

# AS160 Modulates Aldosterone-stimulated Epithelial Sodium Channel Forward Trafficking

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**Aldosterone-induced increases in apical membrane epithelial sodium channel (ENaC) density and Na transport involve the induction of 14-3-3 protein expression and their association with Nedd4-2, a substrate of serum- and glucocorticoid-induced kinase (SGK1)-mediated phosphorylation. A search for other 14-3-3 binding proteins in aldosterone-treated cortical collecting duct (CCD) cells identified the Rab-GAP, AS160, an Akt/PKB substrate whose phosphorylation contributes to the recruitment of GLUT4 transporters to adipocyte plasma membranes in response to insulin. In CCD epithelia, aldosterone (10 nM, 24 h) increased AS160 protein expression threefold, with a time-course similar to increases in SGK1 expression. In the absence of aldosterone, AS160 overexpression increased total ENaC expression 2.5-fold but did not increase apical membrane ENaC or amiloride-sensitive Na current ( $I_{sc}$ ). In AS160 overexpressing epithelia, however, aldosterone increased apical ENaC and  $I_{sc}$  2.5-fold relative to aldosterone alone, thus recruiting the accumulated ENaC to the apical membrane. Conversely, AS160 knockdown increased apical membrane ENaC and  $I_{sc}$  under basal conditions to ~80% of aldosterone-stimulated values, attenuating further steroid effects. Aldosterone induced AS160 phosphorylation at five sites, predominantly at the SGK1 sites T568 and S751, and evoked AS160 binding to the steroid-induced 14-3-3 isoforms,  $\beta$  and  $\epsilon$ . AS160 mutations at SGK1 phospho-sites blocked its selective interaction with 14-3-3 $\beta$  and  $\epsilon$  and suppressed the ability of expressed AS160 to augment aldosterone action. These findings indicate that the Rab protein regulator, AS160, stabilizes ENaC in a regulated intracellular compartment under basal conditions, and that aldosterone/SGK1-dependent AS160 phosphorylation permits ENaC forward trafficking to the apical membrane to augment Na absorption.**

## INTRODUCTION

The regulated activity of the distal nephron epithelial sodium channel (ENaC) is an important determinant of sodium balance, extracellular fluid volume, and blood pressure. The physiological significance of ENaC is illustrated most clearly by human genetic diseases in which channel mutations produce clinical defects in renal salt and water transport such as Liddle syndrome and pseudohypoaldosteronism type 1 (Butterworth *et al.*, 2009). In the airways, ENaC is a key regulator of the airway surface liquid volume, and excessive Na absorption via ENaC contributes to cystic fibrosis and perhaps other chronic obstructive pulmonary diseases (Pilewski and Frizzell, 1999; Mall *et al.*, 2008). ENaC dysregulation has been implicated in other renal conditions, notably, salt-dependent hypertension (Pratt, 2005; Vallon and Lang, 2005). Therefore, it is important to generate a more complete understanding of the molecular mechanisms governing ENaC activity.

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Abbreviations used: CCD, cortical collecting duct; ENaC, epithelial Na channel; Nedd4-2, neural precursor cells expressed developmentally down-regulated gene 4 isoform 2; SGK1, serum- and glucocorticoid-regulated kinase 1.

As the rate-limiting step in distal nephron Na transport, ENaC is a principal target in the regulation of Na retention by mineralocorticoids (Wang *et al.*, 2001). In states of volume depletion, decreased renal perfusion results in the release of renin from the juxtaglomerular apparatus, activating the renin-angiotensin-aldosterone pathway. The binding of aldosterone to mineralocorticoid receptors expressed in distal nephron principal cells activates the transcription of a variety of genes to evoke increases in ENaC-mediated Na absorption after a lag of ~1 h (Zabner *et al.*, 1998; Stockand, 2002). Altered serum osmolarity induces the release of vasopressin, and in the renal collecting duct, vasopressin binds to V2 receptors at the basolateral membranes to increase Na transport via cAMP/protein kinase A (PKA) signaling pathways (Schafer, 2002), with a more rapid response (minutes) that does not depend on *de novo* protein synthesis. Despite differences in their rapidity of action, both aldosterone and vasopressin increase Na transport primarily by altering ENaC trafficking to and from the apical surface (Loffing *et al.*, 2001; Morris and Schafer, 2002), resulting in increased apical membrane Na channel density. The molecular mechanisms that regulate these apical ENaC trafficking events are only partially understood.

Aldosterone exerts its control over distal renal Na transport at multiple sites. Cell localization studies show that the steroid redistributes ENaC subunits from intracellular compartments to the apical membranes of principal cells (Masilamani *et al.*, 1999; Loffing *et al.*, 2000; Frindt *et al.*, 2001; Ergonul *et al.*, 2006), particularly in the early phase of the

response. Aldosterone induces the synthesis of a number of proteins, including the channel itself (Robert-Nicoud *et al.*, 2001). Other significant aldosterone-induced proteins that determine apical ENaC density include the serum- and glucocorticoid-induced kinase (SGK1) and the glucocorticoid-induced leucine zipper protein (GILZ; Snyder, 2002; Bhalla *et al.*, 2006; Malik *et al.*, 2006). SGK and GILZ inhibit the ubiquitin-dependent internalization of ENaC via complementary mechanisms that involve the ubiquitin E3 ligase, Nedd4-2. SGK phosphorylates Nedd4-2, blocking its interaction with PY motifs located at the ENaC subunit C-termini. Conversely, GILZ acts by blocking ERK-mediated ENaC phosphorylation, which reduces the channel's affinity for Nedd4-2 binding. These aldosterone-induced, SGK1- and GILZ-regulated phosphorylation events reduce channel ubiquitylation and internalization via clathrin adapter proteins that contain ubiquitin interacting motifs, such as epsin (Wang *et al.*, 2006), to decrease channel endocytosis and increase apical ENaC density.

After its endocytosis, ENaC must be recycled to the apical membrane in order to maintain apical channel numbers at steady-state levels (Butterworth *et al.*, 2005). This hypothesis has led to the identification of the deubiquitylating enzymes (DUBs), UCH-L3 (Butterworth *et al.*, 2007) and USP2-45 (Fakitsas *et al.*, 2007), which maintain apical ENaC density by obviating the channel's ubiquitin-dependent degradation. This biochemical and functional work has established the central role of ENaC recycling, not only in promoting channel surface stability, but also in maintaining the intracellular channel pool(s) that permit a redistribution of ENaC to the apical surface in response to hormonal regulators. Despite these advances, however, we know much more about the regulation of apical channel density by endocytic processes than we do about events in the recycling pathway that maintain cellular channel pools and promote ENaC forward trafficking during transport stimulation.

The phosphorylation-dependent inactivation of Nedd4-2 is stabilized by 14-3-3 protein binding, which assists in blocking ENaC–Nedd4-2 interactions (Bhalla *et al.*, 2005; Liang *et al.*, 2006, 2008). Aldosterone induces the expression of two 14-3-3 protein isoforms,  $\beta$  and  $\epsilon$ , by three- and eight-fold, respectively, during the early phase of the steroid response. Moreover, knockdown of these aldosterone-induced 14-3-3 isoforms almost completely suppresses aldosterone action and its effect on apical ENaC density, leading to the hypothesis that phospho-proteins other than Nedd4-2 bind 14-3-3 proteins to promote ENaC traffic regulation. Using affinity capture methods, we have identified AS160 as a 14-3-3 binding protein in aldosterone-stimulated cortical collecting duct (CCD) epithelia. AS160 is an Akt/PKB phosphorylation substrate with the molecular signature of a Rab GTPase-activating protein (Rab-GAP). Thus, it is expected to maintain Rab proteins with which it associates in their inactive, GDP-bound states. Evidence from the insulin-dependent GLUT4 trafficking literature implicates Akt-mediated AS160 phosphorylation and 14-3-3 protein binding in the recruitment of the GLUT4 glucose transporter to the cell surface of adipocytes (Sano *et al.*, 2003; Miinea *et al.*, 2005; Watson and Pessin, 2006). According to this concept, Akt phosphorylation of AS160 blocks its GTPase activity, permitting the activation of target Rab(s) via GTP loading and the trafficking of GLUT4 carrier vesicles toward the plasma membrane. Accordingly, we examined the role of AS160 in the aldosterone-stimulated forward trafficking of ENaC to the apical surface of CCD epithelia.

