Regulation of Na\(^{+}\) Reabsorption by the Aldosterone-induced Small G Protein K-Ras2A

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Xenopus laevis A6 cells were used as model epithelia to test the hypothesis that K-Ras2A is an aldosterone-induced protein necessary for steroid-regulated Na\(^{+}\) transport. The possibility that increased K-Ras2A alone is sufficient to mimic aldosterone action on Na\(^{+}\) transport also was tested. Aldosterone treatment increased K-Ras2A protein expression 2.8-fold within 4 h. Active Ras is membrane associated. After aldosterone treatment, 75% of K-Ras was localized to the plasma membrane compared with 25% in the absence of steroid. Aldosterone also increased the amount of active (phosphorylated) mitogen-activated protein kinase kinase likely through K-Ras2A signaling. Steroid-induced K-Ras2A protein levels and Na\(^{+}\) transport were decreased with antisense K-ras2A oligonucleotides, showing that K-Ras2A is necessary for the natriferic actions of aldosterone. Aldosterone-induced Na\(^{+}\) channel activity, was decreased from 0.40 to 0.09 by pretreatment with antisense ras oligonucleotide, implicating the luminal Na\(^{+}\) channel as one final effector of Ras signaling. Overexpression of K-Ras2A increased Na\(^{+}\) transport approximately 2.2-fold in the absence of aldosterone. These results suggest that aldosterone signals to the luminal Na\(^{+}\) channel via multiple pathways and that K-Ras2A levels are limiting for a portion of the aldosterone-sensitive Na\(^{+}\) transport.

Aldosterone is the primary hormone regulating salt and water homeostasis in humans. Thus, this steroid hormone is critical to proper maintenance of blood volume and pressure. Whereas the systemic and tissue actions of aldosterone are clear, namely volume expansion by induction of Na\(^{+}\) reabsorption across renal principal cells, the cellular signal transduction mechanism initiated by this steroid remains unclear and controversial. Similar to the actions of other steroids on diverse target tissues, the natriferic actions of aldosterone on Na\(^{+}\) reabsorbing epithelia are mediated by steroid-induced transcription and subsequent translation of new proteins (1–3). The final step in this aldosterone-mediated process is an increase in activity of luminal Na\(^{+}\) channels (4–6). Several different methods have been used to identify a few putative aldosterone-induced proteins; however, it is unclear whether these proteins are actually relevant to the regulation of NaCl and water homeostasis.

Aldosterone increases Na\(^{+}\) reabsorption at the distal tubule of the nephron through the actions of aldosterone-induced proteins first by increasing the activity of existing luminal Na\(^{+}\) channels (<4 h) and then by promoting synthesis of new luminal Na\(^{+}\) channel and serosal Na\(^{+}\)/K\(^{+}\) ATPase proteins (>6 h; (1–3). Spindler et al. (7) demonstrated recently that the mRNA of the small, monomeric GTP-binding (smG) protein, K-Ras2A, increased within 60 min. in response to aldosterone. Transcript levels increased to a maximum of about 5-fold 3–4 h after treatment and then decreased toward pretreatment levels. However, it remains to be determined whether K-Ras2A protein levels, like that of its mRNA, increase in response to aldosterone. In addition, it is unclear whether K-Ras2A protein is relevant to Na\(^{+}\) transport in epithelia.

Several laboratories have reported that the activity of the epithelial Na\(^{+}\) channel (ENaC) is regulated by GTP (8–11). In addition, factors that regulate localization of smG proteins to the inner leaflet of the plasma membrane, such as methyl esterification, also modulate Na\(^{+}\) reabsorption and epithelial Na\(^{+}\) channel activity (2, 12–15). These observations suggest that K-Ras2A levels, localization, and activity may be relevant to aldosterone-stimulated Na\(^{+}\) reabsorption. Further supporting this notion are the recent observations of Mastroberardino and colleagues (16) showing that co-expression of constitutive-active K-Ras2A with ENaC in the heterologous X. laevis oocyte expression system appeared to increase Na\(^{+}\) channel activity. Nonetheless, it remains to be determined whether K-Ras2A can alter Na\(^{+}\) transport and ENaC activity in epithelial tissue capable of vectorial transport.

