Ubiquitin-protein ligase WWP2 binds to and downregulates the epithelial Na\(^+\) channel

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THE EPITHELIAL Na\(^+\) CHANNEL (ENaC) is a key regulator of Na\(^+\) movement across a number of epithelia, such as kidney collecting duct, colon, and lung (9). The channel complex is composed of three homologous subunits: \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC (3, 4, 18, 19). Disruption of ENaC through mutation or misregulation results in loss of blood pressure control and changes in salt balance. Liddle’s syndrome, an inherited form of hypertension, is caused by dominant gain of function mutations in the COOH-terminal region of the \(\beta\) - or \(\gamma\)-Na\(^+\) channel subunit gene, whereas loss of function mutations in any of the subunit genes cause pseudohypoaldosteronism type I, a neonatal salt-wasting disorder (reviewed in Ref. 16). Therefore, ENaC functions to maintain the body’s salt and water content and to regulate blood pressure.

A major control mechanism for determining Na\(^+\) channel activity is modulation of the number of channels present at the plasma membrane. All three Na\(^+\) channel subunits contain a conserved amino acid motif in their COOH termini (PPPXY) that is mutated or deleted in Liddle’s syndrome. This PY motif mediates binding to the WW domains of Nedd4 and Nedd4–2, which decrease Na\(^+\) current by means of ENaC ubiquitination and degradation (1, 7, 10, 14, 27, 29).

Nedd4 and Nedd4–2 are members of a family of E3 ubiquitin-protein ligases that share a similar domain structure. Family members are characterized by the presence of a catalytic HECT (homologous to E6-AP COOH terminus) domain that facilitates ubiquitin attachment to substrate proteins. In addition, they contain multiple WW domains that mediate binding to PY motifs, and many contain an NH\(_2\)-terminal C2 (calcium/lipid binding) domain that may be important for localization (reviewed in Ref. 12). Although previous work found that Nedd4 and Nedd4–2 bind to and inhibit ENaC in heterologous expression systems (7, 11, 14, 26, 27), it is not known whether they regulate ENaC in native epithelia. Moreover, it is possible that additional family members may regulate epithelial Na\(^+\) absorption. A potential candidate is WW domain-containing protein 2 (WWP2), an E3 ubiquitin ligase that shares a similar domain structure to Nedd4 and Nedd4–2, including an NH\(_2\)-terminal C2 domain, four WW domains, and a COOH-terminal HECT domain (Fig. 1). WWP2 shares ~45% amino acid identity with both human Nedd4 (hNedd4) and hNedd4–2, which are in turn ~62% identical to each other. In this work, we test the hypothesis that WWP2 binds to ENaC and alters Na\(^+\) absorption.

METHODS

Molecular biology. A full-length WWP2 cDNA and a partial hNedd4–2 cDNA were provided by Dr. G. Pirozzi (20).

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hNedd4–2 was found to correspond to the GenBank entry KIAA0439; therefore, a full-length hNedd4–2 cDNA was PCR amplified from human kidney cDNA (Clontech, Palo Alto, CA) on the basis of the KIAA0439 sequence. To prepare glutathione S-transferase (GST) fusion proteins containing individual WW domains of WWP2 and hNedd4–2, pairs of primers surrounding each WW domain were used in standard PCR reactions. PCR fragments were cloned into the pGEX2TK or pGEXKG vector. Fusion proteins were induced with 1 mM isopropyl β-D-pyranoside for 2–3 h, and fusion proteins were isolated from sonicated bacterial lysates by using glutathione-Sepharose beads (Amersham-Phar- macia Biotech, Buckinghamshire, UK). DNA sequences were confirmed with Big Dye terminator sequencing and analysis on an ABI 377 sequencer.

**Northern blot analysis.** A human mRNA master blot was obtained from Clontech. A 1.3-kb BamHI-SalI fragment from the 5′ region of WWP2 and a 0.9-kb SalI-EcoRI hNedd4–2 fragment were used for sequential binding to the master blot, with the blot being stripped between hybridizations. The probes were 32P labeled by random priming according to the manufacturer’s directions (Roche Molecular Biochemicals, Mannheim, Germany). The blot was hybridized overnight in Church’s buffer (0.5 M Na2HPO4, 1% BSA, 1 mM EDTA, pH 8.0, 7% SDS) (5), washed extensively in 0.5 × standard sodium citrate/1% SDS, and exposed to Kodak Biomax film.

**Cell culture, transfection, and binding assays.** COS-7 cells were maintained in low-bicarbonate DMEM media supplemented with 10% FCS, and antibiotics and cells were grown in 5% CO2.

