Nedd4-2 isoforms ubiquitinate individual epithelial sodium channel subunits and reduce surface expression and function of the epithelial sodium channel

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Am J Physiol Renal Physiol 294: F1157–F1165, 2008. First published March 5, 2008; doi:10.1152/ajprenal.00339.2007.—We previously reported the existence of multiple isoforms of human Nedd4-2 (Am J Physiol Renal Physiol 285: F916–F929, 2003). When overexpressed in M-1 collecting duct epithelia, full-length Nedd4-2 (Nedd4-2), Nedd4-2 lacking the NH2-terminal C2 domain (Nedd4-2ΔC2), and Nedd4-2 lacking WW domains 2 and 3 (Nedd4-2ΔWW2,3) variably reduce benzamil-sensitive Na+ transport. We investigated the effect of each of the Nedd4-2 isoforms on cell surface expression and ubiquitination of ENaC subunits. We find that αENaC when transfected alone or with β and γENaC is expressed at the cell surface and this membrane expression is variably reduced by coexpression with each of the Nedd4-2 isoforms. Nedd4-2 reduces the half-life of ENaC subunits and enhances the ubiquitination of α, β, and γENaC subunits when expressed alone or together suggesting that each subunit is a target for Nedd4-2-mediated ubiquitination. As has been reported recently, we confirm that the surface-expressed pool of ENaC is multi-ubiquitinated. Inhibitors of the proteasome increase ubiquitination of ENaC subunits and stimulate Na+ transport in M-1 cells consistent with a role for the ubiquitin-proteasome pathway in regulating Na+ transport in the collecting duct.

AMILORIDE-SENSITIVE Na+ REABSORPTION occurs via epithelial Na+ channel (ENaC) in the transporting epithelia of the connecting tubules and collecting ducts of the kidney. ENaC exists as a heteromultimeric protein complex at the apical membrane of the epithelial cells and consists of three structurally related subunits, α, β, and γ. Aldosterone is one of the more important hormones that regulate distal nephron Na+ transport and its actions appear to be mediated via ENaC. The identification of ENaC mutations that lead to severe inherited hypertension from enhanced Na+ reabsorption or to profound hypotension from salt wasting underscores the importance of the ENaC in the distal nephron in extracellular fluid volume homeostasis and the control of blood pressure (7, 15, 27).

Each of the α, β, and γ subunits of ENaC contains a PY motif in its COOH terminus which serves as an endocytosis motif. Heterozygous mutation of the PY motif of β or γENaC leads to persistence of ENaC at the cell surface and increased Na+ reabsorption and hypertension in Liddle’s syndrome, an autosomal dominant form of severe hypertension (7, 15, 27). In more recent studies, the PY motif of ENaC was shown to interact with WW domains of the HECT domain-type E3 ubiquitin ligases. Several members of this broad family including Nedd4, Nedd4-2, WWP1, and WWP2 have been shown to inhibit ENaC function in heterologous expression systems (12, 28, 32). Inhibition of Nedd4-2 enhances ENaC activity in a lung epithelial cell line, H441, and when ENaC is overexpressed, in a rat thyroid epithelial cell line, FRT (30). Nedd4-2 has emerged as a likely ubiquitin ligase that regulates ENaC in the collecting duct as well since Nedd4-2 can be phosphorylated and inhibited by Sgk1, a kinase that may mediate some of the actions of aldosterone (6, 29). Furthermore, in the collecting duct and connecting tubule, dietary Na+ intake and aldosterone reduce the abundance of Nedd4-2 and induce some isoforms of 14-3-3, a group of regulatory proteins that reduces Nedd4-2 interaction with ENaC, positioning Nedd4-2 as a central player in the regulation of ENaC in the collecting duct and connecting tubule (3, 10, 17, 18).

In our previous studies, we reported on the existence of many isoforms of human Nedd4-2 that arise from alternate transcription and translation and also from variable splicing of some internal exons (11, 12). In the present study, we further analyze Na+ transport with each of three hNedd4-2 isoforms in a collecting duct epithelial cell line, M-1. We examine the impact of hNedd4-2 isoforms on the kinetics, the cell surface expression, and the ubiquitination of ENaC.

