The amiloride-sensitive epithelial sodium channel (ENaC) plays a critical role in fluid and electrolyte homeostasis and consists of α, β, and γ subunits. The carboxyl terminus of each ENaC subunit contains a PPxY motif which is believed to be important for interaction with the WW domains of the ubiquitin-protein ligase, Nedd4. Disruption of this interaction, as in Liddle’s syndrome, where mutations delete or alter the PPxY motif of either the β or γ subunits, has been proposed to result in increased ENaC activity. Here we present evidence that KIAA0439 protein, a close relative of Nedd4, is also a potential regulator of ENaC. We demonstrate that KIAA0439 WW domains bind all three ENaC subunits. We show that a recombinant KIAA0439 WW domain protein acts as a dominant negative mutant that can interfere with the Na⁺-dependent feedback inhibition of ENaC in whole-cell patch clamp experiments. We propose that KIAA0439 and Nedd4 proteins either play a redundant role in ENaC regulation or function in a tissue- and/or signal-specific manner to down-regulate ENaC.

The amiloride-sensitive epithelial sodium channel (ENaC) plays a critical role in fluid and electrolyte homeostasis and is widely expressed in absorptive epithelia such as the renal collecting duct, the colon, the lung, and sweat and salivary ducts (1–4). It consists of α, β, and γ subunits, which are thought to assume a multimeric structure at the membrane (5, 6). The carboxyl terminus of each ENaC subunit contains a PPxY sequence (the PY motif), which when mutated or deleted in either the β or γ ENaC subunits leads to Liddle’s syndrome, an autosomal dominant form of hypertension (7–11). Therefore, mutating just one PY motif from a single subunit of the multimeric ENaC complex is sufficient to lead to a disease phenotype. In vitro systems, identical mutations to those that cause Liddle’s syndrome have been shown to increase amiloride-sensitive Na⁺ current (8, 11–15). This increase is believed to result from the presence of increased numbers of active Na⁺ channels in the cell membrane (12, 16–18), although an increase in channel open probability may also contribute (12, 17, 19).

The PY motifs in the carboxyl termini of ENaC subunits are believed to be necessary for interaction with the WW domains of Nedd4 (20), a widely expressed ubiquitin-protein ligase (20–24). Nedd4 has been proposed to down-regulate Na⁺ channel activity in response to increases in intracellular Na⁺ (17, 25, 26) by ubiquitination of the channel (16, 25, 27) leading to its endocytosis and degradation (16, 27, 28). The detailed mechanisms by which Nedd4 interacts with Na⁺ channels remain, however, unclear. Nedd4 consists of a ubiquitin-protein ligase domain, multiple WW domains, and a Ca²⁺ and lipid binding domain (20–22). The ubiquitin-protein ligase domain of Nedd4 is required for ubiquitination of ENaC (16, 24, 27, 28). The Ca²⁺ and lipid binding domain has been shown to mediate Ca²⁺-dependent redistribution of Nedd4 from the cytoplasm to the cell membrane (29) and has recently been demonstrated to associate in a Ca²⁺-dependent manner with annexin XIIIb, a protein concentrated in apical rafts (30).

In addition to Nedd4, there are now a number of proteins known to share a similar modular structure to Nedd4. Some of the Nedd4 family members have been implicated in ubiquitin-mediated regulation of several cellular proteins (31). The Nedd4-like protein that is most homologous to human Nedd4 is human KIAA0439, encoded by an unpublished cDNA sequence in the GenBank™ data base (accession no. AB007899). These observations prompted us to investigate whether KIAA0439 could also regulate ENaC function. Our hypothesis was further supported by our observation that a Nedd4-like Xenopus protein, claimed to be the homolog of mammalian Nedd4 (32), had been shown to regulate ENaC activity in Xenopus oocytes (28). A close examination of the homology and sequence alignment of this protein with the Nedd4-like proteins in the data base suggested that the Xenopus protein is more closely related to KIAA0439 than to Nedd4 (see below). Given that KIAA0439 and Nedd4 are highly related but distinct proteins in mammals, the results obtained by Abriel et al. (28) suggest that mammalian KIAA0439 could also play a key role in the regulation of ENaC. In this communication we present evidence that mammalian KIAA0439 and Nedd4 may have overlapping or redundant roles in the regulation of ENaC function.

**EXPERIMENTAL PROCEDURES**

**Sequence Retrieval and Homology Analysis—**Nedd4 family members in the GenBank™ data base were identified by protein and nucleotide BLAST and TBLASTN programs using the NCBI server. Multiple sequence alignments and the construction of phylogenetic trees were carried out using the Bionavigator software packages Protpars and Protml at the Australian National Genome Information Services server.
The near full-length sequence of mouse KIAA0439 cDNA was compiled from a number of EST sequence entries in the GenBankTM data base.