## MATERIALS AND METHODS

### Antibodies

Antibodies specific for AS160, Nedd4-2, and SGK1 were purchased from Millipore (Billerica, MA). Phosphorylation site-specific antibodies that selectively recognize phospho-sites S318, S341, T568, S570, S588, T642, S666, and S751 of AS160 have been characterized (Geraghty *et al.*, 2007) and were kindly provided by Dr. Carol MacKintosh (University of Dundee). Antibodies specific for 14-3-3 isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), as follows:  $\beta$  (A-15),  $\gamma$  (C-16),  $\epsilon$  (T-16),  $\theta$  (C-17), and  $\sigma$  (N-14), and their specificity in this system was previously characterized (Liang *et al.*, 2006). Secondary antibodies against mouse or rabbit were obtained from GE Healthcare (Piscataway, NJ). Anti-goat secondary antibodies were purchased from Jackson ImmunoResearch, (West Grove, PA). Secondary antibodies against sheep and antibodies to  $\beta$ -actin and the hemagglutinin (HA) epitope were obtained from Sigma-Aldrich (St. Louis, MO).

A rabbit polyclonal antibody targeting an epitope at the extracellular loop of  $\alpha$ -ENaC has been described (Liang *et al.*, 2006). This antibody was used to provide biochemical data on the levels of total ENaC expression (cell lysate) and apical membrane ENaC (cell surface biotinylation) in response to aldosterone and experimental perturbations, as described below. Because of the number of such blots, cropped regions that include only the molecular mass of full-length  $\alpha$ -ENaC are provided in the figures, but blots covering the full molecular weight range demonstrate the specificity of this antibody (Supplemental Figure 1, A and B). Importantly, the detection of surface ENaC after biotinylation and streptavidin pull-down was in excellent agreement with amiloride-sensitive short-circuit current ( $I_{sc}$ ) across mouse mpkCCDC14 (hereafter mCCD) epithelia, indicating that this approach provides a biochemical marker for apical ENaC function. In addition, similar blots were performed using antibodies to  $\beta$ -ENaC (Santa Cruz, D-3), and the results paralleled those obtained from blotting of the  $\alpha$ -subunit (Supplemental Figure 2).

### Preparation of DNA and Short Hairpin RNA Constructs

FLAG-AS160WT and FLAG-AS1604P constructs (S318A, S588A, T642A, and S751A) were the gift of Dr. Gustav Lienhard (Dartmouth University). An additional phospho-site mutation was introduced into the 4P construct (T568A) to generate FLAG-AS1605P using the QuikChange II XL site-directed mutagenesis kit from Agilent (Santa Clara, CA). The short hairpin RNA (shRNA) used for AS160 knockdown was created using the GeneClip U1 Hairpin Cloning System from Promega (Madison, WI) according to the manufacturer's instructions, and the target sequence 5'-GACTTAACATC-CAACGA-3'.

### Cell Culture and Transfection

mCCD cells were kindly provided by A. Vandewalle and M. Bens (Institut National de la Santé et de la Recherche Médicale, Paris, France); the cells were grown in flasks (passage 30–40) in defined medium as described (Vinciguerra *et al.*, 2003). The growth medium was composed of equal volumes DMEM and Ham's F12, plus 60 nM sodium selenate, 5  $\mu$ g/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5  $\mu$ g/ml insulin, 20 mM D-glucose, 2% vol/vol FCS, and 20 mM HEPES, pH 7.4 (reagents from Invitrogen, Gaithersburg, MD; and Sigma-Aldrich). The cells were maintained at 37°C in 5% CO<sub>2</sub>/95% air, and the media were changed every second day.

For transepithelial current measurements, mCCD cells were subcultured onto six-well plates and were transfected with 4  $\mu$ g cDNA/well when they were ~80% confluent using 10  $\mu$ l of Lipofectamine 2000 per well, according to the manufacturer's protocol. Twenty-four hours after transfection, cells were subcultured onto permeable filter supports (0.4- $\mu$ m pore size, 0.33-cm<sup>2</sup> surface area; Transwell, Corning Costar, Corning, NY), where they polarized after 4–5 d as detected using "chopstick" electrodes (Millipore): open-circuit voltage was typically ~50 mV, and transepithelial resistance ~2 k $\Omega$ ·m<sup>2</sup>. Marker gene (enhanced green fluorescent protein [EGFP]) expression gave a transduction efficiency of 70–80% at the time of assay (Liang *et al.*, 2006). For the biochemical experiments, mCCD epithelia were polarized on 4.5- or 44-cm<sup>2</sup> filters (Corning Costar) for ~5 d before use. To establish a regulatory baseline, the growth medium bathing cells on filters was replaced with a minimal medium of DMEM/F12 (without drugs or hormones) for at least 24 h before experiments. Thereafter, mCCD epithelia were either maintained without additives or treated with aldosterone (10 nM, Sigma-Aldrich) for the indicated times.

### 14-3-3 Affinity Chromatography

The isolation of 14-3-3 binding proteins was performed as described (Pozuelo Rubio *et al.*, 2004; Dubois *et al.*, 2009). Briefly, mCCD epithelia were polarized on 44-cm<sup>2</sup> filters. Cells ( $n = 10^9$ ) were harvested and extracted in 20 ml lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 10 mM  $\beta$ -glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM benzamide, 0.2 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, and 0.1%

(vol/vol) 2-mercaptoethanol. The broken cells were centrifuged at  $27,000 \times g$  for 20 min, and the supernatant was diluted with 20 ml of buffer A (25 mM Tris/HCl, pH 7.5, at 4°C, 100 mM NaCl, and 25 mM NaF). The extract was mixed end-over-end for 1 h at 4°C with 6 ml of Sepharose linked to 6 mg each of BMH1/BMH2 (the *Saccharomyces cerevisiae* 14-3-3 isoforms). The mixture was poured into an Econo-Pac column of 1.5 cm diameter (Bio-Rad, Hercules, CA), the flow-through sample was collected for later use, and the column was washed three times with 500 mM NaCl in buffer A. Samples were collected from the beginning, middle, and end of each salt wash and combined to form three samples for later use: first, second and third wash. The column was "mock-eluted" using 12 ml of 25 mM Tris-HCl, pH 7.5, 25 mM NaF, and 150 mM NaCl containing 1 mM of the control peptide (WFYpSPFLE; peptides are from the Peptide Synthesis Facility, University of Pittsburgh), which does not bind 14-3-3 proteins (Pozuelo Rubio *et al.*, 2004; Dubois *et al.*, 2009), and the eluate collected. The column was then washed with 20 ml of 25 mM Tris-HCl, pH 7.5, 25 mM NaF, 100 mM NaCl, which was discarded. The column was eluted with 12 ml of buffer A containing the consensus 14-3-3 binding peptide, ARAApSAPA at 1 mM (Pozuelo Rubio *et al.*, 2004; Dubois *et al.*, 2009). Samples from the flow-through, each salt wash, and from both phosphopeptide elutions were concentrated to  $\sim 150 \mu\text{l}$  using Vivaspin 10000 concentrators (Vivaproducts, Littleton, MA), and 30  $\mu\text{l}$  of the concentrate was run on SDS/PAGE using 4–15% gels (Bio-Rad) in preparation for immunoblotting.

### 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 PVDF membranes. Unbound sites were blocked for 1 h at room temperature with 5% (wt/vol) skim milk powder in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The blots were incubated with primary antibodies (dilutions: 1:1000 for anti-AS160, phospho-site specific anti-AS160, anti-SGK1, anti- $\alpha$ -ENaC, and anti-14-3-3 isoform; 1:2000 for anti-Nedd4-2; and 1:3000 for anti- $\beta$ -actin) 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  $\mu\text{g/ml}$  horseradish peroxidase-conjugated secondary antibodies in TBST with 5% milk, followed by three TBST washes. The reactive bands were visualized with enhanced chemiluminescence (PerkinElmer, Waltham, MA) and exposed to x-ray film (Eastman Kodak, Rochester, NY).  $\beta$ -Actin expression provided an internal control. Immunoblot data were scanned and band densities quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>).

### Surface Protein Biotinylation

mCCD cells cultured on filter supports were washed (5 min), with ice-cold PBS with agitation on ice, to remove growth media. The apical membrane was biotinylated using 0.5 mg/ml S-S-biotin (Thermo Fisher, Waltham, MA) in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM  $\text{Na}_2\text{B}_4\text{O}_7$ , pH 9) for 20 min. Labeling was quenched by adding a double volume of FBS-containing medium to the apical compartment. Monolayers were then washed three times with ice-cold PBS, with agitation on ice, and the cells were harvested. Cells were lysed in lysis buffer (0.4% deoxycholic acid, 1% NP-40, 50 mM EGTA, 10 mM Tris-Cl, pH 7.4) at room temperature for 10 min. The protein concentration of the postnuclear supernatant was determined, and 200  $\mu\text{g}$  of protein was combined with a streptavidin bead slurry (Thermo Fisher) and incubated overnight at 4°C. Samples from the streptavidin beads were collected in 4 $\times$  sample buffer containing 10%  $\beta$ -mercaptoethanol and incubated for 20 min at room temperature after washing three times with lysis buffer. Samples were heated at 95°C for 3 min, separated by SDS-PAGE, and blotted as above to determine the density of ENaC at the apical membrane surface of mCCD cells.

