The functional expression of the amiloride-sensitive epithelial sodium channel (ENaC) in select epithelia is critical for maintaining electrolyte and fluid homeostasis. Although ENaC activity is strictly dependent upon its α-subunit expression, little is known about the molecular mechanisms by which cells modulate α-ENaC gene expression. Previously, we have shown that salivary α-ENaC expression is transcriptionally repressed by the activation of Raf/extracellular signal-regulated protein kinase pathway. Here, we work further investigates the molecular mechanism(s) by which α-ENaC expression is regulated in salivary epithelial Pa-4 cells. A region located between −1.5 and −1.0 kilobase pairs of the α-ENaC 5′-flanking region is demonstrated to be indispensable for the maximal and Ras-repressible reporter expression. Deletional analyses using heterologous promoter constructs reveal that a DNA sequence between −1355 and −1269 base pairs functions as an enhancer conferring the high level of expression on reporter constructs, and this induction effect is inhibited by Ras pathway activation. Mutational analyses indicate that full induction and Ras-mediated repression require a glucocorticoid response element (GRE) located between −1323 and −1309 base pairs. The identified α-ENaC GRE encompassing sequence (−1334/−1306) is sufficient to confer glucocorticoid receptor/dexamethasone-dependent and Ras-repressible expression on both heterologous and homologous promoters. This report demonstrates for the first time that the cross-talk between glucocorticoid receptor and Ras/extracellular signal-regulated protein kinase signaling pathways results in an antagonistic effect at the transcriptional level to modulate α-ENaC expression through the identified GRE. In summary, this study presents a mechanism by which α-ENaC expression is regulated in salivary epithelial cells.

Sodium balance is important for the maintenance of body electrolyte, extracellular volume, and blood pressure. The amiloride-sensitive epithelial sodium channel (ENaC) is expressed in airway epithelium, distal segments of kidney tubule, skin, bladder, colon, and sweat and salivary glands. In addition, ENaC is a member of the expanded degenerin/ENaC superfamily and consists of at least three homologous subunits, α, β, and γ (2-5). Compelling functional and biochemical evidence suggests that all three subunits form a heteromultimeric complex and contribute to the optimal epithelial sodium reabsorption activity of the amiloride-sensitive sodium channel (3, 6, 7). However, when expressed individually in a Xenopus oocyte system, only the α-subunit can produce an amiloride-sensitive current. Both β- and γ-subunits are not functional on their own, but augment the channel activity of α-ENaC (3, 8). Thus, the expression of a functionally active sodium channel is dependent upon the presence of the α-ENaC subunit. Indeed, different phenotypes were observed in α-ENaC(−/−) and γ-ENaC(−/−) transgenic mice that were generated by homologous gene targeting. For example, 50% of α-ENaC(−/−) mice develop respiratory distress and die within 24 h after birth from an inability to clear the lung fluid (9), whereas none of the γ-ENaC(−/−) mice die within the same time period (10).

Although the composition, structure, and activity of ENaC have been well studied, the molecular basis for the regulatory mechanisms underlying the gene expression of ENaC subunits remains unclear. Results from studies by us and others have suggested that the transcriptional control of ENaC gene expression occurs in a subunit- and tissue-specific manner (Ref. 11; reviewed in Refs. 12 and 13). For instance, the transcription of β- and γ-ENaC genes is up-regulated by the steroid hormone aldosterone in the colon, whereas the α-ENaC mRNA is constitutively expressed. In contrast, dexamethasone treatment is reported to up-regulate the steady-state mRNA levels of all three subunits in the colon and fetal lung. Moreover, vasopressin increases only β- and γ-ENaC mRNA levels without altering α-ENaC mRNA level in RCCD2, rat cortical collecting duct cell line (14). We have previously demonstrated that TPA treatment represses the α-ENaC mRNA level (11). Taken together,

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The Gene Expression of the Amiloride-sensitive Epithelial Sodium Channel α-Subunit Is Regulated by Antagonistic Effects between Glucocorticoid Hormone and Ras Pathways in Salivary Epithelial Cells*

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these studies also outline a putative pathway(s) by which extracellular signals regulate cell-specific ENaC expression. Therefore, studies that more precisely define the interaction between the cellular transcriptional machinery and signaling pathways are needed to provide a framework for understanding the mechanisms underlying the regulation of ENaC expression and the resultant electrolyte homeostasis.

Nuclear hormone receptors modulate gene transcription, upon binding of their cognate ligands, by activation as well as repression (15). One of the best studied members of the nuclear hormone receptor superfamily is the glucocorticoid receptor (GR), which plays an important role in physiology and developmental biology. Transactivation by GR requires binding of receptor homodimers to specific palindromic sequences in the cis-regulatory region of target genes, namely glucocorticoid response elements (GREs). In recent years, it has become apparent that a variety of other factors may have a profound effect on the transactivation potential of GR. For example, the phosphorylation status of GR (16) and the formation of composite or complex GREs (17, 18) can affect classical GR transactivation activity. Thus, although the GR is expressed in virtually all mammalian cell types, it is possible that the expression of a distinct set of GR-responsive genes is cell context-specific and may be modulated by other signaling pathways(s).

Ras proteins are important signaling intermediates that convey signals initiated at the cell surface to various effector pathways in the cytoplasm. Ras exerts effects on cell transformation and proliferation, cytoskeletal structure, differentiation, and apoptosis. These changes may be mediated by multiple effectors, including Raf-1, Ras-GDS, phosphatidylinositols (PI) 3-kinase, and other Ras-binding proteins (reviewed in Ref. 19), often at the transcriptional level. Even though both Ras effector pathways and gene regulation are characterized in molecular detail, the interface between canonical Ras signaling and transcriptional repression remains poorly described. Clearly, there are mechanisms, when appropriately cued, whereby signal transduction cascades operating via Ras activation would negatively modulate transcriptional events in the nucleus, ultimately leading to transcriptional repression.

