Differential Activities of Glucocorticoid-induced Leucine Zipper Protein Isoforms*

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Glucocorticoid-induced leucine zipper protein (GILZ) is expressed in both epithelial and immune tissues and modulates a variety of cellular functions, including proliferation and epithelial sodium channel (ENaC) activity. A number of reports have described various GILZ activities, focusing on a single isoform with molecular mass of ~17 kDa, now termed GILZ1. In GILZ immunoblots using a newly developed antiserum, we detected multiple species in extracts from cultured kidney cells. Mass spectrometric analysis revealed that one of these represented a previously uncharacterized distinct isoform of GILZ, GILZ2. Rapid amplification of cDNA ends was used to clone cDNAs corresponding to four isoforms, which, in addition to GILZ1 and GILZ2, included new isoforms GILZ3 and GILZ4. Heterologous expression of these four GILZ isoforms in cultured cells revealed striking functional differences. Notably, GILZ1 was the only isoform that significantly stimulated ENaC-tured cells revealed striking functional differences. Notably, Heterologous expression of these four GILZ isoforms in cultured cells revealed striking functional differences. Notably, GILZ1 and GILZ2, included new isoforms GILZ3 and GILZ4. Heterologous expression of these four GILZ isoforms in cultured cells revealed striking functional differences. Notably, GILZ1 was the only isoform that significantly stimulated ENaC-mediated Na+ current in a kidney collecting duct cell line, although GILZ2 and GILZ3 also stimulated ENaC surface expression in HEK 293 cells. GILZ1 and GILZ3, and to a lesser extent GILZ2, inhibited ERK phosphorylation. Interestingly, GILZ4, which had no effect on either ENaC or ERK, potently suppressed cellular proliferation, as did GILZ1, but not GILZ2 or GILZ3. Finally, rat and mouse tissues all expressed multiple GILZ species but varied in the relative abundance of each. These data suggest that multiple GILZ isoforms are expressed in most cells and tissues and that these play distinct roles in regulating key cellular functions, including proliferation and ion transport. Furthermore, GILZ inhibition of ERK appears to play an essential role in stimulation of cell surface ENaC but not in inhibition of proliferation.

Glucocorticoid-induced leucine zipper (GILZ)3 is a small leucine zipper protein of ~17 kDa. As its name implies, GILZ was first discovered as a dexamethasone-induced transcript in murine thymocytes, which it protects from apoptosis induced by treatment with anti-CD3 antibody (1). It is a member of the TSC22D (transforming growth factor β1-stimulated clone 22 domain) family of proteins that are widely expressed and appear to impact multiple biological processes (2–5). TSC22D1 (or, simply, TSC22) was first isolated based on its rapid and transient transcriptional induction by transforming growth factor β1 (6). It is a potential tumor suppressor gene and has been shown to down-regulate cell proliferation and induce apoptosis in human salivary gland (7, 8). Its expression in human fetal tissues (9) and, more recently, its detection at sites of epithelial-mesenchymal interactions during mouse embryogenesis (10) suggest an important role for this protein during vertebrate development. A similar role has been identified for the TSC22 homologue, bunched, in developing Drosophila larvae (11). TSC22D2 and TSC22D4 are expressed in renal cortex, medulla, and papilla and are involved in adaptation of these cells to hypertonicity (4). These two transcripts are significantly up-regulated by hyperosmolality, and overexpression of a specific splice variant of TSC22D2 (TSC22D2-4) in mLMD3 cells confers protection against osmotic stress-induced cell death (4).

TSC22D3 (also known as GILZ) is expressed in numerous tissues and is rapidly induced by glucocorticoids in T lymphocytes, in which it inhibits anti-CD3-induced interleukin-2 production, interleukin-2 receptor expression, Fas and Fas-ligand up-regulation, and cell death consequent to CD3-induced activation (1, 12–14). Moreover, GILZ expression is down-regulated by anti-CD3 stimulation, further suggesting that GILZ contributes to the control of T-cell activation and development (12, 13). Recent evidence also supports a role for GILZ in the antiproliferative effects of glucocorticoids in T lymphocytes (15). Glucocorticoids also up-regulate GILZ expression in macrophages, and GILZ overexpression, in turn, inhibits production of inflammatory mediators and proinflammatory chemokines as well as Toll-like receptor expression (16). Although the role of GILZ in immune cell function has been well studied, its role outside of the immune system, until recently, was unknown. In kidney cortical collecting duct (CCD), as well as in mppCCD14 cells, a highly differentiated mouse CCD cell line, its expression was shown to be robustly induced by aldosterone

* The abbreviations used are: GILZ, glucocorticoid-induced leucine zipper protein; CCD, cortical collecting duct; CD, collecting duct; EGF, epidermal growth factor; ENaC, epithelial sodium channel; ERK, extracellular signal-regulated kinase 1/2; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ORF, open reading frame; GRE, glucocorticoid response element; FHE, forkhead response element; RACE, rapid amplification of cDNA ends; mGILZ, mouse GILZ; BrdUrd, bromodeoxyuridine; P1, promoter 1; P2, promoter 2.

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In this cell line, GILZ markedly augments epithelial sodium channel (ENaC)-mediated sodium (Na⁺) transport (18), a process that is central to Na⁺ homeostasis. Transepithelial Na⁺ transport involves the regulated functioning of many different pathways and elements that are interestingly shared in several remarkably diverse cellular processes, such as cell proliferation, differentiation, and apoptosis. Importantly, GILZ appears to stimulate ENaC cell surface expression at least in part by inhibiting extracellular signal-regulated kinase 1/2 (ERK). In the course of experiments aimed at characterizing GILZ function and expression in a kidney collecting duct cell line (mpkCCD₁₄) using a newly developed antiserum, it became clear that several protein species were expressed, including one prominent species with a molecular mass of ~28 kDa, substantially larger than the originally identified GILZ isoform, GILZ1. Mass spectrometric analysis indicated that this species probably represented a distinct GILZ isoform, and we therefore set out to identify and functionally characterize cDNAs corresponding to this and other expressed GILZ isoforms.

