Regulation of the Epithelial Sodium Channel by N4WBP5A, a Novel Nedd4/Nedd4-2-interacting Protein*

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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, lung, and sweat and salivary ducts (1–3). ENaC is composed of three homologous subunits termed α, β, and γ that contain two transmembrane domains, a large extracellular loop, and short intracellular amino and carboxyl termini. The carboxyl terminus of each ENaC subunit contains a PY motif/WW domain interaction and appears to be associated with specific intracellular vesicles, we propose that N4WBP5A functions by regulating Nedd4/Nedd4-2 availability and trafficking. Because N4WBP5A is highly expressed in native renal collecting duct and other tissues that express ENaC, it is a likely candidate to modulate ENaC function in vivo.

The apically localized 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, lung, and sweat and salivary ducts (1–3). ENaC is composed of three homologous subunits termed α, β, and γ that contain two transmembrane domains, a large extracellular loop, and short intracellular amino and carboxyl termini. The carboxyl terminus of each ENaC subunit contains a PY motif (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 (4–6). Therefore, mutating just one PY motif from a single subunit of the multimeric ENaC complex is sufficient to lead to a disease phenotype. Studies in the Xenopus oocyte system show that the mutations in ENaC subunits, similar to those that cause Liddle’s syndrome, result in increased amiloride-sensitive Na+ current (5–10). This increase is mostly attributed to the presence of increased numbers of active Na+ channels in the cell membrane, although an increase in channel open probability (Po) may also be a contributory factor (7, 10–12).

The PY motifs in the carboxyl termini of ENaC subunits have been shown to interact with the WW domains of the two highly related ubiquitin-protein ligases, Nedd4 and Nedd4-2. Disruption of this interaction, as in Liddle’s syndrome where mutations delete or alter the PPX motif of either the β or γ subunits, has been shown to result in increased ENaC activity and arterial hypertension. Here we present evidence that N4WBP5A, a novel Nedd4/Nedd4-2-binding protein, is a potential regulator of ENaC. In Xenopus laevis oocytes N4WBP5A increases surface expression of ENaC by reducing the rate of ENaC retrieval. We further demonstrate that N4WBP5A prevents sodium feedback inhibition of ENaC similar to those that cause Liddle’s syndrome, resulting in increased amiloride-sensitive Na+ current (5–10).

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‡‡§§ The abbreviations used are: ENaC, amiloride-sensitive epithelial sodium channel; rENaC, rat ENaC; RLU, relative light units; PY motif, PPX motif; MBS, modified Bar’s saline; GST, glutathione S-transferase; BFA, brefeldin A; Nedd4, neuronal precursor cells expressed developmentally down-regulated protein 4; N4WBP5A, Nedd4 WW motif-binding protein 5A; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HA, hemagglutinin; TM, transmembrane; CFTR, cystic fibrosis transmembrane conductance regulator.

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the amino-terminal domain and three putative transmembrane domains in the carboxyl-terminal half of the protein (29). A closely related protein identified in data base searches, which we have named N4WBP5A, also contains two PY motifs and the three transmembrane domains. We show here that, similar to N4WB5P, N4WBP5A binds to Nedd4. Because Nedd4 is implicated in ENaC control, we were interested to test whether N4WBP5A has a role in Nedd4-mediated ENaC regulation. We report that N4WBP5A is highly expressed in collecting duct cells of the kidney and other tissues that express ENaC. We show that N4WBP5A interacts with Nedd4-2, in addition to Nedd4, and this interaction is mediated via PY motif/WW domain interactions. Most importantly, we demonstrate that in Xenopus oocytes, N4WBP5A has the ability to enhance ENaC surface expression by interfering with the Nedd4/Nedd4-2-mediated regulation of ENaC.

EXPERIMENTAL PROCEDURES

Sequence Analysis—Hydropathy plot (Kyte and Doolittle) of the N4WBP5A protein was estimated using the ProtScale Software at ExPASy Molecular Biology Server (SIB), and transmembrane (TM) domains were predicted using the TMHMM version 2.0 software (Center for Biological Sequence Analysis, Denmark).

Plasmids and cDNA Constructs—The coding region of N4WBP5A was amplified by reverse transcriptase-PCR, with a carboxyl-terminal FLAG tag, and cloned into the EcoRI and HindIII sites of pCDNA3 (Invitrogen) to generate pCDNA3-N4WBP5A-FLAG. The generation of pCXN2-Nedd4 has been described previously (29). pcDNA3-Nedd4-2-FLAG was generated by PCR amplification of the mouse Nedd4-2 open reading frame with a FLAG tag engineered at the amino terminus and cloned into the EcoRI and XhoI sites of pCDNA3 (Invitrogen). PCR mutagenesis was used to create N4WBP5A-5Y by altering tyrosine residues 56 and 82 to alanine.