The human α-ENaC gene has recently been cloned and characterized (20). A rat genomic DNA fragment harboring the α-ENaC promoter, first exon, partial first intron, and 5′-flanking region has been isolated and used very recently to investigate the α-ENaC gene regulation by us (11). Since more than one transcription initiation site has been identified in both human and rat α-ENaC genes accounting for the polymorphism of α-ENaC transcript size (21),2 we have adopted a numbering convention that assigns the number +1 to the first base of the translation start codon. Previously, we have shown that the regulatory elements in a 1.4-kb (−1573 to −154 bp relative to translation initiator ATG) α-ENaC DNA fragment are sufficient to mediate the basal and the extracellular signal-regulated protein kinase (ERK) pathway-modulated expression of α-ENaC/reporter construct in transient transfection assays (11). In this study, we identified a 508-bp DNA fragment in the 5′-flanking region of the rat α-ENaC gene that is required for transcriptional activation of the α-ENaC promoter and which also confers Ras-mediated repression. By using heterologous promoter constructs and deletion analyses, we demonstrate that a functional GRE and GR are required for both transcriptional activation and Ras-mediated repression. This is the first report of an α-ENaC enhancer with a dual effect, suggesting that its involvement in the transcriptional regulation of α-ENaC gene expression in salivary epithelial cells is via the cross-talk between Ras- and GR-mediated signaling pathways. This mechanism may also be responsible for the expression of the α-ENaC gene in different tissues.

MATERIALS AND METHODS

Cell Culture and DNA Constructs—The rat parotid epithelial cell line Pa-4, also known as parotid C5 cell line (22), was maintained as described previously (23). Dexamethasone was obtained from Sigma, resuspended to 1 mM in ethanol, and stored in −80 °C. The anti-glucocorticoid, ZK98,299 (24, 25), a generous gift from Dr. M. Stallcup (University of Southern California, Los Angeles, CA), was stored at 80 °C as a 1 mM stock solution in ethanol.

A series of reporter constructs encoding the chloramphenicol acetyltransferase (CAT) reporter gene were derived from α-ENaC/CATs, as described previously (11). The −1.0α-ENaC/CAT, −0.7α-ENaC/CAT, and −0.5α-ENaC/CAT (Fig. 2) were made by a double digest of α-ENaC/CATs with SphI (vectors/HindIII, KpnI, and PstI, respectively). Vector-containing fragments were isolated, blunted-ended by T4 DNA polymerase (Promega, WI), and self-ligated. In addition, a 0.5-kb HindIII fragment of α-ENaC (−1573 to −1066 bp) was excised from α-ENaC/CATs and ligated into a HindIII-linearized −0.7α-ENaC/CAT plasmid DNA in both sense and antisense orientations to generate (−1573/−1066)-0.7α-ENaC/CAT and (−1066/−1573)-0.7α-ENaC/CAT reporter constructs, respectively. A 0.2-kb DNA fragment extending from internal PstI (from vector via previous engineering) to PstI (internal) was deleted from (−1066/−1573)-0.7α-ENaC/CAT to construct the (−1066/−1573)-0.5α-ENaC/CAT plasmid.

A series of unidirectional deletion mutants harboring the luciferase reporter gene (Fig. 3) were constructed as follows. The 0.5-kb HindIII α-ENaC DNA fragment (−1573 to −1066 bp) was PCR-amplified using primer pairs designed according to the α-ENaC DNA sequence and cloned into pCR2.1 vector with the Original TA Cloning® kit (Invitrogen). The extent of deletion in each individual clone was determined by DNA sequence analyses. Site-specific mutations within or adjacent to the GRE in p(−1144/−1066)GL2-P were introduced by Transformer™ site-directed mutagenesis kit (CLONTECH) to generate Mt A and Mt B (Fig. 6a). A α-ENaC/CATs plasmid with point mutations on GRE site (GRE Mt; Fig. 7) was constructed by replacing a SosC/HindIII fragment containing the GRE site with the same fragment from Mt B described above that carries mutated GRE.

Reporter constructs, p(−1334/−1306)GL2-P and p(−1334/−1306)GL2-P, containing one and two copies of α-ENaC GRE, respectively, were generated as follows: a pair of complementary and 5′-phosphorylated oligomers, which correspond to −1334 to −1306 bp of α-ENaC, were annealed by incubating 200 pmol of each oligomer in a buffer containing 50 mM Tris-HCl (pH 7.6) and 10 mM MgCl2, followed by heating to 85 °C for 2 min and step wise cooling at 65 °C (15 min), 37 °C (15 min), 25 °C (15 min), and 4 °C (15 min). The annealed oligos, which have a built-in 5′-phosphorylated XhoI overhang, were ligated to XhoI-linearized pGL2-P plasmid DNA.

The DNA sequences and mutations introduced in all constructs were verified by sequence analyses utilizing T7 Sequenase 7-deaza-GTP Sequencing Kit (Amersham Pharmacia Biotech) as instructed by the manufacturer. Expression plasmids harboring Ras mutants of Ras V12, Ras S35, Ras G37, and Ras C40 were generous gifts from Dr. D. Johnson (University of Southern California, Los Angeles, CA), while glucocorticoid receptor (GR) expression construct and a tyrosine aminotransferase GRE containing reporter construct, TAT(GRE)3TK/Luc, were kindly provided by Dr. M. Stallcup (University of Southern California). The glucocorticoid receptor homodimers to specific partial palindromic sequences

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were serum-starved 24 h after the start of transfection by replacing growth medium with 0.05% serum-containing medium for 8 h, followed by adding 10^{-7} M dexamethasone in the same medium. All plates were harvested 16 h thereafter. The CAT reporter gene analysis was carried out as described previously (22), whereas firefly and Renilla luciferase activities were measured using Dual-Luciferase reporter assay kit (Promega) according to the manufacturer’s instruction. All transient transfection assays were carried out at least three times independently.

