14-3-3 Isoforms Are Induced by Aldosterone and Participate in Its Regulation of Epithelial Sodium Channels*

Received for publication, February 13, 2006, and in revised form, March 30, 2006 Published, JBC Papers in Press, April 12, 2006, DOI 10.1074/jbc.M601360200

Xiubin Liang, Kathryn W. Peters, Michael B. Butterworth, and Raymond A. Frizzell

From the Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Aldosterone increases sodium absorption across renal collecting duct cells primarily by increasing the apical membrane expression of ENaC, the sodium entry channel. Nedd4-2, a ubiquitin-protein isopeptide ligase, tags ENaC with ubiquitin for internalization and degradation, but when it is phosphorylated by the aldosterone-induced kinase, SGK1, Nedd4-2 is inhibited and apical ENaC density and sodium absorption increase. We evaluated the hypothesis that 14-3-3 proteins participate in the aldosterone-mediated regulation of ENaC by associating with phosphorylated Nedd4-2. Mouse cortical collecting duct (mCCD) epithelia cultured on filters expressed several 14-3-3 isoforms; this study focused on an isoform whose expression was induced 3-fold by aldosterone, 14-3-3β. In polarized mCCD epithelia, aldosterone elicited significant, time-dependent increases in the expression of α-ENaC, SGK1, phospho-Nedd4-2, and 14-3-3β without altering total Nedd4-2. Aldosterone decreased the interaction of α-ENaC with Nedd4-2, and with similar kinetics increased the association of 14-3-3β with phospho-Nedd4-2. Short interfering RNA-induced knockdown of 14-3-3β blunted the aldosterone-induced increase in α-ENaC expression, returned α-ENaC-Nedd4-2 binding toward pre-aldosterone levels, and blocked the aldosterone-stimulated increase in transepithelial sodium transport. Incubation of cell extracts with a selective phospho-Nedd4-2 antibody blocked the aldosterone-induced association of 14-3-3β with Nedd4-2, implicating SGK1 phosphorylation at Ser-328 as the primary site of 14-3-3β binding. Our studies show that aldosterone increases the expression of 14-3-3β, which interacts with phospho-Nedd4-2 to block its interaction with ENaC, thus enhancing sodium absorption by increasing apical membrane ENaC density.

The first step in sodium transport across epithelial cells lining the distal nephron is facilitated by the epithelial sodium channel, ENaC, α a constituent of the apical (lumen-facing) membrane. These channels are identified by their sensitivity to the diuretic, amiloride, by their high selectivity to sodium over potassium, and by their single channel conductance of ~5 picosiemens (1, 2). Cloning of ENaC identified its three homologous subunits, α, β, and γ (3–5), and has facilitated studies of sodium transport regulation. The physiological importance of ENaC is illustrated by human genetic diseases in which dysfunction of the channel results in clinical defects in salt and water balance (2). Mutations in the genes encoding the α-, β-, or γ-subunits occur in families with pseudohypoaldosteronism, type 1, characterized by a loss of ENaC function (6), or with genetic hypertension, which results from a gain in ENaC function (7). Liddle syndrome is an autosomal dominant form of hypertension that is sensitive to amiloride, reflecting a chronic elevation of ENaC activity.

Acute and chronic regulation of ENaC contribute to salt and water homeostasis by controlling the rate of sodium absorption across renal collecting duct epithelia where ENaC constitutes the rate-determining step in sodium absorption (8). Chronic regulation of ENaC in response to reduced extracellular fluid volume is mediated by aldosterone, the primary mineralocorticoid of vertebrates. A lag of approximately 1 h precedes the response to aldosterone, and this involves binding of the hormone to mineralocorticoid receptors in the cytosol and their translocation to the nucleus. The resulting induction of gene transcription alters the levels of transport and regulatory and metabolic proteins to elicit a concerted increase in distal nephron sodium absorption (9, 10).

During sodium repletion (low aldosterone), the β- and γ-subunits of ENaC are present in distal nephron principal cells, but the low expression of α-ENaC is limiting for channel function because the α-ENaC subunit is essential for the assembly of functional sodium channels that can traffic to the apical surface (11). Thus, stimulation of α-ENaC transcription by aldosterone permits assembly of the heterotrimERIC channel in the endoplasmic reticulum and its trafficking to the apical membrane, providing an important control point for enhancing sodium absorption.

Once ENaC is delivered to the apical surface, its residency and turnover kinetics are governed by endocytic processes and associated protein interactions that involve recognition of PY motifs within the ENaC subunit C termini. These motifs constitute binding sites for the tryptophan-rich WW domains of the E3 ubiquitin ligase, Nedd4-2. ENaC binding and ubiquitination by Nedd4-2 cause the channel to engage with the endocytic machinery (12, 13). By promoting channel internalization and degradation, these protein interactions reduce apical ENaC surface density and maintain a low rate of sodium absorption during salt repletion. Accordingly, genetic mutation of the PY motifs in ENaC, or their elimination by premature stop codons, obviates the interaction of the channel with Nedd4-2, accounting for the increase in apical membrane ENaC density observed in patients with Liddle hypertension (14).

