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Human Nedd4 interacts with the human epithelial Na^+ channel: WW3 but not WW1 binds to Na^+-channel subunits

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The epithelial Na^+ channel (ENaC) regulates Na^+ absorption in epithelial tissues including the lung, colon and sweat gland, and in the distal nephrons of the kidney. When Na^+-channel function is disrupted, salt and water homoeostasis is affected. The cytoplasmic regions of the Na^+-channel subunits provide binding sites for other proteins to interact with and potentially regulate Na^+-channel activity. Previously we showed that a proline-rich region of the α subunit of the Na^+ channel bound to a protein of 116 kDa from human lung cells. Here we report the identification of this protein as human Nedd4, a ubiquitin-protein ligase that binds to the Na^+-channel subunits via its WW domains. Further, we show that WW domains 2, 3 and 4 of human Nedd4 bind to the α, β and γ Na^+-channel subunits but not to a mutated β subunit. In addition, when co-expressed in *Xenopus* oocytes, human Nedd4 down-regulates Na^+-channel activity.

Key words: ENaC, WW domain, ubiquitin-protein ligase.

INTRODUCTION

The epithelial Na^+ channel (ENaC) located in the distal nephrons of the kidney, and in the lung, sweat glands and colon is an essential component of the body's salt-and-water-regulation system [1]. The Na^+ channel is composed of three subunits called α, β and γ that share similar primary sequences [2–5]. Each subunit has cytosolic N- and C-termini, two transmembrane domains and a large extracellular domain [6–8].

In humans, disruption of normal Na^+-channel activity results in disease. Loss-of-function mutations in one of the Na^+-channel subunits cause the neonatal salt-wasting disorder pseudohypoaldosteronism type I [9]. Targeted disruption of the β or γ subunits in mice produces a similar phenotype [10,11]. Conversely, in Liddle's syndrome mutations in the β or γ subunits cause severe high blood pressure [12–14]. Na^+ channels containing the Liddle's mutation expressed *in vitro* show at least twice the activity of normal channels and the turnover rate of mutant channels appears to be decreased [15,16].

Regulation of channel activity and localization can be achieved through binding of proteins to the cytoplasmic portions of the Na^+-channel subunits. All three subunits of the Na^+ channel contain proline-rich sequences in their C-terminal domains. Such regions can mediate binding to WW and SH3 (Src homology 3) domains, small hydrophobic protein domains (38 and 60 amino acids respectively) that bind to proline-containing motifs. WW domains bind to ligands containing either PPXY [17] or PPLP [18,19] sequences, whereas the minimal binding motif for SH3 domains is PXXP [20].

Previously we reported that, *in vitro*, the Na^+-channel α subunit bound to a subset of SH3 domains [21]. Also, the SH3 domain of spectrin binds to the α subunit and localizes it to the apical membrane of epithelial cells [22]. In addition, Staub et al. [23] reported that the WW domains of the ubiquitin-protein ligase Nedd4 [neuronal precursor cell expressed developmentally down-regulated (gene 4)] bind to the PPPXY (PY) motifs found in the C-terminus of all three subunits, and that the α and γ subunits are ubiquitinated [24]. The PY motif of either the β or the γ subunit is disrupted in Liddle's syndrome and it has been hypothesized that failure of Nedd4 to bind to Na^+-channel subunits and mediate ubiquitination prevents channels from being tagged for degradation. This would result in an increased number of active Na^+ channels in the plasma membrane and lead to increased blood pressure, as observed in Liddle's syndrome.

In addition to SH3-domain proteins, we identified a 116-kDa protein that bound to the C-terminal region of the α subunit [21]. Binding was localized to an 18-amino acid peptide containing the PY motif. Here we identify p116 to be human Nedd4 (hNedd4). Interestingly, hNedd4 contains four WW domains, whereas rat and mouse Nedd contain three WW domains. Here we characterize hNedd4 WW domain binding to the human Na^+-channel subunits and show that hNedd4 decreases Na^+-channel activity upon co-expression in *Xenopus* oocytes.

EXPERIMENTAL PROCEDURES

Cell culture

H441 cells were maintained in RPMI 1640 medium. Madin–Darby canine kidney (MDCK) and cos-7 cells were maintained in Dulbecco's modified Eagle's and low-bicarbonate Dulbecco's modified Eagle's media respectively. All media were supplemented with 10% fetal calf serum and antibiotics and cells were grown in 5% CO_2.

Cloning and production of fusion proteins

hNedd4 was cloned by PCR of human kidney cDNA (Clontech, Palo Alto, CA, U.S.A.) using the following primers: 5′ primer, 5′-GGAATTCATGAATGGAGTTCTTACA-3′; and 3′ primer, 5′-GGAATTCCTAATCAACTCCATCAAA-3′, and ligated into the pMT3 vector. Also, hNedd4 was subcloned into the mammalian expression vector pHM6 (Roche Molecular Biochemicals, Mannheim, Germany) which contains a haemagglutinin (HA) tag.