**EXPERIMENTAL PROCEDURES**

Production of Anti-GILZ Antiserum—A rabbit polyclonal antiserum recognizing mouse GILZ was generated to the peptide sequence corresponding to amino acids 111–125 in GILZ1. Peptide synthesis, purification, verification of peptide sequence (by mass spectrometry), conjugation to carrier (keyhole limpet hemocyanin), injection of animals (New Zealand White rabbits), and subsequent boosters were all carried out by Quality Controlled Biochemicals (Hopkinton, MA). Following extensive laboratory characterization, selected bleeds were affinity-purified and used for all subsequent analyses.

mpkCCD₁₄ Cell Culture and Electrophysiological Measurements—mpkCCD₁₄ 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 (18, 19). 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 ohm cm². They were then maintained in steroid hormone-free, serum-free medium (18) for at least 24 h prior to treatment with aldosterone (10⁻⁶ M, apical and basolateral sides) or an equal volume of vehicle as control for specified periods of time. Following electrophysiological and biochemical measurements, cells were harvested and processed for protein analysis. U0126 treatment (10 μM) was performed as described previously (18). All experiments were performed in parallel with appropriate vehicle controls (aldosterone was dissolved in ethanol; U0126 was dissolved in Me₂SO). Transepithelial resistance and potential difference across the cell monolayers were measured using a mini-volt ohmmeter (MilliCell ERS; Millipore Corp.) at the specified time periods following treatment. The equivalent short circuit current (Iₑₛ) was calculated using Ohm's law (18, 19). Amiloride (1 μM) added to the medium completely inhibited this current, thereby indicating its ENaC dependence (data not shown).

5'-Rapid Amplification of cDNA Ends (RACE) PCR and Cloning of GILZ Isoforms—Full-length cDNA corresponding to various mouse GILZ (mGILZ) isoforms were cloned by rapid amplification of cDNA ends using the SMART-RACE cDNA amplification kit (Clontech), according to the manufacturer's instructions. Total RNA isolated from mpkCCD₁₄ cells was used for reverse transcription, followed by 5'-RACE-PCR (using 5'-TTA CAC CGC AGA ACC ACC AGG GCC TTC CGG GG-3' as the gene-specific primer) and subsequent cloning into the TOPOII TA cloning vector (Invitrogen). Following sequence analyses, the ORFs were subcloned into the in vitro expression vector, pMO (described previously (18)). An N-terminal Myc tag (EQKLISEEDL) was added to facilitate analysis of expression.

In Silico Analysis of the Tsc22d3 Gene—The obtained 5'-RACE products were searched via BLAST against the Mus musculus genome on the NCBI site on the World Wide Web, and a region 12 kb upstream and 2 kb downstream of the two most distal matched BLAST results was used for further in silico analysis. Exons were defined by the exact matched BLAST results to the 5'-RACE products and previously reported Tsc22d3 mRNA sequences (available on the World Wide Web), and introns were defined as the genomic sequence between individual exons. The GC content of the Tsc22d3 gene was analyzed using two World Wide Web-based programs, “CpG Island Searcher” (available on the World Wide Web) (20) and “DNA Base Composition Analysis Tool” (available on the World Wide Web).

Initial promoter description of the Tsc22d3 gene was performed previously by other workers (21–23), identifying functional glucocorticoid response elements (GREs) and forkhead response elements (FHREs). For further analysis of the promoter for other putative transcription factor binding sites, TFSearch (available on the World Wide Web), an application on the Helmholtz Network for Bioinformatics (available on the World Wide Web), and MatInspector (available on the World Wide Web) (24) were used.

mpkCCD₁₄ Transient Transfections—mpkCCD₁₄ cells (at 70–80% confluence) were transfected with various Myc-mGILZ constructs (5 μg of DNA/2 × 10⁶ cells) or the empty vector alone (vector control) as specified, using a high efficiency electroporation protocol (nucleofection; Amaxa Biosystems Inc.) according to the manufacturer’s instructions.

Mass Spectrometric Analysis—GILZ was immunoprecipitated from aldosterone-treated (10⁻⁶ M) mpkCCD₁₄ cell lysates using the new anti-GILZ antibody. Immune complexes were resolved by SDS-PAGE, and protein bands were visualized by Coomassie staining. The 28-kDa band of interest was carefully excised and prepared for in-gel digest with trypsin followed by liquid chromatography tandem mass spectrometric analysis, according to techniques standardized by the Mass Spectrometric Facility at the University of California (San Francisco, CA) (available on the World Wide Web). Liquid chromatography tandem mass spectrometric analysis per se was performed by Custom Biologies (Toronto, Canada), according to procedures standardized by the company. Purified eluted tryptic peptides were detected by an LCQ DECA XP mass spectrometer (Thermo), equipped with an electrospray ionization...
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HEK 293 Cell Culture and Transient Transfections—Human Embryonic Kidney (HEK 293) cells were regularly maintained in plastic tissue culture flasks at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. For the transfection experiments, cells were seeded on 6-well dishes and allowed to grow overnight in antibiotic-free medium. They were then transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Following 24 h of transfection, cell lysates were harvested for protein analysis.

For experiments involving immunofluorescent staining of cell surface and total cellular ENaC, cells were seeded on poly-L-lysine-coated coverslips in 6-well dishes (∼1.8 × 10^5 cells/well) and allowed to grow until optimal cell density was reached. Cells were then transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen) and optimized DNA concentrations (200 ng each of α-, β-, and γ-FLAG-xENaC and 250 ng of various GILZ constructs/well). 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 (18, 25). Cell surface ENaC was visualized using FLAG monoclonal antibody M2 (Sigma) and goat anti-mouse secondary antibody conjugated to Alexa-594 fluorophore (Molecular Probes, Inc., Eugene, OR) followed by nuclear co-staining with 4′,6-diamidino-2-phenylindole. Images were obtained at ×630 magnification using a 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, a blinded observer counted the number of cells with detectable surface ENaC, divided by the number of 4′,6-diamidino-2-phenylindole-positive cells, in at least three independent experiments of cells subjected to live cell staining. The typical number of ENaC-positive cells in controls ranged from 40 to 50 per 100 cells.