The stimulatory effect of aldosterone on sodium absorption is mediated primarily by increases in the number of active sodium entry channels in the apical membranes of collecting duct cells (1, 15). Alterations in ENaC-Nedd4-2 interactions contribute to increased apical ENaC density during aldosterone stimulation. This

* This work was supported by National Institutes of Health Grants DK54814 and DK72506 and by grants from the Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Cell Biology and Physiology, University of Pittsburgh School of Medicine, 3500 Terrace St., Pittsburgh, PA 15261. Tel: 412-648-9498; Fax: 412-648-8330; E-mail: frizzell@pitt.edu.

2 The abbreviations used are: ENaC, epithelial sodium channel; CCD, cortical collecting duct; Nedd4-2, neural precursor cells expressed developmentally down-regulated gene 4 isoform 2; SGK1, serum- and glucocorticoid-regulated kinase 1; siRNA, short interfering RNA; mCCD, murine CCD; E3, ubiquitin-protein isopeptide ligase; RT, reverse transcription; HA, hemagglutinin; RNAi, RNA interference; IP, immunoprecipitation.
14-3-3 Isoforms Are Aldosterone-induced Proteins

action of the steroid involves post-translational modifications in Nedd4-2 by the serum- and glucocorticoid-induced kinase, SGK1, a member of the phosphatidylinositol 3-kinases whose expression is induced as part of the early response of cells to mineralocorticoids. After its induction by aldosterone, SGK1 phosphorylates residues corresponding to its consensus motif, RXRXX(S/T), and recent studies have demonstrated that Nedd4-2 contains three phosphorylation sites for the kinase (13, 16). The phosphorylation of Nedd4-2 by SGK1 interferes with its binding to ENaC, blocking channel endocytosis and increasing ENaC surface expression and sodium transport rate. Exactly how Nedd4-2 phosphorylation interferes with its ability to bind ENaC, however, has remained unclear.

14-3-3 proteins constitute a family of highly conserved regulatory molecules that generally bind to phosphorylated residues (motifs) within their protein targets. In this way, they play important roles in a wide range of cellular processes, including signal transduction, cell cycling, protein biogenesis, and membrane trafficking (17, 18). There are seven mammalian isoforms of 14-3-3 (β, ε, γ, η, θ, σ, and ζ), each encoded by a distinct gene, and they may function as homo- or heterodimers (19). Varied motifs have been associated with 14-3-3 binding, but two high affinity, phosphorylation-dependent motifs (RSXpSXP and RXXpSXP) were identified classically as 14-3-3 protein-binding sites (20–23). These clearly resemble the SGK1 phosphorylation motifs on Nedd4-2 (see above); therefore, we sought to determine the role of 14-3-3 proteins in ENaC regulation.

Since this project was initiated, two other groups have reached this conclusion, and their studies have implicated Nedd4-2 as a substrate for 14-3-3 protein binding in response to SGK1 phosphorylation (24, 25). Those studies, performed primarily in Xenopus oocytes and HEK cells, demonstrated interactions of the 14-3-3-η and σ isoforms with Nedd4-2 and increases in ENaC activity when these isoforms were co-expressed (discussed further below). The present study focuses on the role of 14-3-3 proteins in the response of mCCD epithelia to aldosterone. We evaluated 14-3-3 isoform expression in polarized mCCD cells, determined the influence of aldosterone on 14-3-3 isoform expression, and evaluated the impact of an aldosterone-induced isoform, 14-3-3β, on the protein interactions involved in the stimulation of transepithelial sodium transport by the steroid hormone.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies specific for 14-3-3 isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) as follows: β (A-15), γ (C-16), ε (T-16), η (C-17), σ (N-14), ζ (K-12), and ρ (C-16). Santa Cruz Biotechnology indicates isoform specificity of their 14-3-3 antibodies, except for cross-reactivity of anti-14-3-3η with two other isoforms. However, no signal for this isoform was detected at either the RNA or protein level (see below). Anti-Nedd4-2 phosphopeptide antibody was generously provided by Dr. Oliver Staub, University of Lausanne, Switzerland; it was generated, affinity-purified, and characterized as described (26). Antibodies to SGK1 and Nedd4-2 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). For α-ENaC, a rabbit polyclonal antibody was raised against an epitope of the extracellular loop corresponding to the human amino acid sequence YRYPEIKEEELDRITEQT. Cysteine was added at the N terminus for coupling to keyhole limpet hemocyanin; the mouse sequence in this region differs by one amino acid. Secondary antibodies against mouse or rabbit were obtained from Amersham Biosciences. Antibodies to β-actin and the HA epitope were obtained from Sigma.

**Cell Culture**—mCCDcl14 cells (provided by A. Vandewalle and M. Bens, INSERM, Paris, France) were grown in flasks (passage 30–40) in defined medium as described previously (27). The growth medium was composed of equal volumes of Dulbecco’s modified Eagle’s medium and Ham’s F-12, plus 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% v/v fetal calf serum, and 20 nM HEPES, pH 7.4 (reagents from Invitrogen and Sigma). The cells were maintained at 37 °C in 5% CO2, 95% air, and the media were changed every 2nd day.

