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WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome

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The amiloride-sensitive epithelial sodium channel (ENaC) plays a major role in sodium transport in kidney and other epithelia, and in regulating blood pressure. The channel is composed of three subunits (αβγ) each containing two proline-rich sequences (P1 and P2) at its C-terminus. The P2 regions in human β and γ ENaC, identical to the rat βγrENaC, were recently shown to be deleted in patients with Liddle's syndrome (a hereditary form of hypertension), leading to hyperactivation of the channel. Using a yeast two-hybrid screen, we have now identified the rat homologue of Nedd4 (rNedd4) as the binding partner for the P2 regions of β and γrENaC. rNedd4 contains a Ca^{2+} lipid binding (CaLB or C2) domain, three WW domains and a ubiquitin ligase (Hect) domain. Our yeast two-hybrid and *in vitro* binding studies revealed that the rNedd4-WW domains mediate this association by binding to the P2 regions, which include the PY motifs (XPPXY) of either βrENaC (PPPNY) or γrENaC (PPPRY). SH3 domains were unable to bind these sequences. Moreover, mutations to Ala of Pro616 or Tyr618 within the βrENaC P2 sequence (to PPANY or PPPNA, respectively), recently described in Liddle's patients, led to abrogation of rNedd4-WW binding. Nedd4-WW domains also bound to the proline-rich C-terminus (containing the sequence PPPAY) of αrENaC, and endogenous Nedd4 co-immunoprecipitated with αrENaC expressed in MDCK cells. These results demonstrate that the WW domains of rNedd4 bind to the PY motifs deleted from β or γENaC in Liddle's syndrome patients, and suggest that Nedd4 may be a regulator (suppressor) of the epithelial Na^+ channel.

Keywords: epithelial Na^+ channel/Nedd4/PY motif/ubiquitin ligase/WW domains

Introduction

The amiloride-sensitive sodium channel, located at the apical membrane, provides the rate-limiting step for Na^+ transport in high-resistance epithelia. It plays a major role in fluid absorption in the kidney and colon (Rossier and Palmer, 1992; Garty, 1994; Benos *et al.*, 1995) as well as in fluid clearance from the alveolar space at birth and during pulmonary edema (Saumon and Basset, 1993; O'Brodovich, 1995). Proper regulation of this channel is crucial for maintenance of Na^+ balance, blood volume and blood pressure. It is highly regulated by hormones like aldosterone and vasopressin, as well as by PKA/cAMP, PKC, Ca^{2+} and G proteins (Garty, 1994; Benos *et al.*, 1995). Recently, the molecular structure of the rat amiloride-sensitive epithelial Na^+ channel (rENaC) isolated from rat colon has been deduced (Canessa *et al.*, 1993, 1994a; Lingueglia *et al.*, 1993, 1994). It consists of three homologous subunits (αβγrENaC) highly conserved between different species, including rat (rENaC), human (hENaC) and *Xenopus laevis* (xENaC) (McDonald *et al.*, 1994, 1995; Voilley *et al.*, 1994; Puoti *et al.*, 1995). Each subunit consists of two transmembrane domains, a large extracellular domain and two short cytoplasmic tails (Canessa *et al.*, 1994b; Renard *et al.*, 1994; Snyder *et al.*, 1994). Little is known about the structure–function relationships of ENaC. However, each of the three subunits possesses two conserved proline-rich sequences (P1 and P2) within their C-terminal tails. Specific proline-rich sequences, conforming to the minimal consensus of PXXP (Feng *et al.*, 1994; Yu *et al.*, 1994) are known to play an important role in protein–protein interactions via SH3 domains (Mayer and Baltimore, 1993; Cohen *et al.*, 1995). We have previously shown that the P2 region in the C-terminus of αrENaC binds to the SH3 domain of α-spectrin *in vitro* and in living cells, and that microinjection of αrENaC C-terminus led to exclusive localization of the protein to the apical membrane of polarized alveolar epithelial cells, with distribution that parallels that of α-spectrin (Rotin *et al.*, 1994). The proline-rich regions in the C-termini of β and γrENaC, however, do not bind α-spectrin (S.Dho and D.Rotin, unpublished results).

Recently, Liddle's syndrome, a hereditary form of systemic hypertension characterized by hyperactivity of the epithelial sodium channel, has been linked by a genetic linkage analysis to the locus encoding the β-subunit of hENaC (Shimkets *et al.*, 1994). In five affected Liddle syndrome kindreds, mutations were found in the C-terminal region of the β-subunit, introducing premature stop codons or frameshift mutations, and invariably leading to the deletion of the conserved proline-rich (P2) region. More recently, a family suffering from Liddle's syndrome has been found to carry a C-terminal truncation (and thus deletion of the proline-rich region) within the γ-subunit of the channel (Hansson *et al.*, 1995a). So far, there have not been any Liddle patients identified with such C-terminal deletions within the α-subunit. Expression of C-terminal truncated β or γrENaC (which share sequence identity to hENaC in the proline-rich P2 regions) in *Xenopus* oocytes led to an increase in amiloride-sensitive Na^+ current relative to intact β or γ subunits, suggesting that these C-terminal truncations cause an increase in the number of open channels at the plasma membrane (Schild