MATERIALS AND METHODS

Materials. Dexamethasone, selenium, transferrin, triiodothyronine, epidermal growth factor, chloroquine, D-glucose, N-acetyl-Leu-Leu-norleucinal (ALLN), and Z-Leu-Leu-Leu-al (MG132) were all pur chased from Sigma (St. Louis, MO) and cycloheximide was from EMD Chemicals (San Diego, CA). All cell culture media were obtained from Invitrogen Life Technologies (Gaithersburg, MD). Anti-FLAG M2 and anti-actin were obtained from Sigma; anti-V5, anti-myc, and anti-Xpress from Invitrogen; anti-P4D1 from Cell Signaling Technology (Danvers, MA); and anti-α tubulin and HRP-conjugated goat anti-mouse IgG and anti-mouse IgM were from Santa Cruz Biotechnology (Santa Cruz, CA).

Transfection. Human embryonic kidney cell line HEK293 was cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. Human α, β, and γENaC cDNAs were subcloned in pcDNA3 (Invitrogen) with COOH-terminal FLAG V5, and myc epitopes, respectively. Coding regions of all three hNedd4-2 isoforms were sub cloned into expression vectors as described previously (12), and an HA-tagged ubiquitin expression vector was a gift from Dr. D. Bohmann. Nedd4-2CS was generated by mutating cysteine to alanine at position

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942 of hNedd-2 using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HEK293 cells were transiently transfected with α, β, and γENaC, hNedd-2, and/or HA-ubiquitin using Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions for adherent cells. Twenty-four hours following transfection, cells were directly lysed for Western blotting or immunoprecipitation or first used for cell surface biotinylation assays and then lysates were prepared. In some experiments, cells were treated with MG132 (1 μM), a membrane-permeable inhibitor of the proteasome, for 4 h before lysis/biotinylation. In other experiments, cycloheximide (20 μg/ml), an inhibitor of eukaryotic translation, was added to transfected cells in serum-free media for various times before cell lysis. Western blot analysis and immunoprecipitation. Transfected cells were washed twice in PBS followed by lysis in 150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 10 μM ALLN, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) at 4°C. Protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were diluted in SDS-PAGE sample loading buffer (30% glycerol, 50 mM Tris-HCl, pH 6.8, 7.5% SDS, 50 mM dithiothreitol, bromophenol blue), boiled separated on a 7% gel, and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Membrane was blocked with PBS-Tween 20 (PBST; 0.05% Tween 20 in PBS) containing 5% milk for 1 h followed by overnight incubation in 5% milk-PBST with appropriate primary antibody (anti-FLAG 1:3,000; anti-actin 1:1,000; anti-Xpress 1:5,000; anti-HA 1:3,000; anti-FK1 1:1,000; anti-P4D1 1:1,000; or anti-α tubulin 1:1,000). Membranes were then washed with PBST and incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h followed by several washings with PBST. Chemiluminescence detection of proteins was done using Supersignal West Pico or Femto chemiluminescent substrate (Pierce) and the image was captured using VisionWorksLS Image Acquisition and Analysis Software and the EC3 imaging system (UVP, LLC, Upland, CA). Stripping and reprobing of membranes with different antibodies were performed with Restore Western Blot Stripping Buffer (Pierce) as per manufacturer’s instructions.

For immunoprecipitation, 1 mg of protein lysates was incubated overnight with the appropriate antibody in 1:250 dilutions with end-over-end rotation at 4°C. The cell lysate/antibody mixture was incubated with 80 μl (40 μl bead bed volume per sample) of protein G Sepharose (GE Healthcare, Piscataway, NJ), washed twice in PBS followed by lysis in 150 mM NaCl, 50 mM Tris, pH 7.5) at 80°C for 10 min, diluted in immunoprecipitation buffer, and subjected to Neutavidin precipitation and analyzed by SDS-PAGE.

Measurement of short-circuit current. M-1 cells were cultured in DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin. For transepithelial potential measurements, M-1 cells were grown on filters and short-circuit currents (Isc) and transepithelial voltage and resistance were measured at 37°C in Ussing chambers (12). In some experiments, cells on filters were treated with MG132 (1 μM) or chloroquine (100 μM) for varying time periods before measurement of Isc.

Adenovirus transduction. Recombinant adenovirus expressing Nedd4-2 and Nedd4-2Δ2C2 was constructed as described previously (12). Adenoviral vectors expressing Nedd4-2CS and Nedd4-2Δ2WW2,3 were generated by the vector core facility at the University of Iowa. M-1 cells were transduced with adenovirus as previously described (12).

Statistical analysis. The results are presented as means ± SD (or SE) with comparison between groups performed by one-way ANOVA and Student’s t-test. Significant values were considered at P < 0.05 (SigmaStat, SPSS, Chicago, IL).

