A Novel Role for Glucocorticoid-induced Leucine Zipper Protein in Epithelial Sodium Channel-mediated Sodium Transport*

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Rama Soundararajan 1,2, Ting Ting Zhang 1,3, Jian Wang, Alain Vandewalle, and David Pearce 4,5

From the 1 Division of Nephrology, Department of Medicine, University of California at San Francisco, San Francisco, California 94143-0532, 2 INSERM, Unité 478, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, BP 416, 75870 Paris Cedex 18, France, and the 3 Department of Cellular and Molecular Pharmacology, University of California at San Francisco, San Francisco, California 94143

The steroid hormone aldosterone stimulates sodium (Na⁺) transport in tight epithelia by altering the expression of target genes that regulate the activity and trafficking of the epithelial sodium channel (ENaC). We performed microarray analysis to identify aldosterone-regulated transcripts in mammalian kidney epithelial cells (mpkCCD 144). One target, glucocorticoid-induced leucine zipper protein (GILZ), was previously identified by serial analysis of gene expression (SAGE) however, its function in epithelial ion transport was unknown. Here we show that GILZ expression is rapidly stimulated by aldosterone in mpkCCD 144 and that GILZ, in turn, strongly stimulates ENaC-mediated Na⁺ transport by inhibiting extracellular signal-regulated kinase (ERK) signaling. In Xenopus oocytes with activated ERK, heterologous GILZ expression consistently inhibited phospho-ERK expression and markedly stimulated ENaC-mediated Na⁺ current, in a manner similar to that of U0126 (a pharmacologic inhibitor of ERK signaling). In mpkCCD 144 cells, GILZ transfection similarly consistently inhibited phospho-ERK expression and stimulated transepithelial Na⁺ transport. Furthermore, aldosterone treatment of mpkCCD 144 cells suppressed phospho-ERK levels with a time course that paralleled their increase of Na⁺ transport. Finally, GILZ expression markedly increased cell surface ENaC expression in epithelial growth factor-treated mammalian kidney epithelial cells, HEK 293. These observations suggest a novel link between GILZ and regulation of epithelial sodium transport through modulation of ERK signaling and could represent an important pathway for mediating aldosterone actions in health and disease.

Hormone-regulated sodium (Na⁺) transport in tight epithelia is essential for the control of circulatory volume, blood pressure, and extracellular fluid composition in vertebrates. The epithelial Na⁺ channel, ENaC, constitutes the rate-limiting step for Na⁺ transport, and its activity, trafficking, and expression are important targets of regulation, most notably by the mineralocorticoid hormone aldosterone, which acts primarily through the mineralocorticoid receptor to alter the transcription of a set of target genes (1). Several studies using candidate gene approaches (2, 3) or unbiased screens (4–8) have been undertaken to discern the mechanistic basis of aldosterone action. As with other steroid-regulated processes (9), two major classes of target genes have been identified: early and late. Early response genes appear to be required for initiation of the response, whereas the late response genes participate in consolidation (10). The latter include components of the ion transport machinery itself (for example, ENaC subunits and subunits of the Na,K-ATPase) and genes that encode regulatory proteins that likely act to limit the extent of the aldosterone response (for example, activators of the mitogen-activated protein kinase cascade, such as epidermal growth factor (EGF) or its receptor, epidermal growth factor receptor (11)). The former category encodes primarily signaling molecules, implicated in pathways that control ENaC activity and/or trafficking (1). The best characterized of these targets is the serine-threonine kinase serum and glucocorticoid-induced kinase-1 (SGK1) (1), which acts to increase apical membrane ENaC, at least in part, by inhibiting the ubiquitin ligase Nedd4-2, itself a modulator of ENaC trafficking and degradation (12, 13).

Recently, several lines of evidence have suggested that aldosterone-regulated mediators other than SGK1 provide stimulatory input into this system (1). Most notably, although SGK1 knock-out mice have aldosterone resistance, their phenotype is substantially less severe than that of either mineralocorticoid receptor or αENaC knock-out mice (1, 14) or of adrenalectomized wild type animals (15), suggesting that renal ENaC function and renal mineralocorticoid action are partially but not completely dependent on SGK1. The observation that short term aldosterone treatment induces an accumulation of SGK1 in the entire aldosterone-sensitive distal nephron, whereas the apical insertion of ENaC takes place only in the connecting tubule and early collecting duct, indicates that other factors are probably required as well to drive ENaC cell surface expression and activity and, thus, Na⁺ reabsorption (15). It is also notable that inhibitors of phosphatidylinositol 3'-kinase (which is responsible for activation of SGK1) do not completely abolish aldosterone-induced Na⁺ transport (16), further supporting the idea that aldosterone may act through multiple pathways to stimulate Na⁺ reabsorption, some of which are SGK1- and phosphatidylinositol 3'-kinase-

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1 These authors contributed equally to this work.

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3 Present address: Massachusetts College of Pharmacy and Health Sciences, 179 Longwood Ave., Boston, MA 02115.

4 To whom correspondence should be addressed: 600 16th St., Rm. N274H, Genentech Hall, Mission Bay Campus, Box 2140, University of California at San Francisco, San Francisco, CA 94107-2140. Tel.: 415-476-7015; Fax: 415-502-8644; E-mail: pearced@medicine.ucsf.edu.

5 The abbreviations used are: ENaC, epithelial sodium channel; GILZ, glucocorticoid-induced leucine zipper protein; mGILZ, mouse GILZ; CCD, cortical collecting duct; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase (1/2); FRBPS, FK506-binding protein 5; MEK, mitogen-activated protein kinase/ERK kinase; SGK1, serum- and glucocorticoid-induced kinase-1; HEK cells, human kidney epithelial cells; RT, reverse transcription; IRES, internal ribosomal entry site; EGFP, enhanced green fluorescent protein.
independent. Other regulators, including K-Ras (17) and Ks-WNK1 (18), have been identified and shown to stimulate Na⁺ transport; however, aldosterone regulation has not been demonstrated in animals, and their physiological importance has not yet been established.

