Identification of Murr1 as a Regulator of the Human δ Epithelial Sodium Channel*

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The δ epithelial sodium channel (δENaC) subunit is related to the α-, β-, and γENaC subunits that control salt homeostasis. δENaC forms an amiloride-sensitive Na⁺ channel with the β and γ subunits. However, the in vivo function of δENaC is not known. To gain insight into the function of δENaC, a yeast two-hybrid screen of a human brain cDNA library was carried out using the C- and N-terminal domains of δENaC. A novel δENaC-interacting protein called Murr1 (mouse U2af1Rs1 region) was isolated in the C-terminal domain screen. Murr1 is a 21-kDa protein mutated in Bedlington terriers suffering from copper toxicosis. The interaction of Murr1 and δENaC was confirmed by glutathione S-transferase pulldown assay and communoprecipitation. To test the functional significance of the interaction, Murr1 was coexpressed with δβγENaC in Xenopus oocytes. Murr1 inhibited amiloride-sensitive sodium current in a dose-dependent manner. In addition, depletion of the last 59 amino acids of δENaC abolished the inhibition. Murr1 also bound to the β- and γENaC subunits and inhibited αβγENaC sodium current. Therefore, these results suggest that Murr1 is a novel regulator of ENaC.

The amiloride-sensitive epithelial Na⁺ channel (ENaC) is a key regulator of sodium movement across epithelia and consists of three similar subunits: α-, β-, and γENaC (1, 2). Mutations in these subunits cause loss of blood pressure control and changes in salt balance (3–8).

An additional human sodium channel subunit, δENaC, was reported in 1995 (9). Among ENaC family members δENaC has the highest amino acid identity (∼37%) to δENaC and to a recently described ε-subunit from Xenopus laevis (10). A δENaC gene appears to be present in chimpanzee (GenBank™ accession number O46547) and in rabbit (11), but there is no evidence for a rat or mouse δENaC gene. Similar to αENaC, when δENaC is expressed alone in Xenopus oocytes a small, amiloride-sensitive Na⁺ current is induced (9). This current is increased 50-fold by coexpression with the β- and γENaC subunits, and the properties of the δ and δβγ channels were identical (9). The highest expression levels of δENaC mRNA were detected in brain, testis, ovary, and pancreas, indicating that the primary function of δENaC may not be in epithelia (9).

ENaC subunits are members of the degenerin/ENaC gene family. Other family members such as brain Na⁺ channel 1, acid-sensing ion channel, and dorsal root acid-sensing ion channel are expressed in neurons of the central and peripheral nervous systems (12). These channels are stimulated by acidic pH and have been implicated in touch sensation, synaptic plasticity, and pain perception (12). A brain channel matching the properties of the δENaC channel has not been reported.

δENaC shares a common predicted topology to the α-, β-, and γENaC subunits: a large extracellular loop separated by two membrane-spanning domains leaving the short N- and C-terminal domains located inside the cell (13). The C-terminal domains of the αβγENaC subunits provide binding sites for the Nedd4 family of ubiquitin ligases. Nedd4 decreases the surface expression of ENaC, probably by mediating ubiquitination and internalization of the channel, thus controlling sodium movement across epithelia (14, 15). Nedd4 binding to the αβγENaC subunits is mediated by WW domains in Nedd4 and a conserved PY motif (PPPXY) in the C-terminal domain of the αβγENaC subunits. However, this motif is not conserved in δENaC (9), suggesting that the δENaC subunit may be regulated by binding proteins other than Nedd4 family members. Analysis of the amino acid sequence of the C- and N-terminal domains of δENaC shows that the N-terminal domain is particularly proline-rich (Fig. 1A, bottom). Short proline-rich sequences are known to bind interaction domains such as the WW or SH3 domains (16). The C-terminal domain also contains prolines that might provide a binding site for these domains. Alternatively, δENaC may contain novel binding motifs, and identification of proteins binding to novel motifs might provide information on δENaC function. Therefore, we screened a human brain cDNA library with the N- and C-terminal domains of δENaC to identify interacting proteins. Here we report that Murr1, a protein implicated in copper transport (17), interacts with the C-terminal domain of δENaC. In addition, we found that Murr1 binds to β- and γENaC but not to αENaC. Coexpression of Murr1 with either the δβγ or αβγENaC subunits in Xenopus oocytes resulted in sodium current inhibition, and the inhibition of δβγENaC was abolished by C-terminal truncation of δENaC.