Northern Blot Analysis—Total RNA (RNA) was isolated from Pa-4 cells using Trizol reagent (Life Technologies, Inc.) as instructed by the manufacturer. The quantity and quality of RNA were determined by both spectrophotometric analysis and fractionating RNA on an agarose/ formaldehyde gel (1.3% (w/v)/6% (v/v)), followed by staining with ethidium bromide to compare the ratio of 28 S to 18 S ribosomal RNAs. For Northern analyses, equal amounts of RNA (18 μg/sample) were loaded onto an agarose/formaldehyde gel, fractionated by size, transferred to a 0.2-μm nylon membrane (ICN Biomedicals, Inc.), and UV-cross-linked. All blots were prehybridized for 1 h in QuikHyb (Stratagene). The hybridization was carried out according to manufacturer’s instructions with 32P-labeled α-ENaC probes that were prepared from an isolated rat α-ENaC cDNA fragment (bases 1-905) using a Random Primed DNA labeling kit (Roche Molecular Biochemicals). All blots were also reprobed with rat β-actin to ensure the quality and quantity of mRNA between lanes were comparable. After hybridization, these blots were washed in a 0.1× SSC (0.15 μ NaCl and 0.015 μ sodium citrate) solution containing 0.5% SDS at 60 °C for 2 h with three changes of washing solution. The radioactive signal was visualized by autoradiography, where blots were exposed to films at ~80 °C overnight, using BioMax TranScript-LE (Eastman Kodak Corp.) to improve sensitivity. Blots were also quantitated through the use of electronic autoradiography with an Instantimager 228 (Packard Instrument Co).

The amount of α-ENaC message was quantitatively compared between lanes after normalizing against that of β-actin.

RESULTS

Ras Inhibits α-ENaC/Reporter Activity via Multiple Effector Pathways—The Ras pathway has been recently reported to modulate epithelial sodium channel activity and expression in Xenopus oocytes (26). Moreover, we have shown that a downstream effector pathway of Ras, Raf/ERK, can down-regulate the expression of both the endogenous α-ENaC gene and transiently transfected reporter constructs harboring 1.4 kb (~1573/-154 bp relative to ATG) of the rat α-ENaC 5′-flanking region (11). In order to more precisely evaluate the ability of Ras and its different effector pathways to modulate α-ENaC expression in mammalian cells, salivary epithelial Pa-4 cells were cotransfected with an α-ENaC/CAT reporter construct and an expression plasmid encoding a constitutively activated form of Ras (Ras V12) or a “single-effector” mutant, i.e. Ras S35, Ras G37, and Ras C40. The ability of each Ras mutant to modulate α-ENaC/CATs reporter activity was assessed.

Among the three Ras “single-effector” mutants, S35 is able to activate Raf-1 but not Ras-GDS or PI 3-kinase, while G37 activates Raf-GDS but not Raf-1 or PI 3-kinase, and C40 activates PI 3-kinase but not Raf-1 or Ras-GDS (27). As shown in Fig. 1, Ras V12 markedly repressed α-ENaC/CAT activities when cotransfected into Pa-4 cells (lanes 3 and 4 versus lanes 1 and 2). While the C40 mutant had no effect on α-ENaC/CATs activities, mutants S35 and G37 appeared less effective than Ras V12 in our system (Fig. 1). Although it is possible that the known differences in the activity and expression level of these mutant proteins (28) may have contributed to these results, it is more likely that both Ras-GDS and Raf/ERK signaling pathways are involved in down-regulating α-ENaC expression.

Previously, we have shown that MEK inhibitor, PD 98059, was able to block the inhibition of α-ENaC/CATs activity by Raf/ERK activation (11). However, the MEK inhibitor had a limited effect on Ras V12-mediated inhibition, suggesting that an effector pathway(s) other than Raf/ERK may also have been involved in modulating α-ENaC expression. Since Ras V12 elicited the maximum repression on α-ENaC/CATs reporter activity, we used Ras V12 to elucidate the mechanism(s) that down-regulate α-ENaC expression.

Identification of a Ras-repressible Enhancer Required for α-ENaC Expression—The results shown above and our previous published data (11) suggest that elements confined within the α-ENaC/CATs construct are essential for recapitulating the basal and Ras-mediated regulation of α-ENaC expression. To elucidate the cis-element(s) conferring Ras-mediated repression of the α-ENaC/CATs, we constructed a series of reporter plasmids in which truncated fragments of the α-ENaC promoter/enhancer were placed upstream of the promoterless CAT reporter gene. As shown in Fig. 2, removal of the first 508 bp from the parental construct, α-ENaC/CATs, reduced the level of CAT activity approximately 10-fold in Pa-4 cells transiently transfected with −1.0α-ENaC/CAT. Since basal activity was so low, the ability of Ras V12 to repress reporter activity cannot be accurately assessed.