### $I_{\text{SC}}$ Recordings

Epithelia cultured on filter supports were mounted in modified Ussing chambers (Corning Costar), and the cultures were continuously short circuited by an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA) as previously described (Butterworth *et al.*, 2005). Trans-epithelial resistance was calculated using Ohm's law from the current response to a periodic 2.5-mV bipolar pulse. The bathing solution consisted of (in mM): 120 NaCl, 25  $\text{NaHCO}_3$ , 3.3  $\text{KH}_2\text{PO}_4$ , 0.8  $\text{K}_2\text{HPO}_4$ , 1.2  $\text{MgCl}_2$ , 1.2  $\text{CaCl}_2$ , and 10 D-glucose, and the chambers were maintained at 37°C and gassed continuously with a mixture of 95%  $\text{O}_2$ -5%  $\text{CO}_2$ , which fixed the pH at 7.4. Amiloride (10  $\mu\text{M}$ ) 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  $\pm$  SEM. *p*-values were calculated by ANOVA followed by unpaired *t* test as appropriate. *p* < 0.05 was considered to be statistically significant.

## RESULTS

### Identification of AS160 as a 14-3-3 Binding Protein in mCCD Epithelia

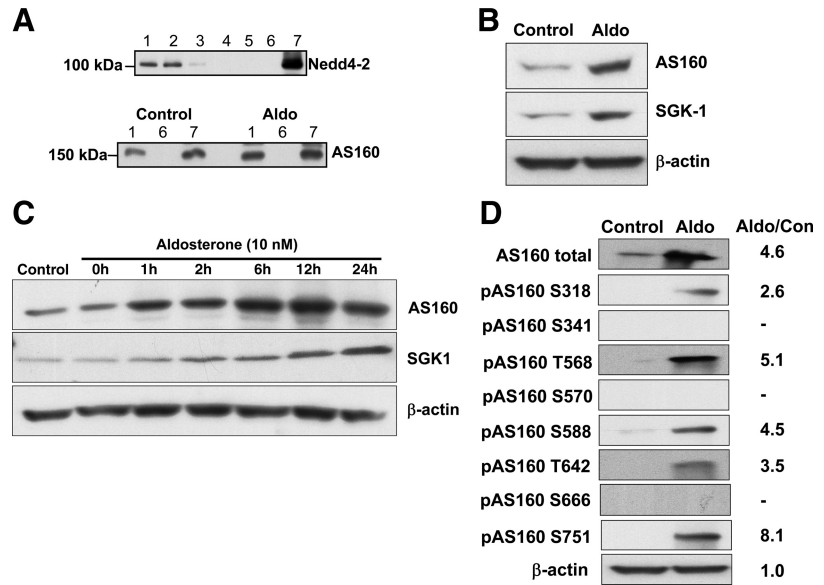
We previously showed that the SGK-mediated phosphorylation of Nedd4-2 promoted its association with two aldosterone-induced 14-3-3 isoforms and that this interaction blocked ENaC-Nedd4-2 binding as a means of augmenting apical channel density in mCCD epithelia (Liang *et al.*, 2006, 2008). An affinity-capture approach, described in *Materials and Methods*, was used in attempts to identify other 14-3-3 binding proteins in polarized, aldosterone-treated mCCD epithelia that might be significant in ENaC traffic regulation. Proteins in the 14-3-3 column eluates were resolved by gradient SDS-PAGE and transferred to nitrocellulose, and the membrane overlaid with the digoxigenin-labeled 14-3-3 proteins that were used to construct the affinity column. This process revealed numerous 14-3-3 binding proteins with molecular weights ranging from  $\sim 30$  to  $>250$  kDa (data not shown). The membrane was then stripped and blotted for Nedd4-2 as a positive control. Figure 1A shows enrichment of this known 14-3-3 binding protein in the specific eluate by a 14-3-3 consensus binding peptide (lane 7), after three salt-buffer (lanes 3–5) and negative-control peptide washes (lane 6). Based on the GLUT4 trafficking literature (see *Introduction*) eluates from control and aldosterone-treated mCCD epithelia were resolved and probed for AS160. As shown in Figure 1A, bottom, the lysate (lane 1), control peptide (lane 6), and consensus peptide eluates indicate that AS160 was enriched in an aldosterone-dependent manner. The ability of the capture method to enrich 14-3-3 binding partners depends on the ability of the 14-3-3 proteins immobilized on the column to compete with endogenous 14-3-3 proteins in the lysate that are already bound to their targets. This is expected to vary with target affinity and isoform specificity and will determine relative target enrichment.

### Aldosterone Induces AS160 Expression and Phosphorylation

To explore the aldosterone-dependent regulation of AS160, cell lysates obtained from control and aldosterone-treated mCCD monolayers were resolved by SDS-PAGE and probed for AS160 by immunoblot (Figure 1B). Under basal conditions, the level of AS160 expression in polarized mCCD epithelia was similar to that observed in the mouse adipocyte 3T3-L1 cell line, which is commonly used in studies of insulin-stimulated GLUT4 trafficking (data not shown). Aldosterone (10 nM, 12 h) induced a significant, 3.2-fold increase in AS160 expression. When examined at 0, 1, 2, 6, 12, or 24 h of steroid treatment, the increase in AS160 was time-dependent and roughly paralleled that observed for the steroid-induced kinase, SGK1 (Figure 1C).

Eight serine or threonine residues on AS160 have been shown to be phosphorylated by a number of agonists (Geraghty *et al.*, 2007). To assess changes in the phosphorylation status of AS160 during aldosterone action, we used eight phosphorylation site specific antibodies, kindly provided by Dr. Carol Mackintosh. These affinity-purified antibodies were raised against mouse phospho-peptides corresponding to phosphorylation sites on AS160 identified by mass spectrometry (Sano *et al.*, 2003; Geraghty *et al.*, 2007); they do not recognize the corresponding nonphosphorylated peptides (Geraghty *et al.*, 2007). Their ability to selectively detect the phosphorylation of AS160 *in vitro* at each of these sites by various protein kinases, including Akt and SGK1, has been previously validated using the purified proteins (Geraghty *et al.*, 2007). As shown in Figure 1D, aldosterone (10 nM,





**Figure 1.** Aldosterone induces AS160 expression and phosphorylation. mCCD epithelia were treated with aldosterone (10 nM, 24 h), and cell lysates were collected. (A) Proteins isolated by 14-3-3 affinity chromatography as described in *Materials and Methods* were subjected to immunoblot for Nedd4-2 or AS160. For Nedd4-2, mCCD epithelia were aldosterone treated; for AS160, both control (steroid deprived) or aldosterone-treated conditions were used. Lanes: 1, cell lysate; 2, column flow-through; 3–5, salt washes; 6, nonspecific peptide; 7, consensus 14-3-3 binding peptide. (B) AS160 and SGK1 expression were monitored by immunoblot in control or aldosterone-treated mCCD epithelia (10 nM, 12 h). (C) Time-course of aldosterone induced increases in AS160 and SGK1 expression. (D) Phospho-specific antibody labeling of AS160 in lysates from control and aldosterone-treated mCCD epithelia. See Geraghty *et al.* (2007) for antibody validation and text for discussion.

12 h) significantly increased AS160 expression as above; moreover, the steroid elicited significant phosphorylation at sites S318, T568, S588, T642, and S751. This pattern of aldosterone-induced phosphorylation is identical to that obtained with SGK1 *in vitro* (Geraghty *et al.*, 2007). In that work, phosphorylation at S318, S588, and T642 of AS160 was observed also in response to Akt, whereas the most significant phospho-specific signals found for aldosterone, at T568 and S751, were unique to SGK1 versus Akt (Geraghty *et al.*, 2007). Significant AS160 phosphorylation signals were not detected at sites S341, S570, or S666 in mCCD cells in response to aldosterone, although they were utilized by other kinases in the above study; this result also parallels the SGK1 data from *in vitro* experiments. Thus, the AS160 regulatory sites that are sensitive to SGK1 and Akt activation pathways show both overlapping and unique features.

#### AS160 Knockdown Increases Apical ENaC in the Absence of Aldosterone

The physiological significance of AS160 in ENaC traffic regulation was evaluated under basal and aldosterone-stimulated conditions in knockdown experiments performed with shRNA targeting AS160 expression, and the results were compared with those obtained with a control, scrambled shRNA. The transfection conditions permitted assays of protein expression and transepithelial current in polarized CCD cells (Liang *et al.*, 2006). The shRNA-induced reduction in AS160 expression in different experiments averaged 60–90% under aldosterone-stimulated and basal conditions, respectively, whereas the scrambled shRNA had no effect relative to untreated controls.