Expression Plasmids and Production of GST Fusion Proteins—The expression construct used to generate protein containing all three WW domains of mouse Ned4 fused to glutathione S-transferase (GST) has been described previously (25, 26). Other WW domain-GST constructs were generated by amplifying WW domain regions from cDNAs for the various Ned4 family members by PCR using appropriate primers

S.D.S.-PAGE and Far-Western Analysis—32P-labeled protein probes were produced by directly labeling the appropriate GST fusion protein using kinase A (New England Biolabs). Glutathione beads containing bound fusion protein were incubated with protein kinase A and γ-[32P]ATP in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol for 60 min at 4 °C. Beads were washed five times in phosphate-buffered saline, and labeled protein was eluted with glutathione buffer. To prepare WW domain protein filters, ~1 μg of each induced GST fusion protein lyase was resolved on S.D.S.-PAGE gels and transferred to nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked in Hyb 75 (33) and then hybridized with α, β, or γ ENaC 32P-labeled protein probes for 4 h at 4 °C in Hyb 75. Membranes were washed three times in Hyb 75 and exposed to x-ray film.

Whole-cell Patch Clamping—Isolated granular duct cells were prepared by collagenase digestion of mouse mandibular glands from male mice as described previously (34). The standard bath solution (pH 7.4) contained 145 mM NaCl, 5.5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 1 mM NaH₂PO₄, 7.5 mM HEPES, and 10 mM glucose. The standard (zero Na⁺) pipette solution (pH 7.2) contained 150 mM N-methyl-D-glucamine (NMDG)-glutamate, 1 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, and 10 mM glucose. In the 70 mM Na⁺ pipette solution, Na⁺ was adjusted by substituting Na⁺-glutamate for NMDG-glutamate. Standard whole-cell patch clamp techniques were used (25, 34). After establishing the whole-cell configuration, the bath solution was replaced with a solution containing 145 mM Na⁺-glutamate, 5 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 1 mM EGTA, and 10 mM glucose (pH 7.4). The amiloride-sensitive current was measured as described previously (25, 34). The chord conductance was measured between 80 mV and the zero current potential of the amiloride-sensitive Na⁺ current. Results are presented as the mean ± S.E. Statistical significance was assessed using Student's unpaired t test. All experiments were performed at 20–22 °C.

RESULTS AND DISCUSSION

KIAA0439 and Ned4 Are Distinct Proteins in Mammals—There are eight predicted human members of the Ned4 family of proteins in the current GenBankTM data base. A sequence comparison using multiple alignment programs indicates that the human Ned4-like proteins can be clustered into four subgroups. The first of these subgroups contains Ned4 and KIAA0439, which are highly related to each other (~78% identity) (Fig. 1A). In this phylogenetic scheme, WWP1, WWP2, and AIP-4 (human homolog of mouse Itch protein) form the second subgroup, and Smurf1 and Smurf2 form the third subgroup of Ned4-like proteins (Fig. 1A). KIAA0322 is the only protein in the fourth subgroup as it is distantly related to the other three subgroups (Fig. 1A). The first two subgroups are more related to each other than to the last two subgroups. We then compared the available sequences of Ned4 and KIAA0439 orthologs from various species to ensure that these two proteins evolved as distinct proteins in mammals. As shown in the phylogenetic tree in Fig. 1B, the Xenopus Ned4-like protein, published previously as the Xenopus homolog of Ned4 (32), is more highly related to the mouse and human KIAA0439 proteins than to the mouse, rat, and human Ned4 proteins. The current EST data base also contains partial cDNA sequences for the porcine and bovine Ned4 and KIAA0439 proteins (data not shown). Additionally, there are partial chicken ESTs in the GenBankTM (accession nos. AJ397021, BE012727, and AJ399234) that encode two distinct but closely related Ned4-like proteins that appear to represent avian Ned4 and KIAA0439 proteins. A BLAST search of the recently completed Drosophila melanogaster genomic sequence suggests that there are three Ned4-like proteins in the fly. One of these (which we call Drosophila Ned4) appears to be a common ancestor for both the Ned4 (~73% identity) and KIAA0439 (~67% identity) proteins. Because the gene cloned by Rehman and Pratt (32) is more closely related to KIAA0439 than to Ned4, it is possible that there is another protein in Xenopus that is the true ortholog of Ned4. However, so far we have not been able to clone such a protein and there are currently no Xenopus ESTs that correspond to Ned4.