For both biochemical and electrophysiological experiments, mCCD cells were subcultured onto permeable filter supports. Transepithelial currents were measured across Transwell filters (0.4-μm pore size, 0.33-cm² surface area; Corning Costar). For the biochemical experiments, mCCD epithelia were polarized on 45-cm² filters (Corning Costar) for ~7 days prior to use. Development of a polarized monolayer was assessed by recordings of open circuit voltage (typically ~50 mV) and transepithelial resistance (typically ~2 kilohms cm²) using “chopstick” electrodes (Millipore). To ensure a regulatory base line, the growth medium bathing cells on filters was replaced with a minimal medium of Dulbecco’s modified Eagle’s medium/F-12 (without drugs or hormones) for at least 24 h prior to experiments. Thereafter, mCCD epithelia were either maintained without additives or treated with aldosterone (10 nM; Sigma) for 0, 6, 12, 24, and 48 h. This concentration of aldosterone activates mineralocorticoid but not glucocorticoid receptors in CCD cells (28), and it corresponds to plasma aldosterone levels that are reached during severe salt restriction (29).

**RNA Expression**—Total RNA was extracted from polarized CCD cells and reverse-transcribed to single-stranded cDNA as described previously (30). Semi-quantitative RT-PCR was performed to analyze gene expression in polarized mCCD epithelia. According to published protocols, cDNA was amplified using 20 pmol of specific primers for α-ENaC, SGK1 (31), 14-3-3 isoforms (32), Nedd4-2, and β-actin (31). Primer sequences and PCR conditions are provided in Table 1. Preliminary experiments were performed to determine the optimal number of PCR cycles that yielded products in the linear range of amplification. The abundance of each detected transcript was normalized to that of β-actin.

**Co-immunoprecipitation and Antibody Competition Assays**—Protein assays (BCA; Pierce) ensured that equivalent amounts of protein were used for Western blot analysis and immunoprecipitation. Pre-cleared mCCD cell lysates (~1 mg of protein) were mixed with the appropriate primary antibodies for 1.5 h at 4 °C in lysis buffer (0.4% deoxycholate acid, 1% Nonidet P-40, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4). Twenty five μl of washed protein A- or G-Sepharose beads was added to each sample and incubated for 1 h at 4 °C with gentle rotation. Immunocomplexes were washed with lysis buffer four times and precipitated by centrifugation at 12,000 × g for 10 s. The immunocomplexes were resuspended in SDS sample buffer and subjected to immunoblotting (see below). For the phospho-Nedd4-2 competition assay, the phosphopeptide antibody was added to lysates from polarized mCCD cells for 2 h at 4 °C before mixing with precipitating antibodies; the co-IP was then performed as above. Controls for the immunoprecipitations were performed using a concentration of HA antibody equal to that of the primary precipitating antibody.
14-3-3 Isoforms Are Aldosterone-induced Proteins

**TABLE 1**

| Gene        | Primer sequences | Cycle | Tm (°C) | Size (bp) |
|-------------|------------------|-------|---------|-----------|
| 14-3-3β     | 5’−TTG AGG GTA CAG ACG GAT GAC−3’ | 30    | 58      | 840       |
| 14-3-3γ     | 5’−CAG TCG CTT GGT GAT GAC−3’ | 30    | 58      | 836       |
| 14-3-3ε     | 5’−ATA AAG AGA CAG ACG GAT GAC−3’ | 30    | 58      | 836       |
| 14-3-3η     | 5’−CTC TGG AGA GAG CTG GAC−3’ | 30    | 58      | 980       |
| 14-3-3σ     | 5’−AGA GAG CAG GAG CCT GCA CGA AAT−3’ | 30    | 58      | 1012      |
| 14-3-3θ     | 5’−CTG AGA GAT GAC GAT GAC−3’ | 30    | 58      | 858       |
| 14-3-3ζ     | 5’−CTG AGA GAT GAC GAT GAC−3’ | 30    | 58      | 571       |
| α-ENaC      | 5’−TCG TGG CTT TGG CGT ACC−3’ | 30    | 60      | 493       |
| SGK1        | 5’−TCC CAA CCA CCA CGT AGA−3’ | 30    | 64      | 350       |
| Nedd4-2     | 5’−TGA AGT GAA GGG TGG ACC−3’ | 30    | 64      | 344       |
| β-Actin     | 5’−ATG GAT GAC GAT GAC−3’ | 30    | 64      | 202       |
| Antisense   | 5’−CTT CTT ACT AAT GCA ACC GAC−3’ | 30    | 58      | 539       |

Immunoblot Analyses—Equal amounts of protein from either aldosterone-treated or nontreated polarized mCCD cells or the immunoprecipitates described above were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Unbound sites were blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBST and incubated with primary antibodies (anti-14-3-3 isoform, each 1:1000; anti-phospho-Nedd4-2, 1:500; anti-Nedd4-2, 1:1000; anti-SGK1, 1:1000; anti-α-ENaC, 1:1000; or anti-β-actin, 1:3000) at room temperature for 2 h. The blots were then washed three times for 10 min each with TBST and incubated for 1 h with 2 μg/ml horseradish peroxidase-conjugated secondary antibodies (1:1000; Amersham Biosciences) in TBST with 5% milk, followed by three TBST washes. The reactive bands were visualized with enhanced chemiluminescence (PerkinElmer Life Sciences) and exposed to x-ray film (Eastman Kodak Co.). β-Actin expression provided an internal control.