Subsequent deletions of 310 and 586 bp from −1.0α-ENaC/CAT, designated as −0.7α-ENaC/CAT and −0.5α-ENaC/CAT, respectively, resulted in minor variations of the basal CAT activity when compared with those from −1.0α-ENaC/CAT-transfected cells (Fig. 2). The reason for these variations is not clear, since they were not always reproducible. This dramatic decrease in basal activity indicated that a Ras-repressible enhancer(s) was located within the first 508 bp (~1573/-1066) of the 1.4-kb α-ENaC DNA fragment. To confirm this, we inserted the (~1573/-1066) fragment 5′ to the −0.7α-ENaC/CAT in both sense and antisense orientations and in the antisense orientation upstream of the −0.5α-ENaC/CAT construct (Fig. 2). High levels of basal activity were observed in cells transiently transfected with any of the three constructs (Fig. 2). Moreover, the Ras-mediated repression of reporter gene activity was quantitatively and qualitatively similar to that observed in α-ENaC/CAT-transfected cells. Thus, the DNA fragment ~1573 to −1066 bp of α-ENaC enhanced the α-ENaC basal promoter activity in a way that could be repressed by Ras activation. The DNA sequences between −1066 and −480 bp, on the other hand, were dispensable for modulating α-ENaC expression.

Delineation of the Activating Sequence(s) within the −1573 to −1066 α-ENaC 5′-Flanking Region—To determine whether

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the first 508-bp fragment of the parental α-ENaC/CATs construct alone could modulate α-ENaC expression, the (−1573/−1066) fragment was inserted in both sense (Fig. 3A, construct A) and antisense (Fig. 3A, construct E) orientations upstream of a heterologous minimal SV40 promoter in pGL2-P vector. As shown in Fig. 3B, the (−1573/−1066) fragment (construct A) conferred an approximately 4-fold higher basal activity than that of vector alone (construct I). Moreover, the activity was stimulated in an orientation-independent manner, a hallmark of an enhancer element. Although it is unclear, the 508-bp fragment when in the antisense orientation (construct E) elicited a higher activity than the sense counterpart (construct A; see Fig. 3B). Moreover, coexpression of Ras V12 had little effect on attenuating reporter activity of vector alone, but markedly down-regulated the enhancer activity conferred by the (−1573/−1066) fragment.

Since the expression pattern of the parental, α-ENaC/CATs, construct was functionally reproduced in heterologous promoter constructs containing the (−1573/−1066) fragment, we focused our attention on characterizing this 508-bp distal enhancer. Serial 5′- and 3′-deletion mutants of the (−1573/−1066) fragment were constructed to locate the DNA motif(s) involved in Ras-repressible enhancer activity. As shown in Fig. 3B, deletions outside the −1355 to −1269 bp sequence had little effect on either enhancer activity or Ras repressibility (constructs B, C, F, and G). In contrast, constructs excluding this region (constructs D and H) exhibited a dramatic reduction in enhancer activity. In addition, Ras repression was virtually abolished (Fig. 3B, constructs D and H). These data suggest that the DNA fragment extending from −1355 to −1269 bp confers both enhancer and Ras-mediated repressor activity. Thus, the 87-bp fragment (−1355 to −1269 bp) in the 5′-flanking region appears to function as both an enhancer and a Ras-mediated repressor of α-ENaC expression in parotid epithelial cells.

Glucocorticoid Hormone Transactivates the Reporter Expression from the (−1573/−1066) DNA Fragment—Analysis of the 87-bp fragment by Find Patterns Algorithm of the GCG software package and transcription factor sites data base (29) identified several putative transcription factor-binding sites within this region. A DNA sequence around position −1316 bp (−1323 to −1309 bp; AGAACANNNTGTCCT) with close resemblance to the consensus site for the glucocorticoid hormone receptor (GRE; AGAACANNNTGTCCT) was chosen for further investigation. To investigate whether the (−1323/−1309) DNA sequence could mediate functional induction and was necessary for the Ras-repressible enhancer activity, Pa-4 cells were cotransfected with a glucocorticoid receptor (GR) expression plasmid and a reporter construct shown in Fig. 3A. Addition of 10−7 M dexamethasone enhanced the reporter activity of constructs A and E by approximately 7-fold over those from corresponding vehicle-treated transfected cells (Fig. 3C). Moreover, despite that a high expression level was induced by exogenous GR/dexamethasone treatment, the expression of Ras V12 plasmid was still able to reduce GR/dexamethasone-mediated enhancement in constructs A and E up to 5-fold (Fig. 3D). In general, shortening the α-ENaC DNA fragment 5′ to −1355 bp and 3′ to −1269 bp neither altered dexamethasone induction nor Ras-mediated repression of reporter activity (Fig. 3, C and D, constructs B, C, F, and G). However, deletion of the region containing the identified GRE virtually abolished GR/dexamethasone-mediated enhancement as well as Ras-mediated repression of the minimal SV40 promoter (Fig. 3, C and D, constructs D and H). These data are consistent with the results shown in Fig. 3B, suggesting that deletions outside the GRE have no effect on reporter activity, whereas deletions of the

Fig. 2. Effect of progressive 5′-deletions and internal deletions on basal activity and Ras-mediated repression of α-ENaC promoter. Salivary Pa-4 cells were transiently transfected with 1.2 μg of DNA of each α-ENaC/CATs deletion constructs (as depicted in the left panel) in the presence and absence of 0.7 μg of cotransfected expression plasmid harboring Ras V12 as indicated. Transfections and CAT assays were performed as described in Fig. 1. The region between −1573 and −1066 bp in either sense (construct E) or antisense (constructs F and G) orientation apparently acts as an enhancer, of which effect is repressed by Ras pathway activation. A representative CAT assay from three independent experiments is shown (right panel).
GRE abolish both enhancer and repressor properties of the 508-bp fragment.