Abbreviations used: ENaC, epithelial Na^+ channel; hENaC, human ENaC; αhENaC, α subunit of hENaC; GST, glutathione S-transferase; SH3, Src homology 3; PY motif, PPPXY motif; MDCK, Madin–Darby canine kidney; HA, haemagglutinin; hNedd4, human Nedd4; WWP, WW-domain-containing protein.

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Individual hNedd4 WW-domain constructs were amplified using PCR and the following pairs of primers: WW1, 5′-CGGGATCCCATTTGCAGCAACAACAA-3′ and 5′-CGGAATTCCTAAATGTTGCCATTCTCAGCAT-3′; WW2, 5′-CGGGATCCCCTGTGCTTTTGCCTACTTC-3′ and 5′-CGGAATTCCTAGCTTGAGGTCAGCTGACTGG-3′; WW3, 5′-CGGGATCCACCCAGCCATCTGAAATTGA-3′ and 5′-CGGAATTCCTATGATGTCTTTCCTCTCAGAT-3′; and WW4, 5′-CGGGATCCTCACTTGATACTTCCAATGA-3′ and 5′-CGGAATTCCTAGGGCACTGCTGGTCCAGT-3′.

The C-terminal regions of the human Na^+-channel subunits (α, β and γ) were amplified using the following primer pairs: αhCterm, 5′-TGGTCTCCAGGCCGAGGGGG-3′ and 5′-GGGCCCCCCCAGAGGACAGGTG-3′; βhCterm, 5′-AGTTACGCTGGCCCACCGCC-3′ and 5′-TTAGATGGCATCACCCTCAC-3′; and γhCterm, 5′-CAGGCTCCCCCATGTCCA-3′ and 5′-TCAGAGCTCATCCAGCATC-3′. All PCR fragments were cloned into the pGEX2TK vector. Fusion proteins were induced with 1 mM isopropyl β-D-thiogalactoside for 2–3 h, and fusion proteins were isolated from sonicated bacterial lysates using glutathione–Sepharose beads (Amersham Pharmacia Biotech, Bucks, U.K.). DNA sequences were confirmed using Big-dye terminator sequencing and analysis on an ABI 377 sequencer. The glutathione S-transferase (GST) fusion proteins containing hNedd4 WW domains also underwent matrix-assisted laser-desorption ionization (MALDI) mass analysis to confirm their molecular masses.

Binding assay and immunodepletion

MDCK cells were grown to 80% confluence and metabolically labelled by overnight incubation with 27 μCi/ml EasyTag™ Express [^{35}S]methionine/cysteine labelling mix (NEN, Boston, MA, U.S.A.). MDCK cells were lysed in RIPA buffer (58 mM NaCl, 5 mM EDTA, 10 mM Tris/HCl, pH 7.2, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 10 μg/ml PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1 μg/ml pepstatin A) for 1 h at 4 °C, centrifuged and the lysates pooled. For immunodepletion, samples of labelled lysate were: (i) precleared by incubation with GST attached to glutathione–Sepharose beads twice for 1 h each at 4 °C, and then bound to specific GST-fusion proteins to detect p116 as a positive control; (ii) immunodepleted by incubation with anti-Nedd4 antibody conjugated to Protein A–agarose, precleared as above and then incubated with specific GST-fusion proteins; or (iii) immunodepleted either in the absence of antibody or with anti-actin (A2066, Sigma, St Louis, MO, U.S.A.) conjugated to Protein A–agarose, precleared and then incubated with specific GST-fusion proteins. After binding with specific GST-fusion proteins the beads were washed twice in RIPA buffer, twice in RIPA buffer containing 0.25 M NaCl and then twice in RIPA buffer containing 0.5 M NaCl. After addition of 5 × SDS sample buffer (150 mM Tris, pH 6.8, 5% SDS, 0.08% Bromophenol Blue, 25% glycerol) containing 5.5 mM 2-mercaptoethanol, the beads were boiled for 5 min and analysed by electrophoresis through 8% polyacrylamide gels. Gels were stained in Coomassie Brilliant Blue R-250, destained, incubated in En^{3}Hance (NEN) for 1 h and then in double-distilled H_2O for 30 min, dried down and exposed to Biomax film (Kodak, Rochester, NY, U.S.A.).