Binding assays were carried out as described (7). Briefly, COS-7 cells were transfected with cDNA constructs by using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The cDNAs encoding Na+ channel subunits contained the FLAG epitope (DYKDDDDK) and were cloned into either pcDNA3 or pMT3 as described (28). A γ-subunit containing a hemagglutinin (HA) tag (as described in Ref. 2) was used in one experiment (see Fig. 3B). A β-subunit containing the Y620A mutation also contained a FLAG epitope inserted immediately after the most COOH-terminal residue and was subcloned into pMT3. Approximately 22 h after transfection, the cells were washed in PBS and lysed in 0.5 ml of buffer (150 mM NaCl, 50 mM Tris, pH 7.4) containing (in μg/ml) 10 phenylmethylsulfonly fluoride, 2 aprotinin, 2 leupeptin, and 1 pepstatin A, as well as 1% Triton X-100, for 1–2 h at 4°C. After centrifugation at 12,000 rpm for 5 min, the lysates were sequentially precleared with glutathione-Sepharose beads and then GST attached to glutathione-Sepharose beads. Next, precleared lysates were incubated with specific WW domain-containing GST fusion proteins and washed extensively, and bound proteins were separated by electrophoresis through 8% polyacrylamide gels. After Western blot transfer to nitrocellulose (Schleicher & Schuell), membranes were incubated with either 1.4 μg/ml anti-FLAG M2 and then anti-mouse horseradish peroxidase (both from Sigma, St Louis, MO) or with 7.5 μM/ml anti-HA horseradish peroxidase (3F10, Roche Molecular Biochemicals). Detection was achieved by enhanced chemiluminescence.

**Expression in Fischer rat thyroid cells and electrophysiology.** We expressed ENaC with WWP2 in Fischer rat thyroid (FRT) epithelia as previously described (25). FRT cells were grown on permeable filter supports (0.4 μm pore size, 12 mm diameter; Millicell PCF) in F12-10 Con’s media (Harlan) with 5% fetal calf serum (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C. One day after seeding, cells were cotransfected with α-, β-, and γ-ENaC (0.07 μg each), and WWP2, WWP2–ΔC2 (a WWP2 construct lacking the C2 domain), or hNedd4–2 (0.02–0.8 μg). To disrupt binding to the WWP2 WW domains, the PY motifs of each ENaC subunit were mutated to P2LP6 motifs (27). Total DNA was held constant (1 μg/Millicell) using cDNA-encoding green fluorescent protein. The plasmids were mixed with TFX 50 (7.9 μg/Millicell; Promega, Madison, WI) in 360 μl/Millicell serum-free F-12 Con’s media for 15 min and transferred to the apical surface of the monolayer. One hour later, the apical media was replaced with F-12 Con’s media containing 5% fetal calf serum and amiloride (10 μM).

Two days after transfection, we measured transepithelial Na+ transport in modified Ussing chambers (Warner Instrument). The apical and basolateral surfaces were bathed in (in mM) 135 NaCl, 1.2 CaCl2, 1.2 MgCl2, 2.4 K2HPO4, 0.6 KH2PO4, and 10 HEPES (pH 7.4) at 37°C and bubbled with O2. Amiloride-sensitive short-circuit current was determined by the difference in current with and without amiloride (10 μM) in the apical bathing solution.

**RESULTS**

**WWP2 is widely expressed.** To determine whether WWP2 is expressed in the same tissues as ENaC, Northern blot analysis was performed. We found that WWP2 was widely expressed in both adult and embryonic human tissues (Fig. 2A). WWP2 was expressed in epithelia that express ENaC, including kidney, lung, and colon. Thus WWP2 is expressed in appropriate locations to regulate ENaC function. However, given the large number of tissues expressing WWP2, including multiple regions of the brain, fetal organs such as kidney and lung, endocrine glands, and reproductive organs, it is likely that WWP2 binds to and regulates additional proteins. As a comparison, we investigated the expression pattern of hNedd4–2. We found that hNedd4–2 and WWP2 were expressed in most of the same tissues (Fig. 2B). Thus, similar to WWP2, it is likely that hNedd4–2 regulates proteins in addition to ENaC. The extensive tissue expression of WWP2 and hNedd4–2 is similar to that observed for hNedd4 (7), although some differences in the relative expression levels were observed, for example, a higher relative level of WWP2 compared with hNedd4–2 in adrenal gland and aorta.