Cell Proliferation Assays—HEK 293 cells were seeded on 96-well tissue culture plates at a concentration of 1 × 10^5 cells/well and allowed to grow overnight in the absence of antibiotics. They were then transfected with 200 ng of the specified GILZ constructs or the empty vector (control) using Lipofectamine 2000. 24 h following transfection, the cells were treated with 10 μM 5-bromo-2′-deoxyuridine (BrdUrd) for an additional 24 h. Cell proliferation was then assessed by (colorimetric) enzyme-linked immunosorbent assay using a commercially available BrdUrd incorporation kit according to the manufacturer’s instructions (Roche Applied Science). All transfections were performed in triplicate, and all experiments were repeated at least four times with similar results.

NF-κB Luciferase Reporter Assays—CV1-b mouse kidney epithelial cells (Cell Culture Facility, University of California, San Francisco) were regularly maintained in plastic tissue culture flasks at 37 °C in Dulbecco’s modified Eagle’s medium H-16 supplemented with 5% fetal bovine serum and 100 units/ml penicillin/streptomycin, as described previously (26). For NF-κB luciferase reporter assays, cells were seeded on 12-well dishes, allowed to grow to optimal cell density, and co-transfected with 200 ng of the specified GILZ constructs, 250 ng of a reporter vector (NF-κB-LUC) containing tandem repeats of the murine NF-κB site in which the promoter drives the expression of the firefly luciferase (a kind gift from Dr. Warner Greene (Gladstone Institutes)), and 20 ng of the constitutively transcribed RSV-β-galactosidase plasmid, Δ6RL (which serves as an internal control for transfection efficiency (27)), using Lipofectamine, according to the manufacturer’s instructions (Invitrogen). 48 h after transfection, cell lysates were harvested and processed to assess protein content, luciferase, and β-galactosidase activity, as described previously (26, 27). Since activation of corticosteroid receptors may strongly influence the Rous sarcoma virus promoter, values are presented normalized to total protein content. Each transfection was performed in triplicate, and each experiment was repeated at least three times with similar results.

Immunoprecipitation and Western Blot Analyses—Protein lysates from cells/tissue samples were prepared as previously described (18). Immunoprecipitation of transfected FLAG-c-Raf was carried out using anti-FLAG-agarose conjugate (Sigma) according to the manufacturer’s instructions. Immunoblotting was performed as previously described (18), using either our new anti-GILZ antibody or one of the following commercially available antibodies, as specified throughout. Phospho-ERK antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA). Total ERK antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-SK1 (serum and glucocorticoid-induced kinase-1) antibody was a kind gift from Prof. Gary Firestone (University of California, Berkeley, CA). Anti-Myc antibody (Santa Cruz Biotechnology) was used to assess heterologous expression of Myc-tagged GILZ isoforms. Anti-FLAG antibody was purchased from Sigma. Blots were stripped and reprobed for Actin to ensure equality of protein loading (anti-actin antibody, Chemicon International, Temecula, CA).

Peptide Competition Assay—In order to check the specificity of signals obtained, the anti-GILZ antibody was preincubated with a peptide competition.
overnight with a 50 μg/ml concentration of the immunizing peptide and then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used as primary antibody for overnight incubation with the protein blot (at the same dilution as the original antibody).

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

RESULTS
Identification of Novel GILZ Isoforms in Cultured Kidney Collecting Duct (CD) Cells and Animal Tissues—In order to begin to characterize endogenous GILZ protein in CD cells, we developed a rabbit polyclonal antiserum to mouse GILZ (see “Experimental Procedures”). As shown in Fig. 1A, the antiserum recognizes GILZ in whole cell lysates of HEK 293 kidney epithelial cells transiently transfected with Myc-tagged or untagged forms of mGILZ. Preimmune serum or antiserum precleared with the immunizing peptide (Peptide Competition Assay) gave little-to-no signal, thereby verifying the authenticity of the new antiserum. As a further test of GILZ specificity, we analyzed possible cross-reactivity with its closest relative in the TSC22D family, mTSC22D1 (Fig. 1B). The antiserum was found to specifically recognize GILZ but not TSC22D1. Fig. 1, A and B, shows that this new antiserum specifically recognizes heterologously expressed GILZ. We next determined if the new antiserum would recognize endogenous GILZ in mouse kidney epithelial cells. For this purpose, mpkCCDc14 cells were grown on Transwell filters and treated with 10⁻⁶ M aldosterone for specified periods of time, as described under “Experimental Procedures.” Harvested protein lysates were subjected to SDS-PAGE. Western blot analysis indicated that the new antiserum did indeed recognize a 17 kDa band, which comigrated with transfected mGILZ, (Fig. 1C, panel 1); however, several additional bands were apparent, including a prominent ~28 kDa band, which was also consistently aldosterone-induced (Fig. 1, C and D). A peptide competition assay suggested that these signals were specific (Fig. 1C, panel 2). Similar to SGK1 (Fig. 1C, panel 4), the 17-kDa form of GILZ (from here on referred to as GILZ1) was maximally induced at around 6 h of hormone treatment, followed by a declining trend in protein expression (Fig. 1, C and D).

The pattern of expression of immunoreactive GILZ in Western blots from aldosterone-treated mpkCCDc14 cells seemed to suggest either selective proteolysis from a larger protein, the formation of stable multimers (e.g. through disulfide linkages), or the existence of multiple GILZ isoforms (from distinct genes or splice variants). The 28-kDa form, in particular, seemed to be present in appreciable levels under “basal” (no hormone) conditions and to be considerably up-regulated by aldosterone (Fig. 1C, panel 1). In cells transfected with GILZ1, we saw no appreciable increase in the other forms, suggesting that they do not result from post-translational modification of GILZ1. Mass spectrometric analysis (see “Experimental Procedures” for