Based on these results, it appears that in Pa-4 cells p(−1066/−1573)GL2-P reporter construct expression can be modulated by two signaling pathways; whereas GR activation stimulates reporter expression, Ras activation antagonizes GR-mediated enhancement. To examine this hypothesis, Pa-4 cells were transiently cotransfected with the reporter construct p(−1066/−1573)GL2-P and a GR expression plasmid or an empty vector. The luciferase activity was increased approximately 1.5-fold by treatment with the GR agonist, dexamethasone (Fig. 4, lane 5 versus lane 4), suggesting that endogenous GR activation was able to increase \( \alpha \)-ENaC enhancer activity modestly via a GRE located between −1573 and −1066 bp. No transcriptional stimulation by dexamethasone was observed in pGL2-P (Fig. 4, lane 3), which lacks the cloned \( \alpha \)-ENaC enhancer fragment. Co-

**Fig. 3.** Functional analyses of the \( \alpha \)-ENaC enhancer element by transient transfection assays with heterologous promoter constructs in Pa-4 cells. A, schematic diagram of a series of p(−1573/−1066)GL2-P and p(−1066/−1573)GL2-P deletion mutants. The end points of truncated DNA fragments in p(−1573/−1066)GL2-P and p(−1066/−1573)GL2-P constructs and their deletion mutants are shown. Construct I represents the vector only, pGL2-P. S and AS depict the sense and antisense orientations of the insert. B, Ras pathway activation down-regulates the enhancer activity in transiently transfected Pa-4 cells cultured in serum-containing growth media. Pa-4 cells were transiently transfected with 0.9 µg each of the constructs shown in diagram (A) with or without 0.5 µg of Ras V12 expression plasmid where indicated. The transient transfection was carried out as described in Fig. 1. Both firefly and Renilla luciferase activities were measured simultaneously using Dual-Luciferase® assay system (Promega). The relative luciferase activity from firefly luciferase reporter gene shown in each group of transfected cells was determined and normalized with the indicator Renilla luciferase activity. Each value shown is the mean ± S.E. based on three independent transfection experiments. C, GR/dexamethasone (Dex) transactivates the \( \alpha \)-ENaC distal enhancer in cells cultured under serum-free conditions. Pa-4 cells were cotransfected with 0.9 µg of each construct shown in diagram (A) and/or 0.5 µg of GR expression plasmid as described in B. Sixteen hours after the start of transfection, the cells were serum-starved for 8 h, followed by the addition of \( 10^{-7} \) M dexamethasone to the culture medium, and incubated overnight. The reporter and indicator luciferase activities were determined as described in B. The level of induction, expressed as fold induction, for each construct is calculated by dividing the normalized reporter luciferase activity in extracts from GR-transfected/dexamethasone-treated cells by that of corresponding GR-transfected/vehicle-treated cells. Error bars were calculated as in Fig. 3B. D, Ras pathway activation represses GR transactivation activity. Pa-4 cells were cotransfected with 0.9 µg of each construct shown in diagram (A) and 0.5 µg of GR expression plasmid in combination with or without 0.5 µg of Ras V12 expression plasmid as indicated. The transfection, treatment protocol, and dual luciferase assay were carried out as described in C. The relative luciferase activity for each reporter construct in extracts, after normalization, from GR-transfected/dexamethasone-treated cells and Ras- plus GR-cotransfected/dexamethasone-treated cells, respectively, is shown. Error bars represent the standard error of the mean for each construct based on three independent transfection experiments.
without obvious cytotoxicity. Cells treated with 10^{-8} M dexamethasone alone for 16 h exhibited an approximately 6-fold increase in α-ENaC mRNA level, which was completely blocked by the cotreatment with 1 μM ZK98.299 (Fig. 5A). β-Actin mRNA expression was used to normalize α-ENaC mRNA level in control and treated Pa-4 cells, and to assure the viability of the treated cells (Fig. 5, A and B).

A modest decrease in the basal α-ENaC mRNA level by ZK98.299 treatment was also observed (Fig. 5A), demonstrating the ability of the remaining glucocorticoid hormone, after switching to serum-free conditions, to modulate α-ENaC expression. Since TPA has been shown to induce the Raf/ERK, Ras effector pathway, in Pa-4 cells (11), we investigated whether TPA could block dexamethasone-mediated induction of the endogenous α-ENaC expression. Cells treated with 10^{-7} M dexamethasone for 16 h exhibited a marked increase in the steady-state α-ENaC mRNA level by approximately 7-fold (Fig. 5B, lane 3). Consistent with the effect of ZK98.299, TPA treatment attenuated both basal and dexamethasone-induced α-ENaC mRNA levels in Pa-4 cells (Fig. 5B, lanes 2 and 4). These data are consistent with the results shown above on the regulatory effects on α-ENaC/reporter activity by GR and Ras V12 expression plasmids, and establish the physiological relevance of this study. Together, these data suggest that glucocorticoid hormone induces α-ENaC expression through the putative GRE and that the GR-mediated transactivation can be antagonized by GR antagonist as well as TPA-mediated Raf/ERK activation.

Point Mutations in GRE Abolish Antagonistic Modulation by Glucocorticoid Hormone/Ras Pathways—Our data indicate that the Ras-mediated repressibility and dexamethasone-stimulatory effect are not operated exclusively via distinct positive and negative regulatory elements. Based on sequence analysis of the −1334 to −1306 bp DNA fragment, a second putative transcription factor binding site AP-1 was identified 15 bases upstream of the GRE (Fig. 6, from the center of putative AP-1 site to the center of putative GRE). AP-1 is activated by the Ras signaling pathway (19), and is a well established antagonist of GR-mediated transcription (17, 18). Therefore, we postulated that Ras activation may repress GR/GRE association by activating AP-1 and thereby stimulating a mutual competition between AP-1 and GR for their respective response element. Second, although we showed that GR-mediated transcription is necessary for endogenous α-ENaC gene expression (Fig. 5A), whether or not this GR transactivation is mediated via the identified GRE is unknown. To test these hypotheses directly, Pa-4 cells were transiently transfected with one of three distinct reporter constructs: wild type, Mt A, and Mt B. Mt A differs from wild type by three nucleotide substitutions at positions −1330, −1328, and −1327, abolishing the putative AP-1 element, whereas Mt B leaves the AP-1 site unaltered but replaces five nucleotides within the putative GRE (Fig. 6A, upper panel).