mCCD Transfection with siRNA—Control siRNA and siRNA for 14-3-3β were obtained from Dharmacon Inc. (Chicago) as SMARTpool® reagents. This contains four SMARTdesign selection siRNAs targeting four regions of the same mRNA without targeting related gene family members (see Fig. 5 for verification). In vitro transfections with 14-3-3 or control siRNAs were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In brief, mCCD cells were seeded on filters and were transfected with siRNAs when they were 80% confluent. A total of 100 pmol of siRNA was used for 5 × 10⁵ cells in 2 ml of culture medium. The filters were washed 24 h after transfection and then maintained under control conditions or treated with aldosterone (10 nM) for 48 h. Thus, 3 days elapsed between siRNA transfection and the biochemical or functional assays to evaluate the influence of 14-3-3 knockdown on protein expression, protein interactions, or sodium transport. In addition to the siRNA control, the results were compared with data obtained from untreated CCD cells.

Short Circuit Current Recordings—Epithelia cultured on filter supports were mounted in modified Ussing chambers (Costar), and the cultures were continuously short circuited by an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City) as described previously (33). The bathing solution consisted of (in mM) the following: 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 10 D-glucose. Chambers were maintained at 37 °C and gassed continuously with a mixture of 95% O₂, 5% CO₂, which fixed the pH at 7.4. Amiloride (10 μM) was added to the apical bath to determine ENaC-mediated transepithelial currents.

**Statistical Analysis**—Data were obtained from experiments performed 3–4 times, and values are presented as mean ± S.E. p values were calculated by analysis of variance followed by unpaired t test as appropriate. A p value <0.05 was considered to be statistically significant.

![FIGURE 1. Aldosterone regulates the expression of 14-3-3 isoforms.](image-url)
RESULTS

Aldosterone Regulates the Expression of 14-3-3 Isoforms—The expression of 14-3-3 isoforms in mCCD cells was determined by RT-PCR and Western blotting. cDNA generated from polarized mCCD epithelia under basal, nonstimulated conditions was used with isoform-specific primers (Table 1) to perform semi-quantitative PCR (Fig. 1A). Products of appropriate size and sequence corresponded to the 14-3-3 isoforms β/γ, ε, θ, and σ; 14-3-3η and -ζ were not detected. In other experiments, cell lysates were obtained from both control and aldosterone-treated mCCD monolayers; the lysates were resolved by SDS-PAGE and probed for 14-3-3 proteins using the seven isoform-specific antibodies described above. The results obtained under basal conditions, shown in Fig. 1B, agree qualitatively with the evaluation of isoform expression at the RNA level. Isoforms β, γ, and σ were most abundantly expressed in mCCD epithelia under basal conditions; relatively weak expression of the ε and θ isoforms was observed. As in the PCR study, the 14-3-3η and -ζ isoforms were not detected at the protein level using the available antibodies (not shown).

The aldosterone sensitivity of 14-3-3 protein expression was determined in mCCD epithelia treated with 10 nM aldosterone for 24 h (Fig. 1B). This treatment increased the expression of the 14-3-3β and -ε isoforms by 2.8- and 8.2-fold, respectively. The β isoform predominated in the presence of aldosterone even though the fold increase in 14-3-3ε was greater. This is the first demonstration of steroid sensitivity of 14-3-3 protein expression, and it implies that these isoforms may play an important role in the functional response of CCD epithelia to aldosterone. Significant changes were not observed in the expression of 14-3-3γ, -θ, or -σ, and as under basal conditions, 14-3-3η and -ζ were not detected following treatment with aldosterone. These data indicate selectivity of the steroid response, and they imply that there is specificity among the expressed isoforms regarding their binding partners in mCCD epithelia.

14-3-3 Proteins Interact Directly with Nedd4-2 in Vivo—As discussed above, prior studies have suggested that 14-3-3η and -σ interact physically with Nedd4-2 in heterologous expression systems (24, 25). We examined the interaction of the five expressed 14-3-3 isoforms with Nedd4-2 in co-immunoprecipitation experiments performed using lysates from polarized mCCD epithelia. Protein complexes were isolated from epithelia maintained under basal or aldosterone-treated mCCD monolayers; the lysates were resolved by SDS-PAGE and probed for 14-3-3 proteins using the seven isoform-specific antibodies described above. The results obtained under basal conditions, shown in Fig. 1B, agree qualitatively with the evaluation of isoform expression at the RNA level. Isoforms β, γ, and σ were most abundantly expressed in mCCD epithelia under basal conditions; relatively weak expression of the ε and θ isoforms was observed. As in the PCR study, the 14-3-3η and -ζ isoforms were not detected at the protein level using the available antibodies (not shown).

The aldosterone sensitivity of 14-3-3 protein expression was determined in mCCD epithelia treated with 10 nM aldosterone for 24 h (Fig. 1B). This treatment increased the expression of the 14-3-3β and -ε isoforms by 2.8- and 8.2-fold, respectively. The β isoform predominated in the presence of aldosterone even though the fold increase in 14-3-3ε was greater. This is the first demonstration of steroid sensitivity of 14-3-3 protein expression, and it implies that these isoforms may play an important role in the functional response of CCD epithelia to aldosterone. Significant changes were not observed in the expression of 14-3-3γ, -θ, or -σ, and as under basal conditions, 14-3-3η and -ζ were not detected following treatment with aldosterone. These data indicate selectivity of the steroid response, and they imply that there is specificity among the expressed isoforms regarding their binding partners in mCCD epithelia.