RESULTS

Nedd4-2 isoforms differentially reduce Na⁺ transport in collecting duct epithelia. We identified several hNedd4-2 isoforms including the full-length protein that has a C2 domain and all four WW domains (Nedd4-2), an isoform lacking a C2 domain (Nedd4-2ΔC2), and an isoform lacking two WW domains (Nedd4-2ΔWW2,3) and had begun to examine their function in Xenopus laevis oocytes and in M-1, collecting duct epithelial cells. Transient transfection and expression of Nedd4-2ΔC2 in M-1 cells reduced basal benzamil-sensitive Isc, whereas Nedd4-2ΔWW2,3 was without apparent effect, although these were not directly compared (12). To improve expression of Nedd4-2 isoforms without disrupting monolayer integrity on filters, we constructed recombinant adenoviral vectors expressing various hNedd4-2 isoforms. Short-circuit currents were measured 24 h post viral transduction and again following dexamethasone stimulation. Our results indicate that Nedd4-2 isoforms inhibit Na⁺ transport and that there are differences between these isoforms which are more pronounced and significant following dexamethasone treatment (Fig. 1A). As we have seen in X. laevis oocytes previously, Nedd4-2ΔC2 is the most potent inhibitor of Na⁺ transport with Nedd4-2ΔWW2,3 being the least effective (12). A Western blot analysis performed on transduced M-1 lysates using an anti-Xpress antibody demonstrated equal expression of all three isoforms confirming that differences between isoforms could not be explained by variations in Nedd4-2 expression (Fig. 1B).

Nedd4-2 isoforms reduce surface expression of αENaC in HEK293 cells. To understand the role of Nedd4-2 isoforms in regulating ENaC, we overexpressed Nedd4-2 isoforms in HEK293 along with single or multiple subunits of individually tagged ENaC subunits. αENaC expressed at the cell membrane was detected by affinity purification of biotinylated surface proteins. Interestingly, αENaC when expressed alone was just as abundant at the cell surface as when expressed with β and γENaC, suggesting that at least in this heterologous expression system, αENaC does not require coexpression with β and γENaC subunits to traffic to or from the cell surface (Fig. 2A). We observed a dramatic reduction in cell surface expression of ENaC with each of the Nedd4-2 isoforms and in a dose-dependent manner, with Nedd4-2ΔC2 being more potent than Nedd4-2 (Fig. 2A). These results are consistent with a role for Nedd4-2 in ubiquitination and degradation of ENaC.

Nedd4-2 overexpression decreases ENaC stability. To determine whether Nedd4-2 increases degradation of ENaC, we examined the effect of Nedd4-2 on steady-state levels of αENaC in HEK293 cells after arresting protein translation with cycloheximide. We demonstrate that the half-life of αENaC is shorter when Nedd4-2 is overexpressed compared with its absence confirming that Nedd4-2 increases ENaC turnover (Fig. 2B).

Inactive Nedd4-2 does not reduce surface expression or function of αENaC. To determine whether the ubiquitin ligase function of Nedd4-2 was necessary for the effect of Nedd4-2 on membrane expression of αENaC, we created a catalytically
inactive Nedd4-2 by mutating a HECT domain cysteine residue at position 942 to serine (Nedd4-2CS). Compared with wild-type Nedd4-2, ubiquitin ligase-deficient Nedd4-2 had no effect on surface expression of ENaC in HEK293 cells (Fig. 3A). To validate the surface biotinylation assay, we reprobed surface blots for actin, an abundant intracellular cytoskeletal protein and demonstrated its absence from the biotinylated fraction of cellular proteins. We then transfected HEK293 cells with tagged ENaC subunits, HA-tagged ubiquitin, and Nedd4-2 or Nedd4-2 CS to test the effect of Nedd4-2 on ubiquitination of αβγENaC. Immunoprecipitation of ENaC followed by immunoblotting for HA-tagged proteins demonstrated the characteristic high molecular mass smear of ubiquitinated proteins. The data show that Nedd4-2 robustly stimulates ubiquitination of the ENaC multimer while Nedd4-2CS did not appear to increase ubiquitination (Fig. 3B).