**WW domains of WWP2 and hNedd4–2 bind to ENaC subunits.** To test whether individual WWP2 WW domains bind to α-, β-, and γ-ENaC, in vitro binding experiments were carried out. GST fusion proteins containing a single WWP2 WW domain were mixed with lysate from COS-7 cells expressing one of the ENaC subunits (containing a FLAG epitope), and in-
The results show that WWP2 WW domains bound selectively to the Na\(^+\) channel subunits. WW domain 1 of WWP2 did not bind to α-, β-, or γ-ENaC, in contrast to WW domains 2 and 3, which bound to all three subunits. Interestingly, WW domain 4 bound to β-ENaC but not to γ-ENaC. GST alone did not bind to any ENaC subunit, indicating that the WW domain-ENaC interactions were specific. In addition, none of the WWP2 WW domains bound to the ENaC subunit that contained a mutation in the PY motif (Y620A). Thus the PY motif mediates the binding of WWP2 to ENaC. As a comparison, we also tested the ability of the four hNedd4–2 WW domains to individually bind to Na\(^+\)/H\(^+\) channel subunits. Contrary to our results for WWP2 and our previous work with hNedd4 (7), all four hNedd4–2 WW domains bound to α-, β-, and γ-ENaC (Fig. 3B). The four WW domains of WWP2, hNedd4, and hNedd4–2 are all classified as type I WW domains, which are predicted to bind PY motifs (15). Thus additional sequence determinants must also contribute to the binding between specific WW domains and PY motifs.

WWP2 inhibits ENaC. We tested the hypothesis that the binding of WWP2 to ENaC alters Na\(^+\)/H\(^+\) current. Expression of α-, β-, and γ-ENaC in FRT epithelia generated transepithelial short-circuit Na\(^+\)/H\(^+\) current that was inhibited by amiloride (25). When coexpressed with ENaC, WWP2 produced a dose-dependent decrease in Na\(^+\) current (Fig. 4A). We found similar results in Xenopus laevis oocytes (not shown). Thus WWP2 inhibits ENaC-mediated Na\(^+\) currents.

WWP2 contains a C2 domain at the NH\(_2\) terminus. To test its functional requirement, we expressed WWP2–C2. We found that WWP2–C2 decreased Na\(^+\)/H\(^+\) current more potently than wild-type WWP2 but less than hNedd4–2, which lacks a C2 domain (Fig. 4A). Thus the C2 domain reduces the ability of WWP2 to inhibit ENaC.

To test whether inhibition requires the binding of WWP2 to ENaC, we mutated the PY motifs of α-, β-, and γ-ENaC, which form the binding sites for the WWP2 WW domains. For this experiment, we used WWP2–ΔC2, because it inhibited ENaC more potently than wild-type WWP2 but less than hNedd4–2, which lacks a C2 domain (Fig. 4A). Thus the C2 domain reduces the ability of WWP2 to inhibit ENaC.

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DISCUSSION

ENaC is located in the apical membrane of specialized epithelial cells. The Na\(^+\) channel is ubiquitinated (30), and residues constituting a WW domain binding sequence (PY motif) are necessary for correct internalization and degradation of the channel (10). Loss or
mutation of the WW binding domain, seen in Liddle’s syndrome patients, prevents binding of ubiquitin-protein ligases to ENaC subunits. By disrupting channel ubiquitination, the half-life of ENaC at the plasma membrane is extended, resulting in excessive Na⁺ re-absorption and hypertension.

It is uncertain which ubiquitin-protein ligase(s) regulate ENaC surface expression in vivo. Previous work has focused on two members of this family, Nedd4 and Nedd4–2, both of which bind and down-regulate ENaC in heterologous cells (1, 7, 10, 14, 27, 29). However, it has recently become clear that these proteins are part of a large family of related ubiquitin-protein ligases (11, 14, 20), suggesting that additional family members might regulate ENaC function. Here, we provide the first evidence that WWP2 is a candidate to regulate ENaC; WWP2 mRNA was present in Na⁺-transporting epithelia, WWP2 bound to Na⁺ channel subunits by means of its WW domains, and it inhibited epithelial Na⁺ absorption.

WWP2 contains four WW domains. Although previous work suggested that WWP2 might bind ENaC by means of its WW domains (11, 20), no analysis of the effect of WWP2 on ENaC activity has been reported. Our binding studies showed that specific WWP2 WW domains bound to ENaC subunits. WW domains 2 and 3 bound to all three ENaC subunits, whereas WW domain 4 bound to α- and β- but not γ-ENaC. In contrast, WW domain 1 did not bind to any ENaC subunit. This binding pattern showed important differences from those of hNedd4 and hNedd4–2. First, all three ENaC subunits bound to WW domains 2, 3, and 4 of hNedd4 and hNedd4–2 (Fig. 3B and Refs. 7 and 26). Second, we found that hNedd4–2 WW domain 1 also bound to the ENaC subunits, in contrast to WW domain 1 of WWP2 and hNedd4, which did not bind (Fig. 3A and Refs. 7 and 26). Thus we have demonstrated differential binding between ENaC and WW domains of the three ubiquitin ligases that might underlie functional differences in their ability to inhibit ENaC. For example, binding of all four hNedd4–2 WW domains to ENaC subunits might partly explain the observation that hNedd4–2 inhibits Na⁺ current more potently than either WWP2 or hNedd4 (Fig. 4A and Ref. 27).