The N4WBP5A-GST bacterial expression construct was generated by PCR amplification of the N4WBP5A cDNA encoding the amino-terminal 135 residues, lacking the initiation methionine, and cloned into pGEX-2TK (Amersham Biosciences) such that the N4WBP5A sequence was fused to the carboxyl-terminal of GST. The Nedd4-2-GST bacterial expression construct was generated by PCR amplification of a region of Nedd4-2, residues 110–226, located between WW domains 1 and 2 and cloned into the BamHI/EcoRI sites of pGEX-2TK such that the Nedd4-2 sequence was fused to the carboxyl-terminal of GST. Single WW-domain GST constructs were generated by PCR amplification of each WW domain followed by cloning into either the EcoRI or BamHI/EcoRI sites of pGEX-2TK (Amersham Biosciences). PCR mutagenesis was used to mutate each WW domain such that the second conserved tryptophan was altered to phenylalanine and the conserved proline altered to alanine. The expression construct containing all four WW-domain GST was generated by PCR amplification and cloning into the EcoRI site of pGEX-2TK.

The three subunits of wild-type rat ENaC (rENaC) (30) or of β3δENaC rENaC (β3δENaC) (30) or of β3δENaC mRENaC (β3δmRENaC) (31) were expressed in oocytes as described previously (21). Oocytes were brought to room temperature, rehydrated in 1× PBS for 5 min, and then blocked for 30 min with 5% normal goat serum in PBS with 1% bovine serum albumin (BSA). The blocking solution was replaced, then rehydrated in 1× PBS, fixed in 4% paraformaldehyde, and permeabilized with 1% Triton X-100. Oocytes were incubated for 1 h at room temperature with an affinity-purified anti-FLAG antibody (32). Oocytes were then washed and mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Negative control experiments were carried out by preincubating the N4WBP5A antisem with an excess of an antigenic peptide, by using the preimmune serum and by omitting the primary antibody. Immunofluorescence was visualized using a Zeiss Axioplan 2 microscope with 20–100× objectives. Images were acquired using a Hamamatsu digital camera and processed using the software package KS300 version 3.0 (Carl Zeiss Ltd., Welwyn Garden City, UK).

Isolation of Oocytes and Injection of cRNA—Xenopus laevis oocytes were prepared and injected as described (32, 33). Defolliculated oocytes were injected with various cRNA combinations. For each ENaC subunit or Kir1.1a channel, 1 ng of cRNA was used, whereas 20 ng of cRNA were used for N4WBP5A, Nedd4-2, and CFTR. Injected oocytes were routinely kept in “high sodium” modified Barth’s saline (88 mM NaCl, 2.4 mM KCl, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 mM Hepes, 3 mM glucose, 1 mM HEPES (Tris) containing 4% (v/v) dimethyl sulfoxide (DMSO) or alternatively in “low sodium” modified Barth’s saline (1 mM NaCl, 40 mM KCl, 60 mM N-methylgluconamine, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 mM Hepes, adjusted to pH 7.6 with HCl).

Two-electrode Voltage Clamp Experiments—Unless stated otherwise, oocytes were studied 2 days after injection using the two-electrode voltage clamp technique as described previously (32, 33). Oocytes were

Northern Blot Analysis—A full-length mouse N4WBP5A cDNA was used to probe a multiple tissue Northern (MTN) blot containing poly(A)+ RNA from mouse tissues (CLONTECH). A β-actin cDNA probe (CLONTECH) was used as a loading control probe.

Lines M-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, and HEK 293T cells were grown in RPMI 1640, 10% fetal calf serum at 37 °C with 5% CO2. Transfections were performed using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. For co-transfection of HEK 293T cells 1 μg of each plasmid was used.

Immunoprecipitations and Far Western Analysis—Immunoprecipitations of HEK 293T cells were carried out the day after transfection, and M-1 cells were seeded at a density of 3 × 106 cells/100-mm dish, and immunoprecipitations were carried out when cells reached confluency. Immunoprecipitations were performed by harvesting cells in lysis buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM EDTA, Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals)), preclearing lysates with protein G-Sepharose (Amersham Biosciences) for 2 h, incubating lysates overnight at 4 °C with the appropriate antibody (5 μg/ml), and then for 2 h at 4 °C with protein G-Sepharose. Immunoprecipitates were washed two times in lysis buffer and once in PBS, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride. The following primary antibodies were used: anti-FLAG M2 monoclonal antibody (1:1000, Sigma, anti-Nedd4-2 (0.25 μg/ml), and anti-Nedd4 monoclonal antibodies (0.75 μg/ml). Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (1:2000, Amersham Biosciences) were used, and detection of bound antibody was achieved using ECL (Amersham Biosciences). Equivalent loadings of purified GST fusion proteins were resolved on SDS-PAGE gels run in duplicate. One gel was stained with Coomassie, whereas the duplicate gel was transferred to nitrocellulose membrane (Schleicher and Schuell). A 32P-labeled N4WBP5A probe was produced by direct labeling of the GST fusion protein with protein kinase A (New England Biolabs). Glutathione beads containing bound fusion protein were incubated with protein kinase A and [32P]ATP in a buffer containing 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 12 mM MgCl2, and 1 mM dithiothreitol for 60 min at 4 °C. Beads were washed 5 times in PBS, and labeled protein was eluted with glutathione buffer. The membrane was blocked in Hyb 75 (28) and hybridized with the 32P-labeled N4WBP5A protein probe for 4 h at 4 °C in Hyb 75. The membrane was washed three times in Hyb 75 and exposed to x-ray film.
Regulation of ENaC by N4WBP5A