We then generated an adenoviral vector expressing Nedd4-2 CS, expressed this in M-1 cells, and measured amiloride-sensitive

Fig. 1. Nedd4-2 isoforms differentially regulate ENaC activity in M-1 cells. A: M-1 cells transduced with adenovirus expressing Nedd4-2, Nedd4-2ΔC2, Nedd4-2ΔWW2,3, or empty virus and short-circuit current (Isc) measured in Ussing chambers under control conditions and after treatment with 100 nM dexamethasone (dex) for 24 h. M-1 cells overexpressing all 3 isoforms had significantly lower currents compared with empty virus (***P < 0.001; Tukey’s pairwise multiple comparison, SigmaStat; n = 15; means ± SE). Following steroid treatment, currents were significantly different in between each of the Nedd4-2 isoforms (###P < 0.001 compared with Nedd4-2ΔWW2,3; $P < 0.05 compared with Nedd4-2ΔWW2,3; P < 0.05 compared with Nedd4-2; Tukey’s pairwise multiple comparison, SigmaStat; n = 15; means ± SE). B: representative Western blot of transduced M-1 cells demonstrates relatively equal expression of each of the Nedd4-2 isoforms. Full-length Nedd4-2 migrates at ~116 kDa, Nedd4-2ΔC2 (N4-2ΔC2) migrates at ~101 kDa, and Nedd4-2ΔWW2,3 (N4-2ΔWW2,3) migrates at ~83 kDa.

Fig. 2. Surface expression and the half-life of ENaC are greatly reduced by Nedd4-2. A: Nedd4-2 isoforms or a control empty vector were transfected with ENaC subunits as indicated into HEK293 cells and biotinylated surface proteins (top) and total cellular lysates (bottom) were blotted with an anti-FLAG antibody, the epitope carried by the αENaC subunit. Each of the Nedd4-2 isoforms reduces surface expression of αENaC in a dose-dependent manner with Nedd4-2ΔC2 being most potent. Representative of at least 2 separate experiments. B: ENaC subunits were cotransfected with Nedd4-2 in HEK293 cells. Cells were treated with 20 μg/ml cycloheximide to arrest translation and at indicated time points lysates were prepared and immunoblotted with an anti-FLAG antibody and after stripping reprobed with anti-α tubulin antibody. Overexpression of Nedd4-2 decreases αENaC stability.
Isc. Compared with an adenoviral vector expressing Nedd4-2, Nedd4-2 CS mutant had no significant effect on ENaC-mediated Na\(^+\) transport, although both Nedd4-2 forms were equally expressed when analyzed by Western blotting (Fig. 3, C and D). The lack of effect of a ubiquitin-ligase-deficient Nedd4-2 on Na\(^+\) transport in M-1 cells correlated with preserved surface expressed ENaC in HEK cells. Each of the Nedd4-2 isoforms enhances ubiquitination. Since the ubiquitin ligase function of Nedd4-2 appeared to be required for its effect on Na\(^+\) transport, we studied the effect of Nedd4-2 isoforms on ubiquitination of ENaC. Ubiquitinated proteins appear as high molecular mass smear. Compared with active Nedd4-2, Nedd4-2-CS has no effect on ubiquitination of ENaC in HEK293 cells. C: adenoviral vectors expressing active or inactive Nedd4-2 (Nedd4-2-CS) or a control empty virus were transduced into M-1 cells. Compared with active Nedd4-2, Nedd4-2-CS has no effect on amiloride-sensitive Isc in M-1 cells (*P < 0.05; Dunn’s pairwise multiple comparison, SigmaStat; n = 6; means ± SD). D: representative Western blot of transduced M-1 cells demonstrates relatively equal expression of the Nedd4-2 isoforms.

Each of the Nedd4-2 isoforms enhances ubiquitination. Since the ubiquitin ligase function of Nedd4-2 appeared to be required for its effect on Na\(^+\) transport, we studied the effect of Nedd4-2 isoforms on ubiquitination of ENaC subunits. Immunoprecipitation of ENaC followed by immunoblotting for ubiquitinated proteins demonstrated that ubiquitination of ENaC subunits can be detected even in the absence of overexpressed Nedd4-2 and that ENaC is ubiquitinated even when expressed alone. Importantly, ubiquitination of the αβγ complex increased in the presence of each of the Nedd4-2 isoforms, although we could not detect a noticeable difference among three isoforms (Fig. 4).

Each subunit of ENaC can be ubiquitinated which is enhanced by Nedd4-2. We next addressed the question of whether the β and γENaC subunits can be ubiquitinated and if this ubiquitination was also enhanced by Nedd4-2. Flag-tagged αENaC, V5-tagged βENaC, and myc-tagged γENaC subunits were expressed together or individually along with HA-tagged ubiquitin in the presence and absence of Nedd4-2 in HEK293 cells and ubiquitinated proteins were detected by immunoprecipitation and blotting. We observed that both β and γENaC subunits can be ubiquitinated when expressed alone and that this was further stimulated by Nedd4-2 (Fig. 5, A and B). Together, the data in Figs. 4 and 5 clearly show that each of the three ENaC subunits can be ubiquitinated and that this can occur even when these subunits are expressed alone and that in each case ubiquitination is enhanced by overexpression of Nedd4-2.