[FIGURE 1. Characterization of new anti-GILZ antibody. A, anti-GILZ antibody recognizes mGILZ. Shown is Western blot analysis (IB) using the new antibody in HEK 293 cells transiently transfected with either the empty vector (vector control) or tagged (N- and C-terminally Myc-tagged), or untagged forms of mGILZ. As a control, the blot was stripped and reprobed with preimmune serum. Further, the anti-GILZ antibody was preincubated with the immunizing peptide, cleared, and then used for immunoblotting (peptide competition assay). B, anti-GILZ antibody is specific for GILZ. Western blot analysis in HEK 293 cells transiently transfected with either the empty vector, 4 or 8 μg of mGILZ, or 4 or 8 μg of its closest relative in the TSC22D family, mTSC22D1 (full-length mTSC22D1 cDNA was cloned from mpkCCDc14 cells using reverse transcription-PCR). Actin was used as a loading control. C, anti-GILZ antibody recognizes endogenous GILZ in mpkCCDc14 kidney epithelial cells. Western blot analysis for GILZ in mpkCCDc14 cells treated with aldosterone (10⁻⁶ M) for specified periods of time or transiently transfected with either the empty vector (vector control) or mGILZ (as a positive control for GILZ expression). Peptide competition assay was performed to check the specificity of signals obtained. The blot was also stripped and reprobed with anti-SGK1 antibody to verify aldosterone action in this cell line. Actin was used as a loading control. D, graphical representation of GILZ densitometry showing relative -fold increase in protein expression for GILZ1 and -2 (shown normalized to corresponding 0 h controls).}
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The in vivo relevance of GILZ isoforms other than GILZ1 was further supported by Western blots performed on extracts from multiple different mammalian tissues. Fig. 2, A–C, show representative blots performed on extracts from various fetal and adult tissues (not aldosterone-treated). Multiple sized bands were identified, two of which correspond in size to GILZ1 and -2, respectively (Fig. 2, B and C, last lane). Most tissues examined had readily detectable amounts of a band consistent with GILZ2. GILZ2 was found in several tissues, including kidney, although at lower levels than GILZ2, and, with the exception of lung, the band became apparent only upon overexposure of the blots (data not shown). CD constitutes only about 5% of the kidney cortex, and expression of GILZ1 is expected to be low in the absence of aldosterone or a low sodium diet. It is interesting that lung, in which ENaC activity is highly glucocorticoid-regulated, exhibited the highest levels of GILZ1.

Cloning of Multiple GILZ Isoforms—Our results thus far pointed to the existence of at least two distinct GILZ isoforms, GILZ1 and -2, probably arising from alternative splicing of a common GILZ gene. The annotated gene structure map (available on the World Wide Web) predicted additional transcriptional products resulting in proteins of sizes consistent with bands seen on Western blots of mpkCCD\textsubscript{e14} extracts (Fig. 1C). In order to explore this possibility and to clone full-length cDNAs of putative GILZ isoforms expressed in mpkCCD\textsubscript{e14} cells, we used 5'-RACE (see “Experimental Procedures” for details). Fig. 3A shows a multiple sequence comparison of four distinct GILZ isoforms identified by our screen. We pulled out the original isoform, GILZ1, as well as a cDNA with sequence consistent with GILZ2/TSC22-related inducible leucine zipper-3c. The 606-bp-long ORF of GILZ2 encodes a protein of 201 amino acids, with a predicted (unmodified) molecular mass of 22.1 kDa. In addition to GILZ1 and -2, we identified two other products, one transcript that encodes a small protein of only 43 amino acids and another that encodes a protein of 80 amino acids. These are referred to hereafter as GILZ3 and -4, respectively. Fig. 3B shows a schematic alignment of the four isoforms. GILZ1 and -2 differ only in their N-terminal sequences and are identical from amino acid 41 onward. (Note that the numbering refers to amino acid positions on GILZ1). Interestingly, GILZ3 shares the first 40 amino acids with GILZ1 and prematurely terminates after amino acid 43. GILZ4 is identical to the last 80 amino acids of GILZ1 and has been previously reported as “TSC22-related inducible leucine zipper-3a” (GenBank\textsuperscript{TM} accession number AF201287). All members of the TSC22D family described thus far seem to share the conserved TSC22/dip/bun signature box and the leucine zipper domain. GILZ3 is an exception to this pattern. This isoform results from a premature stop codon, resulting in a truncated version of the protein lacking the TSC22 box or the leucine zipper domain (Figs. 3 and 4). To our knowledge, this is the first report of the new GILZ isoform, GILZ3.

GILZ Genomic Structure Analysis—Fig. 4 shows a schematic of the mouse Tsc22d3 gene located on the X chromosome (constructed based on the gene structure data available in ENSEMBL and NCBI PUBMED and also our cDNA sequence data). In principle, six exons can produce four different transcriptional products (of ORF lengths 414, 606, 132, and 243 nucleotides each) with translational potential (of 137, 201, 43, and 80 amino acids, respectively). These isoforms are termed GILZ1, -2, -3, and -4, respectively. GILZ1 and -2 share exons 5 and 6, making up the TSC22/dip/bun domain, leucine zipper, and C terminus, but differ in their N-terminal exons(s) and 5'-untranslated region. Although isoform 4 also shares exons 5 and 6 and has a unique 5'-untranslated region, only its start codon is in exon 5, resulting in a translational truncated variant of isoforms 1 and 2. Isoform 3 consists of exons 3 and 6, skipping exons 4 and 5, resulting in a frameshift and subsequent stop codon, encoding a 43-amino acid long translation product. A CpG island and a series of Sp1 sites appear to define a TATA-less promoter, designated as promoter 1 (P1). The large intron B, spanning almost 55 kb, separates exons 1 and 2 from exons
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A.

![Graph showing the four isoforms of GILZ identified so far.](Image)

**FIGURE 3.** The four isoforms of GILZ identified so far. **A**, alignment of the protein sequences of all four mGILZ isoforms, GILZ1, -2, -3, and -4. B, schematic representation of the above. The numbers represent amino acid (AA) positions on GILZ1. The regions in black represent regions of identity among the four naturally occurring mGILZ isoforms and an artificial construct, Δ40-GILZ1 (in red). The dotted region in GILZ3 represents the untranslated portion of the transcript. The region in blue (GILZ1 residues 111–125) represents the antigenic stretch recognized by our anti-GILZ antibody. The conserved TSC22/dip/bun signature box (TSC22) and the leucine zipper domain (LZ) are also shown.