N4WBP5A is a putative transmembrane protein expressed in multiple tissues including kidney. A, representation of the N4WBP5A sequence showing the PY motifs PY1 and PY2 (black boxes) and transmembrane domains TM1–3 (shaded boxes). B, hydrophobicity of the N4WBP5 protein was estimated using the Protocolscale software (Kyte and Doolittle) and transmembrane domains (TM) were predicted using the TMHMM version 2.0 software. C, upper panel, the mouse multiple tissue Northern blot (CLONTECH) containing 2 μg of each poly(A)" RNA was hybridized to a mouse N4WBP5A cDNA probe (upper panel). Two transcripts of 2.4 and 2.1 kb were detected. Lower panel, the same blot was hybridized to a human β-actin probe (CLONTECH) to serve as a loading control. D, indirect immunofluorescence micrographs of N4WBP5A-specific distribution in 4-μm cryosections from rat kidney. Upper panel, renal cortex exhibits extensive N4WBP5A-specific fluorescence in distal convoluted tubules (d) and collecting ducts (cd). The figure also shows that glomeruli (g) and proximal tubules (*) are negative for N4WBP5A. Lower panel, N4WBP5A immunoreactivity is absent when the N4WBP5A antiserum is pre-absorbed with an excess of antigenic peptide. Magnification ×340.
N4WBP5A can be detected in the cytosol mainly at the basolateral side but also in perinuclear regions and within vesicle-like structures, with a punctate distribution pattern. Outer and inner medullary collecting ducts also exhibit strong intracellular N4WBP5A immunoreactivity (data not shown). Fig. 1D also shows that no N4WBP5A-specific immunofluorescence was detected in the glomeruli, proximal convoluted and proximal straight tubules, thin descending and ascending limbs, and in the parietal Bowman's epithelium. No significant fluorescence can be observed in sections incubated with peptide-absorbed antiserum (Fig. 1D, lower panel) when the preimmune serum was used and when the primary antibody was omitted (not shown).

**N4WBP5A Interacts with Ned4 and Ned4-2**—Like Ned4, Ned4-2, a close relative of Ned4, has been shown recently (19, 20, 23) to mediate ENaC regulation. Ned4-2 contains four WW domains, of which WW3 and WW4 interact with the ENaC subunits (20). To test whether N4WBP5A physically interacts with Ned4 and Ned4-2, we carried out immunoprecipitation experiments in HEK 293T cells ectopically expressing these proteins. HEK 293T cells were co-transfected with Ned4 or Ned4-2-FLAG and vector or pcDNA3-N4WBP5A-FLAG. Immunoprecipitations were performed with anti-Ned4ab1 (N4) antibody that cross-reacts with both Ned4 and Ned4-2 or rabbit preimmune serum, and the blot was probed with antifLAG or an anti-Ned4 monoclonal antibody. Both Ned4 and Ned4-2 were found to interact with N4WBP5A (Fig. 2A). To determine whether endogenous N4WBP5A and Ned4/Ned4-2 interact, immunoprecipitations from the mouse cortical collecting duct cell line M-1 were carried out. M-1 cells are known to endogenously express ENaC (35). By using the Ned4-2 antibody, but not preimmune serum from the same animal, both Ned4-2 and N4WBP5A were co-immunoprecipitated (Fig. 2B, lower panel). Because both antibodies (against Ned4-2 and against N4WBP5A) were raised in rabbits, it is currently not possible to do co-localization immunocytochemistry using double staining. However, we analyzed N4WBP5A and Ned4-2 localization in M-1 cells using side by side immunocytochemistry. As shown in Fig. 2C, Ned4-2 is localized to the Golgi and throughout the cytoplasm, whereas N4WBP5A appears as punctated staining throughout the cytosol. Thus, both proteins are present in the cytoplasm of M-1 cells with some apparent overlap of the staining consistent with the co-immunoprecipitation data.

To characterize further the interaction between N4WBP5A and Ned4-2, we produced GST fusion proteins containing either the wild-type or mutant Ned4-2 WW domains. These proteins were used in far Western experiments using the aminoterminal domain of N4WBP5A containing both PY motifs as a probe (Fig. 2D). The fusion protein containing all four WW domains showed strong interaction with N4WBP5A (Fig. 2D, last lane). All four individual WW domains, but not their mutant counterparts, were also able to bind N4WBP5A. This result strongly suggests that the interaction between Ned4-2 and N4WBP5A is mediated via PY/WW domain interactions as is the interaction between N4WBP5 and Ned4 (29) and N4WBP5A and Ned4-2.

**Coexpression of N4WBP5A Specifically Stimulates ENaC Currents**—Because N4WBP5A interacts with Ned4 and Ned4-2 and is highly expressed in nephron segments where ENaC is also present, we tested whether N4WBP5A can affect ENaC activity in the *Xenopus* oocyte system. As shown in Fig. 3 co-expression of N4WBP5A with αβγENaC had a large and sustained stimulatory effect on ENaC currents. ∆I_{\text{amp}} was as-

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*L. Shearwin-Whyatt and S. Kumar, unpublished data.
The stimulatory effect of N4WBP5A was specific for ENaC. The lack of effect of N4WBP5A on Kir1.1a or CFTR suggests that the effect of N4WBP5A on cAMP-activated CFTR currents (data not shown). The lack of effect of N4WBP5A on Kir1.1a K⁺ currents or on cAMP-activated CFTR currents (data not shown). The lack of effect of N4WBP5A on Kir1.1a or CFTR suggests that the stimulatory effect of N4WBP5A was specific for ENaC.