**Fig. 3.** Inactivation of the catalytic domain of Nedd4-2 abolishes its effect on ENaC. A: active or inactive Nedd4-2 (Nedd4-2-CS) or a control empty vector was transfected with ENaC subunits as indicated into HEK293 cells and biotinylated surface proteins and total cellular lysates were blotted with an anti-FLAG antibody, or an actin antibody. The data demonstrate that Nedd4-2 CS does not affect surface expression of ENaC. Actin was immunoblotted in total and surface fractions to confirm that intracellular proteins are excluded in affinity-purified surface protein preparations. B: active or inactive Nedd4-2 (Nedd4-2-CS) or a control empty vector was transfected with ENaC subunits and HA-tagged ubiquitin (HA-Ub) as indicated into HEK293 cells. ENaC subunits were immunoprecipitated with an anti-FLAG antibody and immunoblotted with anti-HA to detect ubiquitinated ENaC. Ubiquitinated proteins appear as high molecular mass smear. Compared with active Nedd4-2, Nedd4-2-CS has no effect on ubiquitination of ENaC in HEK293 cells. C: adenoviral vectors expressing active or inactive Nedd4-2 (Nedd4-2-CS) or a control empty virus were transduced into M-1 cells. Compared with active Nedd4-2, Nedd4-2-CS has no effect on amiloride-sensitive Isc in M-1 cells (*P < 0.05; Dunn’s pairwise multiple comparison, SigmaStat; n = 6; means ± SD). D: representative Western blot of transduced M-1 cells demonstrates relatively equal expression of the Nedd4-2 isoforms.

**Fig. 4.** Each of the Nedd4-2 isoforms enhances the ubiquitination of ENaC. Nedd4-2 isoforms or a control empty vector were transfected with ENaC subunits and HA-Ub as indicated into HEK293 cells. ENaC subunits were immunoprecipitated with an anti-FLAG antibody and immunoblotted with anti-HA to detect ubiquitinated ENaC. Ubiquitinated proteins appear as high molecular mass smear. αENaC when expressed alone can be ubiquitinated and ubiquitination of the ENaC complex is increased in the presence of each Nedd4-2 isoform.
tinated by Nedd4-2. To detect ubiquitinated ENaC at the cell surface, tagged ENaC subunits were transfected alone or together with or without Nedd4-2 and HA-Ub as indicated into HEK293 cells. ENaC subunits were immunoprecipitated with an anti-V5 (β) or anti-myc (γ) antibody and immunoblotted with anti-HA to detect ubiquitinated ENaC subunits. A: ENaC subunit β is ubiquitinated when expressed alone or together and when βENaC is expressed alone, this ubiquitination is enhanced by coexpression of Nedd4-2. B: ENaC subunit γ is ubiquitinated when expressed alone or together and as with βENaC, this is enhanced by coexpression of Nedd4-2. Similar results were observed from at least 3 independent experiments for each subunit.

Cell surface fraction of expressed αENaC is monoubiquitinated. We then performed experiments to determine whether αENaC at the cell surface is modified by the addition of monoubiquitin chains at multiple residues (multiquitination) or by the addition of polyubiquitin chains. HEK293 cells were cotransfected with increasing amounts of ENaC subunits and 24-h posttransfection cells were treated with MG132, and then surface proteins were biotinylated and cell lysates were prepared. Expressed cellular αENaC was isolated from the total protein by immunoprecipitation with anti-FLAG antibody and the cell surface-specific fraction of αENaC was captured by Neutravidin affinity purification. Total as well as surface fractions of αENaC were separated by SDS-PAGE, blotted, and probed first with an anti-polysubiquitin antibody (FK1) and after stripping, reprobed with P4D1, an antibody that detects polyubiquitin as well as monoubiquitin chains attached to substrate proteins. We show the characteristic high molecular mass smear of ubiquitinated proteins captured by FK1 antisera in whole cell lysates but not in surface fractions.
whereas reblotting the same membrane with P4D1 shows the high molecular mass smear in both the fractions (Fig. 6, B and C). This result indicates that αENaC when expressed in HEK293 cells is monoubiquitinated but not polyubiquitinated at the cell surface, confirming results recently reported by others (33).