3–6 and contains multiple response elements (six GREs and three FHREs), most of them having been reported as being functional (21–23). Four GREs and three FHREs, combined with a TATA-box 32–25 bp 5’ from exon 3, form a transcriptionally active cluster, which is designated as promoter 2 (P2). It is interesting to note that no GREs were identified upstream of P1, suggesting that the GREs in intron B upstream of P2 mediate glucocorticoid and mineralocorticoid responsiveness of both promoters. It is also of note that there are two Elk-1 binding sites present in the CpG island and two more in exon 6, suggesting regulation of GILZ transcription by Elk-1. Since Elk-1 is activated by phosphorylated (or active) ERK (28), this raises the interesting possibility of a feedback control of GILZ expression by ERK, which will be the subject of future studies. A consensus AP-1 binding site was identified in P2 but is of uncertain significance, since the transcription rates of isoforms 1 and 2 were reported to be unaltered by basic fibroblast growth factor (29), a potent activator of AP1.

Effect of GILZ Isoforms on Transepithelial Na⁺ Transport and ENaC Cell Surface Expression—Previous evidence in a variety of experimental systems has established that aldosterone-induced changes in ENaC current are due, at least in part, to effects on ENaC surface expression (30). Moreover, our previous data (18) demonstrated an effect of GILZ1 on ENaC-mediated current in mpkCCDc14 cells and on ENaC surface expression in HEK 293 cells. In order to begin to assess the functions of the new isoforms on ENaC relative to that of GILZ1, we compared their effects on Na⁺ current and surface expression in these two model systems (18). First, we transiently transfected mpkCCDc14 cells with GILZ expression constructs as specified in Fig. 5, using a high efficiency electroporation protocol (18). For electrophysiological measurements, cells were transfected with either the empty vector (pMO) or one of four GILZ isoforms (GILZ1 to -4) or Δ40-GILZ1 and seeded at high density on Transwell filters. After forming a stable high resistance monolayer, cells were maintained for 24 h in serum- and corticosteroid-free medium containing EGF to activate ERK, and equivalent short circuit current was determined, as described (18). Fig. 5 shows a graphical representation of these data. We observed that although GILZ1 consistently and significantly stimulated equivalent current, the other isoforms and Δ40-GILZ1 did not affect ENaC current appreciably.

We showed previously that GILZ1 stimulates ENaC surface expression in HEK 293 cells by inhibiting the Raf-MEK-ERK pathway (18). In order to compare directly the effect of the new GILZ isoforms on ENaC plasma membrane expression, we transfected 293 cells with FLAG-ENaC, alone or in combination with one of the four GILZ isoforms or Δ40-GILZ1, recovered for 24 h, and treated with EGF for 1 h. Parallel cultures were then subjected to live cell staining (to detect cell surface ENaC) or permeabilized staining (to detect total cellular ENaC). Fig. 6 shows quantitation of the fraction of cells with detectable cell surface ENaC. As already described (18), EGF treatment markedly decreased cell surface ENaC. Also, cells expressing GILZ1 had completely restored cell surface ENaC levels, suggesting that GILZ1 regulates ENaC activity by modulating its trafficking. GILZ2, GILZ3, and Δ40-GILZ1 also stimulated cell surface ENaC expression to varying degrees, although less potently than GILZ1. Lysates from transfected cells were found by Western blot to have similar expression levels of various isoforms, and permeabilized staining showed no detectable changes in total cellular ENaC (data not shown). It is notable that there is a significant discrepancy between GILZ2 and GILZ3 effects on current in mpkCCDc14 cells and their effects on cell surface expression in HEK 293 cells (compare Figs. 5 and 6). The basis for this discrepancy is uncertain, but it could reflect a failure of
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FIGURE 4. Genomic structure of the mouse Tsc22d3 gene. A schematic diagram shows a region of 60,971 bp on the mouse X chromosome and the Tsc22d3 gene. Six exons can produce four different transcription products (of ORF lengths 414, 606, 132, and 243 nucleotides each) with translational potential (of 137, 201, 43, and 80 amino acids, respectively). These isoforms are termed GILZ1, -2, -3, and -4, respectively. The black boxes represent the translated regions, and the gray boxes represent the untranslated regions of the Tsc22d3 gene. The Cpg island locating exons 1 and 2, together with 12 potential Sp1 binding sites is designated as P1. Four GREs and three FHREs in the large second intron B, combined with a TATA-box 32 to 25 bp 5′ to 3′, respectively, and the CpG island representing exons 1 and 2, together with 12 potential Sp1 binding sites is designated as P2. The transcriptionally active cluster, which is designated as P2. The diamonds in intron B represent potential GREs, and the black circles represent potential FHREs. The numbers at the borders of the gray and black boxes indicate the nucleotide positions read from 5′ to 3′, whereas the numbers above the introns refer to the sizes of the respective introns. The NCBI accession numbers (in green) under the different GILZ isoform notations refer to previously reported mRNA transcripts available in the database.

FIGURE 5. Effect of GILZ isoforms on transepithelial Na⁺ transport in kidney epithelial cells. mpkCCDc14 cells were transfected with either the empty vector alone (pMO, vector control), one of the four GILZ isoforms, or Δ40-GILZ1. After transfection, cells were allowed to grow on Transwell filters until monolayers reached resistance greater than 1000 ohms-cm². They were then maintained in medium devoid of steroid hormones (see “Experimental Procedures” for details) for 24 h before analysis of ENaC activity. Shown is a graphical representation of the electrophysiological recordings obtained. **, p < 0.01 compared with control.

expression, suggesting that residues between amino acids 40 and 58 (GILZ1 numbering) are important for stimulation of ENaC. Additional experiments are required to test these possibilities.