The broad aim of the present study, therefore, was to identify and characterize key genes (other than SGK1) that stimulate ENaC-mediated Na⁺ transport and mediate the early response to aldosterone. Toward this end, we performed microarray analysis in the aldosterone-responsive cortical collecting duct (CCD) cell line, mpkCCDc14, which exhibit typical electrophysiologic features of a tight epithelium, express functional ENaC, and respond to aldosterone (1 μM) with increased amiloride-sensitive Na⁺ currents (19). This manuscript and associated additional data deposited in the NCBI Gene Expression Omnibus (GEO) data base (www.ncbi.nlm.nih.gov/geo) report the results of this screen and characterization of one up-regulated transcript, glucocorticoid-induced leucine zipper protein (GILZ). This corticosteroid early response gene was identified previously as an aldosterone-regulated transcript by serial analysis of gene expression (SAGE) and shown to be rapidly and robustly stimulated by aldosterone in mpkCCDc14 cells (5) as well as in native CCD (20). However, it has not been shown to stimulate (or inhibit) Na⁺ current, and a role in epithelial ion transport has not been established.

In addition to its early marked induction by aldosterone, two independent lines of evidence suggested that GILZ might represent an important mediator of aldosterone action in collecting duct cells. 1) GILZ has been found to act as an inhibitor of ERK1/2 signaling in T-lymphocytes (21). In this context, GILZ interacts with and blocked activation of Raf, the mitogen-activated protein kinase kinase implicated in ERK1/2 activation. 2) ERK activation, for example by EGF (in cultured CD cells) (11, 22) or by progesterone (in ENaC-expressing oocytes) (23), inhibits Na⁺ current and has been suggested to serve as a late brake, providing feedback inhibition to limit aldosterone-stimulatory effects (11). Furthermore, along these same lines, although short term (<1 h) blockade of ERK activation with pharmacologic inhibitors diminishes Na⁺ current in CD cells (24), longer term (>4 h) inhibition stimulates it (22, 25). Finally, in contrast to other neuron segments, ERK1/2 appears to be constitutively active in distal nephron cells (24, 26–28), consistent with tonic inhibition of Na⁺ transport by the ERK pathway.

Taken together, the above observations suggested that GILZ might function as a mineralocorticoid-induced inhibitor of ERK signaling implicated in the early induction of ENaC-mediated Na⁺ transport. We, therefore, examined its ability to inhibit ERK1/2 and stimulate Na⁺ current in oocytes and cultured cells.

MATERIALS AND METHODS

Cell Culture and Electrophysiological Measurements—mpkCCDc14 cells were maintained in plastic tissue culture flasks in a modified Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) medium (regular medium) as described previously (16, 29). For electrophysiological and biochemical experiments, the cells were seeded and grown in regular medium on collagen-coated filters (Transwell, pore-size 0.4 μm, Corning Costar) until the cell monolayers reached transepithelial resistance greater than 1000 ohms-cm². They were then maintained in hormone-free, serum-free medium (29) for at least 24 h before treatment with aldosterone (10⁻⁶ M, apical and basolateral sides) or equal volumes of vehicle as the control for specified periods of time. After electrophysiological measurements, cells were harvested and processed for RNA or protein analysis.

For the EGF experiments, cell monolayers exhibiting high transepithelial resistance were maintained (for 72 h) in modified medium supplemented with 1× serum substitute (Celox Laboratories, Inc.) (medium deprived of growth factors (including EGF), mitogens, insulin, transferrin, and dexamethasone) and then treated with EGF (Sigma; 20 ng/ml; apical and basolateral sides) or U0126 (Cell Signaling; 10 μM; apical and basolateral sides) for a period of 24 h. Some groups were pretreated with U0126 for 2 h before EGF treatment.

All experiments were performed in parallel with appropriate vehicle controls (aldosterone was dissolved in ethanol; U0126 was dissolved in Me₃SO). Transepithelial resistance and potential difference across the cell monolayers were measured using a mini-volt-ohmmeter (MilliCell ERS, Millipore) at specified time periods after treatment. The equivalent short circuit current (Iₛₑₜ) was calculated using Ohm’s law (29). Amiloride (1 μM) added to the medium completely inhibited this current, thereby indicating its ENaC dependence (data not shown). The use of equivalent current as a surrogate for true short circuit current for our specific purposes is well established (30, 31).

Microarray Analysis—Total RNA was isolated from aldosterone- or vehicle-treated cells (after electrophysiological measurements, as above) using standard procedures and made free of residual genomic DNA contamination using the DNase treatment and removal kit (Ambion, Austin, TX). Microarray analysis was performed after the protocol recommended by the University of California at San Francisco Genomics Core Laboratories (the original document can be found at www.array.ucsf.edu). Briefly, first strand cDNA was synthesized with 20 μg of total RNA, 1 μg of oligo dT, 830 μM each dATP, dCTP, and dGTP, 166 μM dUTP, 666 μM 5-(3-aminomethyl)-dUTP (Ambion), 0.1 μM dithiothreitol, 1 μl of RNase inhibitor (Amersham Biosciences), 600 units of RT (Promega Corp.), 10× RT buffer, and diethyl pyrocarbonate-treated water to a final volume of 30 μl for 2 h at 42 °C. After hydrolysis with 10 μl of 1 N NaOH and 10 μl of 0.5 M EDTA, cDNA was purified and recovered using Microcon 30 (Millipore Corp., Bedford, MA). Cy3 or Cy5 (Amersham Biosciences) was then coupled with cDNA in 0.05 M NaHCO₃ (pH 9.0) in the dark at room temperature for more than 1.5 h. After clean-up with CyScribe GFX purification kit (Amersham Biosciences), the labeled cDNA was hybridized with the microarray slide at 50 °C for >40 h. Arrays were printed by University of California at San Francisco Genomics Core Laboratories using 40-mer oligo library (Qiagen, Valencia, CA; Mouse Version 2.0). Data were collected using Genepix 4.0 scanner.