KIAA0439 mRNA Is Expressed in Multiple Tissues—The Ned4 transcript and protein are widely expressed during embryonic development and in adult tissues (22, 35–37). To test the possibility that KIAA0439 may have a tissue-specific expression, we analyzed KIAA0439 mRNA expression by Northern blotting. As shown in Fig. 2, an ~4.0-kb KIAA0439 transcript was present in all tissues examined. The expression was particularly high in the liver and kidney followed by brain, heart, and lung. Longer exposures (not shown) revealed relatively low but detectable levels of transcript in spleen, skeletal

2 Primer sequences are available upon request.
Regulation of ENaC by KIAA0439

muscle, and testis. It is interesting to note that skeletal muscle, which shows strong expression of Nedd4 mRNA (22), has very low levels of KIAA0439 transcript. However, like KIAA0439, Nedd4 expression is high in liver and kidney (22). In addition to the tissues shown in Fig. 2, an analysis of the GenBank data base suggests that the KIAA0439 transcript is also present in the adult thymus, ovary, skin, urinary bladder, and mammary gland, as well as many embryonic tissues, because many of the EST clones in the data base were derived from cDNA libraries prepared from these tissues. The KIAA0439 transcript was also present in mouse mandibular gland cells (from which the mouse KIAA0439 probe was derived), which were used in the patch clamp experiments (see below).

KIAA0439 WW Domains Bind All Three Subunits of ENaC—To investigate the ability of members of the Nedd4 family of proteins to interact with ENaC subunits, a far-Western assay was used. First, the WW domains of Nedd4-like proteins (Fig. 3) were amplified by either PCR or reverse transcriptase-PCR and cloned into an appropriate pGEX vector. The majority of GST-WW domain fusion proteins, except for KIAA0439 WW domains, were present in the insoluble fraction, and therefore it was decided to use this fraction of Escherichia coli lysates for far-Western analysis. Equivalent amounts of GST-WW domain fusion proteins (as determined by SDS-PAGE and Coomassie staining) (Fig. 4A) were electrophoresed by SDS-PAGE and transferred to nitrocellulose. Filters were probed with 32P-labeled GST-ENaC subunit fusion proteins (α, β, or γ) and exposed to x-ray film. As identical results were obtained when either the human or mouse ENaC proteins were used as probes, only one set of data (using human ENaC proteins) is shown in Fig. 4. The α ENaC subunit bound with equal strength to the WW domains of human Nedd4 and KIAA0439 and only weakly to the WW domains of KIAA0322, Smurf1, WWP2/AIP2, AIP4, and Itch (Fig. 4B). Similar results were obtained when an identical filter was probed with β ENaC (Fig. 4C). γ ENaC also bound strongly to both human Nedd4 and KIAA0439 but not at all to WW domains from other Nedd4-like proteins (Fig. 4D). These results show that in vitro, ENaC subunits bind with strong affinity to the WW domains of human Nedd4 and KIAA0439 but weakly or not at all to the other Nedd4 family members tested. The number of WW domains present in these fusion proteins is unlikely to impact upon the results described here as WWP2/AIP2, AIP4, and Itch all had the same number of WW domains (four) to human Nedd4 and KIAA0439. This observation suggests that in vivo, KIAA0322, Smurf1, WWP2/AIP2, AIP4, and Itch are unlikely to target ENaC for ubiquitination. It raises the possibility, however, that like Nedd4, KIAA0439 also regulates the activity of ENaC.

WW Domains of KIAA0439 Block the Inhibitory Effect of High Na+—Using whole-cell patch clamp techniques, we have previously shown that GST fusion proteins containing all three WW domains of mouse Nedd4, together or separately, when added to the pipette solution can overcome the inhibitory effect of high Na+ on amiloride-sensitive Na+ current (25, 26). The GST-Nedd4-WW domain proteins thus act as dominant negative mutants, presumably by displacing or precluding the in-
Fig. 5. Na\(^+\) feedback inhibition of the amiloride-sensitive Na\(^+\) current and the effects of GST-KIAA0439 WW fusion protein. The whole-cell currents in salivary duct cells studied with: A, 0 mM Na\(^+\) pipette solution (n = 5); B, 70 mM Na\(^+\) pipette solution (n = 5); C, 70 mM Na\(^+\) pipette solution plus 100 \(\mu\)g/ml GST-KIAA0439 WW fusion protein (n = 6). For each set of experiments we show representative whole-cell recordings taken prior to and following the addition of 100 \(\mu\)M amiloride to the bath solution, together with the steady-state I-V relations. D, shows the steady-state I-V relations of the amiloride-sensitive current measured using each of the three pipette solutions (●, 0 mM Na\(^+\); ○, 70 mM Na\(^+\); □, 70 mM Na\(^+\) plus GST-KIAA0439 WW), calculated by subtraction of the I-V relation when the bath solution contained amiloride from the control I-V relation.