N4WBP5A Increases Surface Expression of ENaC—The stimulation of ΔI_{ambi}, in oocytes co-expressing N4WBP5A may be due to an increased Pₗ of ENaC or to an increase in the overall number of ENaC channels expressed at the cell surface. To investigate this question we assessed ΔI_{ambi} and surface expression of extracellular HA-tagged ENaC (ENaC-HA) using a chemiluminescence assay (33, 34). The results shown in Fig. 4 demonstrate that in ENaC-HA/N4WBP5A oocytes surface expression and ΔI_{ambi} were increased by about 4.2- and 3.7-fold, respectively, compared with ENaC-HA control oocytes. This indicates that an increased number of ENaC channels expressed at the cell surface was sufficient to explain the stimulation of ΔI_{ambi} in oocytes co-expressing ENaC and N4WBP5A. The Western blot shown in Fig. 4 demonstrates that ENaC-HA and ENaC-HA/N4WBP5A oocytes express similar levels of ENaC-HA protein. Thus, increased protein synthesis was unlikely to be the cause of the stimulatory effect of N4WBP5A on ENaC surface expression.

N4WBP5A Reduces the Rate of ENaC Retrieval from the Plasma Membrane—N4WBP5A may increase surface expression of ENaC by enhancing its delivery to the cell surface or by inhibiting ENaC retrieval. To assess the rate of ENaC retrieval, we inhibited delivery of new channels to the plasma membrane by adding 18 μM brefeldin A (BFA) to oocytes 2 days after injection with cRNA. BFA is a fungal metabolite that inhibits the secretory pathway of newly synthesized proteins without affecting clathrin-mediated endocytosis (42). Fig. 5 illustrates the effect of BFA on ΔI_{ambi} in ENaC and ENaC/N4WBP5A oocytes. In ENaC oocytes ΔI_{ambi} decreased by about 85% within 4 h after addition of BFA (Fig. 5A), which is in good agreement with data reported previously (12). In non-treated ENaC oocytes ΔI_{ambi} continued to increase throughout the 24-h period examined which suggests that the channel insertion exceeded channel retrieval during this period. After removal of BFA, ΔI_{ambi} partially recovered demonstrating that the effect of BFA was non-toxic, specific, and reversible upon removal of the drug (Fig. 5A). In contrast to its dramatic inhibitory effect on ΔI_{ambi} in ENaC oocytes, BFA had essentially no effect on ΔI_{ambi} in ENaC/N4WBP5A oocytes (Fig. 5B). The resistance of ΔI_{ambi}...
to BFA in oocytes co-expressing N4WBP5A indicates that N4WBP5A stabilizes ENaC channels in the plasma membrane probably by preventing their endocytosis.

Stimulation of $\Delta I_{\text{app}}$ by N4WBP5A Requires Sodium Loading of the Oocytes—An increase in intracellular Na$^+$ concentration is known to cause feedback inhibition of ENaC by a complex regulatory pathway involving Nedd4/Nedd4-2 and resulting in channel retrieval from the plasma membrane (17, 22, 43). Because N4WBP5A may interfere with Nedd4/Nedd4-2 function, co-expression of N4WBP5A may prevent Nedd4/Nedd4-2-dependent Na$^+$ feedback inhibition and channel retrieval possibly resulting in enhanced ENaC activity. Therefore, we tested the Na$^+$ dependence of the N4WBP5A effect by incubating ENaC/N4WBP5A and ENaC oocytes either in the usual high sodium (88 mM) or in low sodium (1 mM) MBS for 2 days after injection with cRNA. The $I/V$ plots shown in Fig. 6A illustrate that after incubation in high sodium $\Delta I_{\text{app}}$ was significantly higher in ENaC/N4WBP5A oocytes than $\Delta I_{\text{app}}$ in ENaC control oocytes consistent with the data shown in Figs. 3–5. After 2 days in high sodium, $\Delta I_{\text{app}}$ (at −60 mV) was increased by $515 \pm 73\%$ ($n = 160; N = 16; p < 0.001$) in ENaC/N4WBP5A oocytes compared with that in ENaC oocytes. In contrast, the stimulatory effect of N4WBP5A is dependent on maintaining the oocytes in the presence of high extracellular Na$^+$.

It is well known that ENaC-expressing oocytes kept in the presence of high extracellular Na$^+$ will become severely Na$^+$-loaded (30). Indeed, in oocytes maintained in high sodium after injection with cRNA the reversal potential of $\Delta I_{\text{app}}$ was $-1$ and $-2$ mV for ENaC and ENaC/N4WBP5A oocytes, respectively (Fig. 6A). These values indicate that the apparent intracellular Na$^+$ concentration [Na$^+$_app] of these oocytes was essentially equal to that in the bath solution (95 mM). In contrast, in oocytes maintained in low sodium, the reversal potential of $\Delta I_{\text{app}}$ was 21 mV for both ENaC and ENaC/N4WBP5A oocytes (Fig. 6B). By using this reversal potential one can estimate a value of 41 mM for [Na$^+$_app]. It should be pointed out that this value probably reflects the Na$^+$ concentration in a cytosolic compartment close to the plasma membrane and not necessarily the bulk Na$^+$ concentration inside the cell. During voltage clamp experiments, entry of Na$^+$ through ENaC acutely increases the Na$^+$ concentration in the unstirred compartment close to the membrane. Thus, using the reversal potential of