The current study tested the hypothesis that K-Ras2A is an aldosterone-induced protein critical to steroid-sensitive Na\(^{+}\) reabsorption in renal epithelia. Aldosterone increases K-Ras2A expression. K-Ras2A is necessary for aldosterone-stimulation of Na\(^{+}\) transport and ENaC activity. Aldosterone also causes translocation of K-Ras to the plasma membrane. Subsequent to the translocation, K-Ras2A signal transduction leads to activation of the downstream, effector kinase MEK. It is unclear whether MEK activation is associated with Na\(^{+}\) transport. However, overexpression of wild-type K-Ras2A was sufficient to mimic some of the natriferic actions of aldosterone suggesting that this protein may be limiting for some portion of the aldosterone-sensitive Na\(^{+}\) transport. Our results support the notions that K-Ras2A is an aldosterone-induced protein necessary for steroid-regulated Na\(^{+}\) transport and that K-Ras2A ultimately regulates Na\(^{+}\) channel activity.

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‡ The abbreviations used are: smG, small, monomeric GTP-binding; ENaC, epithelial Na\(^{+}\) channel; NPo, channel activity; MEK, mitogen-activated protein kinase kinase; pKras2A, an expression construct containing full-length K-ras2A cDNA.
**Experimental Procedures**

### Tissue Culture

Whole cell lysate was extracted from A6 cells using the following lysis buffer: 50 mM HCl-Tris, 76 mM NaCl, 2 mM EGTA, plus 1% Nonidet P-40, 10% glycerol (pH 7.4), and protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, tosylphenylalanyl chloromethyl ketone, and 1-chloro-3-tosylamido-7-amino-2-heptanone; see Ref. 18). Total Ras protein and K-Ras protein were immunoprecipitated using standard protocols with the commercially available monoclonal antibodies, Ha-Ras (sc-35, Santa Cruz Biotechnology) or v-Ha-ras (OP01, Oncogene Research Products), and c-K-Ras (OP24), respectively. For Western blot analysis, whole cell lysate and immunoprecipitants were separated by SDS-polyacrylamide gel electrophoresis in the presence of reducing reagent (20 mM dithiothreitol) and subsequently transferred to nitrocellulose. Blots then were probed with either anti-c-K-Ras (OP24) or anti-v-Ha-ras (OP01) and appropriate secondary antibody-horseradish peroxidase conjugate. All immunoblotting was performed in Tris-buffered saline with 5% milk and 0.1% Tween 20. Immunoreactive proteins were visualized using the enhanced chemiluminescence system (Amer sham Pharmacia Biotech). Band density was determined using SigmaGel software (Jandel Scientific).

The polyclonal anti-K-Ras2A antibody (from Santa Cruz) was created against an epitope found only in the A splice variant of K-Ras: the A and B variants diverge at the C terminus between amino acids 156–184. The epitope for anti-K-Ras2A antibody is residues 163–179 of K-Ras protein prior to Western blot analysis. The mouse monoclonal c-K-ras antibody (Oncogene) was developed by immunizing with K-Ras and selecting hybridomas for reactivity with K-Ras (epitope localized between residues 54–189); the inability to react with Ha-Ras and N-Ras. This antibody binds both A and B K-Ras splice variants.

Crude membrane and cytosolic fractions were prepared by differential centrifugation. A6 cells were harvested by sonication in 0.25 M sucrose (10 mM HEPES, pH 7.4). Subsequent to removal of cellular debris, nuclei and mitochondria, the microsomal fraction (P100) was separated from cytosol (S100) by centrifugation at 100,000 × g for 90 min. Prior to SDS-polyacrylamide gel electrophoresis and Western blot analysis, p21 translocated into the P100 and S100 fractions was concentrated (with ν-Ha-ras antibody). Immuneactivity was determined as above.