As shown in Fig. 6A, mutations on the putative AP-1 site (Mt A) had no obvious effect on the heterologous promoter activity when Mt A was transfected alone or cotransfected with GR and/or Ras V12 expression plasmids. Thus, the putative AP-1 site was not involved in Ras-mediated GR transrepression in Pa-4 cells. The activity of Mt B in unstimulated conditions was not affected when compared with that of the wild type or Mt A (Fig. 6A). Therefore, an intact GRE site was not required for the identified enhancer to modulate α-ENaC expression in the absence of glucocorticoid hormone. Its presence, however, was indispensable for dexamethasone-induced α-ENaC expression and for the Ras pathway to block the induction, since altering the nucleotide sequences crucial to GRE motif drastically re-

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**Fig. 4. Hormone-dependent activation of α-ENaC enhancer activity by GR.** Salivary Pa-4 cells were transiently transfected with 0.9 μg of vector pGL2-P plasmid or p(−1066/−1573)GL2-P reporter construct with or without 0.5 μg of Ras V12 expression plasmid in combination with 0.5 μg of GR expression plasmids, as indicated. The amount of transfected DNA was kept constant at 2 μg by supplementing with varying amount of PCMV plasmid DNA. After transfection, the cells were treated as described in Fig. 3C with 0.1 μM dexamethasone or vehicle as indicated. The level of induction, expressed as fold induction, is calculated by dividing the normalized reporter luciferase activity in each extract (lanes 2–9, respectively) over that of pGL2-P-transfected/vehicle-treated cells, which is arbitrarily designated as 1 (lane 1) and indicated by a horizontal line. This experiment has been repeated three times, and one representative plot is shown.
duced the GR/dexamethasone-induced reporter activity and the magnitude of Ras-mediated, GR-dependent repression (Fig. 6A). Moreover, AP-1 site elicited no enhancing effect on the expression of Mt B, which lacks the functional GRE site, confirming that Ras activation had no effect on the identified α-ENaC enhancer activity in the absence of GR/dexamethasone transactivation.

The data in Fig. 3 suggest that α-ENaC DNA sequences between −1355 and −1269 bp confer high GR/dexamethasone-induced activity and Ras-mediated repression of GR/dexamethasone-dependent activity. Furthermore, the data in Fig. 6A illustrate that this Ras-repressible enhancer activity is largely abolished when the half-palindrome (nucleotides −1322 to −1318) of the GRE site is mutated, but not by mutations on the adjacent AP-1 sequence. To establish that the identified GRE sequence alone can serve as a GR/dexamethasone-dependent enhancer and confer Ras-repressibility over the GR/dexamethasone-mediated transactivation on a heterologous promoter, α-ENaC DNA sequences of −1334 to −1306 bp were cloned in both sense and antisense orientations into a luciferase reporter vector, pGL2-P, containing a minimal SV40 promoter. In transiently GR-cotransfected Pa-4 cells, the p(−1334/−1306)GL2-P reporter construct exhibited at least a 10-fold increase in luciferase activity in the presence of dexamethasone; a α-ENaC GRE (−1334/−1306) copy number-dependent dexamethasone enhancement was also observed (Fig. 6B). A similar result was also observed from the antisense construct, p(−1306/−1334)GL2-P (data not shown). As illustrated in Fig. 6B, the transcriptional enhancement by GR/dexamethasone on reporter constructs harboring either one copy or two copies of α-ENaC GRE was inhibited by the cotransfected Ras V12 expression plasmid. Similarly, GR/dexamethasone-dependent enhancement and Ras-mediated repression were also observed in Pa-4 cells by a tyrosine aminotransferase (TAT) simple GRE
FIG. 6. Functional analyses of putative GRE as a regulatory element in GR-mediated transactivation and Ras-mediated repression. A, function of the putative GRE in the context of heterologous promoter. DNA sequences of the rat α-ENaC distal enhancer region extending from −1334 to −1306 bp are shown. The GRE- and AP-1-like recognition motifs are boxed. Mt A and Mt B represent mutant variants used in transfection assays. Sequences that are identical to the wild type are shown as dashes, whereas the mutated DNA sequences are shown in letters. Reporter constructs (0.9 µg) was cotransfected with 0.4 µg of Ras V12 and/or 0.4 µg of GR expression plasmids as indicated. Luciferase activities of the wild type and mutant variants were assayed and analyzed as described in Fig. 3. B, the putative GRE in α-ENaC confers glucocorticoid response in the context of heterologous promoter. Three different reporter constructs were tested. Two constructs carry one and two copies of α-ENaC GRE encompassing sequences, −1334 to −1306 bp, upstream of the minimal SV40 promoter-containing luciferase reporter gene and the third construct, TAT/GRE5/TK/Luc plasmid, harbors three copies of simple GRE from tyrosine aminotransferase gene upstream of a minimal TK promoter. Pa-4 cells were cotransfected with 0.9 µg each of the above three constructs in combination with Ras V12 and GR expression constructs as indicated. Transfection with the empty vector pGL2-P is included as a control. The transfection, treatment protocol, and dual luciferase assays were carried out as described in Fig. 3. The luciferase activity in each extract of pGL2-P, pGL2-P/TAT/GRE5/TK/Luc, pGL2-P, pGL2-P/GR, and pGL2-P-Ras transfectected cells was measured. The results shown in Fig. 6 demonstrate the integration of GR and Ras signaling pathways and transcriptional control paradigms that are activated by various stimuli and to assess the integrated response to these stimuli. Previously, we have reported that TPA-mediated ERK activation appears to be required for regulating α-ENaC expression, however, this fails to rule out the possibility that a second, albeit much weaker, GRE exists in the 1.4 kb of α-ENaC 5′-flanking region that could also be inhibited by Ras pathway activation.