We examined total and apical surface expression of  $\alpha$ -ENaC, the latter as a biochemical marker of steady-state channel density (Liang *et al.*, 2008). The results are illustrated by the representative blots of Figure 2A and the data summary of Figure 2B. Under basal (nonstimulated) conditions, AS160 knockdown increased cell surface ENaC about fourfold without significantly altering total ENaC expression. Apical ENaC expression level in cells with reduced AS160 approached, but did not reach, that observed after aldosterone treatment. The magnitude of this effect may be attenuated by ongoing activity of Nedd4-2 on channel endocytosis

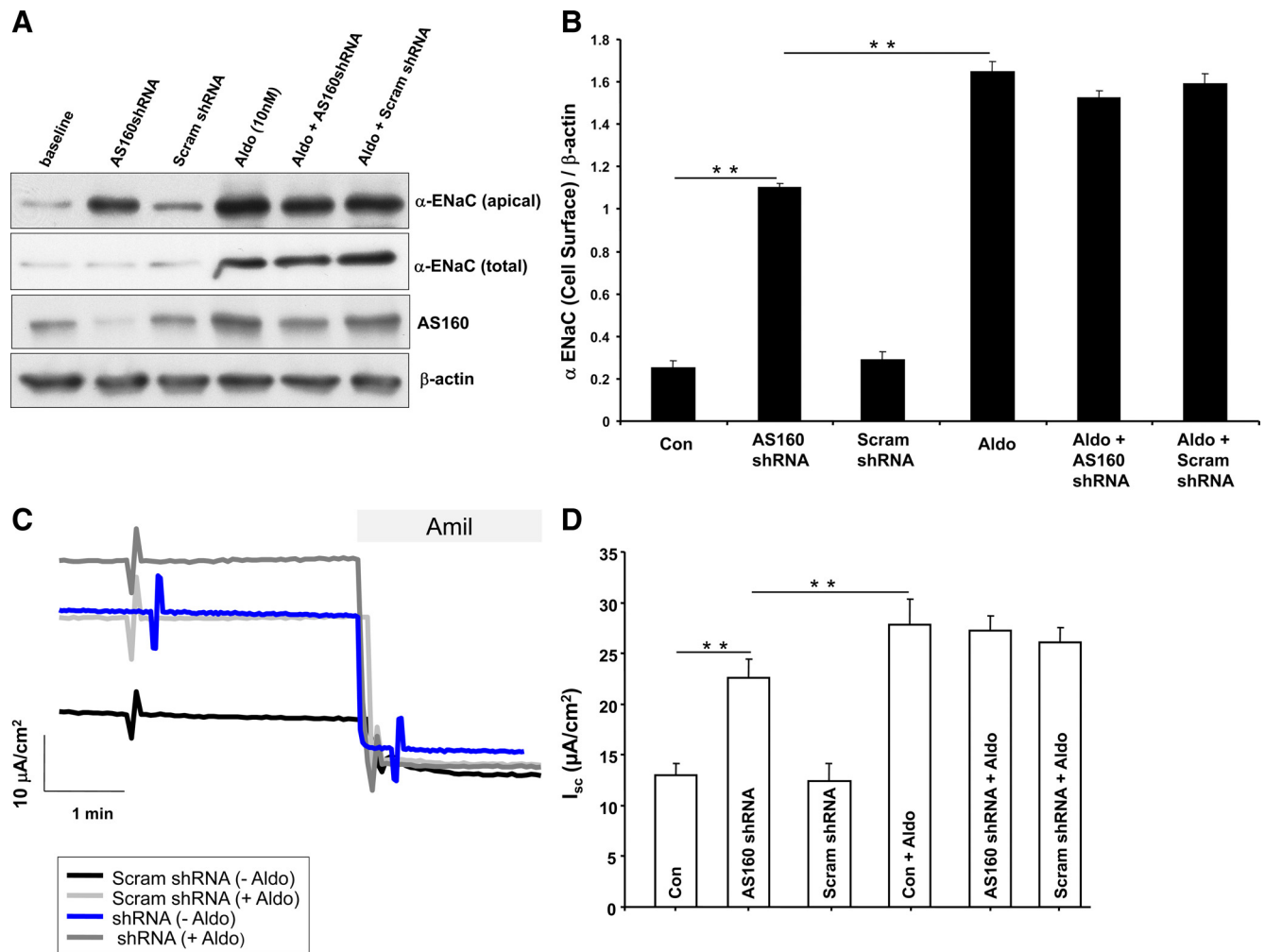
under basal conditions, which would reduce surface ENaC relative to aldosterone where this action of Nedd4-2 is blocked. The effect of AS160 knockdown, to increase apical ENaC in the absence of steroid, was abolished when wild-type (WT) AS160 was expressed exogenously, consistent with a specific effect of the knockdown conditions (data not shown). Aldosterone treatment alone increased total and apical ENaC expression, as previously observed (Liang *et al.*, 2008), and during AS160 knockdown, aldosterone-induced increases in apical ENaC density were attenuated relative to the increases observed with steroid alone. The lack of additive effects suggests that aldosterone and AS160 knockdown redistribute ENaC to the apical membrane from common intracellular compartment(s) where ENaC is localized under basal conditions.

#### Na Transport Parallels Apical ENaC Density during AS160 Knockdown

To determine the functional impact of reduced AS160 expression on ENaC-mediated, transepithelial Na absorption, we determined the amiloride-sensitive  $I_{sc}$  across mCCD epithelia under basal and aldosterone-stimulated conditions. Representative  $I_{sc}$  traces under these conditions are provided in Figure 2C, and the mean data are shown in Figure 2D. Aldosterone treatment elicited about a two- to threefold increase in the amiloride-sensitive  $I_{sc}$  across mCCD epithelia, in agreement with our prior findings (Liang *et al.*, 2006, 2008). Transfection with shRNA targeting AS160 expression increased the magnitude of basal currents toward the levels observed in response to steroid treatment, whereas the control shRNA had no significant effect.  $I_{sc}$  values from epithelia treated to suppress AS160 and treated with aldosterone were not statistically different from those observed with aldosterone alone. Thus, the agreement between apical ENaC and  $I_{sc}$  indicates that the ENaC detected by apical biotinylation in response to AS160 knockdown or aldosterone represents functional channel protein.

#### AS160 Overexpression Increases the Aldosterone-sensitive ENaC Pool

If AS160 stabilizes a pool of intracellular ENaC that is mobilized by aldosterone stimulation, as the data of Figure 2



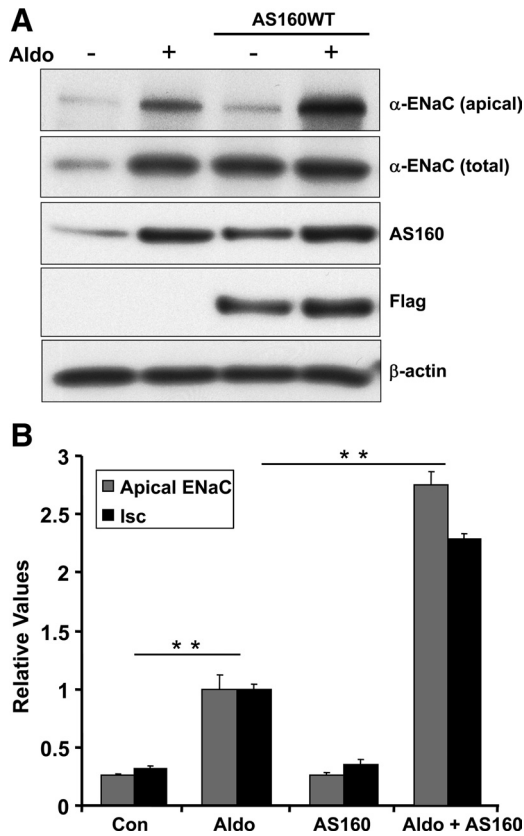
**Figure 2.** AS160 knockdown increases apical ENaC and Na transport in the absence of aldosterone. Preconfluent mCCD cells were transfected with AS160-targeting or scrambled shRNAs and polarized on filters as described in *Materials and Methods*. Controls were treated with Lipofectamine. (A) Expression of the indicated proteins was determined by immunoblot using lysates from mCCD epithelia that had been maintained under control conditions or stimulated with aldosterone for 24 h. Apical ENaC was determined by biotinylation and streptavidin pull-down as described in *Materials and Methods*. (B) Quantitation of apical ENaC as a function of the experimental conditions in part A, from three independent experiments. (C) Time courses of short-circuit current ( $I_{sc}$ ,  $\mu$ A/cm<sup>2</sup>) across mCCD epithelia treated as in A. The abrupt drop in  $I_{sc}$  is elicited by addition of amiloride (10  $\mu$ M) to the apical chamber to define the ENaC-mediated Na transport rate. Current deflections represent transepithelial resistance determinations. (D) Amiloride-sensitive  $I_{sc}$  from eight epithelia for each condition in A and B ( $n = 3$ , apical ENaC;  $n = 6$ , ENaC current assays). Statistical significance between groups identified by line ends;  $**p < 0.01$ .

imply, then it may be possible to augment this channel pool by the exogenous expression of AS160. Representative data are provided in Figure 3A, and mean data from both biochemical and functional experiments are shown in Figure 3B. Under basal conditions, AS160 overexpression increased total ENaC expression  $\sim 2.5$ -fold, to approximately the level observed during aldosterone stimulation. Despite this increase in channel expression, however, apical membrane ENaC, as determined by cell surface biotinylation, was not increased; in addition, Na absorption rate remained at basal levels. When AS160-expressing epithelia were treated with aldosterone, however, surface ENaC increased  $\sim 2.5$ -fold over the level produced by aldosterone treatment alone, indicating that ENaC accumulated in the intracellular pool was recruited to the apical surface in response to the steroid. These changes in apical ENaC density were paralleled by increases in the transepithelial ENaC currents across mCCD epithelia. Similar to the knockdown study, the overexpression experiments are consistent with the concept that AS160

stabilizes ENaC within an intracellular compartment under basal conditions, whereas aldosterone stimulation enables the accumulated intracellular ENaC to progress to the apical surface.