### Anti-MEK 1/2 and anti-phospho-MEK 1/2 antibodies are commercially available (PhosphoPlus MEK 1/2 (Ser-217/Ser-221) Antibody Kit, New England BioLabs, Inc.). Both antibodies were used for Western blot analysis per the manufacturer’s instructions. For these experiments, A6 cell lysate was prepared with lysis buffer supplemented with 50 mM NaF, 2 mM Na3VO4, and 0.1 mM okadaic acid to decrease protein phosphatase activity.

## Molecular Biological Methods

### Cloning of Xenopus laevis K-Ras2A cDNA—Full length X. laevis K-ras2A cDNA was obtained using a reverse transcription-polymerase chain reaction (CLONTECH Laboratories, Inc.) on single-stranded cDNA prepared from A6 cell mRNA using the FastTrack 2.0 kit (Invitrogen) in conjunction with specific primers (forward primer, 5′-CCGGTAGGCAGAGACAGC-3′; reverse primer, 5′-AATAGAGGGACGGGCGTGATACT-3′) developed using the published X. laevis K-ras2A sequence (GenBank™ accession number Y12715, Ref. 7). The 900-bp product of this reaction was ligated into pCMV-TK (Promega) and subsequently subcloned into pcDNA3.1(zeo−) (Invitrogen) with NotI. Sequence data from this eukaryotic expression construct, pKras2A, was homologous to the published sequence (7) and consistent with the construct containing the full K-ras2A open reading frame. Moreover, in vitro translation of the pKras2A construct as shown in Fig. 1 produced a protein of appropriate size for full-length K-ras2A.

### Regulation of Na+ Reabsorption by K-Ras2A

Expression of K-Ras2A from pKras2A is controlled by the cytomembrane virus promoter.

**Transfection of A6 Cells—** A6 cells were transfected with pKras2A or control plasmid using the LipofectAMINE Plus (Life Technologies, Inc.) system as described previously (17, 18). Subsequent to transfection, A6 cells were selected with zeocin (800 µg/ml) to enrich the cultures in transfected cells. Zeocin-resistant A6 cells were used for experimentation up to two subpassages after transfection. Zeocin-selected cells cultured in complete medium were allowed to reach confluence prior to experimentation. To facilitate quantification of induction of Na+ transport by aldosterone, cells were treated with basic medium (serum and steroid free) for 2 days prior to the readdiction of steroid. This set transport to a basal level and allowed for the investigation of the aldosterone-sensitive signaling pathway that results in increased Na+ reabsorption.

**Antisense Oligonucleotide Strategy—** Phosphorothioate sense (5′-GGGAGTGCAGCCAGTACAGTG-3′) and antisense K-ras (5′-CCACGCTTGACTCGACGATC-3′) oligonucleotides were synthesized by the Emory University Microchemical Facility. The K-ras2A antisense oligonucleotide complemented the translation start site of K-ras2A mRNA (base pairs 5–19). In addition, the more general ras antisense (5′-CTCCGCTTATCCACGACGAC-3′) and nonsense (5′-GAGGCAGTAAAAGTGGCTGATG-3′) oligonucleotides were created. These oligonucleotides complemented K-ras2A, as well as other forms of X. laevis ras, such as Ha-ras. Confluent A6 cells were cultured for 24 h in basic medium and then treated with (5–10 µM) oligonucleotide in basic medium for an additional 24 h. Currents across treated A6 cell monolayers and the activity of ENaC in cell attached patches (see below) were subsequently made before and after addition of aldosterone (1.5 µM, 4 h). Cells then were harvested for protein chemistry analysis (described above).

## Electrophysiology

### Patch Clamp Methods—** A6 cells were prepared for single-channel analysis using standard patch-clamp techniques, described previously (4). Typical pipette and bath solutions contained 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM MgCl2, and 1 mM CaCl2 (pH 7.4). Current recordings of ENaC were made after obtaining gigaseal seals with the patch electrode on the surface of the A6 cell. Unitary current (i) zero for the open state (SO) was determined from the best-fit Gaussian distribution of the amplitude histograms. Channels were considered in an open state (SO) when the current was greater than (n – 1/2k) and less than (n + 1/2k), where n is the number of open channel current levels. The probability of a channel being in the open state (PO) is defined as the time spent in S divided by the total time of the recording. Because the number of channels in a patch is not known with certainty, the activity of all ENaC in a given patch is reported as NP, defined as the sum of the Pn times the respective current level. For all experiments, data was filtered at 100 Hz and collected at 500 Hz.