DISCUSSION

The results presented herein demonstrate that the transcription of the rat α-ENaC gene in salivary epithelial cells depends upon an enhancer located between −1.5 and −1.0 kb upstream from the translation initiation site. Elements within this region are required for Ras pathway-mediated repression of α-ENaC expression (Fig. 2). We have mapped a Ras-repressible enhancer activity of this region to a DNA sequence between −1334 and −1306 bp, which encompasses a GRE site. Our data provide evidence that the overall activity of the α-ENaC promoter/enhancer represents an integrated response to GR/dexamethasone-dependent activation and Ras-mediated repression.

A number of physiological, pathological, and pharmacological stimuli are known to regulate ENaC function (reviewed in Refs. 12 and 13). In order to establish how these stimuli work in concert, it is necessary to define the signal transduction pathways and transcriptional control paradigms that are activated by various stimuli and to assess the integrated response to these stimuli. Previously, we have reported that TPA-mediated ERK activation appears to be required for regulating several biological responses in salivary epithelial cells includ-
Raf/ERK-mediated down-regulation of a

transactivation of We conclude that Ras pathway activation represses GR-mediated

transcription, and treatment protocols were as de-

scribed in Fig. 3, whereas CAT assay was as described in Fig. 1.

- ENaC expression plasmids, as indicated. Following transfection, the cells were ei-

erer serum-starved for 8 h and treated with 10^{-7} M dexamethasone (Dex) over-

night (B), or cultured in the growth me-

dium and treated with dexamethasone over-

ight (A), or cultured in the growth me-

dium and treated with dexamethasone over-

ight (B). Transfection, normaliza-

tion, and treatment protocols were as de-

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tion, and treatment protocols were as de-

scribed in Fig. 3, whereas CAT assay was as described in Fig. 1.

| Ras V12 | GR/Dex |
|---------|--------|
| − + − + | − + − + |
| − − + + | − − + + |

To delineate the mode of a-ENaC gene regulation, we have examined

the ability of different Ras effector pathways, essential cis-elements, and

their cognate trans-factors to regulate a-ENaC expression. A 508-bp fragment located within −1573 and

−1066 bp of the rat a-ENaC gene was found to activate transcription

of a-ENaC promoter (Fig. 2). Moreover, the ob-

served enhancing activity was promoter- and orientation-inde-

pendent, based on its ability to increase the transcription via

either the SV40 minimal promoter or the proximal a-ENaC

promoter in both sense and antisense orientations, the hall-

mark of a classical enhancer. One novel aspect of the identified

a-ENaC enhancer is that both the activation and repression

elements reside within the same region, −1355 to −1269 bp,
*i.e.* deletion of the −1355 to −1269 bp region substantially
decreases both enhancement and Ras-mediated repression (Fig. 3),

implying that elements located within this region enhances the homologous/heterologous promoter activity and

that Ras pathway activation antagonizes its enhancing effect. Moreover, site-specific mutations of nucleotides −1322 to

−1318 abrogate both enhancing and repressing effects (Fig. 6A), strongly suggesting that the enhancement and repression are mediated via the same element. In this regard, we further
demonstrate that a single copy of this element alone is suf-
cient to recapitulate the dexamethasone-stimulated and Ras-

inhibitable effects on the heterologous promoter (Fig. 6B). In

addition, specific mutation on the identified GRE in its native context drastically attenuated the Ras-repressible and GR/dex-

amethasone-inducible a-ENaC/CATs reporter activity (Fig. 7).

We conclude that Ras pathway activation represses GR-mediated

transactivation of a-ENaC via the identified GRE located in nucleotides −1334 to −1306 of the a-ENaC 5′-flanking

region.

This novel finding extends the potential role of Ras path-
way(s) in modulating various biological responses. The exact

mechanism(s) by which Ras blocks GR/dexamethasone-mediated

transactivation used in modulating a-ENaC expression is

unknown. Currently, we are investigating whether Ras path-

way activation down-regulates the GR protein level or attenu-

ates its ability to interact with the a-ENaC GRE in Pa-4 cells.

A similar functional antagonism between the activation of JNK

and ERK kinases and dexamethasone-stimulated GRE/re-

porter activities has recently been reported (35). These authors

showed that phosphorylation of GR at Ser-246 by JNK led to

the inhibition of GR-mediated transcriptional activation,

whereas ERK did so indirectly. Our present work extends the

conclusion by Rogatsky et al. (35) by demonstrating for the first
time that the transcription of the endogenous a-ENaC gene as

well as a-ENaC reporter construct(s) is regulated via the op-

posing effects of GR- and Ras-mediated signaling pathways.

Furthermore, the findings that endogenous a-ENaC expression

is induced by glucocorticoid hormone and attenuated by TPA

treatment (Fig. 5) support the physiological and pharmacolog-

ical relevance of our data. Moreover, it rules out the possibility

that the observed transrepression is a result of transcriptional

squelching caused by overexpression of Ras V12 and/or GR

eXpression plasmids.