Effect of GILZ Isoforms on Cellular Proliferation—GILZ1 has been shown to inhibit proliferation in T-cell-derived 3DO cells, an effect that has been attributed to its inhibition of pERK (15). In view of their distinct actions in modulating ENaC, it was of interest to determine if the new GILZ isoforms have differential effects on proliferation and if those effects correlate with their effects on cellular processes such as ERK activation. Furthermore, the activities of the various isoforms might provide insight into the mechanistic basis of this important GILZ function. We therefore expressed the GILZ isoforms in HEK 293 kidney epithelial cells and assessed serum-stimulated BrdUrd incorporation according to standard methods (see “Experimental Procedures”). As shown in Fig. 7, the isoforms differed markedly in their effects on proliferation. GILZ1 inhibited serum-induced proliferation by about 35%, slightly less than described previously for 3DO cells; GILZ2 appeared to have a modest inhibitory effect (<10%), which did not reach statistical significance; GILZ3 did not inhibit proliferation; and GILZ4 blocked proliferation as strongly as GILZ1. With the exception of GILZ1, these effects were in contradistinction to their effects on plasma membrane ENaC expression. The truncated GILZ1, Δ40-GILZ1, was the most potent inhibitor of mpkCCDc14 proliferation, diminishing BrdUrd incorporation by more than 50%. These results demonstrate that GILZ isoforms have distinct effects on proliferation and further suggest that the key domains required for antiproliferative activity are contained within amino acids 58–137, in contrast to ENaC stimulatory sequences, which are contained in amino acids 1–40 (both GILZ1 numbering). Interestingly, these isoform-specific effects are in contrast to effects on pERK inhibition, as discussed below (Fig. 8), and do not support a role of Ras/Raf inhibition in GILZ inhibition of proliferation.

Effect of GILZ Isoforms on ERK Activation in mpkCCDc14 Cells—The above data suggested that distinct GILZ isoforms are implicated in stimulation of ENaC (GILZ1 and GILZ3) on the one hand and inhibition of proliferation (GILZ1 and GILZ4) on the other. Interestingly, previous work strongly supports the idea that inhibition of Raf-dependent ERK phosphorylation is central to GILZ1 stimulation of ENaC, yet it has also been suggested that Raf inhibition is implicated in its effects on proliferation as well (15). Hence, determining the effects of the isofoms other than GILZ1 to activate ENaC (e.g. by failing to stimulate proteolytic processing) or could be due to cell type differences or a threshold effect (the effects of all isoforms, including GILZ1, on short circuit current are less dramatic than their effects on surface expression). GILZ4 was the only isoform to stimulate neither short circuit current nor cell surface ENaC

In the nucleotide positions read from 5′ to 3′, the NCBI accession numbers refer to the sizes of the respective introns. The NCBI accession numbers (in green) under the different GILZ isoform notations refer to previously reported mRNA transcripts available in the data base.

the isoforms other than GILZ1 to activate ENaC (e.g. by failing to stimulate proteolytic processing) or could be due to cell type differences or a threshold effect (the effects of all isoforms, including GILZ1, on short circuit current are less dramatic than their effects on surface expression). GILZ4 was the only isoform to stimulate neither short circuit current nor cell surface ENaC
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FIGURE 6. Effect of GILZ isoforms on ENaC cell surface expression in kidney epithelial cells. HEK 293 cells were transfected with either the empty vector alone (pMO; vector control), one of the four GILZ isoforms, or Δ40-GILZ1 for an additional 24 h. Cellular proliferation was then assessed as described under “Experimental Procedures.” Shown is a graphical representation of BrdUrd incorporation normalized to control. *** p < 0.001; ** p < 0.01 compared with control.

FIGURE 7. Effect of GILZ isoforms on cellular proliferation in kidney epithelial cells (GILZ1 and -3 and Δ40-GILZ1 significantly inhibit cellular proliferation in HEK 293 cells). HEK 293 cells were transfected with either the empty vector alone (pMO; vector control), one of the four GILZ isoforms, or Δ40-GILZ1, 24 h after transfection, cells were treated with 10 μM BrdUrd for an additional 24 h. Cellular proliferation was then assessed as described under “Experimental Procedures.” Shown is a representative Western blot analysis (IB) for active/phosphorylated (pERK) ERK. Lysates from U0126-treated (10 μM) cells were used as a positive control for reduced ERK phosphorylation. Total ERK was used as a loading control.

FIGURE 8. Effect of GILZ isoforms on ERK activation in kidney epithelial cells (GILZ1 and -3 significantly inhibit ERK activation in EGF-treated mpkCCD14 cells). mpkCCD14 cells were transfected with either the empty vector alone (vector control), one of the four GILZ isoforms, or Δ40-GILZ1 and allowed to grow on Transwell filters as described in the legend to Fig. 5. Lysates from cell monolayers were harvested for protein analysis. Shown is a representative Western blot analysis (IB) for active/phosphorylated (phospho-)ERK. Lysates from U0126-treated (10 μM) cells were used as a positive control for reduced ERK phosphorylation. Total ERK was used as a loading control.

FIGURE 9. Analysis of the ability of various GILZ isoforms to interact with c-Raf (GILZ1 and -2 interact with c-Raf in co-immunoprecipitation assays). HEK 293 cells were transiently transfected with FLAG-c-Raf either alone or in combination with one of the four isoforms of GILZ or Δ40-GILZ1 as shown (all GILZ constructs were Myc-tagged). As a negative control, one set of cells was transfected with the empty vector alone (lane 1 in all blots). Anti-FLAG-agarose beads were used to pull down FLAG-c-Raf. The first panel shows a representative immunoblot (IB) for FLAG-c-Raf after immunoprecipitation (IP). The blot was stripped and reprobed for Myc to detect the interacting isoforms of GILZ. The third panel is an immunoblot showing relative expression of the various GILZ constructs in transiently transfected HEK 293 whole cell lysates.

various isoforms on ERK phosphorylation status was important both for understanding their distinct biology and for clarifying the mechanistic basis of GILZ actions. mpkCCD14 cells were transfected with GILZ isoforms, grown on Transwell filters, and harvested for protein analyses as described (18). Cell lysates were subjected to Western blot analysis with pERK and total ERK antibodies (Fig. 8). As demonstrated previously (18), GILZ1 was found to significantly inhibit ERK phosphorylation. Interestingly, GILZ3 also markedly down-regulated ERK activation. However, GILZ4 and Δ40-GILZ1, which had the most potent effects on proliferation, consistently stimulated pERK formation. Total ERK levels remained unchanged in all samples, consistent with the idea that GILZ specifically regulates activation of existing ERK protein and not its abundance. Taken together, these data suggest an important role for the far N terminus of GILZ1 in mediating its effects on pERK suppression and, moreover, suggest that activities unrelated to ERK inhibition are implicated in the antiproliferative effects.