### Measurement of Trans-epithelial Current—** Trans-epithelial voltages and resistances were collected using the Millicel Ercal Resistance System (Millipore Corp.) as described previously (17, 18). Both voltages and resistances were measured in open circuit conditions to better mimic a real physiological environment. Ohm’s law was used to calculate equivalent short-circuit current (Isc). All current with our conditions is amiloride-sensitive, with the majority being carried by Na+. Thus, in this instance, Isc is a good measurement of trans-epithelial Na+ transport.

A6 cells cultured in complete medium were grown to confluence on permeable supports (0.02 µm Anopore membrane; Nalge NUNC Inter-
K-Ras2A Is a Necessary Protein for Aldosterone-induced Na\(^+\) Transport—Fig. 3 demonstrates that steroid-induction of K-Ras2A protein expression is necessary for aldosterone-stimulated Na\(^+\) reabsorption. Fig. 3A is a typical Western blot probed with anti-K-Ras2A antibody. Both lanes contain protein harvested from confluent A6 cell monolayers that was immunoprecipitated with anti-K-Ras antibody (starting concentration of total protein was identical). The left lane is the precipitant from cells treated with pCon, and the right lane is from cells treated with antisense oligonucleotide. The arrow indicates K-Ras2A. Antisense K-ras2A oligonucleotide (ANTT) for 24 h decreased the amount of aldosterone-induced K-Ras2A protein compared with cells treated with sense oligo (SENSE). The conditions for this Western blot are identical to those in Fig. 2B.Summary graph of four such experiments. Antisense but not sense clearly attenuates the amount of K-Ras2A in A6 cell treated with aldosterone. C, A6 cell monolayers treated with antisense had significantly less aldosterone-induced Na\(^+\) current compared with monolayers treated with sense and monolayers not treated with oligonucleotide. CON, control.

and then treated with aldosterone (1.5 \(\mu\)M) plus sense oligo for an additional 4 h. The right lane is the precipitant from cells treated in the same fashion with antisense oligonucleotide. The arrow indicates K-Ras2A. Antisense K-ras2A oligonucleotide attenuated the aldosterone-induced expression of K-Ras2A protein. Four such experiments are summarized in Fig. 3B. Compared with sense-treated cells, antisense treatment significantly reduced aldosterone-induced K-Ras2A proteins levels 3.1 ± 0.3-fold from 3.7 ± 0.7 to 1.2 ± 0.2 arbitrary density units. This same maneuver as shown in Fig. 3C (see also Table I) reduced aldosterone-induced Na\(^+\) transport across A6 cell monolayers. Aldosterone increased current by 1.8 ± 0.1 \(\mu\)A/cm\(^2\) in control cells (untreated) \((n = 88)\) and by the same amount in sense-treated cells \((n = 83)\). This increase was significantly greater than the aldosterone-induced increase of 1.3 ± 0.1 \(\mu\)A/cm\(^2\) in antisense-treated cells \((n = 101)\). Although the 1.4 ± 0.05-fold reduction in current upon exposure to antisense oligonucleotides is smaller than the reduction in apparent protein density (3.1-fold), there is no significant difference in fractional reduction of protein and current \((z\text{-test}, p = 0.322)\). In addition, if a small fraction of the current was not due to Na\(^+\) transport (even as little as 0.1 \(\mu\)A/cm\(^2\)), then our measurement of the fractional reduction in current would be significantly underestimated. The Apical Na\(^+\) Channel Is One Final Effector of Aldosterone-stimulated Ras Signaling.—The single channel current traces (Fig. 4A) and summary graph (Fig. 4B) demonstrate that ras antisense but not nonsense oligonucleotide decreases aldosterone-stimulated Na\(^+\) channel activity. These current traces are typical of ENaC from A6 cells treated with aldosterone in addition to nonsense (top trace) or antisense (bottom trace) ras