Transient expression in cos-7 cells and GST pulldown assays

Cos-7 cells were plated at a density of 2×10^5 cells/2 ml on 35-mm plates. Then 18–20 h later the cells were transfected with cDNA constructs using Fugene-6 (Roche Molecular Biochemicals) and the manufacturer's protocol. The cDNAs encoding Na^+-channel subunits contained the FLAG-epitope tag (DYKDDDDK) and were cloned into either pcDNA3 or pMT3 as described in [25]. A β subunit containing the Y620A mutation also contained a FLAG epitope inserted immediately after the most C-terminal residue and was subcloned into pMT3. Approx. 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 1% Triton X-100 and protease inhibitors, for 1–2 h at 4 °C. After centrifugation at 13000 ***g*** for 5 min the lysates were pooled, if appropriate, and equal amounts added to 1.5-ml tubes. Two preclearance steps were carried out, where the lysates were incubated first with 40 μl of glutathione-Sepharose beads and then with 50 μg of GST attached to glutathione-Sepharose beads for 1 h each at 4 °C. Next, precleared lysates were added to 1.5-ml tubes containing 50 μg of specific GST-fusion protein and incubated at 4 °C for 3–4 h. After extensive washing in ice-cold lysis buffer (without protease inhibitors), the samples were boiled for 5 min in SDS sample buffer and separated by electrophoresis in 8% polyacrylamide gels. Western transfer on to nitrocellulose (Schleicher and Schuell) was performed for 3–4 h at 100 V. Membranes were blocked with 5% BSA in TTBS (1% Tween-20/150 mM NaCl/50 mM Tris, pH 7.4) overnight at 4 °C. Membranes were then incubated with one of the following antibodies: 1.4 μg/ml anti-FLAG-M2 (Kodak Scientific Imaging Systems, New Haven, CT, U.S.A.), 1 mg/ml anti-Nedd4 (gift of Dr. D. Rotin, Hospital for Sick Children, Toronto, Canada) or 50 ng/ml anti-HA (3F10, Roche Molecular Biochemicals) for 2 h at room temperature, washed extensively in TTBS, then incubated for 1 h in horseradish peroxidase-coupled secondary antibody. After further washing in TTBS detection was achieved by chemiluminescence (either LumiLight from Roche Molecular Biochemicals, or ECL® from Amersham Pharmacia Biotech).

Northern blotting

A human mRNA Masterblot was obtained from Clontech. A ^{32}P-labelled hNedd4 probe was prepared by isolation of a 1-kb fragment (containing all four WW domains) and random priming following the manufacturer's directions (Roche Molecular Biochemicals). The blot was hybridized overnight in Church's buffer (0.5 M Na_2HPO_4/1% BSA/1 mM EDTA, pH 8.0/7% SDS; [26]), washed extensively in 0.5 × SSC/1% SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), and exposed to Kodak Biomax film.

Expression and electrophysiology in *Xenopus* oocytes

α, β and γ subunits of human ENaC (hENaC) cloned into pMT3 (0.2 ng each, as described in [4,5]) were expressed in combination with hNedd4 or secreted alkaline phosphatase (control), cloned into pMT3 (0.2 or 0.8 ng), in *Xenopus* oocytes by nuclear injection of cDNA. Following injection (1 or 2 days), whole-cell current was measured by two-electrode voltage-clamp at −60 mV with oocytes bathed in 116 mM NaCl/2 mM KCl/0.4 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes (pH 7.4). Amiloride-sensitive current was calculated as the difference in current before and after addition of amiloride (100 μM) to the bathing solution. Expression of ENaC with secreted alkaline phosphatase did not alter Na^+-channel current compared with cells expressing ENaC alone.

RESULTS

The α subunit of hENaC (αhENaC) binds to both p116 and Nedd4

We tested the hypothesis that p116, a protein in human lung cell (H441) lysates that binds to the PY motif of αENaC, is Nedd4.

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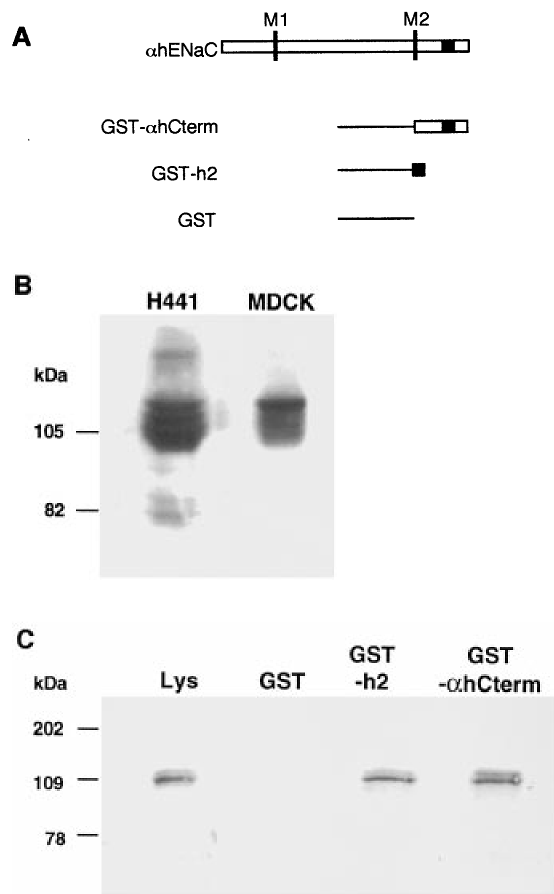

Figure 1 Nedd4 is expressed in H441 and MDCK cells

(**A**) Schematic diagram of the αhENaC protein (M1 and M2 represent membrane-spanning domains), and the GST-fusion proteins used. The GST moiety is represented by a line (not to scale). The αhENaC GST-fusion proteins contain either the full C-terminal region of αhENaC (GST-αhCterm) or 18 amino acids (GST-h2) from within the C-terminal region, where a black box represents the amino acids SQPGPAPSPALTAPPPAY. (**B**) Lysates from both H441 and MDCK cells were electrophoresed and Western blotted with an anti-Nedd4 antibody. The lower-molecular-mass bands observed probably represent degradation products. (**C**) GST-fusion proteins containing the αhENaC C-terminal region (GST-αhCterm) or a short peptide (GST-h2) specifically bound to Nedd4 from H441 cell lysates, but GST alone did not. Lys represents H441 cell lysate that was included as a control for anti-Nedd4 antibody binding. Similar results were observed using MDCK cell lysates (results not shown).