14-3-3 Isoforms Are Aldosterone-induced Proteins

The time course of aldosterone-induced expression of α-ENaC, SGK1, and 14-3-3β and phospho-Nedd4-2. mCCD epithelia were treated with 10 nM aldosterone for the indicated times. Expression of the indicated mRNAs or proteins was determined by RT-PCR (A) and immunoblot (B). C, quantitation of immunoblot data for the indicated proteins, normalized to β-actin expression. Control epithelia were not exposed to aldosterone; values at zero time are from epithelia exposed to steroid and lysed immediately in sample buffer; these values did not differ.
under these conditions (see Fig. 1). Relative to its basal expression level, the 14-3-3γ interaction was particularly weak, suggesting isoform selectivity for Nedd4-2 binding. Nedd4-2 binding to 14-3-3γ and 14-3-3ε was markedly increased by aldosterone, the latter reflecting largely the 8-fold induction of its expression by the steroid (Fig. 1B). The interaction of Nedd4-2 with 14-3-3γ was not affected by aldosterone treatment.

Presumably, the interactions detected in the absence of aldosterone represent binding to phosphorylated Nedd4-2. Previous studies have detected tonic activity of SGK1 in mCCD cells (34, 35). In addition, we detected a small amount of phosphorylated Nedd4-2 in the absence of aldosterone treatment (Fig. 3). This basal level of interaction may also result from lingering effects of dexamethasone, which is present in the mCCD cell growth media and removed 1 day prior to study. The identification of 14-3-3β as an aldosterone-induced protein, and its predominant association with Nedd4-2 during stimulation, prompted us to examine the functional significance of this interaction and its influence on ENaC-mediated sodium transport during the aldosterone response.

**Pattern of ENaC Regulatory Protein Expression during the Action of Aldosterone**—We used RT-PCR and immunoblot analyses to examine the expression of α-ENaC, SGK1, Nedd4-2, and 14-3-3β as a function of time during the stimulation of mCCD epithelia by 10 nM aldosterone. As shown in Fig. 3, A and B, the expression of α-ENaC, SGK1, and 14-3-3β was increased at both the RNA and protein levels during the action of aldosterone, whereas the expression of total Nedd4-2 remained unchanged. At the protein level, blots obtained with a phosphopeptide antibody to Nedd4-2 showed that the increases in SGK1 expression were paralleled by increased phosphorylation of Nedd4-2. Quantitation of the protein data from all experiments, which included normalization to β-actin expression (Fig. 3C), indicates a 14-fold increase in SGK1 over the aldosterone treatment period, while phospho-Nedd4-2, α-ENaC, and 14-3-3β each increased about 3-fold. Thus, the aldosterone-induced expression of 14-3-3β is similar in magnitude and time course to that of other critical components of the regulatory pathways that mediate the steroid response.

**Reciprocal Interactions of α-ENaC and 14-3-3β with Nedd4-2 during the Response to Aldosterone**—As discussed above, a decrease in the association of Nedd4-2 with ENaC is an important aspect of the increase in apical membrane channel density that accompanies the renal response to aldosterone or SGK1 (12, 13). We therefore evaluated the interactions between Nedd4-2 and either α-ENaC or 14-3-3β in co-IP experiments as a function of aldosterone treatment time; again, anti-HA served as a control for the IPs. As shown in Fig. 4A, association of ENaC with Nedd4-2 decreased as a function of treatment time, and this was paralleled by an increase in the binding of 14-3-3β to Nedd4-2. As shown in the lowest row, where the blots were performed using the phosphopeptide antibody, it was the phosphorylated form of Nedd4-2 that increasingly associated with 14-3-3β. In this manner, phosphorylated Nedd4-2 is prevented from associating with ENaC by its interaction with 14-3-3β. This reciprocity in Nedd4-2 binding to ENaC versus 14-3-3 is a time-dependent function of the response to aldosterone.

The data from all experiments are quantified in Fig. 4B (without normalization, see below); they show that Nedd4-2 phosphorylation elicits a shift in its binding partner, from ENaC to 14-3-3β, during the response to aldosterone. In Fig. 4C, the data were quantified with a normalization method that factors in the increases in α-ENaC or 14-3-3β expression because of aldosterone stimulation. Thus, the values provided in Fig. 4C are corrected for the aldosterone-associated elevations of α-ENaC or 14-3-3β. Comparison of Fig. 4, B and C, indicates that the switch in Nedd4-2 binding from α-ENaC to 14-3-3β has two components, one due to the preferential association of 14-3-3β with phospho-Nedd4-2 and the other due to changes in the expression levels of these proteins. For example, without normalization for expression level, the increase in 14-3-3β binding to phospho-Nedd4-2 was 5-fold. With normalization, the increase was much less, ~60% over control, so that the 3-fold
increase in 14-3-3β expression with aldosterone treatment is a significant component of total binding.