EXPERIMENTAL PROCEDURES

DNA Constructs—Full-length and truncated δENaC constructs (all containing the FLAG epitope tag DYKDDDDK) were cloned into pm37 after PCR using the primers described below (all primers from 5’ to 3’).

For full-length δENaC: 5’ primer CCATCGATATGGTGAGCAAGC- AAGC and 3’ primer GGAATTCCTACTGGATCAGTGCTCCGTG- GTCCGGTGCACAGTGTAAG. The C-terminal truncations were all constructed using the same 5’ primer, ACCCGTGCAGCCACCTATGG- ACTACAAGGACGAGTGACAGAACGTCGGAGCAGCATG, and

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§ The abbreviations used are: ENaC, epithelial sodium channel; GST, glutathione S-transferase; HA, hemagglutinin; Murr1, mouse U2af1RSl region; SEAP, secreted extracellular alkaline phosphatase; SC, synthetic complete medium.
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for the following 3′ primers, CCGAATTCGAGGAGCATTCCGGG and CCGAATTCGAGGAGCATTCCGGG for 5′-23 (last 23 amino acids were deleted), CCGAATTCGAGGAGCATTCCGGG and CCGAATTCGAGGAGCATTCCGGG for 5′-57 (first 27 amino acids deleted) and CCGAATTCGAGGAGCATTCCGGG and CCGAATTCGAGGAGCATTCCGGG for 5′-41. Full-length sENaC was also cloned into the vector phM6 (Roche Applied Science) in-frame with the HA epitope tag using the 5′ primer GGAATTCGCCCACTGAGGATCCAGTTGAGGTTTGAACCCCTG and the 3′ primer GGGAATTCGACGCCACCATGGAGGCCGGGGCCA.

Because sequence analysis showed that the isolated Murr1 clones were missing the first 15 base pairs, these missing base pairs were added as part of a PCR primer in a subsequent PCR reaction. Murr1 FL-Flag and Murr1-Flag from a yeast two-hybrid library plasmid was inserted into the 5′ primer CCATCGATGCCACCATGGAGGGCGAGCTTGAGGGTG and the 3′ primer CCATCGATGCCACCATGGAGGGCGAGCTTGAGGGTG to ligate into the vector phM6. For the production of Murr1-GST fusion protein, Murr1 was cloned in full-length GST in-frame with the vector pGEX-3X (AP BioTech) using the 5′ primer CGGGATCCCACGAGGGCGAGCTTGAGGGTGGCA and the 3′ primer GATC. Bacteria were lysed using Bugbuster (Novagen), and the soluble material was removed by centrifugation (5 min, 16,000 × g), and the supernatant was incubated with 25 μg/ml anti-FLAG antibody for 3 h at 4 °C. Then 25 μl of protein G-Sepharose beads was added and incubation continued for 1 h at 4 °C. The beads were washed four times in lysis buffer and resuspended in 2× SDS sample buffer. The samples were analyzed by Western blotting using anti-HA antibody (Sigma).

Expression in Xenopus Oocytes and Immunoprecipitation—Oocytes were taken from Xenopus laevis ovaries and were handled according to the standard protocol. The isolated oocytes were injected with cRNA. The cRNA was synthesized by the in vitro transcription system using SP6 RNA polymerase. The cRNA construct for each protein was injected into oocytes at a concentration of 1–2 ng per oocyte. The oocytes were incubated for 2.5 days at 18 °C in Nectophase solution (85% seawater, 10% Ringer's solution) and were then used for electrophysiological experiments. The oocytes were bathed in Ringer's solution 118 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 25 mM HEPES, pH 7.4, and 5 mM 2-thiogalactopyranoside (2 mg/ml) for 2 h beginning 2 days before the experiment. The oocytes were then transferred into calcium-free Ringer's solution for 1 h and finally transferred to calcium-free Ringer's solution 20 min before the experiment.