Array Data Analysis—After array hybridization and scanning, quality of the array was examined with R_bioconductor. First, the uniformity of spot and labeling and the intensity of labeling were checked with an MA plot (M = log₂(Cy5/Cy3), A = log₂((Cy5+Cy3)/2)). A quantitative assessment such as measurement of signal-to-noise ratio and the median signal intensity was also performed. Only those arrays having reasonable quality were used for further analysis. The statistically significant differentially expressed genes were selected with SAM using the one-class response with data (log₂(Cy5/Cy3) normalized in R_bioconductor). Hierarchical clustering was performed with Acuity 4.0 (Axon Instruments). Four independent microarray experiments were performed.

Real-time Quantitative Reverse Transcription PCR—Total RNA was isolated from aldosterone- or vehicle-treated cells using standard procedures and made free of residual genomic DNA contamination using the DNase treatment and removal kit (Qiagen). Purified RNA was reverse-transcribed with MultiScribe reverse transcriptase (PE Applied Biosystems, Foster City, CA), and PCR reactions were set up using the SYBR Green PCR kit (PE Applied Biosystems) according to the manufacturer’s instructions. Fluorescence changes during real-time quanti-
tative PCR were measured with an ABI Prism 7700 sequence detector (PE Applied Biosystems) as previously described (32). The following primers were used for PCR: 5'-GGG ACA ACG TCC ACC TTC TG (SGK1), 3'-GGG CTG CTT AAT GTG GAC AAG CTG (SGK1), 5'-GGT TGCC ACC CTT AAT CCT TAC AGA T (αENaC), 3'-TCC AGC TCT TCC AGA TCT T (αENaC), 5'-TCC ACA AAG CCC TGG TGA A (FKBP5), 3'-AAA ACC ATA GCG TGG TCC AA (FKBP5), 5'-TGG TGG CCC TAG ACA ACA AGA (GILZ), 3'-TCA CAG GTG TCA TCA GGT GTG T (GILZ), 5'-TTT TTT ACG AAT GAG ACT (cyclophilin), 3'-CAC CCT CCA AAA GAC CAC ATG (cyclophilin). All primers were specifically designed to span intron-exon junctions. The results obtained for different genes are given normalized to the control values relative to cyclophilin. All PCR reactions were performed in triplicate using samples obtained from three independent experiments. A − RT negative control was included with every run. The validity of the method was also independently verified using semiquantitative reverse transcription PCR using a completely different set of primers (data not shown).

Xenopus laevis Oocyte Co-expression Assay—The use of α-, β-, and γ-ENaC pSEasy expression vectors has been described previously (7). Full-length GILZ cDNA was cloned from the mouse cortical collecting duct cell line mpkCCDc14 cells (mGILZ) using RT-PCR into the in vitro expression vector, pMO. An N-terminal Myc tag (EQKLISEEDL) was added to facilitate analysis of expression. After preparation of linearized DNA, capped RNAs were synthesized using SP6 or T7 RNA polymerase (mMessage mMachine kit, Ambion). Stage V–VI Xenopus oocytes were microinjected with a mixture of α-, β-, and γ-ENaC subunit cRNAs (3 ng each) either alone or in combination with mGILZ cRNA (3 ng). As a control, one group of oocytes was microinjected with an equal volume of diethyl pyrocarbonate-treated water. After injection the oocytes were incubated for 24 h in low Na+ Barth’s solution containing 10 mM NaCl, 86 mM N-methyl-D-glutamine (pH 7.4), 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH 7.4). For some experiments, 24 h after microinjection the oocytes were treated with 2 µg/ml progesterone (Sigma) (or an equal volume of ethanol as the vehicle control) for 6 h. Some groups were pretreated with 50 µM U0126 (Cell Signaling) (or an equal volume of Me2SO as vehicle control) for 2 h before treatment with progesterone. Electrophysiological measurements were performed at a holding potential of −100 mV in high Na+ Barth’s solution containing 96 mM NaCl (other ions as above) using a laboratory-built two-electrode voltage clamp (33). Oocytes (six in each group) were continuously superfused in a 200-µl chamber at 6 µl/min. The macroscopic phenamil-sensitive current was defined as the difference between currents measured at −60 mV at the end of a holding potential of −100 mV and the holding potential of −100 mV in high Na+ Barth’s solution. The remaining solution was used directly for Western blot analysis. Preparation of Protein Lysates—After electrophysiological measurements, the mpkCCDc14 cell monolayers were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100) (34) containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 1× Complete protease inhibitor mixture (Roche Applied Science), 1× phosphatase inhibitors Set-1 and Set-2 (Calbiochem), 1 mM Na3VO4 (Calbiochem), and 2 µM Microcystin-LR (Sigma). Lysates were clarified by centrifugation at 10,000 rpm for 15 min at 4 °C and used for Western blot analysis or immunoprecipitation after protein estimation (35). Stored Xenopus oocytes were lysed in lysis buffer (10 mM Tris (pH 7.5), 137 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM Na3VO4) containing 2 mM phenylmethylsulfonyl fluoride and 1× Complete protease inhibitor mixture. Lysates were centrifuged at 12,000 rpm for 15 min at 4 °C to separate out yolk and debris, and the remaining solution was used directly for Western blot analysis.