**14-3-3β Knockdown Reverses the Aldosterone-induced Increase in ENaC Expression and Increases the Association of ENaC with Nedd4-2**—The significance of 14-3-3 protein for the interaction between ENaC and Nedd4-2 was evaluated under basal and aldosterone-stimulated conditions using RNA interference (RNAi) experiments. We employed 14-3-3β-targeted siRNAs to knock down this 14-3-3 isoform in polarized mCCD cells, and we compared the results with those obtained with control scrambled siRNA. The RNAi-induced reduction in 14-3-3β expression in different experiments ranged between 60 and 88% under basal or stimulated conditions, whereas the control siRNA did not affect 14-3-3β expression (Fig. 5A).

First, we showed that 14-3-3β knockdown did not affect the expression of the other 14-3-3 isoforms that are present at significant levels during aldosterone treatment, e and σ (Fig. 5A, 2nd and 3rd rows). Thus, the RNAi effect on 14-3-3β was selective. Next, we evaluated the influence of 14-3-3β knockdown on the expression of α-ENaC; the results are shown by the blots in Fig. 5A, 4th row. Quantitation of the ENaC data from all experiments is provided in Fig. 5B. In nonstimulated cells, a reduction in 14-3-3β did not alter ENaC expression. The lack of an effect of 14-3-3 knockdown under basal conditions may result from the low level of phospho-Nedd4-2 present in these cells in the absence of SGK1 induction (Fig. 3B). With the phosphorylated binding partner for 14-3-3 in short supply, a less than complete reduction in 14-3-3β levels may have minimal effects. Alternately, another 14-3-3 isoform, e.g. 14-3-3σ (Figs. 1 and 2), may stabilize the relatively small amount of phosphorylated Nedd4-2 found in the absence of aldosterone stimulation or compensate for the effects of decreased 14-3-3β. In this regard, Bhalla et al. (25) showed that 14-3-3σ binds Nedd4-2, and tonic activity in this pathway is suggested by the ability of Nedd4-2 RNAi to increase ENaC current in the absence of stimulation (36). Thus, attempts to alter basal ENaC activity may require a more general targeting 14-3-3 isoforms.

In stimulated cells, however, the increase in α-ENaC expression observed with aldosterone treatment was blunted ~75% in cells expressing reduced 14-3-3β. Relative to control cells, the ~25% elevation of α-ENaC remaining after 14-3-3β RNAi may reflect the influence of aldosterone on the transcription of this ENaC subunit under these conditions. Conversely, the significant reduction in α-ENaC observed with 14-3-3β knockdown during aldosterone stimulation likely reflects decreased degradation of the channel associated with its increased residency in the plasma membrane when its interactions with Nedd4-2 are attenuated (12, 37).

Next, we determined the influence of 14-3-3β RNAi on the physical interaction between α-ENaC and Nedd4-2 under basal and stim-
amiloride-sensitive Isc from all experiments of the type shown in Fig. 6 decreased decrease in the interaction of Nedd4-2 with ENaC; this interaction returned to levels near those observed in the absence of steroid. Data from all experiments (Fig. 6B) indicate that aldosterone produced a 75% reduction in the amount of Nedd4-2 that co-immunoprecipitated with α-ENaC. With 14-3-3β knockdown, the aldosterone-induced reduction in ENaC-Nedd4-2 binding was only 30%. Together, these data indicate that 14-3-3β binding to phosphorylated Nedd4-2 is necessary to block the association of Nedd4-2 with ENaC.

**DISCUSSION**

Key to the action of aldosterone is the transcription of a specific set of genes that elicit a long term increase in sodium absorption (1, 2). The induction of SGK1 expression represents a central molecular mechanism governing the activity of ion channels that mediate distal nephron sodium absorption (ENaC) and also potassium secretion (ROMK) (38). Among the targets of SGK1 is the E3 ubiquitin ligase, Nedd4-2, whose phosphorylation decreases its interaction with ENaC, reducing channel ubiquitination, endocytic retrieval, and degradation, thereby increasing apical membrane ENaC density to augment sodium transport. The early
response of SGK1 is widely regarded as a primary event in aldosterone stimulation, both in vitro and in vivo (38–41). In adrenalectomized rats, a significant increase in SGK1 is observed within 2 h of aldosterone administration. On the other hand, chronic (several days) treatment of mice with high levels of aldosterone or a salt restriction protocol produced elevations of SGK1 of 8-fold, assessed by in situ hybridization (29). In this study, SGK1 showed the largest increase in expression in response to aldosterone (~3-fold at 6 h and ~14-fold at 48 h; Fig. 3), similar to increases observed in vivo.

This work, and that of others (24, 25), has identified an important additional mechanism for ENaC control in which the SGK1-mediated inhibition of Nedd4-2 activity relies on the interaction of phospho-Nedd4-2 with 14-3-3 proteins. In the present studies, we detected the expression of five 14-3-3 isoforms in mCCD epithelia by PCR and immunoblot and showed that 14-3-3β and -ε expression was augmented by physiological levels of aldosterone. These are the first studies to identify steroid regulation of 14-3-3 expression, and they imply that there may be mineralocorticoid response elements upstream of the promoters for the β and ε isoforms. We focused on 14-3-3β as the predominant isoform expressed in aldosterone-treated epithelia and found that time-dependent increases in 14-3-3β levels paralleled those of SGK1, α-ENaC, and phospho-Nedd4-2. Total Nedd4-2 was unchanged, as in prior studies (26). Co-immunoprecipitation experiments demonstrated that Nedd4-2 shifted its physical association from ENaC to 14-3-3β, which interacted with the phosphorylated form of Nedd4-2. The physiological significance of this interaction was revealed by selective 14-3-3β knockdown, which reversed the ENaC–Nedd4-2 interaction to nearly pre-aldosterone levels and virtually eliminated the aldosterone-induced stimulation of sodium absorption across mCCD epithelia. 14-3-3β knockdown also blunted the steroid-induced increase in α-ENaC expression, indicating the importance of ENaC stabilization at the apical membrane in this response. Thus, our findings demonstrate that the ability of 14-3-3 proteins to block the interaction between Nedd4-2 and ENaC is a critical component of the stimulation of renal sodium retention elicited by aldosterone.