Two findings suggest this may be the case. First, the size of p116 (116 kDa) is consistent with the apparent molecular mass of rat Nedd4. Second, we found that H441 and MDCK cells both express Nedd4, as shown by Western blotting with anti-rat Nedd4 (see Figure 1B). This antibody bound to a cluster of between two and four proteins of approx. 116 kDa, similar to what was observed by Staub et al. [23]. In the absence of anti-Nedd4 antibody no staining was observed, suggesting that all the bands were specific (results not shown). In addition, GST-fusion proteins (Figure 1A) containing no insert (GST alone), the full C-terminal region of αhENaC (GST-αhCterm) or an 18-amino acid peptide containing the PY motif (GST-h2) were incubated with unlabelled MDCK- and H441-cell lysates. Specific binding of Nedd4 to the α subunit of the Na^+ channel was detected by Western blotting (Figure 1C).

Immunodepletion studies show that p116 is Nedd4

To determine whether p116 is Nedd4 we immunodepleted Nedd4 from cell lysates and asked whether p116 was still available to bind to αhENaC GST-fusion proteins. We performed this experiment in MDCK cells since they are kidney cells, they form an epithelium and they are a common model system for studying epithelial proteins. To determine whether MDCK cells express p116, we incubated αhENaC GST-fusion proteins with extracts of metabolically labelled MDCK cells. The αhENaC GST-fusion proteins bound a protein identical in size to p116 (Figure 2, lanes 4 and 7), indicating that, in addition to H441 cells, MDCK cells also express this protein. We removed Nedd4 from ^{35}S-labelled MDCK-cell lysates using anti-Nedd4 antibody conjugated to Protein A–agarose. Subsequently, immunodepleted lysates were precleared and then incubated with GST alone, GST-αhCterm or GST-h2. Figure 2 shows that when Nedd4 was immunodepleted, no p116 was bound to GST-αhCterm or GST-h2 (lanes 5 and 8). When control immunodepletions were carried out, p116 still bound to both GST-αhCterm and GST-h2 (lanes 6 and 9). GST alone did not bind p116 under any of these conditions (lanes 1–3). This result strongly suggests that p116 is Nedd4.

hNedd4 cloning and mRNA expression

The data suggest that p116 in H441 cells is hNedd4. A cDNA sequence corresponding to hNedd4 (Genbank accession number D42055) was used to clone hNedd4 from human kidney cDNA. Since the exact 5′ end of hNedd4 has not been reported we

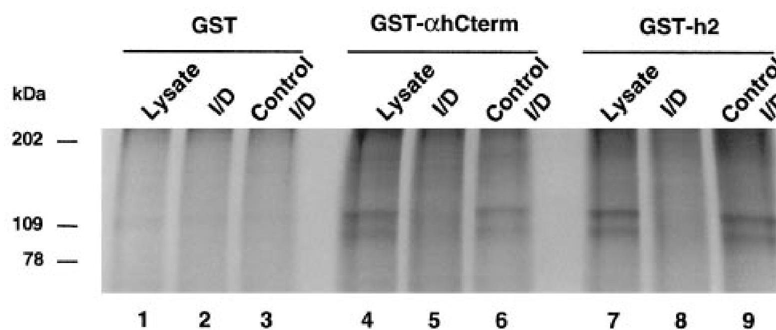

Figure 2 After MDCK cells are immunodepleted of Nedd4, p116 is not available to bind αhENaC GST-fusion proteins

Lane 1, GST lysate, shows that p116 from MDCK cell lysates does not bind to GST alone; however, p116 is observed binding to both GST-αhCterm (lane 4) and GST-h2 (lane 7). After immunodepletion with anti-Nedd4 (I/D), p116 was not bound to GST-αhCterm (lane 5) or GST-h2 (lane 8). Lanes labelled control I/D show a control immunodepletion with no anti-Nedd4 present. The lower immunodepleted band probably represents a degradation product.

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hNedd4 MNGVLTSVQTKTIKKSLNPKWNEEILFRVHPQQHRLLFEVFDENRLTRDDFLGQVDVPLY 60
rNedd4 MSGVLTSVQTKTIKKSLNPKWNEEILFRVLPQQHRILFEVFDENRLTRDDFLGQVDVPLY 167

hNedd4 PLPTENPRLERPYTFKDFVLHPRSHKSRVKGYLRLKMTYLPKTSGSEDDNAEQAEELEPG 120
rNedd4 PLPTENPRMERPYTFKDFVLHPRSHKSRVKGYLRLKMTYLPK-NGSDDENADQAEELEPG 226