RESULTS

Yeast Two-hybrid Screen—To identify proteins binding to sENaC, we performed a yeast two-hybrid screen with the intracellular C- and N-terminal domains of sENaC. Because of the high expression of sENaC in brain (9), we chose to screen a human brain cDNA library. The sENaC C-terminal domain screening yielded 40 putative positive clones showing histidine and adenine protophytrope and β-galactosidase activity; however, the N-terminal domain screen did not yield any positive clones. The sENaC C-terminal domain-interacting clones that activated the reporter genes with the original bait (sENaC C-terminal domain) were not capable of inducing expression of any reporter gene by themselves and did not interact with an unrelated bait (laminin C) (data not shown). Interacting clones were sequenced and subjected to a BLAST search. Three of the clones were identified as the human Murr1 gene. The Murr1 gene was first described by Nabetani et al. (20), who discovered that the imprinted gene U2af1-rs1 lies in an intron of the Murr1 gene, although the Murr1 gene itself does not appear to be imprinted. Thus, Murr1 was named as a gene that locates in the mouse U2af1-rs1 region. Murr1 has recently been implicated in copper metabolism because a mutated form of Murr1 is associated with copper toxicosis in Bedlington terriers (17). However, the function of Murr1 in copper metabolism is not known.

Murr1 appears to be widely expressed in human tissues,
including tissues that express δENaC (17). Therefore we investigated the possibility of an interaction between δENaC and Murr1. The Murr1 gene codes for a 21-kDa protein that does not contain any known interaction domains, suggesting the interaction with δENaC might involve novel binding motifs.

**Murr1 Interacts Specifically with the C-terminal Domain of δENaC**—Expression of δENaC and its truncations in COS7 cells resulted in the appearance of two or more specific bands, most likely because of glycosylation of the extracellular domain. This observation is consistent with the multiple bands, including tissues that express γENaC, and that the probable binding site for Murr1 is located in γENaC subunits. The PY motif residues are in bold. The interaction between amino acids 592 and 615 of δENaC (Fig. 1A) was confirmed by GST pulldown assay. Truncations from the N-terminal domains were removed. However, the inhibition was significantly less than that observed with δβγENaC + Murr1, suggesting that Murr1 may exert its inhibition in part via the β- and/or γENaC C-terminal domains. Next, we coexpressed a truncated δENaC subunit missing the last 59 amino acids (δ59, which did not bind to Murr1 in *vitro*, Fig. 2) with full-length β- and γENaC. Coexpression of Murr1 with the δβγENaC channel did not result in significant downregulation of the sodium channel (96 ± 12%, n = 27 of δβγ + SEAP). This result suggests that Murr1 binds to the C-terminal domain of δENaC to inhibit channel activity and that the contribution of the β and γ subunits to Murr1 inhibition is minimal.

To confirm that both δENaC and Murr1 were expressed in this experiment, sodium channel-expressing oocytes were collected and pooled after current measurement. Murr1 and δENaC were isolated by immunoprecipitation using anti-FLAG antibody. Fig. 5B shows that both proteins were expressed in the oocytes. Therefore, Murr1 binds to the C-terminal region of δENaC to inhibit sodium current.
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**Murr1 Interacts with β- and γENaC in Vitro**—Although Murr1 still inhibited current when the C-terminal domains of the β- and γENaC subunits were removed, (Fig. 5, δβγENaC + Murr1), the decrease was significantly less than that observed with Murr1 inhibition of δβγENaC. Therefore, we investigated whether the α-, β-, or γENaC subunits also interact with Murr1, using a GST pulldown assay. α-, β-, or γENaC, either HA- or FLAG-tagged, were expressed in COS7 cells for 24 h, and the lysates were incubated with Murr1-GST or GST alone. Fig. 6A shows that β- and γENaC, but not αENaC, bind to Murr1-GST in vitro.

Because the β- and γENaC subunits bind Murr1, we tested whether Murr1 could regulate a channel formed by the α-, β-, and γENaC subunits. Fig. 6B shows that Murr1 inhibited αβγENaC current by 53 ± 7% (n = 18). Therefore, Murr1 is able to regulate sodium channels formed by either δβγ or αβγ ENaC subunits.