Relation to Other Work—Since commencing our studies, two papers have appeared that identified the interaction of 14-3-3 proteins with phosphorylated Nedd4-2, as demonstrated here also. Proteomic analyses by Ichimura et al. (24) used lysates from PC12 cells to implicate Nedd4-2 as a 14-3-3β binding partner. They showed that a mutant 14-3-3β inhibited ENaC currents in Xenopus oocytes that were augmented by co-expression of SGK1, but they were unable to increase ENaC currents in response to wild type 14-3-3β co-expression. In addition, they found that the ubiquitination of α- or γ-ENaC, expressed as individual subunits in HEK cells, was reduced by co-expression with 14-3-3β, allowing them to speculate that 14-3-3 proteins would increase sodium transport by interfering with ENaC degradation. However, it seems likely that individual ENaC subunits expressed in a heterologous system will be degraded in the endoplasmic reticulum, so that the relevance of this finding to the action of Nedd4-2 on ENaC at the plasma membrane is difficult to evaluate from these experiments.

In another study, Bhalla et al. (25) examined the interaction of 14-3-3 proteins with Nedd4-2 using a pan-14-3-3 antibody, which did not permit distinction between 14-3-3 isoforms. Wild-type and mutant Nedd4-2 constructs were used to identify the phosphosite(s) for 14-3-3β binding at Ser-444 of Xenopus Nedd4-2, which has an equivalent sequence context to mouse Ser-328, the site of 14-3-3β binding identified here. They were able to show that exogenous 14-3-3β, co-expressed in oocytes, augmented ENaC currents over the level stimulated by the co-expression of SGK1 alone. Importantly, 14-3-3β reduced the ubiquitination of ENaC induced by co-expression of Nedd4-2, and because these experiments involved co-expression of heterotrimeric ENaC channels in HEK cells, 14-3-3β is likely influencing the ubiquitination of channels at the plasma membrane. Together, these studies implicated 14-3-3 proteins as co-factors in SGK1/Nedd4-2-mediated ENaC regulation.

Our work places these largely biochemical and proof-of-concept studies into a more physiological context by examining the role of 14-3-3 proteins in aldosterone regulation of sodium absorption in mCCD epithelia. First, all of our studies were performed using polarized mCCD epithelia that endogenously express ENaC. Second, RT-PCR and isoform-selective antibodies were used to evaluate the expression of endogenous 14-3-3 isoforms. We did not find 14-3-3ε to be expressed in mCCD epithelia, and 14-3-3γ, although expressed at reasonably high levels, was not induced by aldosterone.

Nedd4-2 Phosphorylation and the Action of 14-3-3 Protein—The polyclonal phosphopeptide antibody used in our studies to detect phospho-Nedd4-2 was raised against the mouse sequence Lys-Pro-Arg-Ser-Leu-Ser(P)328-Ser-Pro-Thr-Val, and its specificity for this site has been verified by Flores et al. (26) in several ways. Their approaches included elimination of antibody binding by phosphatase treatment of cell lysates, competition for antibody binding only by phosphopeptide antigen, and most importantly, lack of antibody recognition of an S328A Nedd4-2 mutant. Accordingly, this antibody is selective for Nedd4-2 phosphorylated at Ser-328 and not at other sites. It permitted us to demonstrate that 14-3-3β associated selectively with the phosphorylated form of Nedd4-2 (Fig. 4), and in antibody competition experiments, we were able to show that the antigenic site at Ser-328 was the primary locus of 14-3-3β binding (Fig. 8). In future work then, it should be possible to use this antibody to assess the potential interaction of other 14-3-3 isoforms with phospho-Ser-328.

The SGK1 phosphorylation site at Ser-328 is also a target for cAMP/cAMP-dependent protein kinase phosphorylation (42), implying that at least part of the increase in cell surface ENaC associated with stimulation of sodium absorption by vasopressin is explained by a similar mechanism. Thus, it is likely that the action of cAMP-dependent protein kinase relies also on the ability of 14-3-3 proteins to interfere with the ENaC-Nedd4-2 interaction and to stabilize the channel at the cell surface during the acute regulation of sodium absorption by cAMP agonists.