The results clearly indicate that aldosterone application (1.5 \(\mu\)M, 4 h) to A6 cell monolayers increases expression of K-Ras2A protein. For all Western blots, K-Ras was immunoprecipitated from whole cell lysate containing equal amounts of total protein (a typical Western blot is shown in Fig. 2A). The rationale for immunoprecipitating K-Ras prior to Western blot analysis is that K-Ras is in such low abundance in confluent, terminally differentiated, nondividing A6 cells that direct immunodetection is not feasible without first enhancing the amount of the protein of interest. Subsequent to SDS-polyacrylamide gel electrophoresis and transference, blots containing anti-K-Ras-immunoprecipitated protein from untreated (Fig. 2, CON) and steroid-treated (ALDO) cells were probed with antibody antibody. In Fig. 2, K-Ras2A is indicated by the arrow, and the two heavier proteins are the IgG bands of the immunoprecipitating antibody. Fig. 2B and Table I summarize the densities of K-Ras2A in untreated and treated cells for six such experiments. Aldosterone treatment for 4 h significantly increased K-Ras2A protein levels 2.8-fold from 1.2 ± 0.1 to 3.4 ± 0.4 arbitrary density units, demonstrating that K-Ras2A is an aldosterone-induced protein. K-Ras2A Is Necessary for Aldosterone-induced Na\(^+\) Transport—Fig. 3 demonstrates that steroid-induction of K-Ras2A protein expression is necessary for aldosterone-stimulated Na\(^+\) reabsorption. Fig. 3A is a typical Western blot probed with anti-K-Ras2A antibody. Both lanes contain protein harvested from confluent A6 cell monolayers that was immunoprecipitated with anti-K-Ras antibody (starting concentration of total protein was identical). The left lane is the precipitant from cells treated with sense K-ras2A oligonucleotide (5–10 \(\mu\)M) for 24 h and then treated with aldosterone (1.5 \(\mu\)M) plus sense oligo for an additional 4 h. The right lane is the precipitant from cells treated in the same fashion with antisense oligonucleotide. The arrow indicates K-Ras2A. Antisense K-ras2A oligonucleotide attenuated the aldosterone-induced expression of K-Ras2A protein. Four such experiments are summarized in Fig. 3B. Compared with sense-treated cells, antisense treatment significantly reduced aldosterone-induced K-Ras2A proteins levels 3.1 ± 0.3-fold from 3.7 ± 0.7 to 1.2 ± 0.2 arbitrary density units. This same maneuver as shown in Fig. 3C (see also Table I) reduced aldosterone-induced Na\(^+\) transport across A6 cell monolayers. Aldosterone increased current by 1.8 ± 0.1 \(\mu\)A/cm\(^2\) in control cells (untreated) \((n = 88)\) and by the same amount in sense-treated cells \((n = 83)\). This increase was significantly greater than the aldosterone-induced increase of 1.3 ± 0.1 \(\mu\)A/cm\(^2\) in antisense-treated cells \((n = 101)\). Although the 1.4 ± 0.05-fold reduction in current upon exposure to antisense oligonucleotides is smaller than the reduction in apparent protein density (3.1-fold), there is no significant difference in fractional reduction of protein and current \((z\text{-test}, p = 0.322)\). In addition, if a small fraction of the current was not due to Na\(^+\) transport (even as little as 0.1 \(\mu\)A/cm\(^2\)), then our measurement of the fractional reduction in current would be significantly underestimated. The Apical Na\(^+\) Channel Is One Final Effector of Aldosterone-stimulated Ras Signaling.—The single channel current traces (Fig. 4A) and summary graph (Fig. 4B) demonstrate that ras antisense but not nonsense oligonucleotide decreases aldosterone-stimulated Na\(^+\) channel activity. These current traces are typical of ENaC from A6 cells treated with aldosterone in addition to nonsense (top trace) or antisense (bottom trace) ras
oligonucleotide. Summarized in Fig. 4B are five such experiments. The aldosterone-induced Na⁺ channel activity of 0.40 ± 0.03 in nonsense-treated cells is significantly greater than the NPo of 0.09 ± 0.01 in antisense-treated cells. Although the 4.3 ± 0.19-fold reduction in NPo upon exposure to antisense oligonucleotides is larger than the reduction in apparent protein density (3.1-fold) and current (1.4-fold), there is no statistically significant difference in fractional reduction of protein and NPo (z test, p = 0.758) or of current and NPo (z test, p = 0.072). Note oligonucleotide treatment did not affect single channel amplitude.