FIG. 5. Effect of BFA on $\Delta I_{\text{app}}$ in ENaC control oocytes (A) and in ENaC/N4WBP5A oocytes (B). Two days after incubation in high sodium MBS, oocytes were divided into a control group (open circles) and a BFA-treated group (filled circles). BFA (18 μM) was added at time 0 as indicated by the arrow pointing down, and $\Delta I_{\text{app}}$ was subsequently assessed in 4-h intervals. Each value represents the mean ± S.E.M. $\Delta I_{\text{app}}$ was 21 mV for both ENaC and ENaC/N4WBP5A oocytes, respectively (Fig. 6B).
\[ \Delta I_{\text{app}} \text{ to calculate } [\text{Na}^+]_{\text{app}} \text{ tends to overestimate the true intracellular sodium concentration (32). Nevertheless, these estimates clearly indicate that the intracellular sodium concentration was substantially lower in the cells incubated in low sodium than in those incubated in high sodium.} \\]

On average \[ \Delta I_{\text{app}} \text{ was } 876 \pm 121\% \text{ (n = 30; N = 3) larger in ENaC oocytes incubated in low sodium compared with the corresponding } \Delta I_{\text{app}} \text{ in ENaC oocytes incubated in high sodium.} \] This is consistent with findings from a previous study that concludes that sodium-dependent down-regulation of ENaC can be prevented by maintaining oocytes in the presence of a low extracellular sodium concentration (11). The inability of N4WB5PA to stimulate ENaC activity in oocytes incubated in low sodium and its stimulatory effect on ENaC incubated in high sodium suggest that in the latter oocytes N4WB5PA prevents the sodium-dependent down-regulation of ENaC and thereby increases the number of ENaC channels at the cell surface. Indeed, in ENaC/N4WB5PA oocytes incubated in high sodium, \[ \Delta I_{\text{app}} \text{ reached about the same level (14.76 \pm 2.21 } \mu \text{A; n = 30; N = 3) as in ENaC oocytes incubated in low sodium (19.02 \pm 3.12 } \mu \text{A; n = 30; N = 3).} \] Thus, extracellular sodium removal and co-expression of N4WB5PA have a similar stimulatory effect on ENaC currents.

**N4WB5PA Prevents Sodium Feedback Inhibition of ENaC**—To further investigate whether N4WB5PA interferes with sodium feedback inhibition of ENaC, we combined \( \Delta I_{\text{app}} \) measurements with surface detection of ENaC-HA. Two days after cRNA injection and incubation in low sodium \( \Delta I_{\text{app}} \) in ENaC-HA/N4WB5PA was very similar to that in ENaC-HA control oocytes averaging 7.90 \pm 1.91 } \mu \text{A (n = 10) and 8.17 \pm 1.50 } \mu \text{A (n = 10), respectively (Fig. 7A). Oocytes were subsequently divided into two groups and either transferred to a bath solution containing 95 mM Na\(^+\) or further maintained in the presence of 1 mM extracellular Na\(^+\). In ENaC-HA and ENaC-HA/N4WB5PA oocytes maintained in 1 mM Na\(^+\) \( \Delta I_{\text{app}} \) remained high at a relatively constant level. In contrast, exposure of ENaC-HA oocytes to 95 mM Na\(^+\) reduced \( \Delta I_{\text{app}} \) to 0.66 \pm 0.07 } \mu \text{A (n = 10) within 3 h demonstrating the presence of substantial sodium feedback inhibition of ENaC. Interestingly, in ENaC-HA/N4WB5PA oocytes exposure to 95 mM Na\(^+\) had a much smaller effect with \( \Delta I_{\text{app}} \) remaining at 6.51 \pm 0.77 } \mu \text{A (n = 10) after 3 h and at 5.41 \pm 0.48 } \mu \text{A (n = 10) after 9 h (Fig. 7A). These current measurements suggest that N4WB5PA prevents sodium-stimulated ENaC retrieval from the plasma membrane. This was confirmed by ENaC-HA surface expression measurements summarized in Fig. 7B. As long as the oocytes were maintained in the presence of 1 mM extracellular Na\(^+\), surface expression of ENaC was similar in ENaC-HA oocytes compared with that in ENaC-HA/N4WB5PA oocytes. However, upon exposure to 95 mM extracellular Na\(^+\) surface expression was dramatically reduced in ENaC-HA oocytes, whereas it was largely preserved in ENaC-HA/N4WB5PA oocytes. These findings are consistent with the \( \Delta I_{\text{app}} \) data shown in Fig. 7A and confirm that acute sodium-stimulated ENaC retrieval is prevented by N4WB5PA.