Immunoprecipitation—Immunoprecipitation of transfected Myc-GILZ was carried out using anti-c-Myc-agarose conjugate (Sigma) according to the manufacturer’s instructions with minor modifications. Briefly, cell lysates (1 mg of whole cell lysate) were incubated with 40 µl of the agarose beads overnight at 4 °C. The beads were then washed 4 times in wash buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl) and collected by brief centrifugation at 5000 rpm for 30 s. Immunoblotting for Myc-GILZ was performed as described under “Western Blot Analysis.” Western Blot Analysis—Equal quantities of protein (10 µg of mpkCCDc14 cell lysate or 2 oocyte-lyase equivalents per lane) or immunoprecipitated samples were electrophoresed on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences). Immunoblotting was performed as described previously (36) using horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The blots were first probed for phospho-ERK (E10 monoclonal phospho-ERK1/2 antibody; Cell Signaling, 1:1000 dilution). Each phospho-ERK blot was subsequently stripped and re-probed for total-ERK to ensure equality of protein loading. The total-ERK 1/2 antibody (goat polyclonal antibody, Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:100. In some experiments the blot was stripped yet again and re-probed with anti-Myc antibody (rabbit polyclonal antibody, Cell Signaling, 1:500 dilution) to assess heterologously expressed Myc-tagged GILZ expression. For densitometric analysis, signal intensities of various bands were determined using the NIH Imaging software. The expression levels of specific phospho-ERK bands were inferred upon normalizing their signal intensities to that of total-ERK within the same lane (for quantification of mpkCCDc14 ERK bands, signal intensities corresponding to both ERK1 and ERK2 were added to provide one value). Values so obtained were used to determine mean ± S.E. for a graphical representation. Significance was determined using standard statistical tests as described below. Each figure is representative of at least three independent experiments with similar results.
each of α-, β-, and γ-FLAG-xENaC and 250 ng of pMO-Myc-mGILZ. 24 h after transfection cells were treated with EGF (20 ng/ml) for 1 h. As a control, some groups were pretreated with U0126 (10 μM) for 2 h before treatment with EGF. After treatment the coverslips were removed and processed to detect expression of cell surface ENaC using live cell staining or using permeabilized staining to detect total cellular ENaC, as described previously (37) (please note that the FLAG epitope has been inserted into the extracellular loop of each of the ENaC subunits (38)). Cell surface ENaC was visualized using FLAG monoclonal antibody M2 (Sigma) and goat anti-mouse secondary antibody conjugated to Alexa-594 fluorophore (Molecular Probes) followed by nuclear costaining with 4,6-diamidino-2-phenylindole. Images were obtained at 630× magnification using Zeiss Axioscope epifluorescence microscope with Metamorph imaging software (Universal Imaging) and filter sets for 4,6-diamidino-2-phenylindole (nuclei) and Alexa-594 (FLAG-ENaC). Each experiment was repeated at least three times with similar results. For quantitation (Fig. 6B), a blinded observer counted the number of cells with detectable surface ENaC and divided the number by the number of 4,6-diamidino-2-phenylindole-positive cells in three independent experiments of cells subjected to live cell staining.

Statistical Analysis—Data are represented as the mean ± S.E. In all experiments involving Na+ current measurements, 3–6 samples were tested, and at least 3 independent experiments were performed with the same treatment protocol. Unless otherwise specified, all statistical comparisons were evaluated using a Student’s unpaired two-tailed t test, and significance was defined as a p value <0.05.

RESULTS

Identification of Aldosterone-regulated Genes by Microarray Analysis—To identify aldosterone-regulated mRNAs, mpkCCD14 cells were grown on Transwell filters and treated with aldosterone (10−6 M) or ethanol (vehicle control) for 1 or 6 h and then subjected to microarray analysis (as described under “Materials and Methods”). The complete list of aldosterone-regulated genes obtained in our screen has been deposited and is available at the NCBI Gene Expression Omnibus (GEO) data base (www.ncbi.nlm.nih.gov/geo). The data series accession number is GSE2933, and the platform number is GPL2623. The scaled signal values from all four hybridization experiments (performed with the same treatment protocol) are available online. 49 genes were found to be consistently up-regulated upon aldosterone treatment (>1.5-fold compared with control). FKBP5 (FK506-binding protein 5) and GILZ were two of the most strongly induced genes identified in this screen (up-regulated 4.1- and 2.3-fold, respectively, compared with control). Notably, in addition to FKBP5 and GILZ, SGK1 and αENaC were also identified in the same screen, supporting the authenticity of the microarray analysis. More than 171 aldosterone-repressed genes (down-regulated to <0.6-fold compared with control) were also identified. A select set of stimulated and repressed genes was chosen for further characterization by real-time RTPCR based on the degree of induction/repression and potential significance in regulating Na+ transport.

Real-time PCR performed on 1 and 6 h RNA for four selected induced genes (SGK1, αENaC, FKBP5, and GILZ) confirmed induction (Fig. 1). SGK1 and GILZ were rapidly induced (>3.5-fold stimulation within 1 h), whereas αENaC and FKBP5 were more slowly induced (>5-fold
stabilization by 6 h). In contrast, real-time PCR did not similarly confirm repression of transcripts identified as down-regulated in the microarray (peroxisome proliferator-activated receptor α, epiregulin, necludin, and claudin-10 were selected for real-time PCR analysis) (data not shown). These results are consistent with the idea that microarray analysis can reliably identify aldosterone-induced, but not repressed genes, and shed doubt on direct conclusions drawn regarding global patterns of gene repression based on microarray data. In light of the rapidity and extent of GILZ induction and its potential role in suppression of ERK signaling, it was selected for further characterization.