Effect of Nedd4-2 isoforms on ubiquitination of total and surface ENaC subunits. To determine whether there are qualitative differences in the ability of Nedd4-2 isoforms to ubiquitinate individual ENaC subunits, we expressed individual subunits with each of the Nedd4-2 isoforms. Each of the subunits was ubiquitinated but we were unable to demonstrate a meaningful difference in ubiquitination of total cellular ENaC between each of the Nedd4-2 isoforms (Fig. 7A). We also examined the effect of Nedd4-2 isoforms on ubiquitination of individual ENaC subunits at the cell surface and demonstrated that each of the Nedd4-2 isoforms was able to ubiquitinate surface α (Fig. 7B), β, and γENaC (data not shown). Finally, we confirmed that each of the Nedd4-2 isoforms reduced surface expression of αENaC when expressed alone (Fig. 7C).

ENaC is target for proteosomal degradation. Ubiquitinated ENaC is internalized and is thought to be targeted to the lysosome or proteosome for degradation. To determine whether ENaC is a target for proteosomal or lysosomal degradation, we overexpressed tagged ubiquitin with αβγENaC subunits in HEK293 cells in the presence and absence of MG132 or chloroquine, followed by immunoprecipitation and immunoblotting (Fig. 8A).

We demonstrate that ubiquitinated αENaC is increased in the presence of MG132 but not with chloroquine, suggesting that ENaC is targeted to the proteosome for degradation. To determine whether inhibition of the proteosomal degradation pathway would have an impact on Na⁺ transport, we then tested the effect of MG132 in M-1 cells. Following MG132 treatment, Na⁺ transport increased significantly as early as 1 h and reached a peak (~2-fold) between 2–3 h (Fig. 8B). Due to a significant drop in resistance in MG132-treated monolayers, we could not study the effect of MG132 beyond 4 h. To determine whether Nedd4-2 targets ENaC to the proteosome, we overexpressed Nedd4-2 in M-1 cells and then examined the effect of MG132 on Na⁺ transport. As described earlier, Nedd4-2 reduced basal ISc compared with empty virus. Treatment with MG132 was able to partially reverse the effect of Nedd4-2 on Na⁺ transport in M-1 cells, suggesting that Nedd4-2 reduces Na⁺ transport by targeting ENaC subunits to the proteosome (Fig. 8C). Interestingly, chloroquine appeared to reduce Na⁺ transport in M-1 cells.

Finally, we tested the effect of MG132 or chloroquine on Na⁺ transport after expression of each of the Nedd4-2 isoforms. As shown earlier, Nedd4-2ΔC2 had a larger effect on inhibition of Na⁺ transport compared with Nedd4-2 and with Nedd4-2ΔWW2,3 (Fig. 8D). Compared with Nedd4-2 and with Nedd4-2ΔWW2,3, MG132 was unable to significantly increase Na⁺ transport in cells expressing Nedd4-2ΔC2. Together, the data in M-1 and HEK293 cells suggest that ENaC...
is variably regulated by individual Nedd4-2 isoforms and targeted to the proteasome for degradation.

**DISCUSSION**

Nedd4-2 (also referred to as Nedd4L) is a member of the Nedd4/Rsp5 family of E3 ubiquitin protein ligases with a catalytic HECT domain (homologous to E6-AP COOH terminus) and was originally identified as a developmentally regulated gene highly expressed in the mouse central nervous system (16). These are modular proteins that contain multiple WW domains, which are protein-protein interaction domains, a COOH-terminal HECT domain which carries the ubiquitin ligase function and some members of this family also contain an NH2-terminal C2 domain, which is a Ca2+/lipid-binding domain (1, 26). Members of Nedd4/Rsp5 family are evolutionarily highly conserved and are required for the ubiquitination of a large number of cellular targets and regulate diverse cellular processes including signaling, trafficking of plasma membrane proteins, and degradation of misfolded proteins.

In previous studies, we reported the existence of multiple isoforms of hNedd4-2 that arise from alternate transcription and translation and from variable splicing of some internal exons (11). This leads to Nedd4-2 isoforms with and without a C2 domain and isoforms that vary in the number of WW domains. In vitro binding studies suggested that different WW domains have variable affinities against PY motif of ENaC (2, 8, 9, 11, 14). When coexpressed with ENaC in X. laevis oocytes, we found that Nedd4-2 with and without a C2 domain robustly reduced Na+/H+ transport, although a Nedd4-2 isoform without WW domains 2 and 3 resulted in a smaller fall in Na+/H+ transport that was not statistically significant (12). The effects of Nedd4-2 isoforms on Na+/H+ transport correlated with the