**Lack of Effect of N4WB5PA on ENaC with Liddle’s Syndrome Mutation**—The finding that N4WB5PA reduces sodium feedback inhibition was similar to the observation that mutations causing Liddle’s syndrome reduce sodium-dependent down-regulation of ENaC (11). Moreover, the lack of effect of BFA on ENaC/N4WB5PA oocytes was reminiscent of the previously reported finding (12) that BFA had almost no effect on the activity of ENaC with Liddle’s syndrome mutation because the retrieval of the mutated channel by clathrin-mediated endocytosis was defective. Taken together, our results suggest that in the presence of N4WB5PA the wild-type ENaC channel adopts a similar phenotype as the channel with Liddle’s syndrome mutation. We therefore hypothesized that ENaC with Liddle’s syndrome mutation cannot be stimulated by N4WB5PA because endocytotic retrieval was already compromised in the mutated channel. Indeed, the data shown in Fig. 8 demonstrate that N4WB5PA failed to stimulate significantly \( \Delta I_{\text{app}} \) and channel surface expression in oocytes expressing ENaC with Liddle’s syndrome mutation (\( \left( \alpha_{\text{ENaC}}^{\text{R564X}}, \gamma_{\text{ENaC}}^{\text{H8004}} \right) \)). These findings suggest that the stimulatory effect of N4WB5PA requires the presence of an intact endocytotic retrieval mechanism that can be inhibited by N4WB5PA to enhance ENaC surface expression.

**N4WB5PA Competes with Nedd4-2 for the Regulation of ENaC Endocytosis**—Biochemical data suggest that N4WB5PA binds to Nedd4-2 (Fig. 2). Thus in Xenopus oocytes heterologously expressed N4WB5PA may sequester endogenous \( \alpha_{\text{ENaC}}^{\text{R564X}} \). Sequestration of Nedd4-2 would reduce Nedd4-2-mediated endocytosis and degradation of ENaC resulting in an
cytes were incubated in high sodium MBS for 2 days. Each bar amount of N4WBP5A and Nedd4-2 were co-injected as indicated by the ng of cRNA for ENaC (1 ng for each subunit). In addition different N4WBP5A and Nedd4-2 on ENaC.

Surface expression (filled bars) is expressed in RLU per 15 s per oocyte. 10 oocytes per group were used for the same batch of oocytes /H11006 control oocytes which averaged 2.77 ± 0.05 RLU per 15 s per oocyte. 10 oocytes per group were used for the surface expression of oocytes expressing N4WBP5A and Nedd4-2. Co-injection of 20 ng of Nedd4-2 cRNA reduced expressed ENaC with varying amounts of N4WBP5A and Nedd4-2. Co-injection of 20 ng of N4WBP5A cRNA had the usual stimulatory effect with Nedd4-2. On the other hand, N4WBP5A and with the ability to bind Nedd4-2 efficiently,3 to prevent Nedd4 from stimulating ENaC retrieval. On the other hand, N4WBP5, although structurally similar to other hand, N4WBP5, although structurally similar to N4WBP5A, failed to stimulate ENaC with Liddle syndrome mutation. Surface expression and Δ lat expressed were assessed in oocytes expressing α-HA(amoebae)-ENaC (Liddle-HA oocytes) in the presence or absence of N4WBP5A. Oocytes were incubated in high sodium MBS for 2 days. Surface expression (filled bars) is expressed in RLU per 15 s per oocyte. 10 oocytes per group were used for the Δ lat measurements and for detection of surface expression. In contrast, in the same batch of oocytes Δ lat was increased by 4-fold in oocytes coexpressing ENaC and N4WBP5A (data not shown). Similar results were obtained in a second batch of oocytes. n.s., not significant.

FIG. 8. N4WBP5A fails to stimulate ENaC with Liddle’s syndrome mutation. Surface expression and Δ lat were assessed in oocytes expressing α-HA(amoebae)-γ-HAENaC (Liddle-HA oocytes) in the presence or absence of N4WBP5A. Oocytes were incubated in high sodium MBS for 2 days. Surface expression (filled bars) is expressed in RLU per 15 s per oocyte. 10 oocytes per group were used for the Δ lat measurements and for detection of surface expression. In contrast, in the same batch of oocytes Δ lat was increased by 4-fold in oocytes coexpressing ENaC and N4WBP5A (data not shown). Similar results were obtained in a second batch of oocytes. n.s., not significant.

increased surface expression of ENaC. To confirm a functional competition between N4WBP5A and Nedd4-2 (Fig. 9), we co-expressed ENaC with varying amounts of N4WBP5A and Nedd4-2. Co-injection of 20 ng of Nedd4-2 cRNA reduced Δ lat to 0.97 ± 0.16 μA (n = 10) compared with Δ lat in ENaC control oocytes which averaged 2.77 ± 0.19 μA (n = 10; p < 0.001). This is consistent with the well described inhibitory effect of Nedd4 on ENaC function (43). On the other hand, co-injection of 20 ng of N4WBP5A cRNA had the usual stimulatory effect with Δ lat averaging 7.74 ± 0.78 μA (n = 10; p < 0.001). Interestingly, co-injection of 20 ng of N4WBP5A and 20 ng of Nedd4-2 cRNA resulted in Δ lat that averaged 3.33 ± 0.36 μA (n = 10) and was similar to that in ENaC control oocytes. Thus, the stimulatory effect of N4WBP5A was cancelled by the inhibitory effect of Nedd4-2. Indeed, the stimulatory effect of N4WBP5A was suppressed by Nedd4-2 in a dose-dependent manner (Fig. 9). Thus, it is conceivable that in native epithelia, which express both Nedd4-2 and N4WBP5A, the relative abundance of each protein will be critical for determining ENaC activity by regulating its retrieval rate.