**GILZ Stimulates ENaC Activity in Xenopus Oocytes**—The rate-limiting step in transepithelial Na⁺ transport is ENaC-mediated apical entry, which has been shown to be inhibited by activated ERK1/2 (11, 24). To examine GILZ effects on ENaC-mediated Na⁺ currents in the presence of active ERK, we used progesterone-stimulated *Xenopus* oocytes. In the resting state, stage V-VI *Xenopus* oocytes have little detectable ERK activity, which is markedly stimulated by progesterone (39). We first confirmed that ERK phosphorylation and ENaC inhibition could be induced and were correlated in this system. Stage V-VI oocytes were injected with cRNAs for ENaC subunits. After 24 h of incubation in a low Na⁺ solution, they were treated with 2 μg/ml progesterone or vehicle-control (ethanol) and allowed to incubate further for varying periods of time. At specified time points oocytes were analyzed for phenamil-sensitive current (a measure of ENaC activity) using the two-electrode voltage clamp technique as described under “Materials and Methods.” After electrophysiological measurements, the oocytes were harvested for protein analysis. Fig. 2A shows a representative Western blot for phospho-ERK (p42ERK/ERK2) after varying periods of progesterone/ethanol treatment. We observed the appearance and increase of detectable phospho-ERK after 6 h of progesterone treatment, confirming and extending previous results (23). In contrast, oocytes treated with vehicle had no detectable phospho-ERK, even after 10 h of treatment. Electrophysiological readings obtained (a minimum of six oocytes in each group per experiment) showed that progesterone consistently and significantly inhibited ENaC current (Fig. 2B) and that this precisely paralleled the appearance and increase of phospho-ERK (Fig. 2A), consistent with the previous suggestion that ERK activation down-regulates ENaC activity in *Xenopus* oocytes (23). Because 6 h of progesterone treatment was sufficient to both consistently down-regulate ENaC current and to generate readily detectable levels of active phosphorylated ERK by Western blot using a specific antibody, we confined subsequent oocyte experiments to this particular time point. Importantly, at this time point the integrity of the oocyte membrane is not compromised, and resting potentials are stable, allowing for consistently reliable measurements of ENaC activity.

The results of the above experiments (Figs. 2, A and B) suggested that freshly isolated stage V-VI oocytes, on one hand, and progesterone-treated oocytes, on the other, represented two distinct stages of ERK activation. This, therefore, presented an ideal model system to study the effect of GILZ in the absence and presence of active/phosphorylated ERK. Stage V-VI oocytes were microinjected with ENaC cRNAs with or without GILZ cRNA. After 6 h of treatment with progesterone (2 μg/ml) or vehicle, phenamil-sensitive current was measured by the two-electrode voltage clamp technique. As observed earlier (Fig. 2B), progesterone treatment markedly inhibited ENaC activity (Fig. 2C). Notably, this effect was completely reversed by U0126, a selective inhibitor of MEK1/2, the specific activator of ERK1/2, supporting the cause-and-effect relationship between ERK activation and down-regulation of ENaC activity in *Xenopus* oocytes. Na⁺ currents in progesterone-treated oocytes expressing GILZ were comparable with or slightly greater than those treated with U0126, exhibiting a 9.1-fold greater ENaC activity relative to base line (progesterone-treated oocytes not expressing GILZ). U0126 provided no further activation (Fig. 2C, third set of bars). Although GILZ by itself also tended to stimulate Na⁺ current in resting oocytes lacking detectable phospho-ERK (compare first set of bars in Fig. 2C), this effect was modest (1.6-fold on average) and less consistently observed. The ENaC-stimulatory effect of GILZ was dose-dependent and observed at various cRNA concentrations tested (0.5, 3, and 10 ng, with 3 ng exhibiting maximal ENaC stimulation; data not shown). These experiments demonstrate that GILZ strongly stimulates ENaC activity and suggests that most, if not all, of the effect is through inhibition of ERK activation. Further supporting this conclusion, oocytes subjected to Western blot following two-electrode voltage clamp measurements demonstrated that GILZ markedly inhibited the generation of phospho-ERK (Fig. 2D) in a manner very similar to that observed with U0126.

**ERK Activation Down-regulates Transepithelial Na⁺ Transport in Kidney Collecting Duct Cells**—We next extended our studies to determine whether GILZ affected ERK activity and transepithelial Na⁺ transport in monolayers of cultured kidney epithelial cells. For these purposes, we used mpkCCD14 cells (derived from mouse kidney collecting duct (29)) grown to high resistance on Transwell filters and treated with EGF (20 ng/ml) for 24 h to induce ERK activity. Long term treatment with EGF (24 h or more) to activate ERK in numerous cell types, including renal collecting duct cells, has been well described (11, 25). We first determined the basal and EGF-stimulated level of active/phosphorylated ERK1 and ERK2 (p44 and p42, respectively) in mpkCCD14 cells (Figs. 3, A and B). Fig. 3A shows a representative Western blot, whereas Fig. 3B shows a graphical representation of the same data (densitometric analysis from four independent experiments). As has been described previously for both collecting duct and cultured distal nephron cells (11, 22) but in marked contrast to unstimulated *Xenopus* oocytes (Fig. 2), there was significant basal expression of phospho-ERK1/2 in mpkCCD14 cells. Phospho-ERK1/2 expression was consistently and significantly increased from this basal by EGF (1.7-fold), whereas total-ERK remained unchanged. As in oocytes, U0126 eliminated all detectable phospho-ERK in the presence or absence of EGF without altering total-ERK levels. Electrophysiological measurements performed on the same cells before harvesting for immunoblot demonstrated an inverse relationship between ERK activation and equivalent current; EGF down-regulated an equivalent current 2-fold relative to untreated cells (Fig. 3C), in parallel with the 1.7-fold increase in phospho-ERK protein levels (Fig. 3B). This EGF-induced decrease in Na⁺ transport was prevented by pretreating cells with U0126, further supporting the cause-and-effect relationship between ERK activation and ENaC down-regulation, as was seen in oocytes.