hNedd4 WVVLDQPDAACHLQQQQEPSPLPPGWEERQDILGRTYYVNHESRRTQWKRPTPQDNLTDA 180
rNedd4 WVVLDQPDAATHLQHPPEPSPLPPGWEERQDVLGRTYYVNHESRTTQWKRPSPEDDLTDD 286

hNedd4 ENGNIQLQAQRAFTTRRQISEETESVDNQESSENWEIIREDEATMYSSQAFPSPPPSSNL 240
rNedd4 ENGDIQLQAHGAFTTRRQISEDVDGPDNHESPENWEIVREDENTIYSGQAVQS-PPSGHP 345

hNedd4 DVPTHLAEELNARLTIFGNSAVSQPASSSNHSSRRGSLQAYTFEEQPTLPVLLPTSSGLP 300
rNedd4 DVQVRLAEELDTRLTMYGNPATSQPVTSSNHSSRGGSSQTCIFEEQPTLPVLLPTSSGLP 405

hNedd4 PGWEEKQDERGRSYYVDHNSRTTTWTKPTVQATVETSQLTSSQSSAGPQSQASTSDSGQQ 360
rNedd4 PGWEEKQDDRGRSYYVDHNSKTTTWSKPTMQDDPR------------------------- 449

hNedd4 VTQPSEIEQGFLPKGWEVRHAPNGRPFFIDHNTKTTTWEDPRLKIPAHLRGKTSLDTSND 420
rNedd4 -----------------------------------------SKIPAHLRGKTPVD-SND 492

hNedd4 LGPLPPGWEERTHTDGRIFYINHNIKRTQWEDPRLENVAITGPAVPYSRDYKRKYEFFRR 480
rNedd4 LGPLPPGWEERTHTDGRVFFINHNIKKTQWEDPRMQNVAITGPAEPYSRDYKRKYEFFRR 517

hNedd4 KLKKQNDIPNKFEMKLRRATVLEDSYRRIMGVKRADFLKARLWIEFDGEKGLDYGGVARE 540
rNedd4 KLKKQTDIPNKFEMKLRRANILEDSYRRIMGVKRADFLKARLWIEFDGEKGLDYGGVARE 577

hNedd4 WFFLISKEMFNPYYGLFEYSAT-DNYTLQINPNSGLCNEDHLSYFKFIGRVAGMAVYHGK 599
rNedd4 WFFLISKEMFNPYYGLFEYSATEDNYTLQINPNSGLCNEDHLSYFKFIGRVAGMAVYHGK 637

hNedd4 LLDGFFIRPFYKMMLHKPITLHDMESVDSEYYNSLRWILENDPTELDLRFIIDEELFGQT 659
rNedd4 LLDGFFIRPFYKMMLQKLITLHDMESVDSEYYSSLRWILENDPTELDLRFIIDEELFGQT 697

hNedd4 HQHELKNGGSEIVVTNKNKKEYIYLVIQWRFVNRIQKQMAAFKEGFFELIPQDLIKIFDE 719
rNedd4 HQHELKTGGSEVVVTNKNKKEYIYLVIQWRFVNRIQKQMAAFKEGFFELIPQDLIKIFDE 757

hNedd4 NELELLMCGLGDVDVNDWREHTKYKNGYSANHQVIQWFWKAVLMMDSEKRIRLLQFVTGT 779
rNedd4 NELELLMCGLGDVDVNDWREHTKYKNGYSLNHQVIHWFWKAVLMMDSEKRIRLLQFVTGT 817

hNedd4 SRVPMNGFAELYGSNGPQSFTVEQWGTPEKLPRAHTCFNRLDLPPYESFEELWDKLQMAI 839
rNedd4 SRVPMNGFAELYGSNGPQSFTVEQWGTPDKLPRAHTCFNRLDLPPYESFDELWDKLQMAI 877

hNedd4 ENTQGFDGVD 849
rNedd4 ENTQGFDGVD 887
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Figure 3 Comparison of the human and rat Nedd4 amino acid sequences

The single dashed overline shows the C2 domain, single overlines show WW domains and double overlines show the ubiquitin-protein ligase domain. Note that the sequence comparison is made over the region of hNedd4 used in the experiments reported here.

designed primers for cloning hNedd4 by comparing the hNedd4 amino acid sequence with a functional murine Nedd4 sequence [27], and used the hNedd4 methionine, which corresponded to the predicted murine Nedd4 start methionine. Using 5′ RACE (rapid amplification of 5′ cDNA ends) techniques we have not found cDNA fragments which extend the hNedd4 sequence past the available sequence (F. J. McDonald, unpublished work). hNedd4 is 86% identical to rat Nedd4. However, hNedd4 contains four WW domains compared with only three WW domains in rodent Nedd4 (Figure 3). Therefore it was important to determine which of the four WW domains bind to ENaC subunits and to ask if hNedd4 regulates Na^{+}-channel function.