FIG. 9. Effect of co-expression of different amounts of N4WBP5A and Nedd4-2 on ENaC. Oocytes were all injected with 3 ng of cRNA for ENaC (1 ng for each subunit). In addition different amounts of N4WBP5A and Nedd4-2 were co-injected as indicated by the ratios N4WBP5A:Nedd4-2 (ng/ng) below the bars. After injection oocytes were incubated in high sodium MBS for 2 days. Each bar represents the mean Δ lat of 10 oocytes. n.s., not significant.

The PY Motifs and the TM Domains of N4WBP5A Are Essential for Its Function—N4WBP5A has two PY motifs and three transmembrane (TM) domains (Fig. 1A). We used an N4WBP5A version in which both PY motifs were mutated (N4WBP5A-PY), and a carboxy-terminal truncation mutant that has intact PY motifs but lacks all three TM domains (N4WBP5Astop) to establish the domains that are important for N4WBP5A function. Fig. 10A illustrates that both mutants failed to stimulate ENaC activity, although Western blots confirmed their expression. Δ i avg averaged 1.64 ± 0.27 μA (n = 10) in ENaC control oocytes and 16.11 ± 2.44 μA (n = 10; p < 0.001) in ENaC/N4WBP5A oocytes. In contrast to the stimulatory effect of N4WBP5A, Δ i avg averaged 1.53 ± 0.22 μA (n = 10; p > 0.05) and 1.66 ± 0.27 μA (n = 10; p > 0.05) in ENaC/N4WBP5Astop and ENaC/N4WBP5A-PY oocytes, respectively. These results suggest that the PY motifs of N4WBP5A are required for its stimulatory effect on ENaC. The PY motifs are likely to mediate N4WBP5As interaction with Nedd4-2 thereby preventing Nedd4-2 from down-regulating ENaC. However, our data also show that the presence of the PY motifs, which are preserved in N4WBP5Astop, was not sufficient to prevent Nedd4-2-mediated down-regulation of ENaC. In particular the TM domains were likely to be needed for the correct localization/stabilization of N4WBP5A in the appropriate cellular compartment to enable it to interact with Nedd4-2.

Absence of N4WBP5A Surface Expression and Lack of Effect of the Homologous Protein N4WBP5 on ENaC—To rule out a direct effect of N4WBP5A on ENaC at the level of the plasma membrane, we investigated whether FLAG-tagged N4WBP5A was transported to the cell surface using the chemiluminescence assay (Fig. 10B). The signals detected in ENaC/N4WBP5A, ENaC/N4WBP5A-PY, and N4WBP5A oocytes were very low and similar to the background signal measured in control oocytes expressing ENaC alone. In contrast a 1000-fold higher signal was detected in ENaC-FLAG oocytes that served as positive controls (7). The possibility that N4WBP5A was retained intracellularly by endogenous xNedd4-2 is unlikely, because the N4WBP5A-PY, which has no stimulatory effect and therefore does not seem to interact with Nedd4-2, was also not expressed at the cell surface (Fig. 10B). Interestingly, oocytes coexpressing ENaC and FLAG-tagged N4WBP5, a protein closely related to N4WBP5A and also containing two PY motifs and three transmembrane domains (29), showed a significant chemiluminescence signal (Fig. 10B). These data indicate that N4WBP5A was not transported to the cell surface, whereas N4WBP5 was delivered to the cell surface efficiently. Importantly, co-expression of N4WBP5 with ENaC failed to stimulate significantly Δ i avg which in ENaC/N4WBP5 oocytes averaged 136 ± 22% of that in ENaC control oocytes (n = 80; n = 8). We conclude that N4WBP5A was confined to an intracellular location where it interacted with Nedd4 in a complex manner to prevent Nedd4 from stimulating ENaC retrieval. On the other hand, N4WBP5, although structurally similar to N4WBP5A and with the ability to bind Nedd4-2 efficiently,3 traffics to the plasma membrane but did not appear to affect Nedd4-ENaC interaction. The difference in the trafficking

3 A. Fotia and S. Kumar, unpublished data.
behavior of the two homologues suggested that the intracellular localization of N4WBP5A was important for its interaction with Nedd4 and its stimulatory effect on ENaC surface expression.

**DISCUSSION**

We have described here the regulation of ENaC in Xenopus oocytes by a novel protein N4WBP5A which is highly expressed in native renal collecting duct and other tissues known to express ENaC. We have demonstrated that the increased ENaC activity in the presence of N4WBP5A is due to increased surface expression of ENaC. This effect is likely due to an increased stability of ENaC in the plasma membrane and does not appear to be dependent on the transport of newly synthesized ENaC to the surface.