GILZ Isoform Interactions with c-Raf—GILZ1 has been shown to interact with c-Raf, and this interaction has been shown to contribute to inhibition of c-Raf activation and consequent phosphorylation of ERK (2). Furthermore, we have previously demonstrated a link between increased ENaC cell surface expression in kidney epithelial cells and modulation of ERK signaling by GILZ1 (18). In order to determine if the other isoforms of GILZ can bind c-Raf as well, we performed coimmunoprecipitation assays in HEK 293 cells transiently transfected with FLAG-c-Raf either alone (as a control) or in combination with one of the four isoforms of GILZ or Δ40-GILZ1, as described under “Experimental Procedures” (all GILZ constructs were Myc-tagged). Fig. 9 is a representative co-immunoprecipitation blot testing interaction of different GILZ isoforms with c-Raf. With the exception of GILZ1 (which...
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FIGURE 10. Effect of GILZ isoforms on NF-κB transcriptional activity (GILZ1 and -3 inhibit NF-κB activity in luciferase reporter assays). CV1-b kidney epithelial cells were co-transfected with the NF-κB reporter vector and one of the various GILZ constructs (either GILZ1 to -4 or Δ40-GILZ1) as described under "Experimental Procedures." After 48 h of transfection, cell extracts were obtained and assayed for luciferase activity. Values are expressed as -fold increase/decrease compared with that of control cells normalized to protein concentration. ***, p < 0.001 compared with control.

exhibited much weaker interaction) and GILZ2 (which exhibited much stronger interaction) and none of the other isoforms seemed to bind c-Raf. Whether the interaction with GILZ2 affects c-Raf kinase activity or not remains to be tested; however, its lack of effect on phospho-ERK suggests that it does not (Fig. 8). Interestingly, although GILZ3 strongly inhibits ERK phosphorylation just like GILZ1 (Fig. 8), it does not appear to bind c-Raf, unlike GILZ1 (Fig. 9). It is possible that it affects c-Raf activity indirectly or that it interferes with the functioning of other members of the mitogen-activated protein kinase 1/2 pathway, such as MEK1/2 or Ras. Future studies will address these issues.

Effect of Various GILZ Isoforms on NF-κB Transcriptional Activity—NF-κB is a transcriptional regulator, which plays a key role in the control of genes involved in cell proliferation and apoptosis (31). GILZ1 has been found to inhibit NF-κB transcriptional activity, possibly through direct physical interaction (13, 32). In order to examine similar possible effects of other GILZ isoforms, we co-transfected CV1-b cells with an NF-κB reporter vector (driving luciferase expression) and the various GILZ isoform expression vectors and measured luciferase activity. CV1-b cells have readily detectable levels of GILZ1, whereas GILZ2 and GILZ3 (but not GILZ4) also significantly increased surface expression (Fig. 6). 2) GILZ isoforms differed in inhibition of proliferation. In marked contrast to ENaC stimulation, GILZ2 and GILZ3 (but not GILZ4) also significantly increased NF-κB-mediated gene transcription. GILZ1 and -3 consistently up-regulated it (Fig. 8). 4) GILZ isoforms differed in interaction with c-Raf. GILZ1 bound c-Raf most efficiently, followed by GILZ2. Raf interaction with the other isoforms was much weaker, although GILZ1 had the strongest stimulatory effect, whereas GILZ4 seemed to consistently suppress ERK phosphorylation. In marked contrast to ENaC stimulation, GILZ4 was the most potent inhibitor of proliferation, followed by GILZ1, whereas GILZ2 and GILZ3 had no significant effect (Fig. 7). 3) GILZ isoforms differed in regulation of NF-κB-mediated gene transcription. GILZ1 and -3 consistently inhibited NF-κB-mediated gene transcription, with GILZ1 being the more potent suppressor (Fig. 10).

DISCUSSION

We have cloned cDNAs corresponding to four distinct isoforms of GILZ termed GILZ1 to -4 in order of their discovery, including the original 137-amino acid-long variant reported by Riccardi and co-workers in 1997 (1), which we now term GILZ1 (Fig. 3). The transcripts arise as splice variants from a single gene, Tsc22d3, with two promoters, one upstream of exon 1 and the other within a large intron (intron B, upstream of exon 3 (Fig. 4). Some of these transcripts were predicted by genome sequence analysis, whereas others have been reported in the context of high throughput cDNA sequencing projects (available at the NCBI site on the World Wide Web) or immunocross-reactivity (33). However, with the exception of GILZ1 (TSC22D3-2), their protein products, expression patterns, and functions were unknown. In particular, GILZ2 (TSC22D3-1) was originally identified in porcine brain as a protein that shares immunoreactivity with the sequence-unrelated nonamer neuropeptide, DSIP (delta-sleep-inducing peptide) (33). It was later found to be the most highly glucocorticoid-induced cDNA among over 9000 tested in a gene chip array in human peripheral blood mononuclear cells (34). GILZ4 was identified by microarray analysis as part of a large scale project involving identification and characterization of a family of genes related to TSC22 and was reported earlier as “TSC22-related leucine zipper 3a” (TILZ3a) (35, 36). It is clear from our present data that these various splice variants have distinct functions in the control of ion transport and proliferation and differentially modulate distinct signaling pathways.

In general, the most highly expressed variant was GILZ2, which was expressed at high levels in mpkCCDc14 cells as well as in multiple rat and mouse tissues (Fig. 2). mpkCCDc14 cells also expressed readily detectable levels of GILZ1, as did both rat and mouse lung, and GILZ1 was also detectable by Western blot in rat and mouse kidney obtained from non-aldosterone-treated animals but only with longer exposures. Interestingly, Jurkat (Fig. 2) and 3DO cells (1), both of T-cell origin, expressed higher levels of GILZ1 than GILZ2; however, rodent spleen (Fig. 2) and lymph node (not shown) expressed more GILZ2.