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**Fig. 8.** Effect of Nedd4-2 isoforms and proteasomal inhibitors on ENaC function. A: ENaC subunits were expressed with and without HA-Ub in HEK293 cells and cells were treated with or without 1 μM MG132 or 100 μM chloroquine for 4 h as indicated. Ubiquitinated ENaC subunits were detected by immunoprecipitation with anti-Flag followed by immunoblotting with anti-HA antibody. There appeared to be more ubiquitinated ENaC detectable in the presence of MG132. B: M-1 cells grown in filters were treated with or without 1 μM MG132 and Isc was measured in Ussing chambers. Isc was expressed as fold change compared with the control value corresponding to the same time point. Currents increase significantly within an hour of MG132 and reach a peak between 2–3 h (**P < 0.001; *P < 0.05 Tukey’s pairwise multiple comparison, SigmaStat; n = 8 from 3 different experiments; means ± SE). C: M-1 cells were transduced with adenovirus expressing Nedd4-2 or empty virus and Isc was measured in Ussing chambers under control conditions and after treatment with MG132 or chloroquine for 2 h. M-1 cells overexpressing Nedd4-2 had significantly lower currents compared with empty virus. MG132 but not chloroquine partially reverses the effect of Nedd4-2 on Na+ transport (*P < 0.05 between vehicle and MG132; ¶P < 0.05 between vehicle and chloroquine; Tukey’s pairwise multiple comparison, SigmaStat; n = 12 from 3 experiments; means ± SE). D: M-1 cells were transduced with adenovirus expressing each of the Nedd4-2 isoforms and Isc was measured in Ussing chambers under control conditions and after treatment with MG132 or chloroquine for 2 h. M-1 cells overexpressing Nedd4-2ΔC2 had lower currents compared with Nedd4-2 and Nedd4-2ΔWW2,3. MG132 partially reverses the effect of Nedd4-2 and Nedd4-2ΔWW2,3, but not Nedd4-2ΔC2, on Na+ transport (*P < 0.05 between vehicle and MG132; ¶P < 0.05 between vehicle and chloroquine; Tukey’s pairwise multiple comparison, SigmaStat; n = 16 from 3 experiments; means ± SE).
strength of their interaction when heterologously expressed in COS-7 cells.

In the present study, we extend these observations by examining the effect of Nedd4-2 isoforms on surface expression of ENaC and on ubiquitination and degradation of ENaC. We first tested the effect of Nedd4-2 isoforms expressed with recombinant adenoviral vectors on Na+ transport in M-1 cells. We found that each of the three isoforms substantially inhibited Na+ transport, although Nedd4-2 ΔWW2,3 was the weakest inhibitor (Fig. 1). The prior data in *X. laevis* oocytes may reflect differences in the model system wherein both Nedd4-2 and ENaC were heterologously expressed and reflect inefficiencies in plasmid-based gene transfer while simultaneously maintaining monolayer integrity in M-1 cells (12). We find that adenoviral transduction of M-1 cells leads to high level gene expression with little effects on transcellular resistance in Ussing chambers. The inhibition of Na+ transport with Nedd4-2 correlated with reduction in cell surface expression of ENaC when each of the Nedd4-2 isoforms was expressed. As has been previously reported, the effect of Nedd4-2 on Na+ transport and on surface expression appeared to require its ubiquitin ligase domain (Fig. 3). However, while others reported a dominant negative effect of ENaC at least in *X. laevis* oocytes, we do not see such an effect in M-1 cells (4, 14, 24). This may mean that another ubiquitin ligase that interacts with and has a higher affinity for ENaC may be naturally expressed in M-1 cells.

We examined membrane expression of ENaC using a surface biotinylation assay. We demonstrate that αENaC can efficiently traffic to the cell surface even when expressed without β and γ subunits of ENaC (Fig. 2). These studies would indicate that single subunits are processed and delivered to the cell surface, although these experiments do not help us determine whether the αENaC subunit can be selectively trafficked to the apical membrane of polarized cells. Others reported that individual ENaC subunits form homomultimers and can traffic to COS-7 and HEK293 plasma membranes with high efficiency, although the cell surface subunits appear to be minimally glycosylated and present in detergent-insoluble complexes (25). Whether individual ENaC subunits have any physiological function is not known, although when expressed alone, the αENaC subunit but not the β and γENaC subunit can support small amiloride-sensitive currents (5, 23).