The effect of N4WBP5A is dependent on the presence of high Na\(^{+}\), suggesting that it acts to inhibit the sodium feedback pathway that involves Nedd4/Nedd4-2-mediated retrieval of ENaC from the plasma membrane. Whereas both Nedd4 and Nedd4-2 protein are present in many mammalian tissues, only a single homologue that is more similar to Nedd4-2 than to Nedd4 is known to be present in *Xenopus* (18–20, 27). Recent results demonstrate that in *Xenopus* oocytes that contain endogenous xNedd4-2, *Xenopus*, mouse and human Nedd4-2 proteins, and to a much smaller extent rat and human Nedd4 proteins, can regulate surface expression of ENaC (19, 20, 23). Therefore, the relative abundance of Nedd4-2 and N4WBP5A proteins may affect levels of surface ENaC expression in vivo.

The stimulatory effect on ENaC requires intact PY motifs and transmembrane domains of N4WBP5A. It is likely that the PY motifs are needed to mediate the interaction of N4WBP5A with a WW domain containing protein. Our biochemical evidence that N4WBP5A PY motifs are required for its interaction with Nedd4/Nedd4-2 is consistent with N4WBP5A interfering with the activity of endogenous xNedd4-2. The additional requirement for intact TM domains suggests that these domains of N4WBP5A are also important to mediate Nedd4-2 sequestration. N4WBP5A is highly related to N4WBP5, a protein
largely associated with the Golgi complex (29), whereas N4WBP5A appears to be localized to post-Golgi vesicles. The finding that N4WBP5 had no stimulatory effect on ENaC activity in the Xenopus oocyte system supports the conclusion that the effect of N4WBP5A is specific and that sequestration of Nedd4-2 requires more than the presence of binding domains because both proteins, N4WBP5 and N4WBP5A, bind Nedd4-2 with similar affinities via the PY motif/WW domain interaction.

How might N4WBP5A affect the Nedd4-2-mediated ENaC control? One possibility is that vesicles containing N4WBP5A sequester Nedd4-2 preventing its localization to ENaC at the plasma membrane. When necessary, Nedd4-2 may be released permitting translocation to the plasma membrane where it can associate with ENaC and mediate ubiquitin-dependent down-regulation of the membrane-associated ENaC. This hypothesis is supported by the fact that Nedd4-2 can interact with both the ENaC subunits and N4WBP5A. Our data suggest that N4WBP5A itself is not transported to the cell surface. However, the vesicles containing N4WBP5A may traffic to the cytosolic side of the membrane allowing sequestration of Nedd4-2 when necessary, e.g. during hormonal stimulation of ENaC surface expression.

In addition to the tissues that express ENaC, N4WBP5A is also present in other cell types and tissues. Both Nedd4 and Nedd4-2 are also expressed in many tissues that lack ENaC (14, 18, 19, 28). This suggests that N4WBP5A may also play a role in modulating the capacity of Nedd4/Nedd4-2 to regulate other cellular proteins that are controlled by Nedd4/Nedd4-2-mediated ubiquitination. However, N4WBP5A has no stimulatory effect on CFTR or Kir1.1a, both of which are apically located channel proteins expressed in a range of epithelia. This suggests that the stimulatory effect of N4WBP5A is rather specific for ENaC. Moreover, the lack of effect of the closely related protein N4WP5B on ENaC function and the specific expression of N4WP5B in nephron segments known to express ENaC make N4WP5B a strong candidate for a physiologically relevant regulator of ENaC activity. So far the effect of N4WP5B on ENaC activity is demonstrated in oocytes, and it remains to be determined that it also occurs in native renal epithelial cells. However, the finding that the majority of collecting duct cells show N4WP5B-specific immunoreactivity clearly indicates that ENaC and N4WP5B are co-expressed in native renal collecting duct principal cells. The hormonal regulation of ENaC activity in these cells is essential for the fine-tuning of renal sodium absorption and hence for body sodium balance and the long term regulation of arterial blood pressure. Thus, it will be an interesting topic for future research to investigate whether N4WP5B is a component of the molecular mechanisms involved in ENaC regulation, e.g. by aldosterone.

The regulation of ENaC by N4WP5A described here is somewhat reminiscent of the recently described control of CFTR plasma membrane expression by CAL, a PDZ domain-containing, Golgi-associated protein (44). CAL favors retention of CFTR within the cell by interacting with the carboxyl terminus of CFTR, whereas Na+/K+ exchanger regulatory factor, NHE-RF, promotes CFTR cell surface expression by competing with CAL for binding of CFTR. The similarity between CFTR regulation by CAL and ENaC regulation by N4WP5A suggests that antagonistic competition between regulatory protein ligands may be an important new mechanism for regulating surface expression of membrane proteins.

In conclusion, we have discovered that the recently identified Nedd4/Nedd4-2-binding protein N4WP5A enhances ENaC surface expression most likely by preventing Nedd4-2-mediated channel retrieval from the plasma membrane. N4WP5A also attenuates Na+ feedback inhibition of ENaC which is known to involve the Nedd4/Nedd4-2 pathway (17, 22, 43). The effect of N4WP5A is reminiscent of the action of the aldosterone-induced kinase and the serum- and glucocorticoid-regulated kinase, which also increase surface expression of ENaC probably by PY motif-dependent binding to Nedd4-2 and its phosphorylation (24, 25). Thus, as illustrated schematically in Fig. 11, Nedd4-2 seems to be an integrator of various pathways regulating ENaC activity. It remains a challenge for future research to elucidate the relative importance of N4WP5A and its functional interrelationship with the different pathways and mechanisms involved in ENaC regulation under physiological and pathophysiologic conditions.

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