#### AS160 Phospho-site Mutants Suppress ENaC Forward Trafficking

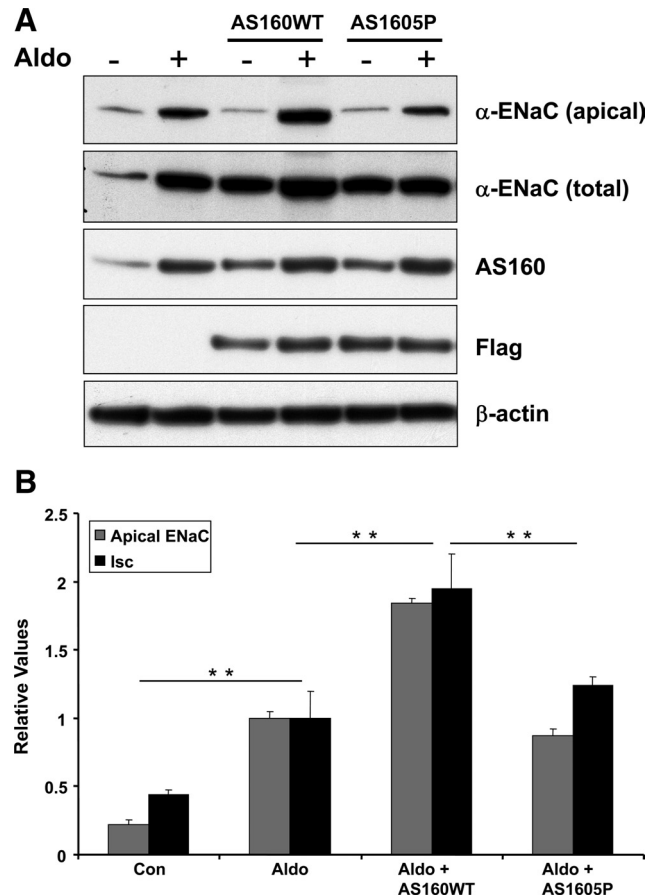
Previous studies of the impact of AS160 phosphorylation sites on adipocyte insulin action identified four Akt phospho-sites whose mutation inhibited insulin-stimulated translocation of GLUT4 to the cell surface (Sano *et al.*, 2003); as noted above, some of these sites overlap with those for SGK (Geraghty *et al.*, 2007). The AS1604P mutant was kindly provided by Dr. Gustav Leinhard (Dartmouth University). Its expression in mCCD epithelia led to a  $\sim 35\%$  reduction in cell surface ENaC density in aldosterone-stimulated epithelia relative to cells exogenously expressing WT AS160 (data not shown). However, the *in vivo* phosphorylation data of Figure 1D identify sites T568 and S751, which are not mu-



**Figure 3.** AS160 overexpression increases the apical ENaC and Na transport responses to aldosterone. mCCD cells were transfected with AS160 cDNA; controls were treated with Lipofectamine as described in *Materials and Methods*. (A) Proteins from control or FLAG-AS160WT-transfected CCD epithelia were maintained under control conditions or treated with aldosterone (10 nM, 24 h), and the indicated proteins were determined by immunoblot; apical ENaC by surface biotinylation. (B) Quantitation of apical ENaC and amiloride-sensitive  $I_{sc}$  for mCCD epithelia treated as in A; values normalized to those from aldosterone-treated epithelia (set at 1.0;  $n = 6$ , each assay). Statistical significance between groups identified at line ends; \*\* $p < 0.01$ .

tated in the 4P mutant, as most responsive to SGK1-dependent phosphorylation. Therefore, T568 was mutated to alanine to produce a 5P mutant, as noted in *Materials and Methods*.

Typical and composite data from these experiments are shown in Figure 4, A and B. Under basal conditions, the AS1605P mutant had no significant effect on cell surface ENaC, despite producing a significant increase in total ENaC expression, as observed for the overexpression of WT AS160 (Figure 3 and here). Because phosphorylation at these sites is expected to suppress AS160 GAP activity during stimulation, it is not surprising that exogenous AS1605P stabilized intracellular ENaC under basal conditions. However, the increase in apical ENaC evoked by the expression of WT AS160 in the presence of aldosterone was not observed for the 5P mutant. Similar data were obtained in adipocytes, where expression of the Akt phospho-site mutant (4P) inhibited insulin-stimulated GLUT4 trafficking ~80% (Sano *et al.*, 2003). AS1605P did not reduce apical ENaC significantly below the level observed for aldosterone alone, however, suggesting that the mutant does not have a dominant interfering action on the activity of endogenous AS160.

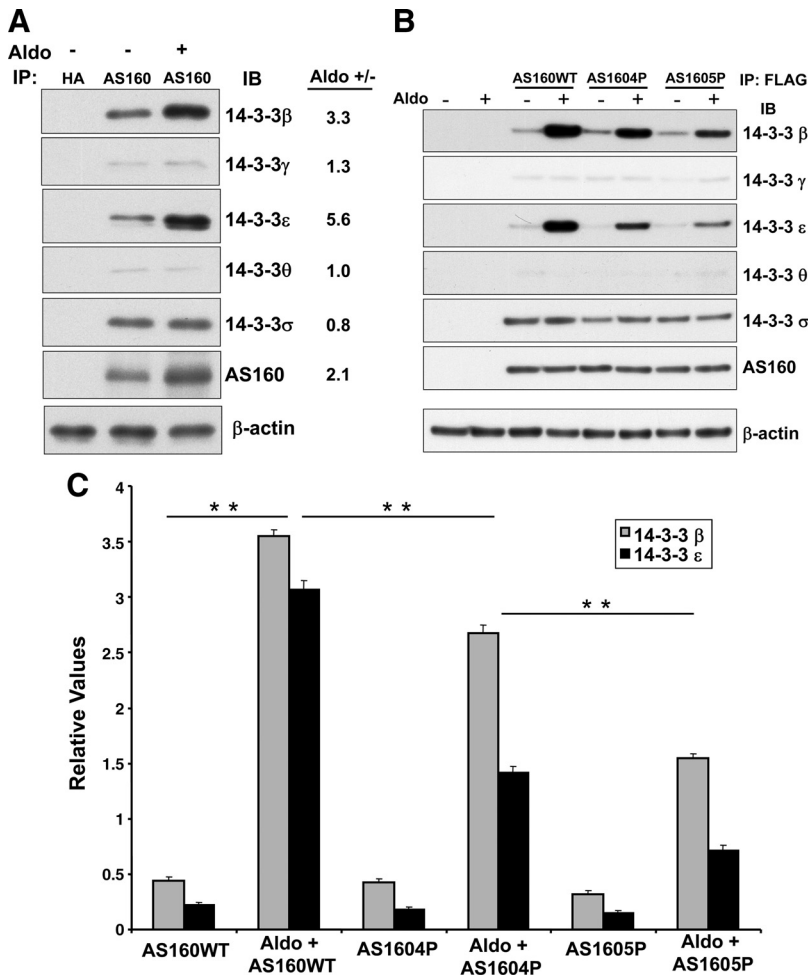


**Figure 4.** Aldosterone's action on apical ENaC and Na transport is suppressed by AS160 mutations at SGK1 phospho-sites. Experiments performed as in Figure 3 used epithelia transfected with either FLAG-AS160WT or FLAG-AS1605P. (A) Indicated proteins in mCCD lysates were detected by immunoblot; apical ENaC by surface biotinylation. (B) Quantitation of apical ENaC and amiloride-sensitive  $I_{sc}$  for from epithelia treated as in A; values are normalized to those from aldosterone-treated epithelia (set at 1.0;  $n = 3$ , apical ENaC;  $n = 6$ , ENaC current). Statistical significance between groups identified at line ends; \*\* $p < 0.01$ .