**GILZ Stimulates Transepithelial Na⁺ Transport and Inhibits ERK Activation in mpkCCD14 Cells**—To directly examine GILZ effects on ENaC-dependent transepithelial Na⁺ current in mammalian kidney epithelial cells, we transiently transfected mpkCCD14 cells with the GILZ expression vector pIRES2-EGFP-Myc-mGILZ (4 μg per 2 × 10⁶ cells), a bicistronic plasmid that encodes Myc-tagged GILZ followed by an internal ribosomal entry site (IRES) and the coding sequence for EGFP. Fig. 4A depicts phase-contrast and epifluorescence images of these cells grown on 6-well tissue culture dishes at 200× magnification. We consistently observed comparable (60–70%) transfection efficiency in cells transfected with either pIRES2-EGFP-Myc-mGILZ (panel a) or the empty vector expressing EGFP alone (panel b). Untransfected cells or mock-transfected cells (no-DNA control) had no detectable GFP expression and were morphologically indistinguishable from trans-
FIGURE 2. GILZ stimulates ENaC activity in Xenopus oocytes. A, progesterone treatment induces significant ERK activation in Xenopus oocytes by 6 h. Stage V-VI Xenopus oocytes were co-injected with cRNA for all three ENaC subunits (3 ng each). 24 h after incubation in a low Na⁺ medium (see “Materials and Methods” for details), they were treated with 2 μg/ml progesterone (or an equal volume of ethanol vehicle control) for varying periods of time. Phenamil-sensitive current (as a measure of ENaC activity) was recorded in these oocytes using the two-electrode voltage clamp technique at a holding potential of −100 mV. The oocytes were then harvested for protein analysis. Shown is a representative Western blot for active/phosphorylated (Phospho-) ERK (p42/ERK2) in oocytes treated with progesterone or ethanol (EtOH, vehicle control). Total-ERK was used as a loading control.

B, ERK activation is integrally associated with down-regulation of ENaC activity in Xenopus oocytes. Graphical representation of electrophysiological readings obtained in oocytes treated with progesterone (Prog)/ethanol for specified periods of time is shown. C, GILZ acts similar to U0126 in protecting oocytes from progesterone-induced loss in ENaC activity. Stage V-VI Xenopus oocytes were co-injected with cRNA for all 3 ENaC subunits (3 ng each) either alone or in combination with GILZ cRNA (3 ng). 24 h after incubation in a low Na⁺ medium (see “Materials and Methods” for details), they were treated with 2 μg/ml progesterone (or equal volume of ethanol/Me2SO vehicle control) for 6 h. As a control, one group of oocytes was pretreated with U0126 (50 μM) for 2 h before progesterone treatment. Phenamil-sensitive current (as a measure of ENaC activity) was recorded in these oocytes using the two-electrode voltage clamp technique. Graphical representation of electrophysiological readings were obtained. D, GILZ prevents activation of ERK in a manner similar to U0126. Shown is a Western blot analysis for active/phosphorylated (Phospho-) ERK (p42/ERK2) in GILZ-expressing oocytes (or controls) treated with progesterone or ethanol/Me2SO (vehicle control). Total ERK was used as a loading control. The blot was stripped and reprobed with anti-Myc antibody to ensure GILZ expression in oocytes injected with Myc-tagged GILZ cRNA.
fected cells (data not shown). Importantly, Western blot staining of cell lysates demonstrated that heterologously expressed Myc-mGILZ protein expression persisted for >5 days (Fig. 4B), sufficient for growth and equivalent current measurements on Transwell filters.

For electrophysiological measurements mpkCCD_{14} cells were transfected with pIRES2-EGFP-Myc-mGILZ or pIRES2-EGFP and grown on Transwell filters until a stable high resistance was reached followed by 24 h of maintenance in serum- and corticosteroid-free medium (to study GILZ-specific effects in the absence of other steroid hormone-stimulated factors) containing EGF to activate ERK, at which point equivalent short circuit current was evaluated as in Fig. 3. Fig. 4C shows a graphical representation of these data. We observed that GILZ consistently and significantly stimulated equivalent current (1.9-fold), which is >90% amiloride-inhibitable (indicating that it is ENaC-mediated) in this cell line (29). After electrophysiological measurements, cells were harvested for protein analysis. Fig. 4D is a representative Western blot for phospho-ERK1/2. Our results strongly suggest that GILZ markedly down-regulates phospho-ERK, in parallel with increased transepithelial Na\(^{+}\) transport. Importantly, total-ERK levels remained unchanged in both samples, consistent with the idea that GILZ specifically regulates activation of existing ERK protein and not its abundance.

Aldosterone Suppresses Phospho-ERK Levels in mpkCCD_{14} Cells as It Stimulates Na\(^{+}\) Current—We next examined the time course of phospho-ERK in mpkCCD_{14} cells in response to aldosterone treatment. Aldosterone could in principle stimulate, inhibit, or have no effect on ERK activity, depending on the balance of inhibitory (e.g. by GILZ) and stimulatory (e.g. by K-Ras (40) and epidermal growth factor receptor (11)) factors. mpkCCD_{14} cells were treated with aldosterone (10^{-7} M), and the equivalent current was measured (Fig. 5A) after which cells were
harvested, and phospho-ERK expression was ascertained by immunoblot. Fig. 5B shows a representative phospho-ERK Western blot, and Fig. 5C shows densitometric quantitation of the same (from four independent experiments). Interestingly, phospho-ERK levels began to decrease by 2 h after aldosterone treatment, at which time point Na\(^+\) current began to increase and remained suppressed at 24 h. This time course parallels the aldosterone-induced increase in Na\(^+\) current (Fig. 5A). These results suggest that aldosterone suppresses phospho-ERK protein expression in mpkCCD\(_{14}\) cells, in parallel with induction of GILZ expression.