Mechanism of 14-3-3 Action—Phosphorylation of Nedd4-2 effectively blocks its binding to ENaC (12, 13), producing a functional mimic of Liddle syndrome; however, the mechanism by which Nedd4-2 phosphorylation interferes with its recognition of the PY motifs in ENaC has remained uncertain. This is because the phosphorylation sites for SGK1 on Nedd4-2 lie between the WW domains that interact with ENaC and not within them. Despite multiple phosphorylation sites for SGK1, prior studies indicate that one site is dominant in suppressing the ENaC–Nedd4-2 interaction, Ser-328 in mouse, lying between the 2nd and 3rd WW domains (26). In addition, WW domain 3 is crucial for ENaC binding (43, 44). The reciprocal interactions that we observed, as a function of aldosterone action, between Nedd4-2 and ENaC on one hand and phospho-Nedd4-2 and 14-3-3β on the other hand suggest that 14-3-3 binding physically obstructs the interaction between ENaC and Nedd4-2.
Structural studies show that 14-3-3 proteins function as dimers, having two binding sites for client protein interactions. This often allows them to bring different regions of the same protein into proximity (17, 18). Moreover, intramolecular associations of 14-3-3 may deform their binding partners, and the intramolecular binding of a 14-3-3 may elicit its secondary down-regulation in some deformities. These deformities include 14-3-3, which is especially rigid because of the helical structure, i.e. their conformation is minimally affected by client protein binding. This permits 14-3-3 proteins to physically deform their binding partners, and the intramolecular binding of a 14-3-3 dimer within Nedd4-2 would be expected to alter its ability to interact productively with the ENaC channel according to this hypothesis.

It is possible also that 14-3-3 dimers act as a bridge that brings Nedd4-2 into proximity with other phosphoproteins. Because the ubiquitination of SGK1 may elicit its secondary down-regulation in some systems, and SGK1 has been found to interact with 14-3-3, 14-3-3 proteins could facilitate an interaction between Nedd4-2 and SGK1 (25). The isoform selectivity of this interaction should be examined, because 14-3-3 is located at relatively low levels in mCCD epithelia, and it was not induced by aldosterone. It is possible also that interactions of Nedd4-2 with other regulatory pathways (46), or with proteins that serve for its localization to specific sites within cells, may be influenced by a bridging function of 14-3-3.

Selective 14-3-3 Isoform Knockdown—Experimentally, the knockdown of 14-3-3β was selective; it did not influence the expression of the other predominant isoforms, 14-3-3ε or -γ (Fig. 5). Yet the knockdown of 14-3-3β alone significantly suppressed the response to aldosterone, despite the parallel induction of 14-3-3ε expression by the steroid. Accordingly, there may not be a large degree of 14-3-3 isoform redundancy in this system.

Although we do not have a clear explanation for the large effect produced by 14-3-3β knockdown, this finding may reflect a binding preference of phospho-Nedd4-2 for a heterodimer of the isoforms whose expression was augmented by steroid treatment. As stated above, 14-3-3 proteins function as dimers, and although a broad range of heterodimerization within this family has been observed among isoforms, it is possible that selective binding is favored by a heterodimer of 14-3-3β and 14-3-3ε. Studies of the structural basis of selective phosphopeptide binding by 14-3-3 proteins have implicated isoform-specific surfaces in the dimer binding clefts as important for the selective discrimination of substrates (47). Other studies imply that a preferred substrate interaction may arise from the formation of an optimal interface between 14-3-3 monomers within the dimer and the conformation that this confers for substrate binding (48). Additional studies will be needed to address this issue.

In summary, the present work has identified two aldosterone-induced 14-3-3 isoforms in mCCD epithelia and demonstrated their isoform-specific association with phosphorylated Nedd4-2. This physical interaction obstructs access of the ubiquitin ligase to the cytoplasmic domains of ENaC subunits, an interaction that would normally lead to ENaC internalization and degradation. Accordingly, in addition to its effect on α-subunit transcription, aldosterone enhances sodium absorption and increases ENaC levels by stabilizing channels at the apical membrane, interfering with their degradation by a mechanism that involves phosphorylation of Nedd4-2, and induction of its 14-3-3 binding partners.
14-3-3 Isoforms Are Aldosterone-induced Proteins

40. Alvarez de la Rosa, D., and Canessa, C. M. (2003) *Am. J. Physiol.* 284, C404–C414
41. Helms, M. N., Fejes-Toth, G., and Naray-Fejes-Toth, A. (2003) *Am. J. Physiol.* 284, F480–F487
42. Snyder, P. M., Olson, D. R., Kabra, R., Zhou, R., and Steines, J. C. (2004) *J. Biol. Chem.* 279, 45753–45758
43. Kanelis, V., Rotin, D., and Forman-Kay, J. D. (2001) *Nat. Struct. Biol.* 8, 407–412
44. Asher, C., Sinha, I., and Garty, H. (2003) *Biochim. Biophys. Acta* 1612, 59–64
45. Chun, J., Kwon, T., Lee, E. J., Kim, C. H., Han, Y. S., Hong, S. K., Hyun, S., and Kang, S. S. (2004) *Mol. Cells* 18, 360–368
46. Plant, P. J., Lafont, F., Lecat, S., Verkade, P., Simons, K., and Rotin, D. (2000) *J. Cell Biol.* 149, 1473–1484
47. Wilker, E. W., Grant, R. A., Artim, S. C., and Yaffe, M. B. (2005) *J. Biol. Chem.* 280, 18891–18898
48. Rimessi, A., Coletto, L., Pinton, P., Rizzuto, R., Brini, M., and Carafoli, E. (2005) *J. Biol. Chem.* 280, 37195–37203