**Overexpression of K-Ras2A Protein Is Sufficient to Produce Some Increase in Na⁺ Reabsorption**—Fig. 5 (and Table 1) shows that cells transiently transfected with pKras2A have more K-Ras2A protein and Na⁺ transport compared with control transfectants. Fig. 5A, top panel, is a typical Western blot probed with anti-K-Ras antibody of the Ras-immunoprecipitant from A6 cells transfected with control plasmid (left lane) or the pKras2A construct (right three lanes). For these experiments, all immunoprecipitations were performed on equal amounts of total protein. K-Ras is indicated by the arrow. The summary graph (Fig. 5A, bottom panel) shows that cells transfected with the pKras2A construct (n = 6) had 3.1-fold more K-Ras2A protein levels compared with control transfectants. The aldosterone-induced Na⁺ current of 2.7 ± 0.1 μA/cm² (n = 36) across K-Ras2A-overexpressing A6 cell monolayers was significantly greater than the 1.5 ± 0.2 μA/cm² (n = 19) of control transfectants, and the 1.8 ± 0.1 μA/cm² across cells that were not transfected. Moreover, as shown in Table 1, the Na⁺ currents across K-Ras2A-overexpressing A6 cell monolayers in the absence of steroid were 2.2-fold greater than those in control transfectant monolayers, suggesting that overexpression of K-Ras2A is sufficient to mimic some of the natriuretic actions of aldosterone. All current across transfected cells was sensitive to 5 μM amiloride.

**Aldosterone Activates K-Ras2A Signal Transduction**—Figs. 6 and 7 show that aldosterone-induced K-Ras2A activates MEK, a well established, downstream effector of the Ras signal transduction pathway. Activation of MEK was, in part, dependent on K-Ras2A protein levels and associated with translocation of K-Ras to the plasma membrane, the cellular locale where this smG protein interacts with its effectors. The typical Western blots of Fig. 6A show that K-Ras protein in A6 cells not treated with steroid is localized to both the cytosol (CON, S100) and particulate fractions (CON, P100). In contrast, K-Ras protein in A6 cells treated with aldosterone for 4 h is localized primarily to the particulate (ALDO, P100) and not the cytosol (ALDO, S100). The summary graph shown in Fig. 6B (n = 3) shows that approximately 75% of the A6 cell K-Ras is localized to the particulate fraction after treatment with aldosterone; in contrast, 25% is localized to the particulate in the absence of steroid. Movement of Ras to the particulate is consistent with activation of this smG protein. Also consistent with activation of Ras are the results shown in Fig. 7, showing that aldosterone addition to A6 cell monolayers induces MEK phosphorylation. The typical Western blot (Fig. 7A) of whole cell lysate prepared from cells treated with vehicle (CON) and steroid (1.5 μM (ALDO) was probed with anti-phospho-MEK 1/2 antibody. Aldosterone activated (phosphorylated) MEK within 15 min. At 4 h, phospho-MEK levels were 3.4 ± 1.6-fold (n = 3) higher in aldosterone-treated A6 cells compared with cells treated with vehicle. As shown in Fig. 7B, inhibition of K-Ras2A protein synthesis with antisense Krass2A oligonucleotide attenuated MEK activation in response to aldosterone treatment for 4 h.

This typical Western blot shows the levels of phospho-MEK (Fig. 7B, top) and total MEK (bottom) in lysate prepared from...
K-Ras2A protein levels are 2.8-fold higher in aldosterone-treated cells (left) and steroid and antisense-treated cells (right).