The functional impact of AS160 phospho-site mutations on ENaC-mediated Na absorption was determined under basal and aldosterone-stimulated conditions, and the mean data are provided also in Figure 4B. As in the studies of WT AS160 overexpression (Figure 3), aldosterone treatment elicited a two- to threefold increase in the amiloride-sensitive  $I_{sc}$ , and overexpression of WT AS160 produced a further two- to threefold increase in  $I_{sc}$ . In epithelia transfected with AS1605P, however, the aldosterone-stimulated current was not significantly different from the level observed with steroid treatment alone. As found for apical ENaC density, the 5P mutant abolished the ability of expressed AS160 to augment the aldosterone-stimulated  $I_{sc}$ . These data indicate that phosphorylation of AS160 in response to aldosterone stimulation is required for ENaC transit to the cell surface and for Na transport stimulation.

#### Aldosterone Increases AS160 Interaction with 14-3-3 $\beta$ and $\epsilon$

Having initially detected AS160 because of its aldosterone-dependent interaction with 14-3-3 proteins, we examined the selectivity of its interaction with the five 14-3-3 isoforms



**Figure 5.** Aldosterone elicits phosphorylation-dependent interactions of AS160 with 14-3-3β and ε. (A) IP of endogenous AS160 using lysates from control and aldosterone (10 nM, 24 h)-treated mCCD epithelia (anti-HA as control), followed by immunoblotting with the indicated 14-3-3-specific antibodies. Values in the right column provide quantitation of the aldosterone/control 14-3-3 isoform intensities in the AS160 precipitates. (B) Experiment performed as in A, but cell lysates were from mCCD epithelia transfected with FLAG-AS160WT, -AS1604P, or -AS1605P; FLAG antibody IPs were performed to detect 14-3-3 interactions with the expressed proteins. The data shows differential 14-3-3 isoform interactions with the 4P and 5P phospho-site mutants. (C) Mean coIP signals for 14-3-3β and ε from three experiments of the type shown in B, normalized to β-actin. Statistical significance between groups identified by line ends; \*\**p* < 0.01.

whose expression we identified previously in polarized mCCD epithelia (Liang *et al.*, 2006). AS160 antibodies were used to isolate protein complexes from epithelia maintained under basal or aldosterone-treated conditions, they were resolved by SDS-PAGE and blotted with isoform selective 14-3-3 antibodies. Representative data are illustrated in Figure 5A. Immunoprecipitations (IPs) performed with an anti-HA IgG as control yielded no 14-3-3 signal. Under basal conditions, endogenous AS160 interacted primarily with the 14-3-3β, ε, and σ isoforms. Much weaker interactions were observed between AS160 and the 14-3-3γ and θ isoforms, as found previously for Nedd4-2 (Liang *et al.*, 2006). Aldosterone treatment markedly increased AS160's interaction with 14-3-3β and ε. That these interactions depend on AS160 phosphorylation is suggested by the lack of aldosterone effect on the AS160-14-3-3σ binding and by the selective association of AS160 with 14-3-3β and ε, which exceeded the fold-increase in AS160 expression with aldosterone. As for Nedd4-2, the interaction of phospho-AS160 with 14-3-3β and ε would maintain its inactive state.

#### 14-3-3 Protein Interactions Depend on AS160 Phosphorylation

Direct evidence that AS160 phosphorylation mediates its interaction with the aldosterone-induced 14-3-3 isoforms is provided by the representative and summary data of Figure 5, B and C. mCCD epithelia overexpressing WT AS160 or the 4P or 5P mutant as FLAG fusion proteins were immunopre-

cipitated and their association with endogenous 14-3-3 isoforms was probed by immunoblot. As found for endogenous AS160 (Figure 5A), expressed WT AS160 interacted selectively with 14-3-3β and ε in aldosterone-treated epithelia. Isoform interactions with the AS1604P and 5P mutants were reduced by comparison; for 14-3-3ε, approaching the level observed under basal conditions. Interactions between AS160 and other 14-3-3 isoforms were not influenced significantly by phospho-site mutations, confirming that these interactions do not depend on aldosterone-mediated AS160 phosphorylation. Together with the phospho-specific antibody data of Figure 1D, these findings indicate that the binding of AS160 to the aldosterone-induced 14-3-3 isoforms depends on its phosphorylation at sites identified for modification by SGK1.

#### DISCUSSION

Accumulating evidence suggests that vesicular ENaC trafficking is controlled by a series of kinase-mediated signaling pathways that regulate the distribution of the channel between intracellular compartments and the apical membrane (Butterworth *et al.*, 2009). These kinases respond to multiple systemic and local signals, and there is a critical need to link these stimuli to the specific kinase-targeted regulators (phospho-proteins) that control ENaC trafficking. However, this goal has been achieved to any significant degree only in the case of the endocytic mediator, Nedd4-2. Nevertheless, there



are undoubtedly multiple steps in the recycling and forward trafficking pathways subsequent to ENaC endocytosis, where decisions about ENaC fate (recycling vs. degradation or intracellular stabilization vs. forward trafficking) impact apical ENaC density. These processes are obscure in comparison with our knowledge of Nedd4-2 regulation. The present work identifies the Rab-GAP, AS160, as a key regulatory node in the aldosterone-dependent control of ENaC forward trafficking.

### AS160 Defines an Aldosterone-regulated Cellular ENaC Compartment

The potential for AS160 to provide this function was based on several factors. First, this Rab-GAP plays a well-recognized role in mediating GLUT4 trafficking to the plasma membrane in response to its insulin/Akt-mediated phosphorylation (Holman and Sakamoto, 2008). Second, 14-3-3 protein interactions are significant in stabilizing the phosphorylated state of AS160 in insulin responsive cells (Ramm *et al.*, 2006; Geraghty *et al.*, 2007). Third, like Nedd4-2, we found AS160 to be enriched in 14-3-3 affinity column eluates derived from aldosterone-treated mCCD epithelia (Figure 1A). Our hypothesis, that additional regulators of ENaC trafficking would be identified based on their agonist-dependent interactions with 14-3-3 proteins, was based on prior studies of the role of these proteins in ENaC-Nedd4-2 interactions (Liang *et al.*, 2006; Liang *et al.*, 2008). Those studies showed that a 50% knockdown of the aldosterone-induced 14-3-3 isoforms,  $\beta$  or  $\epsilon$ , virtually eliminated the Na transport response of mCCD epithelia to aldosterone. One interpretation of this finding is that serial steps in the ENaC recycling pathway, distal to Nedd4-2, are also controlled by 14-3-3 protein interactions. Based on the present findings, 14-3-3 affinity capture has the potential to identify other novel and significant regulators of agonist-regulated, postendocytic ENaC trafficking.

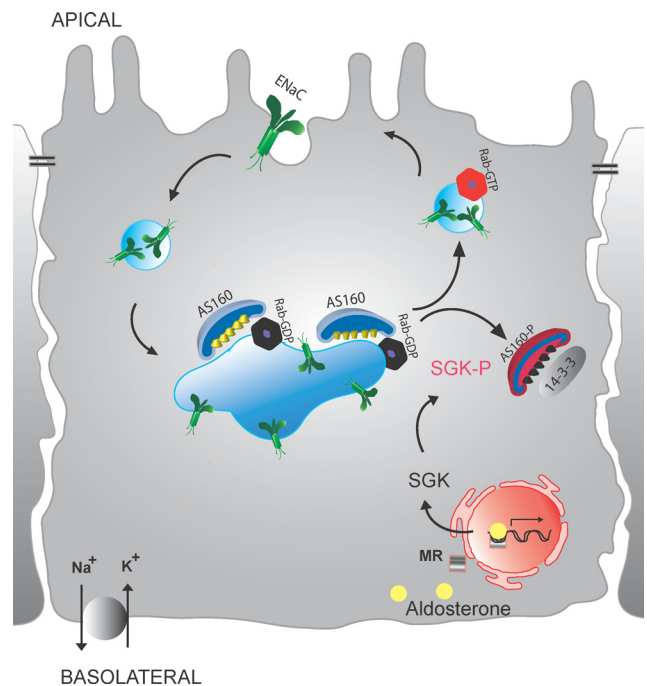
Our data support the concept that AS160 assists in defining an intracellular compartment in which ENaC accumulates under basal conditions and that this compartment is accessed by aldosterone, via SGK-mediated phosphorylation of AS160, to permit the forward trafficking of ENaC to the apical membrane. First, aldosterone was found to induce AS160 expression with a time-course similar to that of SGK1 (Figure 1). Moreover, steroid stimulation generated a pattern of AS160 phosphorylation in polarized mCCD epithelia (Figure 1D) that was virtually identical to that produced by SGK1 from *in vitro* phosphorylation experiments performed with purified proteins (Geraghty *et al.*, 2007). These findings suggested that the SGK1-mediated phosphorylation of AS160 at five serine/threonine sites is largely responsible for transducing the stimulatory action of aldosterone. Two of these sites, T568 and S751, are distinct from those targeted by insulin/Akt in adipocytes (Geraghty *et al.*, 2007). The significance of these sites in aldosterone-regulation was confirmed by the expression of phospho-site mutants, which suppressed steroid-induced increases in apical ENaC density and Na transport (Figure 4) and blocked the interactions of AS160 with the 14-3-3 isoforms that are induced by aldosterone (Figure 5).