FIGURE 4. GILZ stimulates transepithelial Na\(^+\) transport and inhibits ERK activation in kidney epithelial cells. mpkCCD\(_{14}\) cells were transiently transfected with the EGFP-expressing GILZ expression vector (pIRES2-EGFP-Myc-mGILZ; 4 μg per 2 × 10\(^6\) cells) or the empty vector expressing EGFP alone (negative control) using a high efficiency nucleofection protocol developed by Amaxa Biosystems, Inc. A, phase-contrast and epifluorescence images (brightfield and GFP) of cells transfected with the GILZ expression vector (panel a) or empty vector alone (panel b), obtained using Nikon Eclipse TE300 microscope at 200× magnification. B, biochemical verification of the transfected mGILZ protein. Immunoblot (IB) analysis for Myc-GILZ (after immunoprecipitation (IP) using anti-Myc antibody) in cells transfected with either the GILZ expression vector or the empty vector alone. C, GILZ significantly stimulates transepithelial Na\(^+\) transport in kidney epithelial cells. After transfection cells were allowed to grow on Transwell filters until the monolayers reached resistance of greater than 1000 ohms-cm\(^2\). They were then maintained in medium devoid of steroid hormones (see “Materials and Methods” for details) for 24 h before electrophysiological measurements. Shown is a graphical representation of the electrophysiological recordings obtained in cells transfected with either the GILZ expression vector or the empty vector alone. D, GILZ significantly inhibits ERK activation in EGF-treated mpkCCD\(_{14}\) cells. Western blot analysis for active/phosphorylated (Phospho-) ERK (p42/p44) in mpkCCD\(_{14}\) cells transfected with either the GILZ expression vector or the empty vector alone is shown. Total-ERK was used as a loading control.
Finally, we examined GILZ regulation of ENaC cell surface expression in mammalian kidney epithelial cells in the presence of activated ERK1/2. For these purposes, we used transfected HEK 293 cells treated with EGF to activate ERK1/2. These mammalian kidney epithelial cells reliably express and traffic to the plasma membrane a variety of integral membrane proteins, including ion transporters and channels (37, 41). Moreover, the use of EGF to activate ERK1/2 in HEK 293 cells through stimulation of endogenous epidermal growth factor receptor is well described (42, 43). Hence, they offered a useful system for examining potential effects of ERK activation and its inhibition by GILZ on ENaC trafficking. Cells were transfected with FLAG-ENaC alone or in

**FIGURE 5. Aldosterone suppresses phospho-ERK levels in kidney epithelial cells.** Polarized mpkCCD_{14} cells were treated with aldosterone (10^{-6} M) or ethanol (EtOH, vehicle control) for specified periods of time. After electrophysiological measurements, the cells were harvested for protein analysis. A, aldosterone stimulates transepithelial Na\(^{+}\) transport in mpkCCD_{14} cells. Graphical representation of the electrophysiological recordings was obtained (as in Fig. 3). B, equivalent short circuit current. C, Western blot analysis for phospho-ERK after aldosterone treatment. Shown is a representative blot. Total-ERK was used as a loading control. C, graphical representation of the above data (scanning densitometry results from four independent experiments). Relative concentrations of phospho-ERK protein levels are expressed in arbitrary units normalized to total-ERK within the same lane.
combination with GILZ, recovered for 24 h, and treated with EGF for 1 h. Parallel cultures were then subjected to live cell staining (Figs. 6, A and B) or permeabilized (Fig. 6C) staining. Fig. 6A depicts epifluorescence images of cells after live cell staining for FLAG-ENaC, which detects only cell surface epitopes (37), and Fig. 6B shows quantitation of the fraction of cells with detectable cell surface ENaC in each condition (per 100 cells, shown normalized to control cells) from three independent experiments performed. C, epifluorescence images of cells transfected with FLAG-ENaC alone or FLAG-ENaC and GILZ in the presence/absence of EGF and subjected to staining with anti-FLAG antibody after fixation and permeabilization.