When heterologously expressed in HEK 293 or mpkCCDc14 cells, the GILZ isoforms differed markedly in a variety of functions. 1) GILZ isoforms differed in stimulation of ENaC. Only GILZ1 significantly stimulated ENaC-mediated Na+ current in polarized mpkCCDc14 cells grown on Transwell filters (Fig. 5). In contrast, in unpolarized HEK 293 cells grown on glass coverslips, although GILZ1 had the strongest stimulatory effect, GILZ2 and GILZ3 (but not GILZ4) also significantly increased surface expression (Fig. 6). 2) GILZ isoforms differed in inhibition of proliferation. In marked contrast to ENaC stimulation, GILZ4 was the most potent inhibitor of proliferation, followed by GILZ1, whereas GILZ2 and GILZ3 had no significant effect (Fig. 7). 3) GILZ isoforms differed in ERK phosphorylation. GILZ1 and -3 potently suppressed, and GILZ2 modestly suppressed, ERK phosphorylation, whereas GILZ4 seemed to consistently up-regulate it (Fig. 8). 4) GILZ isoforms differed in interaction with c-Raf. GILZ1 bound c-Raf most efficiently, followed by GILZ2. Raf interaction with the other isoforms was not detectable (Fig. 9). 5) GILZ isoforms differed in regulation of NF-κB-mediated gene transcription. GILZ1 and -3 consistently inhibited NF-κB-mediated gene transcription, with GILZ1 being the more potent suppressor (Fig. 10).
inhibit (and indeed stimulated) ERK phosphorylation, 5) did not interact with c-Raf, and 6) did not significantly repress NF-κB transcripitonal activity.

Taken together, these data identify specific functions of GILZ isoforms and strongly suggest distinct mechanisms for these functions. Interestingly, the ability of isoforms to inhibit proliferation did not appear to be correlated with their ability to inhibit ERK phosphorylation in whole cell lysates. Indeed, GILZ4 and Δ40-GILZ1, which were most potent at inhibiting proliferation actually stimulated pERK. This result sheds doubt on the conclusions of a prior report (15), which concluded that GILZ1 inhibits proliferation by inhibiting signaling through Raf-MEK-ERK. It remains possible that compartment-specific pERK inhibition was not detected. Consistent with our earlier results, GILZ1 and -3, which were the most potent inhibitors of pERK, were also the most potent stimulators of surface ENaC. GILZ2, which mildly inhibited pERK, mildly stimulated surface ENaC, and GILZ4, which did not inhibit (but rather stimulated) pERK, did not stimulate surface ENaC at all. One inconsistency in this otherwise tight correlation of pERK inhibition with ENaC stimulation is presented by the artificial truncation Δ40-GILZ1, which mildly stimulated surface ENaC despite its lack of inhibition of pERK. The significance of this observation remains to be determined.

The only variant to stimulate endogenous Na⁺ current in mpkCCD14 cells was GILZ1. Notably, GILZ3, which like GILZ1 markedly inhibited pERK, did not stimulate Na⁺ current, consistent with the interesting possibility that GILZ1 and GILZ3 inhibit pERK in distinct compartments or distinct temporal manners. It is interesting to note in this regard that GILZ1 but not GILZ3 strongly bound c-Raf (Figs. 6, 8, and 9). Although of uncertain significance, this observation suggests that GILZ1 and GILZ3 inhibit ERK phosphorylation by distinct mechanisms. Recent data suggest that in addition to binding Raf, GILZ1 directly binds and inhibits the Raf activator, Ras (15). Although interaction with and inhibition of Ras could explain GILZ3 inhibition of pERK formation without direct interaction with c-Raf, the TSC22 domain of GILZ1, which is absent in GILZ3, was required for interaction with Ras. Hence, insight into the mechanism of GILZ3 inhibition of pERK remains elusive at this time.

The ability of GILZ1 to bind and hence inhibit NF-κB transcriptional activity has been ascribed to its ability to homodimerize through its leucine zipper (32). The C-terminal proline- and glutamic acid-rich region is also believed to be required for this function (32). In the light of these observations, it is difficult to explain the effect of GILZ3 (albeit mild), because it lacks both the leucine zipper and the C-terminal proline- and glutamic acid-rich region. It is possible that GILZ3 affects NF-κB activity indirectly through associations with other protein(s). This remains to be elucidated, as does the effect of GILZ3 on production of cytokines, such as interleukin-2.

Our present data (Fig. 1C) and the data of others (4) show that GILZ2, like GILZ1, is significantly induced by aldosterone in kidney cells. It is also substantially more highly expressed than GILZ1 in cultured renal collecting duct cells and in native kidney (Fig. 2). Interestingly, GILZ2 bears a significant degree of homology to the recently cloned OSTF1 (osmotic stress transcription factor 1), a protein that is involved in the cellular osmotic stress response of gill cells in euryhaline tilapia (37). In this fish, OSTF1 mRNA and protein levels rapidly increased in response to hyperosmotic stress. This finding is reminiscent of SGK1, which is also regulated by aldosterone and osmotic stress in kidney collecting duct cells (38) and other cell types as well (39, 40). The rapid activation kinetics is again characteristic of immediate early genes like SGK1. It is interesting to speculate that GILZ2 may be involved in regulating osmosensory signal transduction pathways in kidney epithelial cells. This remains to be elucidated.

In conclusion, we have shown that there are at least four distinct variants of GILZ/TSC22D3 expressed in a variety of mammalian cells and tissues. Our data suggest that the four isoforms identified are not functionally redundant but rather are involved in distinct aspects of cellular physiology and modulate distinct signaling pathways; notably, GILZ1 appears to be the most potent isoform in stimulation of Na⁺ transport and repression of NF-κB, and GILZ4 appears to be the most potent in inhibition of proliferation. Interestingly, pERK inhibition appears to be required for GILZ regulation of ENaC but not of proliferation. GILZ regulation of the latter appears to proceed by a distinct mechanism, which remains uncertain at this time.

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