Substantial progress has been made in understanding the

mechanisms through which Ras exerts its biological effects. For

example, there are multiple effector pathways stimulated by

Ras activation, which vary in different cells. Recent studies

have shown that the activation of Ras signaling pathway reg-

ulates total RNA and protein synthesis (36–38). A wide array of

promoters have been reported to be up-regulated by Ras

activation as a result of a stimulatory effect on the basal tran-

scriptional apparatus of the cells as well as on specific tran-

scription factors (reviewed in Ref. 39). Using the attenuated

transcription of a-ENaC by the Raf/ERK pathway as a para-

digm, we have uncovered a mammalian signal transduction

mechanism underlying Ras-mediated gene repression in epi-

thelial cells. These results emphasize a fundamental difference

between activation and inhibition of gene expression mediated

by the same pathway, and reinforce the importance of studying

Ras action in both the global and the specific enhancement/ repression of gene expression.

Based on our data, a putative model on the regulation of

a-ENaC expression in the salivary epithelial cells is depicted as

an integrated response to both Ras- and GR-mediated signaling

$$\text{Fig. 7. The a-ENaC GRE confers glucocorticoid response in the context of its own promoter. Salivary Pa-4 cells were transiently transfected with 0.9 \mu g of a-ENaC/CATs (Wt) or its GRE mutant (GRE Mt) with point mutations, as described in Fig. 6A, in the presence or absence of 0.5 \mu g of Ras V12 and 0.5 \mu g of GR expression plasmids, as indicated. Following transfection, the cells were either serum-starved for 8 h and treated with 10^{-7} M dexamethasone (Dex) overnight (A), or cultured in the growth medium and treated with dexamethasone overnight (B). Transfection, normalization, and treatment protocols were as described in Fig. 3, whereas CAT assay was as described in Fig. 1.}$$
We propose that Ras activation leads to the activation of ERK kinases as well as a less well defined Ral-GDS effector pathway. Subsequently, these two effector pathways may work in concert or individually to inhibit GR-stimulated \( \alpha \)-ENaC transcription in a direct and/or indirect fashion. This model is supported by the failure of MEK inhibitor, PD 98059, to fully block Ras-mediated repression, indicating that other effector pathway(s) may participate in parallel with the elucidated Raf/ERK pathway to mediate GR-dependent transcriptional repression of \( \alpha \)-ENaC expression. The results obtained from transient transfection studies employing mutant constructs with homologous (Fig. 2) and heterologous (Fig. 3) promoters support the hypothesis that GR activation is necessary for Ras-mediated repression of \( \alpha \)-ENaC promoter. In addition, part of the Ras-mediated GR transcription is independent of the presence of the adjacent putative AP-1 site (Fig. 6A, Mt A). Moreover, the expression of Ras V12 inhibits the luciferase activity in cells transfected with reporter constructs containing either \( \alpha \)-ENaC- or TAT-GRE (Fig. 6B). These data suggest that Ras-mediated GR/dexamethasone-dependent repression can be a common mechanism modulating the expression of GR-target genes in epithelial cells. It is possible, however, that there is other yet unidentified transcription factor(s) "X" interacting with DNA motif(s) within the cloned 1.4-kb \( \alpha \)-ENaC 5'-flanking region that may likewise be modified by Ras pathway activation to inhibit \( \alpha \)-ENaC expression (Fig. 8).

In conclusion, our data on the regulation of \( \alpha \)-ENaC gene expression by Ras- and GR-mediated pathways in salivary epithelial cells represent the first detailed study on the role(s) of regulatory elements in the transcriptional control of any of the three subunits of ENaC expressed in epithelia. The present work indicates that the cross-talk between Ras- and GR-mediated pathways (Fig. 8). We propose that Ras activation leads to the activation of ERK kinases as well as a less well defined Ral-GDS effector pathway. Subsequently, these two effector pathways may work in concert or individually to inhibit GR-stimulated \( \alpha \)-ENaC transcription in a direct and/or indirect fashion. This model is supported by the failure of MEK inhibitor, PD 98059, to fully block Ras-mediated repression, indicating that other effector pathway(s) may participate in parallel with the elucidated Raf/ERK pathway to mediate GR-dependent transcriptional repression of \( \alpha \)-ENaC expression. The results obtained from transient transfection studies employing mutant constructs with homologous (Fig. 2) and heterologous (Fig. 3) promoters support the hypothesis that GR activation is necessary for Ras-mediated repression of \( \alpha \)-ENaC promoter. In addition, part of the Ras-mediated GR transcription is independent of the presence of the adjacent putative AP-1 site (Fig. 6A, Mt A). Moreover, the expression of Ras V12 inhibits the luciferase activity in cells transfected with reporter constructs containing either \( \alpha \)-ENaC- or TAT-GRE (Fig. 6B). These data suggest that Ras-mediated GR/dexamethasone-dependent repression can be a common mechanism modulating the expression of GR-target genes in epithelial cells. It is possible, however, that there is other yet unidentified transcription factor(s) "X" interacting with DNA motif(s) within the cloned 1.4-kb \( \alpha \)-ENaC 5'-flanking region that may likewise be modified by Ras pathway activation to inhibit \( \alpha \)-ENaC expression (Fig. 8).
ated pathways dictate the overall transcriptional regulation of the \( \alpha\)-ENaC gene. The challenge now is to understand how Ras-mediated signals, transient or persistent, are directed at distinct effector pathway(s) and the interface between each effector pathway and transcriptional control to convert GR/dexamethasone-mediated transactivation to transrepression in different cell types. Studies on the signaling pathway(s) and transcriptional mechanism(s) governing \( \alpha\)-ENaC gene expression provide a unique paradigm for understanding the interplay between different signaling pathways as well as the biological consequences of such events. It is conceivable that these events may allow cells to respond accordingly to changing cues in different cellular environments. Moreover, the results obtained from this study should be useful for future studies on the expression of the \( \alpha \) and other subunits of ENaC in various epithelial cells. Finally, it may aid in devising strategies to manage or modulate the concerted expression of all three ENaC subunits simultaneously in epithelia.

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