a reduction of the total pool of Sgk1 (Figure 2B). Similar observations were made previously in A6 cells by Wang *et al.* (26), who observed a slight, statistically nonsignificant decrease of total Sgk1 expression. Therefore, expression of Sgk1 may depend on PI-3K activity.

It is interesting that the data in Figure 2E suggest that basal phosphorylation of Nedd4-2 requires the C2 domain, as the exogenous Nedd4-2 lacking the C2 domain does not display any basal phosphorylation. The C2 domain is a $Ca^{2+}$-dependent phospholipid-binding domain that has been shown to influence localization of the Nedd4-2 paralogue Nedd4–1 (30,31) or Nedd4-2 (32) and to interfere with Nedd4–1–dependent regulation of ENaC in *Xenopus laevis* oocytes (33,34). It therefore is conceivable that deletion of the C2 domain may change the properties or the localization of Nedd4-2 in mpkCCD$_{cl4}$ cells, consequently interfering with the basal phosphorylation by a kinase other than Sgk1.

It now is well established that Sgk1 is an early aldosterone-induced protein that seems to regulate ENaC and control $Na^+$ reabsorption (1,10–13). In support of our previous work (19), we now provide evidence that aldosterone regulates $Na^+$ transport in CCD cells *via* phosphorylation of Nedd4-2. Such phosphorylation then leads to interference with the ENaC/Nedd4-2 interaction, most likely because the phosphorylated Ser238 serves as a binding site for 14-3-3 adaptor proteins, which may sterically hinder the binding of Nedd4-2 to ENaC (35). The change in phosphorylation of Nedd4-2 is modest (approximately two-fold), as compared with the more than seven-fold stimulation of the $Na^+$ currents, indicating that the early response of aldosterone action involves not only Nedd4-2 phosphorylation. Clearly other factors also play a role in aldosterone-controlled $Na^+$ transport. Not only ENaC activity is affected by aldosterone but also the $Na^+$,$K^+$-ATPase (36), which has been shown to be regulated by Sgk1 as well (37). Moreover, there are several consensus phosphorylation sites for Sgk1 on Nedd4-2, which may as well be modified by changing levels of Sgk1 (19,20). Alternatively, Sgk1 may act, in addition to Nedd4-2 phosphorylation, *via* different mechanisms, such as direct binding and phosphorylation of ENaC, as suggested previously (26,38). Such additional mechanisms, which would preserve the sensitivity of aldosterone stimulation to PI-3K inhibition, are compatible with reports showing that Sgk1 increases ENaC activity in oocytes even when the PY motifs on ENaC (binding sites for Nedd4-2) are missing (39,40) or that aldosterone increases the activity of channels missing either the $\beta$- or $\gamma$-subunit PY motif in mpkCCD$_{cl4}$ cells (23) or transgenic mice (41).

In conclusion, our data demonstrate that aldosterone increases Nedd4-2 phosphorylation, simultaneously with Sgk1 expression, both in CCD cells derived from the mouse kidney and *in vivo* in ADX rats. This supports a model in which

aldosterone increases Sgk1 expression, which then will phosphorylate Nedd4-2. Such phosphorylation has been shown to interfere with ENaC/Nedd4-2 interaction, consequently reducing Nedd4-2–dependent ENaC ubiquitinylation, internalization and leading to accumulation of ENaC channels at the plasma membrane.

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