**DISCUSSION**

Several hormones, including aldosterone, insulin, and vasopressin, act through various cellular signaling cascades to modulate ENaC activity and fine-tune epithelial Na⁺ transport (1). The actions of aldosterone, in particular, are largely or possibly solely mediated...
through effects on gene transcription, and a number of genes have been identified whose induction parallels changes in Na⁺ transport (5). These include several of the genes encoding transporters themselves, which tend to be induced relatively slowly and probably play a role in the consolidation ("late") phase of the response (10) (e.g. aENaC, Fig. 1B). However, increasing evidence has supported the idea that members of a second subset of genes respond rapidly and mediate the early phases of the response by modulating existing signaling pathways. SGK1, which transmits phosphatidylinositol 3'-kinase-dependent signals that result in Nedd4-2 inhibition, represents one mediator of this type (44). Taken together with earlier in vivo data (20), our present data suggest that GILZ is a complementary mediator of the early phase of aldosterone action that participates in an analogous way to inhibit ERK signaling. The key lines of evidence supporting this conclusion are as follows. 1) Aldosterone rapidly stimulates GILZ mRNA levels in mpkCCD₁₄₁ cells (Ref. 5 and Fig. 1) and in native rat cortical collecting duct (20). In parallel with increased GILZ expression, aldosterone inhibits phospho-ERK1/2 expression (Fig. 5). 2) GILZ expression markedly inhibits formation of phospho-ERK2 in Xenopus oocytes treated with progesterone (a well characterized activator of ERK in oocytes) while simultaneously blocking ERK-dependent inhibition of Na⁺ current (Fig. 2). The net effect represents a 9.1-fold greater Na⁺ current in GILZ-expressing than in non-expressing oocytes. In this regard, it is notable that a specific inhibitor of mitogen-activated protein kinase kinase (MEK1/2), U0126, had a similar effect on both Na⁺ current and phospho-ERK levels, strongly suggesting that the ENaC stimulatory effect of GILZ was largely due to its effects on the ERK pathway and not due to any unrelated parallel action (Fig. 2). 3) heterologous expression of GILZ in EGFR-treated mpkCCD₁₄₁ cells caused parallel down-regulation of activated ERK and up-regulation of transepithelial Na⁺ transport (Fig. 4). As in oocytes, a specific MEK inhibitor had a non-additive effect, similar to GILZ expression, further supporting the idea that they act in the same pathway, upstream of ERK1/2 (Fig. 3). As in oocytes, most of the stimulatory effect of GILZ in mpkCCD₁₄₁ cells appears to reflect inhibition of ERK signaling and, hence, dis-inhibition of Na⁺ transport. 4) EGF, which is known to act through epidermal growth factor receptor to stimulate ERK1/2 in HEK 293 cells, markedly decreased cell surface ENaC expression, and heterologous expression of GILZ in EGF-treated HEK 293 cells completely reversed this effect (Figs. 6, A and B). Together, these data strongly support the view that GILZ, through inhibition of the ERK cascade, contributes to the early stimulation of ENaC-mediated Na⁺ transport by mineralocorticoids, thereby identifying a new molecular mechanism involved in aldosterone-induced transepithelial Na⁺ transport. It is notable in this regard that there appears to be substantial basal ERK1/2 activity in cultured CD cells in the absence of corticosteroid hormones (Fig. 3A, first lane) (24) and in rat and human CD (27, 28). Interestingly, there are also reports that aldosterone can itself activate ERK signaling in A6 and Madin-Darby canine kidney cells (40, 45). In consideration of the present results, aldosterone-activation of the ERK pathway would appear to be a variable late-phase negative-feedback response manifest in some cell types or conditions, which might temper otherwise prolonged/uncontrolled Na⁺ reabsorption (11). In mpkCCD₁₄₁ cells the predominant effect appears to reflect GILZ-mediated inhibition of ERK, likely acting in concert with SGK1-induced inhibition of Nedd4-2 to stimulate Na⁺ current. The ERKs constitute a family of evolutionarily conserved protein kinases critical for several cellular responses such as proliferation, differentiation, and apoptosis (46). Because these signaling pathways influence numerous aspects of cell function, it is essential to understand how modulators of this pathway function. GILZ, which belongs to the TSC22 (transforming growth factor β-stimulated clone 22) family of leucine zipper proteins, was initially thought to function as a transcription factor (47). Indeed, it has been shown to antagonize the function of transcription factors (AP1 and NFkB, classic final mediators of the ERK pathway) that drive cell proliferation (48, 49) and could therefore be considered as promoting differentiation and differentiation-related functions such as Na⁺ transport in CCD principal cells (as the present study demonstrates). However, GILZ has also been found recently to inhibit Ras-dependent activation of Raf (21), the mitogen-activated protein kinase kinase kinase that functions in ERK1/2 activation. In view of the inverse effect of GILZ on phospho-ERK levels and Na⁺ current and similarity of the GILZ effect to that of the MEK inhibitor, U0126, it seems likely that GILZ stimulates Na⁺ transport by inhibiting Raf or potentially some other pathway component upstream of ERK. An effect on AP1 or NFkB (downstream of ERK) in this context seems less likely.

Interestingly, the present results are reminiscent of the mechanism by which SGK1 stimulates ENaC activity, which also involves inhibiting an inhibitor: Nedd4-2 (1, 13). Nedd4-2 enhances ENaC internalization, likely through ubiquitination of the channel itself (13). SGK1 phosphorylates Nedd4-2, thus triggering recruitment of 14-3-3 proteins (50, 51), which diminish the ability of the ubiquitin ligase to interact with and/or ubiquitinate ENaC. ERK-mediated ENaC inhibition appears to proceed through phosphorylation of the channel, which stimulates interaction with Nedd4 (52), leading to a decrease in channel surface expression and subsequent degradation (Fig. 6) (53). We believe the latter to be a late effect that is secondary to enhanced internalization. Although this mechanism remains to be further elucidated, it is notable that in HEK 293 cells a 1-h treatment with EGF markedly diminished cell surface ENaC without any detectable change in the total cellular pool. The effect of GILZ on ENaC appears to be largely if not solely mediated by its inhibition of ERK1/2. Hence, our present data support the view that the effects of ERK and GILZ, like those of Nedd4-2 and SGK1 (13, 15), are primarily on levels of plasma membrane channel expression. A recent report suggested that in oocytes, ERK primarily decreases ENaC Po and has little effect on membrane expression (54). The basis for this discrepancy is not clear at this time; however, these mechanisms are not mutually exclusive, and it will be interesting to determine which is operative under physiological conditions; perhaps both.

If the mechanism in CD cells proceeds in a manner analogous to that in lymphocytes, it would appear that GILZ inhibited ERK by disrupting the interaction of Ras and Raf (21). However, several components of the signaling cascade are together in multi-protein complexes, and it may be that Raf inhibition is only part of the mechanism of ENaC regulation. This remains to be explored. In any case, taken together with earlier reports, the present data suggest that during the early phase of aldosterone one action, the parallel effects of SGK1 and GILZ converge on the ENaC-Nedd4/Nedd4-2 interaction, abrogating it by simultaneously increasing Nedd4-2 phosphorylation and decreasing channel phosphorylation. This study highlights the importance of “dis-inhibition” as a key mechanism in the control of ENaC function, probably through modulation of trafficking and degradation (55).
impact on ERK activity. To our knowledge, this is the first direct demonstration of a role for GILZ or any other endogenous mitogen-activated protein kinase inhibitor in stimulating Na" transport. We suggest that GILZ and SGK1 together coordinately mediate cross-talk between steroid and growth factor signaling pathways, thereby providing context appropriate regulation of a variety of cellular activities, including Na" transport.

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