The summary data in Table I show 1) that aldosterone increases K-Ras2A protein levels and Na⁺ transport within 4 h; 2) that inhibition of aldosterone-induced K-Ras2A protein synthesis with antisense oligonucleotides leads to an associated decrease in induced Na⁺ transport; and 3) that transient transfection with pKras2A increases K-Ras2A protein levels and Na⁺ transport in the absence and presence of aldosterone.

DISCUSSION

The experiments described tested the hypothesis that K-Ras2A is an aldosterone-induced protein required for steroid-regulated Na⁺ transport. Aldosterone was shown to increase K-Ras2A protein levels within 4 h, suggesting that this Ras is an aldosterone-induced protein. Moreover, K-Ras2A was shown to be critical to aldosterone regulation of ENaC activity and Na⁺ reabsorption. Overexpression of K-Ras2A increased Na⁺ transport; however, K-Ras2A overexpression alone was not as strong an activator of Na⁺ transport as aldosterone, suggesting that aldosterone signals to ENaC through pathways independent or complementary to the Ras pathway. Electrophysiological data suggests that K-Ras2A supports the activity of luminal Na⁺ channels; however, the mechanism of this action remains to be determined. Aldosterone-activated K-Ras2A localized to the plasma membrane, where it subsequently activated MEK. However, it is unclear whether MEK activation is associated with vectorial Na⁺ transport or ENaC activity. We believe these results show that induction of K-Ras2A by aldosterone is necessary for steroid-sensitive Na⁺ reabsorption and propose that aldosterone activates the luminal Na⁺ channel, in part, via K-Ras2A signaling as shown in Fig. 8.

K-Ras2A Is an Aldosterone-induced Protein—It is well established that aldosterone regulates Na⁺ transport by first inducing gene expression and subsequent protein synthesis (1–3). However, these aldosterone-induced proteins remain controversial and not well characterized. In a recent publication, Spindler et al. (7) reported that in A6 cells, aldosterone increased K-ras2A mRNA levels, supporting the possibility that this transcript codes an aldosterone-induced protein. Transcription of K-ras2A mRNA was a primary effect of aldosterone.

The current results of Fig. 2 (see also Table I) showing that K-Ras2A protein levels are 2.8-fold higher in aldosterone-treated A6 cells compared with untreated controls show directly for the first time that K-Ras2A is an aldosterone-induced protein. This observation and that of Spindler et al. (7) suggest that one primary action of aldosterone on epithelia is to increase K-Ras2A protein levels via transcriptional control of K-ras2A mRNA expression. The time course of increased K-ras2A mRNA transcription and synthesis of K-Ras2A protein are consistent with the early actions of aldosterone. This mechanism also is supported by the results of Figs. 3 and 4 (see below). K-Ras2A similar to other smg proteins is a component of cell proliferative/differentiation pathways. K-Ras2A-initiated proliferative/differentiation pathways may contribute to the later trophic actions of aldosterone through activation of the MAP Kinase cascade, which then would regulate secondary gene expression. The results of Figs. 6 and 7 are consistent with this notion.

K-Ras2A, a Modulator of ENaC Activity, Is Necessary for Steroid-sensitive Na⁺ Transport—The results from antisense experiments (Figs. 3 and 4, Table I) and those localizing K-Ras (Fig. 6) support the idea that K-Ras2A protein is critical for aldosterone-stimulated Na⁺ transport and ENaC activity. These findings are also consistent with those of Eaton et al. (2), Rokaw et al. (13) and Ismailov et al. (12) showing that addition of GTP and S-adenosyl-L-methionine to the intracellular face of membranes containing the epithelial Na⁺ channel activates these ion channels. Small G proteins are activated when conjugated with GTP, and S-adenosyl-L-methionine metabolism is involved in a reversible posttranslational modification of smg proteins that regulates their activity and localizes them to the plasma membrane (20, 21). Studies by us (2, 17, 18) and other laboratories (14, 15) demonstrate that aldosterone, in addition to increasing K-Ras2A protein levels, also increases the activity of isoprenylcysteine-O-carboxyl methyltransferase, the enzyme that uses S-adenosyl-L-methionine as a methyl donor to modify Ras protein. Thus, it is likely that aldosterone induces expression of K-Ras2A protein simultaneously with activation of the smg protein methylating enzyme, isoprenylcysteine-O-carboxyl methyltransferase. This would lead to an increase in the cellular pool of methylated-Ras. Because methylated-Ras is active and localized to the inner leaflet of the plasma membrane (20, 21), regulation of the levels of this protein may be one regulatory site for control of Na⁺ transport (see Figs. 2, 6, and 8).