The differences in amino acid sequence that allow WW domain 1 of hNedd4–2 (but not WWP2 or hNedd4) to bind to ENaC are not known. Such differences might occur in residues that bind to ENaC, or they might disrupt the overall conformation or charge of the domain. WW domain 1 of WWP2 is 45% identical in amino acid sequence to WW domain 1 of both hNedd4 and hNedd4–2, whereas WW domain 1 of hNedd4 and hNedd4–2 are more similar to each other (63% identity). We recently identified an arginine residue
Although the hNedd4 action more potently when the C2 domain was deleted. Increased ENaC inhibition by hNedd4. Similar to activity (26). In fact, removal of the C2 domain inrequired for hNedd4 to mediate inhibition of ENaC.

Our Northern blot analysis showed that WWP2 is expressed in a variety of tissues not known to express ENaC, suggesting that WWP2 is likely to interact with additional proteins. Consistent with this hypothesis, atrophin-1 and LMP2A, a latent membrane protein from Epstein-Barr virus infected cells, contain PY motifs that bind to WWP2 (13, 31). WWP2 also binds to a PY motif in the CIC-5 chloride channel, and a dominant-negative WWP2 mutant increased CIC-5 surface expression and Cl− current (24). Thus it seems likely that WWP2 regulates the surface expression of proteins in addition to ENaC.

The function of the C2 domain in WWP2 and related ubiquitin-protein ligases is uncertain. Previous work suggested that the Nedd4 C2 domain may mediate its movement to the plasma membrane in the presence of elevated Ca2+ and through an interaction with annexin XIIb (21, 22). However, the C2 domain was not required for hNedd4 to mediate inhibition of ENaC activity (26). In fact, removal of the C2 domain increased ENaC inhibition by hNedd4. Similar to hNedd4, we found that WWP2 inhibited Na+ absorption more potently when the C2 domain was deleted. Although the hNedd4−2 protein does not appear to contain a C2 domain (Fig. 1), the cDNA contains a sequence encoding a potential C2 domain upstream of the start codon. It is possible that additional forms of hNedd4−2 containing this C2 domain may exist. Thus an emerging theme is that the C2 domain represses the function of ubiquitin-protein ligases. In future studies, it will be important to define the mechanisms involved and the potential role of this domain in the Ca2+-dependent regulation of ENaC.

Our work and previous studies suggest that three related human E3 ubiquitin-protein ligases (WWP2, hNedd4, and hNedd4−2) are candidates to regulate ENaC. However, the relative role of each is unclear. Although it is possible that the three ubiquitin-protein ligases are functionally redundant, several findings suggest that they might have distinct functions. First, the differential binding of their WW domains to ENaC subunits suggests that they may regulate ENaC through different mechanisms. For example, WW domain 1 of WWP2 and hNedd4 (which do not bind ENaC) may form binding sites to recruit other adaptor or signaling proteins into the complex. Second, some ubiquitin-protein ligases have a C2 domain but others do not [e.g., Nedd4−2 and splice forms of WWP1 (8)]. This might result in differences in their Ca2+-dependent regulation or localization. Third, the three ubiquitin-protein ligases differ in their ability to be phosphorylated by serum and glucocorticoid-regulated kinase (SGK), a serine/threonine kinase that regulates ENaC downstream of aldosterone. SGK phosphorylates hNedd4−2 and reduces its binding to ENaC, providing a molecular pathway to explain the rapid aldosterone-mediated upregulation of the Na+ channel (6, 27). In contrast, SGK does not phosphorylate hNedd4, because it lacks SGK phosphorylation sites (27). Interestingly, WWP2 also lacks such phosphorylation sites, suggesting that similar to hNedd4, it is not a direct target for SGK regulation.

In Na+−transporting epithelia, WWP2 and related ubiquitin-protein ligases may reside in distinct subcellular locations or in different cell types. Moreover, they might be modulated by different mechanisms to provide unique ENaC regulatory mechanisms. Thus multiple ubiquitin-protein ligases might work in concert to regulate epithelial Na+ absorption. However, each of these mechanisms would be defective in Liddle’s syndrome, because the PY motifs to which they bind are deleted or mutated. Further work to uncover the mechanisms by which WWP2, hNedd4, and hNedd4−2 individually regulate ENaC will be necessary to understand the role of ENaC in both normal and disease conditions.

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