**DISCUSSION**

In contrast to the relatively well-characterized αβγENaC subunits, the δENaC subunit has been poorly investigated. The most apparent difference between δENaC and αβγENaC is tissue distribution. Whereas the α, β, and γ subunits are predominantly expressed in epithelial tissues such as kidney, colon, or lung (1, 2), δENaC expression is highest in brain, testis, ovary, and pancreas (9). Although δENaC is expressed at low levels in kidney tissue (9), it is unlikely that δENaC can compensate for αENaC, because loss of function mutations in αENaC cause the salt-wasting disorder, pseudohypoaldosteronism type 1 (3). This observation suggests that in the kidney, δENaC is not expressed at a high enough level to compensate for αENaC, that αENaC and δENaC are expressed in different cell types, or that channels formed by αENaC and δENaC are functionally unique and perhaps regulated by different cellular pathways.

To begin to investigate the function and regulation of δENaC we performed a yeast two-hybrid screen with the N- and C-terminal domains of δENaC to isolate δENaC binding partners. Such binding partners might represent novel regulators or subunits of a δENaC channel. Murr1, a gene implicated in copper metabolism (17), was identified as a novel δENaC binding protein.

The Murr1 gene is mutated in inherited autosomal recessive copper toxicosis in Bedlington terriers. Affected dogs showed a
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FIG. 5. Murr1 mediates inhibition via the δENaC C-terminal domain. A, amiloride-sensitive Na+ current for Murr1-FLAG (70 pg) or SEAP (237 pg) transiently expressed in X. oocytes with δβγENaC, δβγγENaC, or δβγENaC (237 pg each subunit). Currents were recorded at −60 mV and normalized to 1. Bars represent means ± S.E. (n = 17–27). * indicates a statistically significant difference (p < 0.001) from the SEAP control. ** indicates a statistically significant difference (p < 0.001) of δβγ-FLAGγ + Murr1-FLAG from δβγ + Murr1-FLAG and δβγγ + Murr1-FLAG. *** indicates statistical significant difference (p < 0.001) of δβγ + Murr1-FLAG from δβγγ + Murr1-FLAG. B, Western blot with anti-FLAG antibody of δFLAG (upper panel) and Murr1-FLAG (lower panel) immunoprecipitated with anti-FLAG antibody from X. oocytes expressing δFLAGγ + Murr1-FLAG, δβγENaC + Murr1-FLAG, and δβγγ + Murr1-FLAG or un.injected oocytes (lane 1). The δENaC subunit was not FLAG-tagged. L denotes antibody, light chain; H denotes antibody, heavy chain.

FIG. 6. In vitro binding of Murr1 to δβγENaC and downregulation of αβγENaC by Murr1. A, Western blot analysis of δ, α-, β-, and γENaC expressed in COS7 cells and bound with Murr1-GST fusion protein (lanes labeled Murr1) or GST alone. The lane labeled lys shows successful expression of each construct in COS7 cells (arrows); GST lanes indicate that none of the expressed proteins bound to GST fusion protein alone. The upper bands in the βENaC and γENaC Murr1 lanes represent nonspecific binding of the anti-HA antibody. B, amiloride-sensitive Na+ current for αβγENaC (237 pg each) transiently expressed in X. oocytes with Murr1-FLAG (70 pg) or SEAP (237 pg) as a negative control. Currents were recorded at −60 mV and normalized to 1. Bars represent means ± S.E. (n = 15–18). * indicates a statistically significant difference (p < 0.001) from the SEAP control.

Two-electrode voltage-clamp studies in X. oocytes revealed that Murr1 is a potent inhibitor of δβγENaC sodium current. This effect might be because Murr1 alters the trafficking of subunits to or from the plasma membrane, it might have a direct effect on channel gating, or it might be an adaptor linking the channel to another regulatory protein. ENaC subunit trafficking is known to be influenced by syntaxins (25, 26), which decrease surface expression of ENaC presumably by interfering with its insertion into the membrane (27), and Nedd4 family members, which facilitate ubiquitination and internalization of ENaC (28).