Nedd4 was described originally as a developmentally down-regulated gene in brain tissue [28]; however, Nedd4 is also

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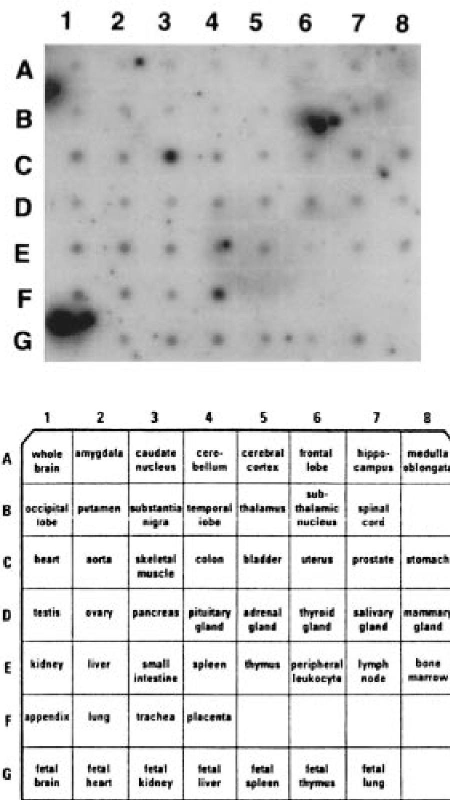

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	sub-thalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	

Figure 4 hNedd4 Northern-blot analysis

A Clontech Master RNA blot was hybridized with a ^{32}P-labelled hNedd4 probe. Human-tissue sources of the mRNAs on the Master RNA blot are shown below. Note that very dark off-centre spots in a number of the squares represent non-specific binding. This same Master blot was probed with two different DNA probes [for the WW-domain-containing proteins (WWPs) WWP2 and WWP4] prior to this experiment. Each of the three probes showed differential expression, demonstrating that the signal shown here for Nedd4 is specific.

expressed in adult tissues [29]. By Northern-blot analysis we found that hNedd4 is widely expressed (Figure 4). In fact Nedd4 was expressed in all adult tissues examined, including tissues that contain epithelia and express ENaC. Previously it was also shown that rat Nedd4 and the Na$^+$-channel subunits co-localize in kidney, lung and colon epithelial cells [29]. Interestingly, Nedd4 is also expressed in a number of tissues that do not express ENaC, raising the possibility that Nedd4 also modulates the function of proteins other than ENaC. In addition, hNedd4 was detected in a number of human fetal tissues including kidney and lung (Figure 4).

ENaC subunits bind to hNedd4 expressed in cos-7 cells

To investigate the interactions between hNedd4 and the Na$^+$-channel subunits the hNedd4 cDNA was subcloned into the pHM6 vector, which contains an HA tag, and hNedd4 was expressed transiently in cos-7 cells. Using immunoprecipitation and Western blotting with an anti-HA antibody, a 116-kDa protein corresponding to hNedd4 was specifically expressed in and isolated from Nedd4-expressing cells (results not shown).

To confirm that hNedd4 bound to the α subunit and to ask if hNedd4 also bound to the β and γ ENaC subunits, GST pulldown assays were performed. Human Nedd4 was transiently expressed in cos-7 cells and the cell lysates were incubated with

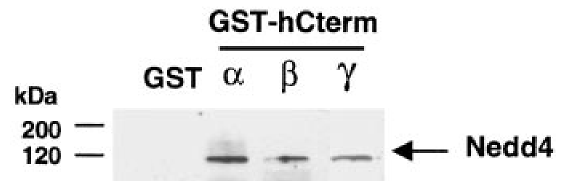

Figure 5 The C-terminal regions of the α, β and γ Na$^+$-channel subunits bind to hNedd4 expressed in cos-7 cells

HNedd4 was transiently expressed in cos-7 cells and cell lysates were incubated with GST alone or with GST-fusion proteins containing the C-terminal regions of the α, β or γ Na$^+$-channel subunits. Bound proteins were separated by SDS/PAGE, and hNedd4 binding was detected by Western blotting with an anti-HA antibody.

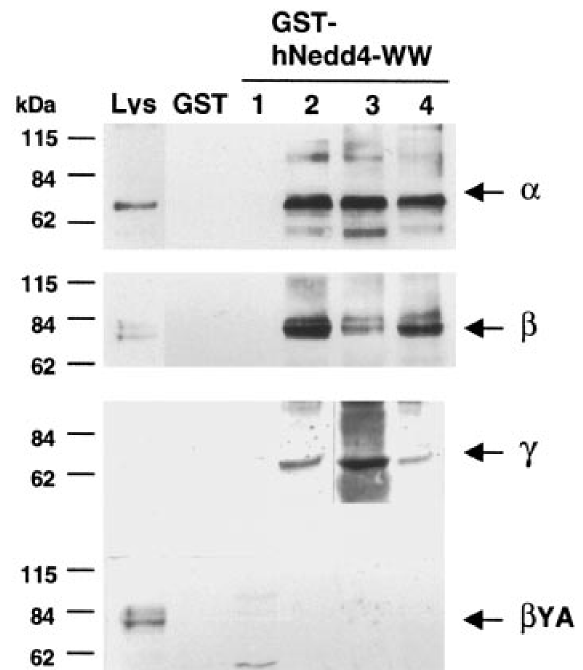

Figure 6 hNedd4 WW-domain binding to Na$^+$-channel subunits expressed in cos-7 cells

GST-fusion proteins containing the four individual hNedd4 WW domains (lanes 1–4) or GST alone were incubated with cos-7 cell lysates expressing FLAG-tagged α, β, γ or βY620A (βYA) Na$^+$-channel subunits. Bound proteins were separated by SDS/PAGE. Binding was detected through Western blotting with the anti-FLAG-M2 antibody. To show expression of the Na$^+$-channel subunits 50 μl of cos-7 cell lysate was taken before binding and included in the Western-blot analysis as a positive control in lanes labelled Lys. Note that γ was not able to be detected in the lysate in this experiment.