Fig. 6. A GST-KIAA0439 WW domain protein restores the inhibitory effects of high Na\(^+\) in mouse mandibular duct cells. The effects of adding either a GST-Nedd4 WW domain fusion protein or a GST-KIAA0439 WW domain fusion protein (both at 100 \(\mu\)g/ml) on chord conductance of the amiloride-sensitive Na\(^+\) current are shown. The addition of either the GST-Nedd4 WW domain protein or the GST-KIAA0439 WW domain protein completely overcomes the inhibition of amiloride-sensitive Na\(^+\) current by the inclusion of 70 mM Na\(^+\) in the pipette solution.

The interaction between ENaC subunits and endogenous Nedd4. To examine a potential role for KIAA0439 in the Na\(^+\) feedback pathway, we decided to test whether KIAA0439 WW domains would generate a similar effect. We first confirmed that the amiloride-sensitive Na\(^+\) current in mouse mandibular duct cells is inhibited by increasing the Na\(^+\) concentration in the pipette solution. As shown in Fig. 5A, when the 0 mM Na\(^+\) pipette solution was used, the addition of 100 \(\mu\)M amiloride to the bath solution inhibited an inwardly rectifying current (Fig. 5D), which we have previously shown to be Na\(^+\)-selective and to have the characteristics of the amiloride-sensitive Na\(^+\) conductance in intact salivary ducts (38). This current was not present when the 70 mM Na\(^+\) pipette solution was used (Fig. 5, B and D). The inclusion in the 70 mM Na\(^+\) pipette solution of 100 \(\mu\)g/ml GST-KIAA0439 fusion protein containing all four WW domains completely reversed the inhibitory effect of increased Na\(^+\) in the pipette solution (Fig. 5, C and D). Fig. 6 summarizes the results of the experiments in Fig. 5 together with the results of additional control experiments. From these data it is evident that the inclusion of 100 \(\mu\)g/ml GST-KIAA0439 WW domain fusion protein in the 70 mM Na\(^+\) pipette solution overcomes the inhibition of the amiloride-sensitive Na\(^+\) current. The effect of this fusion protein is similar to that produced by the inclusion of a similar concentration of GST-Nedd4 WW domain fusion protein (Fig. 6). The addition of 100 \(\mu\)g/ml GST-KIAA0439 WW domain fusion protein to the 0 mM Na\(^+\) pipette solution had no effect on the amiloride-sensitive Na\(^+\) current (Fig. 6), indicating that the effects of the fusion protein are due to inhibition of Na\(^+\) feedback inhibition rather than to a nonspecific stimulatory effect on the amiloride-sensitive conductance. Under similar conditions, control GST protein has no effect on the ENaC activity with high or low Na\(^+\) in the pipette solution (25). The effects of GST-WW domain fusion proteins are not due to nonspecific binding of the KIAA0439 WW domains to the PY motifs in ENaC subunits, as individual Nedd4 WW domains fused to GST, which can interact with ENaC PY motifs in vitro, do not influence ENaC function in patch clamp experiments (26). Furthermore, the GST-Nedd4 WW domain proteins, carrying a single mutated WW domain and two WW domains in the wild-type configuration, lose their ability to affect amiloride-sensitive ENaC current, irrespective of which of the WW domains is mutated (26). These results can be interpreted to suggest that WW domains of KIAA0439 act by displacing either the endogenous Nedd4 protein or the endogenous KIAA0439 protein. As both Nedd4 and KIAA0439 are expressed in salivary gland cells, and both proteins are able to bind ENaC subunits, either possibility may explain the effect seen in Figs. 5 and 6.
Although there are clear-cut differences in the levels of transcript in different tissues, both Nedd4 and KIAA0439 are expressed in many of the same tissues, suggesting that Nedd4 and KIAA0439 may be functionally redundant. Alternatively, Nedd4 and KIAA0439 may function in different tissues in response to different regulatory signals. Neither of these possibilities can be ruled out as yet, as all of the data currently available on the function of Nedd4 (and KIAA0439) in ENaC regulation, it will be necessary in the future to utilize a cell system in which the activity of one of the genes is ablated, for example, by gene knock-out technology.

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