We compared each of three hNedd4-2 isoforms in their ability to reduce cell surface expression of αENaC in heterologous expression system (Fig. 2). We observe that all three isoforms are able to downregulate cell surface expression of αENaC without any significant change in total αENaC. Nedd4-2 ΔC2 is most competent in reducing surface expression of αENaC. Previous patch-clamp experiments using *X. laevis* expression system demonstrated that removal of C2 domain of hNedd4 led to an enhanced ability to decrease sodium channels at the cell surface, suggesting that the C2 domain may interfere with Nedd4 function (14). As described by others, the C2 domain of Nedd4-2, in contrast, does not appear to significantly alter the effect of Nedd4-2 on ENaC expression or function (14). We compared each of the three hNedd4-2 isoforms in their ability to ubiquitinate the αβγENaC complex. We were unable to demonstrate any differences between these isoforms (Fig. 4). This may be because ubiquitinated ENaC in an overexpression system may represent the composite of that ubiquitinated intracellularly during processing and that ubiquitinated at the cell surface in response to expressed Nedd4-2. Second, differences in ubiquitination patterns between these isoforms may have differing effects on internalization of ENaC or vary the fate of ubiquitinated ENaC between the degradative and recycling pathways and measurements of total or surface ubiquitinated ENaC can mask real differences in the fate of ENaC. Our results show that there are measurable differences in the ability of these isoforms to reduce surface expression of ENaC and to modulate Na+ transport, although we know little about the relative contribution of these alternate forms to Na+ transport in the native collecting duct.

Our studies demonstrate that each of the Nedd4-2 isoforms reduces surface expression of αENaC and that this is mediated via its ubiquitin ligase activity (Figs. 2 and 3). Ubiquitination is an important mechanism for the degradation of intracellular as well as cell surface proteins including ion channels. The interaction of the WW domains of Nedd4-2 with the PY motif of ENaC is thought to bring the ubiquitin-protein ligase domain into close proximity with lysine residues in the NH2 terminus of the channel subunits, leading to ubiquitination of the subunits. In previous studies, the α and γ subunits, but not the β subunit, were considered to be targets for ubiquitination, although this was not consistently examined (3, 10, 31). We demonstrate that ENaC subunits can be ubiquitinated when expressed as a multimeric protein (Fig. 4) as has been demonstrated very recently by others (34). The FLAG epitope on αENaC was used to immunoprecipitate αENaC and as β and γENaC subunits interacting with α these subunits are also likely to be present in this complex, the demonstrated ubiquitination likely corresponds to the sum of the ubiquitination of all ENaC subunits (Fig. 4). To confirm that Nedd4-2 could modify each ENaC subunit, we also examined ubiquitination of individual subunits with each of the Nedd4-2 isoforms (Fig. 7).

ENaC can be modified by the addition of monoubiquitin at multiple lysine residues to form a multiubiquitinated complex or by addition of polyubiquitin chains to a single lysine residue (13, 21). Some proteins are subject to both forms of posttranslational modification. While polyubiquitinated proteins are typically targeted to the proteasome, monoubiquitinated proteins may be targeted to the lysosome or simply be tagged for recycling endosomes. As has been recently reported, we demonstrate that while the cytosolic ENaC is polyubiquitinated, the small fraction of ENaC that is expressed at the cell surface is multiubiquitinated (33).

Although ubiquitination of ENaC is now well-recognized, neither the intracellular location nor the fate of ubiquitinated ENaC is well-established. Misfolded or unassembled proteins in the endoplasmic reticulum are generally removed by the proteasomal pathway as part of the quality control machinery of the cell. However, the synthesis of αβγENaC subunits is not coordinately regulated with αENaC being regulated by corticosteroids in the connecting tubule and collecting duct while βγ subunits are regulated by corticosteroids in the colon. Under salt-replete conditions, there is little detectable αENaC in the distal nephron, although there is abundant β and γENaC in an intracellular location that appears to be protected from proteasomal degradation despite the absence of αENaC in the protein complex. Upon salt depletion or under conditions of aldosterone excess, αENaC is rapidly synthesized and the fully assembled αβγ heterotrimer now moves to the apical membrane (19, 20). It would appear therefore that under physiolog-
ichanical conditions, the role of ubiquitin ligases may be primarily to regulate the abundance of ENaC that reaches the cell surface. Our data suggest that the proteasomal pathway regulates ENaC abundance in M-1 cells and in HEK293 cells. We also demonstrate that inhibition of the proteasomal pathway, but not the lysosomal pathway, increases Na\(^+\) transport in M-1 cells. Similar results have been reported in A6 cells, a distal nephron cell line derived from X. laevis kidney (22).

In summary, we find that α, β, and γ subunits of ENaC can be ubiquitinated when expressed alone as well as when expressed as a complex. The cell surface expression of ENaC and the function of ENaC are reduced by each of the Ned4-2 isoforms, with Ned4-2 ΔC2 having the strongest effect. Interaction with Ned4-2 and the subsequent ubiquitination of ENaC subunits appear to target these proteins for proteasomal degradation.

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