Second, the overexpression of AS160 increased ENaC expression in mCCD epithelia, but in the absence of aldosterone, this increase in channel protein did not translate to increases in apical ENaC density or Na transport. With aldosterone treatment, however, the intracellular ENaC accumulated in response to exogenous AS160 expression generated a two- to threefold increase in apical ENaC density and Na current, above the levels observed with aldosterone

alone (Figure 3). Thus, the steroid has access to the intracellular ENaC pool that is stabilized by nonphosphorylated AS160. As might be anticipated, however, aldosterone does not have access to cellular ENaC stabilized by AS160 bearing mutations at the sites phosphorylated by SGK1.

The two- to threefold increase in AS160 expression produced during aldosterone stimulation would serve to augment the capacity of the aldosterone-responsive intracellular compartment by expanding this trafficking pool. Perhaps more importantly, it would assist also in sequestering the elevated apical ENaC levels that are associated with aldosterone, once its stimulation wanes. The increase in AS160 expression could result from its aldosterone-induced transcriptional regulation or from stabilization of the protein by 14-3-3 binding. There is precedence for this action of 14-3-3 proteins (Benjamin *et al.*, 2006; Gherzi *et al.*, 2006). In addition, aldosterone treatment increased the levels of expressed FLAG-AS160 but not that of FLAG-AS160<sup>5P</sup> (see Figures 2A and 4B), suggesting a posttranslational stabilization mechanism that is related to AS160 phosphorylation.

Third, knockdown of AS160 expression mimicked the action of aldosterone, permitting ENaC progression to the apical surface in the absence of steroid (Figure 2). Intracellular ENaC level was inversely proportional to AS160 expression level: exogenous AS160 increased cellular ENaC, whereas AS160 knockdown increased surface ENaC without altering total channel expression, consistent with a decrease in the Rab-GAP has an inhibitory action on ENaC forward trafficking under nonstimulated conditions. In addition,



**Figure 6.** Model for AS160 action in aldosterone-dependent ENaC trafficking. Under basal conditions, AS160 Rab-GAP activity maintains the Rab protein(s) involved in apical ENaC recycling in the GDP-loaded, inhibited state, stabilizing ENaC within a regulated cellular compartment. Aldosterone stimulates the transcription of SGK1, which phosphorylates AS160 at multiple sites (see text), blocking its GAP activity and permitting Rab-GTP loading, with Rab activation and trafficking of ENaC toward the apical surface. Binding of the aldosterone-induced  $\beta$  and  $\epsilon$  14-3-3 isoforms stabilizes phospho-AS160, maintaining its inhibited state.



AS160 knockdown compromised the ability of aldosterone to increase apical ENaC density and Na transport, consistent with the concept that a physiological action of the steroid is to access the cellular ENaC compartment that is stabilized by nonphosphorylated AS160 under basal conditions.

#### *Aldosterone-dependent 14-3-3 Protein Interactions*

The aldosterone-dependent interaction of AS160 with 14-3-3 proteins mimicked that observed previously for Nedd4-2 (Liang *et al.*, 2006, 2008); specifically, the increase in 14-3-3 binding to AS160 in coIP experiments was restricted to the steroid-induced isoforms, despite the presence of other 14-3-3 orthologues. This finding implies that phosphorylated AS160 and Nedd4-2 exhibit specificity for interactions with 14-3-3 $\beta$  and  $\epsilon$ . As shown by our prior work (Liang *et al.*, 2008), this interaction involves the binding of an obligatory 14-3-3  $\beta/\epsilon$  heterodimer. The structural basis of the preferred heterodimer interaction involves electrostatic interactions formed at the  $\beta$  -  $\epsilon$  interface, detected in crystal structures of the dimer complex (Gardino *et al.*, 2006). Identifying the basis of substrate specificity for the  $\beta$  -  $\epsilon$  dimer in AS160 and Nedd4-2 interactions, and perhaps for other steroid-dependent regulators, will likely require structural studies of these complexes or their relevant components. Increases in 14-3-3 $\beta$  and  $\epsilon$  association with AS160 approximated the fold increases in the steroid-induced expression of these isoforms (Liang *et al.*, 2006), which would be consistent with increased association via mass action. However, the AS160/14-3-3 coIP performed with the AS160P mutant (Figure 5), showed a marked suppression of aldosterone-dependent interactions with 14-3-3 $\beta$  and  $\epsilon$ , indicating the dependence of binding on AS160 phosphorylation.

AS160 harbors eight phosphorylation sites that produce distinctive patterns of phosphorylation in response to at least four protein kinases (Geraghty *et al.*, 2007). On the other hand, structural studies show that 14-3-3 proteins function as dimers, providing two binding sites for client protein associations. Most 14-3-3 interactions are intramolecular (Fu *et al.*, 2000), and they often involve the initial binding to a high-affinity site, implicated for phospho-Ser328 of Nedd4-2 in our prior studies (Liang *et al.*, 2006). This initial interaction is thought to provide the avidity needed for the targeting of a second phospho-site of lower affinity. It is possible in the present context that the unique SGK1 sites of AS160, relative to those for Akt (Figure 1D), will provide a dominant 14-3-3 regulatory interaction. Nevertheless, insulin/Akt provides an acute agonist pathway for ENaC stimulation (Lee *et al.*, 2007), so that differential patterns of AS160 phosphorylation and 14-3-3 protein binding induced by aldosterone/SBK1 versus insulin/Akt may determine selectivity of agonist actions that are not fully apparent at present. These may include the modulation of selective Rab isoform activities or the regulation of AS160 activity at different intracellular trafficking compartments, which may lead to additivity or synergy in agonist effects. It will be interesting to assess the role of AS160 in mediating the actions of various agonists that impact ENaC trafficking.

#### *A Model for AS160 Regulation of ENaC Trafficking*

Our data are consistent with a model (Figure 6) in which the nonphosphorylated Rab-GAP, AS160, stabilizes ENaC within an intracellular compartment due to its ability to maintain one or more associated Rab proteins in their inhibited, GDP-bound form. Aldosterone stimulation of Na transport, associated with increased apical ENaC density, involves the transcriptional induction of SGK1, which phosphorylates AS160 at kinase-specific sites to block its GAP activity and permit Rab protein

GTP loading. This relieves the AS160-mediated suppression of ENaC forward trafficking to the apical surface. Selective binding of the aldosterone-induced 14-3-3  $\beta/\epsilon$  heterodimer stabilizes AS160 in its phosphorylated form, hindering its interaction with the Rab protein(s) under its control. This disinhibition may involve displacement of the Rab-GAP from the regulated intracellular ENaC compartment, as altered cellular localization of targets is frequently associated with 14-3-3 protein binding (Fu *et al.*, 2000; Benjamin *et al.*, 2006). Recent findings suggest that Rab11b plays an important role in ENaC recycling (Butterworth *et al.*, 2008). Whether this, or other, Rab isoforms interact with AS160 as a function of aldosterone action will require further study.