GST-fusion proteins containing the C-terminal regions of the human α, β or γ Na$^+$-channel subunits. Figure 5 shows that in control incubations GST alone did not bind to hNedd4, whereas the GST-fusions containing the C-terminal regions of the α, β and γ Na$^+$-channel subunits all bound to hNedd4.

Individual WW-domain binding to ENaC subunits

Staub et al. [23] reported previously that rat Nedd4 WW domains 1, 2 and 3 bind to the β and γ Na$^+$-channel subunits. Human Nedd4 contains a fourth WW domain situated between the second and third WW domains of rat Nedd4 (Figure 3). Note

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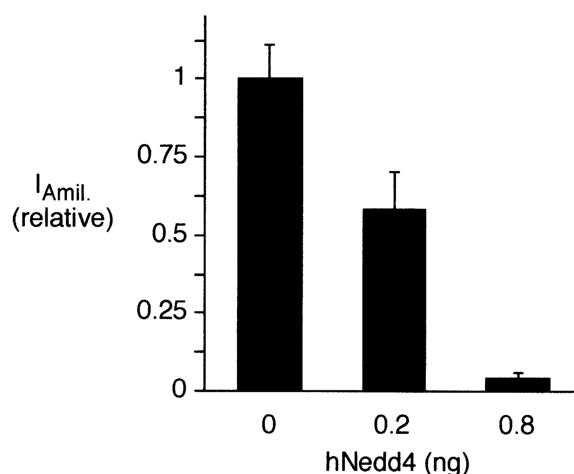

Figure 7 Down-regulation of Na^+-channel activity by hNedd4

Amiloride-sensitive Na^+ current (I_{Amil}) for ENaC co-expressed in oocytes with 0.2 or 0.8 ng of Nedd4 or secreted alkaline phosphatase (0 ng, control), as indicated. Data are expressed relative to current recorded from control oocytes expressing ENaC with secreted alkaline phosphatase, and are means ± S.E.M. for $n = 12$–14.

that WW4 of hNedd4 is homologous to rat WW3 whereas human WW3 appears to be unique. To ask which of the four WW domains of hNedd4 bound to the Na^+-channel subunits, GST-fusion proteins containing individual WW domains from hNedd4 were prepared. For these experiments full-length α, β or γ Na^+-channel subunits containing the FLAG-epitope tag were expressed transiently in cos-7 cells. Cell lysates were then incubated with GST-fusion proteins containing each of the hNedd4 WW domains, or with GST alone. Since each Na^+-channel subunit contained an epitope tag recognized by the anti-FLAG-M2 antibody, Western blotting was used as the assay for binding. Binding of hNedd4 WW domains to a FLAG-tagged β subunit containing a tyrosine-to-alanine mutation (Y620A), which confers increased Na^+-channel activity equivalent to a Liddle's syndrome mutation [15], was also carried out. The binding results are shown in Figure 6. WW domains 2, 3 and 4 of hNedd4 bound to the α, β and γ Na^+-channel subunits. However, in contrast to rat Nedd4, WW1 did not bind to α, β or γ. None of the WW domains bound to a β subunit containing the Y620A mutation, suggesting that Y620 is required for hNedd4 WW-domain binding.

Human Nedd4 inhibits ENaC activity in *Xenopus* oocytes

To investigate whether hNedd4 interacts with and regulates the human Na^+ channel *in vivo*, we co-expressed hNedd4 with the α, β and γ hENaC subunits in *Xenopus* oocytes. In the absence of hNedd4, an amiloride-sensitive Na^+ current was generated (Figure 7), as previously described [5]. When an equal amount of hNedd4 was co-expressed with the α, β and γ hENaC subunits, Na^+-channel activity was decreased by 50 % (Figure 7). When four times the amount of hNedd4 was co-expressed with the Na^+ channel, current was abolished. Therefore, hNedd4 was able to interact with and down-regulate the Na^+ channel *in vivo*.

DISCUSSION

The identity of p116, a protein previously isolated as an αhENaC-binding protein, was found to be hNedd4 by use of an immuno-depletion experiment and Western blotting. The polyclonal anti-Nedd4 antibody used in this experiment was raised against WW2 of rat Nedd4 [23]. We cannot exclude the possibility that the anti-Nedd4 antibody removed WW-domain proteins in addition to Nedd4. The WW-domain-containing proteins (WWPs) identified by Pirozzi et al. [30] contain WW domains, bind to Na^+-channel peptides and are of a similar predicted molecular mass to Nedd4. However, WW2 of hNedd4 is 82 % identical to rat WW2 (7 amino acid changes out of 38). This contrasts to between 40 and 60 % amino acid identity with the WW domains of WWP1 and 2.