When the C-terminal domains of β- and γENaC were deleted, Murr1 still inhibited the current produced by the δβγγENaC channel, though this current was significantly larger than currents obtained with δβγENaC + Murr1. These results suggest that Murr1 exerts its inhibitory effect primarily via the δENaC subunit. This hypothesis was confirmed by deleting the C-terminal domain of δENaC; Murr1 was unable to inhibit currents obtained with δβγENaC + Murr1.

The function of Murr1 remains unknown because the protein shows no homology to other proteins and does not contain any identifiable amino acid motifs or domains.

Using immunoprecipitation and pulldown data, we have demonstrated that Murr1 interacts with the C-terminal domain of δENaC. Using N- and C-terminal truncations of δENaC, the binding site for Murr1 was located between amino acids 592 and 615 of δENaC. The C-terminal domain of δENaC is only 25% identical to that of αENaC (Fig. 1B), and the PY motif found in the αβγENaC subunits that mediates interaction with Nedd4 family members is not conserved in δENaC. Murr1 does not contain any recognizable protein interaction motifs; thus further work will be required to identify the exact amino acids involved in the interaction of δENaC and Murr1.

homozygous deletion of exon 2 of the Murr1 gene (17). Copper toxicity is characterized by inefficient excretion of copper into the bile, resulting in accumulation of copper in the liver (21). In affected dogs excess copper remains in the lysosomes (22), perhaps because of a defect in lysosomal vesicle trafficking to the bile canalicular membrane. These findings suggest that Murr1 might be involved in ion transport and/or in vesicle trafficking. In humans, the majority of copper toxicity patients have Wilson’s disease. The genetic defect for this disease has been localized to the ATP7B gene, which encodes a P-type ATPase (21). Recently Tao et al. (23) demonstrated that Murr1 interacts directly with the intracellular N-terminal domain of the Wilson disease gene ATP7B, indicating a link between Murr1 and ATP7B. However, investigation of 23 patients with non-Wilsonian hepatic copper toxicity did not identify any mutations or polymorphisms in the Murr1 gene (24). The function of Murr1 remains unknown because the protein shows no homology to other proteins and does not contain any identifiable amino acid motifs or domains.

Using immunoprecipitation and pulldown data, we have demonstrated that Murr1 interacts with the C-terminal domain of δENaC. Using N- and C-terminal truncations of δENaC, the binding site for Murr1 was located between amino acids 592 and 615 of δENaC. The C-terminal domain of δENaC is only 25% identical to that of αENaC (Fig. 1B), and the PY motif found in the αβγENaC subunits that mediates interaction with Nedd4 family members is not conserved in δENaC. Murr1 does not contain any recognizable protein interaction motifs; thus further work will be required to identify the exact amino acids involved in the interaction of δENaC and Murr1.
to inhibit δβγ channel function. However, the β- and γENaC
subunits might also be involved in Murr1-mediated δβγENaC
channel regulation because Murr1 bound both subunits in a
pulldown assay.

Murr1 also reduced αβγENaC sodium current. The effect of
Murr1 on the αβγENaC current was less than that observed for
δβγENaC, possibly because Murr1 does not bind to αENaC.
These results suggest that Murr1 may be a general regulator of
ENaC family members and potentially of other channels or
transporters.

A link between sodium and copper transport has been demon-
strated in fish gill and intestinal epithelia, including evidence for
copper leak through ENaC (29). Although neither Murr1 nor
dENaC appears to contain consensus copper binding sites (30,
31), it is possible that Murr1 links the transport of sodium and
copper across epithelia. Alternatively, Murr1 might be important
for correct trafficking of both sodium channels and copper trans-
porters. Both the ATP7A and ATP7B copper-transporting pro-
teins are localized to the trans-Golgi network in low copper
concentrations. Increasing the copper concentration causes a shift
in the location of ATP7A and ATP7B to the plasma membrane or a
vesicular population (1, 32).

Further experiments such as cellular colocalization, effect on
dENaC trafficking, and single-channel patch clamp analysis
will be necessary to elucidate the mechanism by which Murr1
inhibits both δβγENaC and αβγENaC channel function.

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