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#### REFERENCES

- Benjamin, D., Schmidlin, M., Min, L., Gross, B., and Moroni, C. (2006). BRF1 protein turnover and mRNA decay activity are regulated by protein kinase B at the same phosphorylation sites. *Mol. Cell. Biol.* 26, 9497–9507.
- Bhalla, V., Daidie, D., Li, H., Pao, A. C., LaGrange, L. P., Wang, J., Vandewalle, A., Stockand, J. D., Staub, O., and Pearce, D. (2005). Serum- and glucocorticoid-regulated kinase 1 regulates ubiquitin ligase neural precursor cell-expressed, developmentally down-regulated protein 4-2 by inducing interaction with 14-3-3. *Mol. Endocrinol.* 19, 3073–3084.
- Bhalla, V., Soundararajan, R., Pao, A. C., Li, H., and Pearce, D. (2006). Disinhibitory pathways for control of sodium transport: regulation of ENaC by SGK1 and GILZ. *Am J. Physiol. Renal Physiol.* 291, F714–F721.
- Butterworth, M. B., Edinger, R. S., Frizzell, R. A., and Johnson, J. P. (2009). Regulation of the epithelial sodium channel by membrane trafficking. *Am J. Physiol. Renal Physiol.* 296, F10–F24.
- Butterworth, M. B., Edinger, R. S., Johnson, J. P., and Frizzell, R. A. (2005). Acute ENaC stimulation by cAMP in a kidney cell line is mediated by exocytic insertion from a recycling channel pool. *J. Gen. Physiol.* 125, 81–101.
- Butterworth, M. B., Edinger, R. S., Ova, H., Burg, D., Johnson, J. P., and Frizzell, R. A. (2007). The deubiquitinating enzyme UCH-L3 regulates the apical membrane recycling of the epithelial sodium channel. *J. Biol. Chem.* 282, 37885–37893.
- Butterworth, M. B., Edinger, R. S., Silvis, M. R., Frizzell, R. A., and Johnson, J. P. (2008). Rab11 Is Involved in the Regulated Recycling of the Epithelial Sodium Channel (ENaC). *J. Am. Soc. Nephrol.* 19, 29A.
- Dubois, F., Vandermoere, F., Gernez, A., Murphy, J., Toth, R., Chen, S., Geraghty, K. M., Morrice, N. A., and MacKintosh, C. (2009). Differential 14-3-3 affinity capture reveals new downstream targets of phosphatidylinositol 3-kinase signaling. *Mol. Cell Proteomics* 8, 2487–2499.
- Ergonul, Z., Frindt, G., and Palmer, L. G. (2006). Regulation of maturation and processing of ENaC subunits in the rat kidney. *Am J. Physiol. Renal Physiol.* 291, F683–F693.
- Fakitsas, P., *et al.* (2007). Early aldosterone-induced gene product regulates the epithelial sodium channel by deubiquitylation. *J. Am Soc. Nephrol.* 18, 1084–1092.
- Frindt, G., Masilamani, S., Knepper, M. A., and Palmer, L. G. (2001). Activation of epithelial Na channels during short-term Na deprivation. *Am J. Physiol. Renal Physiol.* 280, F112–F118.
- Fu, H., Subramanian, R. R., and Masters, S. C. (2000). 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.* 40, 617–647.
- Gardino, A. K., Smerdon, S. J., and Yaffe, M. B. (2006). Structural determinants of 14-3-3 binding specificities and regulation of subcellular localization of 14-3-3-ligand complexes: a comparison of the X-ray crystal structures of all human 14-3-3 isoforms. *Semin. Cancer Biol.* 16, 173–182.
- Geraghty, K. M., Chen, S., Harthill, J. E., Ibrahim, A. F., Toth, R., Morrice, N. A., Vandermoere, F., Moorhead, G. B., Hardie, D. G., and MacKintosh, C. (2007). Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. *Biochem. J.* 407, 231–241.

- Gherzi, R., Trabucchi, M., Ponassi, M., Ruggiero, T., Corte, G., Moroni, C., Chen, C. Y., Khabar, K. S., Andersen, J. S., and Briata, P. (2006). The RNA-binding protein KSRP promotes decay of beta-catenin mRNA and is inactivated by PI3K-AKT signaling. *PLoS Biol.* 5, e5.
- Holman, G. D., and Sakamoto, K. (2008). Regulating the motor for GLUT4 vesicle traffic. *Cell Metab.* 8, 344–346.
- Lee, I. H., Dinudom, A., Sanchez-Perez, A., Kumar, S., and Cook, D. I. (2007). Akt mediates the effect of insulin on epithelial sodium channels by inhibiting Nedd4-2. *J. Biol. Chem.* 282, 29866–29873.
- Liang, X., Butterworth, M. B., Peters, K. W., Walker, W. H., and Frizzell, R. A. (2008). An obligatory heterodimer of 14-3-3beta and 14-3-3epsilon is required for aldosterone regulation of the epithelial sodium channel. *J. Biol. Chem.* 283, 27418–27425.
- Liang, X., Peters, K. W., Butterworth, M. B., and Frizzell, R. A. (2006). 14-3-3 isoforms are induced by aldosterone and participate in its regulation of epithelial sodium channels. *J. Biol. Chem.* 281, 16323–16332.
- Loffing, J., Pietri, L., Aregger, F., Bloch-Faure, M., Ziegler, U., Meneton, P., Rossier, B. C., and Kaissling, B. (2000). Differential subcellular localization of ENaC subunits in mouse kidney in response to high- and low-Na diets. *Am J. Physiol. Renal Physiol.* 279, F252–F258.
- Loffing, J., Zecevic, M., Feraille, E., Kaissling, B., Asher, C., Rossier, B. C., Firestone, G. L., Pearce, D., and Verrey, F. (2001). Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J. Physiol. Renal Physiol.* 280, F675–F682.
- Malik, B., Price, S. R., Mitch, W. E., Yue, Q., and Eaton, D. C. (2006). Regulation of epithelial sodium channels by the ubiquitin-proteasome proteolytic pathway. *Am J. Physiol. Renal Physiol.* 290, F1285–F1294.
- Mall, M. A., *et al.* (2008). Development of chronic bronchitis and emphysema in beta-epithelial Na<sup>+</sup> channel-overexpressing mice. *Am J. Respir. Crit. Care Med.* 177, 730–742.
- Masilamani, S., Kim, G. H., Mitchell, C., Wade, J. B., and Knepper, M. A. (1999). Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J. Clin. Invest.* 104, R19–R23.
- Miinea, C. P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peranen, J., Lane, W. S., and Lienhard, G. E. (2005). AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem. J.* 391, 87–93.
- Morris, R. G., and Schafer, J. A. (2002). cAMP increases density of ENaC subunits in the apical membrane of MDCK cells in direct proportion to amiloride-sensitive Na(+) transport. *J. Gen. Physiol.* 120, 71–85.
- Pilewski, J. M., and Frizzell, R. A. (1999). Role of CFTR in airway disease. *Physiol. Rev.* 79, S215–S255.
- Pozuelo Rubio, M., Geraghty, K. M., Wong, B. H., Wood, N. T., Campbell, D. G., Morrice, N., and Mackintosh, C. (2004). 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. *Biochem. J.* 379, 395–408.
- Pratt, J. H. (2005). Central role for ENaC in development of hypertension. *J. Am. Soc. Nephrol.* 16, 3154–3159.
- Ramm, G., Larance, M., Guilhaus, M., and James, D. E. (2006). A role for 14-3-3 in insulin-stimulated GLUT4 translocation through its interaction with the RabGAP AS160. *J. Biol. Chem.* 281, 29174–29180.
- Robert-Nicoud, M., *et al.* (2001). Transcriptome of a mouse kidney cortical collecting duct cell line: effects of aldosterone and vasopressin. *Proc. Natl. Acad. Sci. USA* 98, 2712–2716.
- Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., Garner, C. W., and Lienhard, G. E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J. Biol. Chem.* 278, 14599–14602.
- Schafer, J. A. (2002). Abnormal regulation of ENaC: syndromes of salt retention and salt wasting by the collecting duct. *Am J Physiol. Renal Physiol.* 283, F221–F235.
- Snyder, P. M. (2002). The epithelial Na<sup>+</sup> channel: cell surface insertion and retrieval in Na<sup>+</sup> homeostasis and hypertension. *Endocr. Rev.* 23, 258–275.
- Stockand, J. D. (2002). New ideas about aldosterone signaling in epithelia. *Am J. Physiol. Renal Physiol.* 282, F559–F576.
- Vallon, V., and Lang, F. (2005). New insights into the role of serum- and glucocorticoid-inducible kinase SGK1 in the regulation of renal function and blood pressure. *Curr. Opin. Nephrol. Hypertens.* 14, 59–66.
- Vinciguerra, M., Deschenes, G., Hasler, U., Mordasini, D., Rousselot, M., Doucet, A., Vandewalle, A., Martin, P. Y., and Feraille, E. (2003). Intracellular Na<sup>+</sup> controls cell surface expression of Na, K-ATPase via a cAMP-independent PKA pathway in mammalian kidney collecting duct cells. *Mol. Biol. Cell* 14, 2677–2688.
- Wang, H., *et al.* (2006). Clathrin-mediated endocytosis of the epithelial sodium channel. Role of epsin. *J. Biol. Chem.* 281, 14129–14135.
- Wang, J., Barbry, P., Maiyar, A. C., Rozansky, D. J., Bhargava, A., Leong, M., Firestone, G. L., and Pearce, D. (2001). SGK integrates insulin and mineralocorticoid regulation of epithelial sodium transport. *Am J. Physiol. Renal. Physiol.* 280, F303–F313.
- Watson, R. T., and Pessin, J. E. (2006). Bridging the GAP between insulin signaling and GLUT4 translocation. *Trends Biochem. Sci.* 31, 215–222.
- Zabner, J., Smith, J. J., Karp, P. H., Widdicombe, J. H., and Welsh, M. J. (1998). Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro. *Mol. Cell* 2, 397–403.