Northern-blot analysis (Figure 4) of human adult and fetal mRNA shows that hNedd4 is widely expressed in both adult and fetal tissues, and tissues that lack ENaC. Therefore hNedd4 potentially regulates multiple proteins in different tissues. A number of proteins from different tissues have been identified with PY motifs [31] and these, or other PY-containing proteins, may be potential targets for Nedd4 regulation. In support of this statement GST-fusion proteins containing the WW domains of hNedd4 bind to multiple proteins of varying molecular masses in H441 cells (S. J. Coddington-Lawson and F. J. McDonald, unpublished work).

Previous studies have reported that all three WW domains of rat Nedd4 bind to the rat β and γ Na^+-channel subunits in filter-binding assays; however, binding of individual rat Nedd4 WW domains to the α subunit was not tested [23]. Human Nedd4 differs from rodent Nedd4 in having four WW domains. Thus it was critical to determine which of the four WW domains bind to ENaC subunits. This is clinically relevant since Liddle's syndrome is caused by mutations in human ENaC that disrupt WW-domain-binding sites. Using GST pulldown assays, which show binding of transiently expressed full-length Na^+-channel subunits to GST-fusion proteins containing individual WW domains, we show here that not all the WW domains of hNedd4 bind the α, β and γ subunits of ENaC. Specifically, WW domains 2, 3 and 4 of hNedd4 bind to the α, β and γ Na^+-channel subunits, but WW1 does not bind to any of the Na^+-channel subunits. WW1 in rNedd4 binds the rat β and γ Na^+-channel subunits; however, the equivalent WW1 in hNedd4 does not bind the human β or γ Na^+-channel subunits. Although WW1 does not bind to any of the Na^+-channel subunits, the same GST-WW1 fusion protein binds to a number of unidentified proteins in lung cell extracts (results not shown), suggesting that the hNedd4 WW1 domain is able to bind and regulate proteins other than the Na^+ channel, or that these other proteins are required for regulation of ENaC. There are five amino acid changes between the WW1 of human and rat Nedd4 that might account for the different binding properties. Interestingly, mouse WW1 appears to be functionally more similar to human than to rat WW1, since this mouse domain did not bind to ENaC subunits [32]. Our binding studies *in vitro* suggest that all three Na^+-channel subunits bind to WW domains of hNedd4. However, further experiments will be required to establish which WW domains bind to the Na^+ channel *in vivo*, and whether all the Na^+-channel subunits are bound by hNedd4.

None of the hNedd4 WW domains bind to a βhENaC subunit containing alanine at position 620 in place of tyrosine. It was reported previously that this mutation causes an approx. 2-fold increase in Na^+-channel activity [15]. This residue is therefore critical for correct regulation of the Na^+ channel. Other studies have reported that a subset of WWPs, including Nedd4, require a tyrosine in the binding sites of their substrates [17,23]. Recently Lu et al. [33] reported binding of murine Nedd4 and Pin1 WW domains to phosphoserine- and phosphothreonine-containing peptides. Since our binding experiments utilize full-length Na^+-

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channel subunits, phosphorylation could be required for the binding of some or all of the Nedd4 WW domains, although our protocol did not contain phosphatase inhibitors. However, the lack of WW-domain binding to the mutant Y620A β subunit suggests that the PY motif in the C-terminal region of all three ENaC subunits is the WW-domain-binding site.

Goulet et al. [34] and Abriel et al. [35] reported down-regulation of Na^+-channel activity by rat and *Xenopus* Nedd4, respectively. Here we show that hNedd4 down-regulates activity of the human Na^+ channel. Although the cDNA clone of hNedd4 used here may be incomplete at the 5′ end, the apparent molecular mass of 116 kDa suggests that our hNedd4 is full length. The C2 domain is found at the N-terminus of Nedd4, and rat Nedd4 [as well as murine Nedd4, a more recent addition to Genbank (accession number P46935)] has an extended N-terminal region compared with hNedd4. Deletion of the C2 domain prevents rNedd4 from moving to the plasma membrane [36]. However, the hNedd4 cDNA used here appears to contain all the information necessary for hNedd4 to localize to the membrane, bind to the Na^+ channel and promote down-regulation of ENaC.

Understanding the role of hNedd4 regulation of the Na^+ channel is important in understanding the function of human ENaC in normal and disease conditions. Nedd4 is a ubiquitin-protein ligase and the binding of Nedd4 to the Na^+-channel subunits may mediate the addition of ubiquitin to the α and γ subunits. As a result ENaC may be targeted to the lysosome or proteosome for degradation [24]. Consistent with this is the observation that Nedd4 increased the rate of degradation of ENaC [34]. Therefore, by controlling the rate of turnover of ENaC, Nedd4 could play a critical role in the regulation of renal Na^+ absorption, and hence, the control of blood pressure. However, further investigation will be required to determine whether Nedd4 mediates the ubiquitination of ENaC, and how ubiquitination results in removal of ENaC from the membrane for channel degradation.

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