The single Na⁺ channel current recordings in Fig. 4 support the hypothesis that K-Ras2A regulates aldosterone-signaling
to the luminal Na\(^+\) channel in epithelia. These show directly that inhibition of Ras protein expression with antisense causes a concomitant decrease in aldosterone-stimulation of apical Na\(^+\) channel activity. The specific mechanism whereby K-Ras2A affects the epithelial Na\(^+\) channel to regulate vectorial Na\(^+\) transport remains to be determined.

The observation that overexpression of wild-type K-Ras2A increases Na\(^+\) transport across A6 cell monolayers in the absence of steroid is consistent with the results of Mastroberardino et al. (16) showing that simultaneous expression of constitutive-active K-Ras2A with ENaC in oocytes likely increases ENaC activity. Although these authors demonstrated that a constitutive-active mutant of K-Ras2A likely increased Na\(^+\) channel activity in a heterologous system, it was unclear whether wild-type K-Ras2A was a physiological regulator of Na\(^+\) transport in renal epithelia. The main concern with the results of Mastroberardino et al. (16) is that, because overexpression of constitutive-active K-Ras2A mutants cause oocyte maturation, the observed increase in Na\(^+\) channel activity may have been related to the K-Ras2A-induced oocyte maturation and not a physiological regulation of the Na\(^+\) channel (although another agent, progesterone, which also promotes maturation, did not seem to produce an increase in ENaC activity). Nonetheless, the results of the current study demonstrating that overexpression of K-Ras2A is sufficient to cause some Na\(^+\) transport support these earlier findings in the oocyte. Moreover, the current results also demonstrate directly that K-Ras2A regulates Na\(^+\) transport, likely through modulation of Na\(^+\) channel activity in epithelial cells capable of physiological, vectorial transport.

Overexpression of K-Ras2A increased Na\(^+\) transport in the absence of steroid by 2.2-fold. This result and those obtained with the antisense oligonucleotides suggest that aldosterone signals to the luminal Na\(^+\) channel via multiple pathways and that K-Ras2A levels are limiting for one of the aldosterone-stimulated pathways. Aldosterone-induced Na\(^+\) transport was 1.8-fold greater in K-Ras2A-overexpressing cells compared with controls. We believe this suggests that in K-Ras2A-overexpressing cells after addition of aldosterone, Ras protein is no longer limiting or that it is saturated. It is possible that K-Ras2A protein is not limiting but that the processing of this protein (e.g., methylation) or the binding of K-Ras2A with GTP becomes limiting after addition of aldosterone (see Fig. 8). This thought is consistent with our recent findings that methylation mediated by isoprenylcysteine-O-carboxyl methyltransferase regulates Na\(^+\) transport and is limiting after but not before the addition of aldosterone (18).

It is unclear whether Ras has a role in maintaining the basal activity of the Na\(^+\) channel in the absence of steroid. The observation that K-ras2A antisense oligonucleotide does not greatly affect basal Na\(^+\) transport is consistent with three possibilities: 1) basal transport is independent of the Ras signaling pathway, 2) experimental limitations do not allow for the discrimination of the effects of Ras on basal Na\(^+\) transport, and 3) that other ions besides Na\(^+\) contribute substantially to basal currents.

In summary, the results reported in the current paper support the hypothesis that K-Ras2A is an aldosterone-induced protein necessary for steroid-induced Na\(^+\) transport. One final effector of activated K-Ras2A is the epithelial Na\(^+\) channel.

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