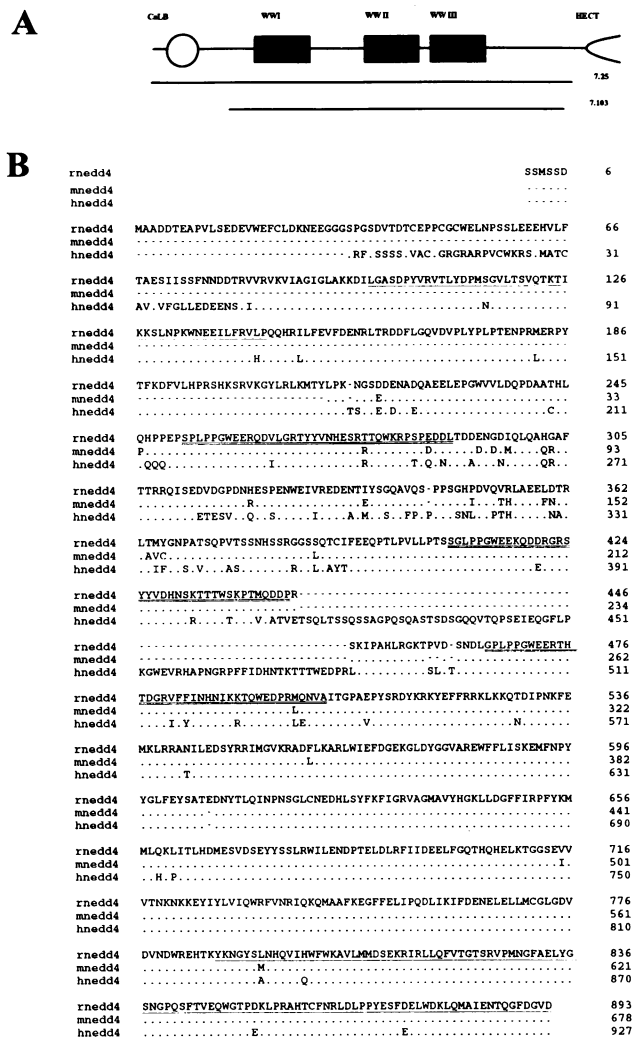

Fig. 1. Nedd4 protein sequence. (**A**) Schematic representation of the rNedd4 protein and the two partial cDNA clones of rNedd4 (7.103 and 7.25) isolated in the two-hybrid screen. (**B**) Protein sequence alignment of rat (r), mouse (m) and human (h) Nedd4 (labelled nedd4). Changes between rat, mouse and human sequences are indicated. Dots represent identities, dashes represent deletions. The N-terminal regions of mNedd4 or hNedd4 are not yet known. The three WW domains are double underlined, the CaLB domain at the N-terminus and the Hect domain at the C-terminus are underlined. The fourth WW domain of hNedd4, less conserved than the other three, is included in amino acids 447–486. The GenBank/EMBL accession number for the DNA sequence is U50842.

et al., 1995). Together, these findings show that the proline-rich C-termini of β and γENaC play an important role in regulating channel activity. It also suggests that there may be a regulatory repressor factor, possibly a protein, which normally interacts with these sequences. To identify such a putative regulatory protein, we carried out a yeast two-hybrid screen of a rat lung library using the proline-rich (P2) region of βrENaC as a bait. We report here that using this screen, we have isolated a cDNA encoding rNedd4, the rat homologue of Nedd4 (Kumar *et al.*, 1992), which contains three WW domains, a CaLB (C2) domain and a ubiquitin ligase (Hect) domain. Moreover, we show by either co-immunoprecipitation, two-hybrid, or *in vitro* binding assays that rNedd4 can

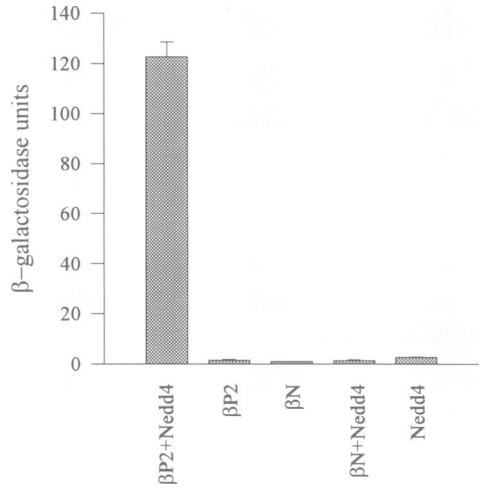

Fig. 2. Interactions of rNedd4 with the P2 region of βrENaC (βP2) in the two-hybrid system. Yeast L40 cells were transformed with βP2 as a fusion protein with LexA, alone or together with the rNedd4 clone 7.103 (called Nedd4) fused to the GAL4 activation domain. Similar transformations were performed with a control fragment corresponding to the N-terminal region of βrENaC (βN) fused to LexA, without or with the rNedd4 clone 7.103. rNedd4 was also transformed alone. Transformants were assayed by a liquid β-galactosidase assay as described in the Materials and methods. Each column is the result of three measurements. Bars represent standard errors.

also bind γrENaC and αrENaC, and that interactions are mediated by binding of the Nedd4 WW domains to the P2 regions (which include the sequence XPPXY, or PY motifs) of the rENaC subunits. We thus propose that by WW domain-mediated binding to regions important for ENaC function, Nedd4 may be a regulator of the epithelial Na^+ channel.

Results

Isolation of rNedd4 by the yeast two-hybrid screen

To identify proteins that interact with the proline-rich region of βrENaC (implicated in channel hyperactivation and deleted in Liddle patients), we performed a yeast two-hybrid screen (Chien *et al.*, 1991) of a rat lung library using the 49 C-terminal amino acids of βrENaC, which include the conserved P2 region (βP2) as a bait. Two independent clones (7.103 and 7.25) were isolated, both encoding partial sequences of the rat homologue of Nedd4 (rNedd4) (Figure 1A). Nedd4 (**N**eural precursor cells **e**xpressed **d**evelopmentally **d**own-regulated) was originally isolated as a partial cDNA clone from a mouse brain library (Kumar *et al.*, 1992). Using the clone 7.25 as a probe, clones encoding the C-terminal region of rNedd4 were subsequently isolated. The deduced sequence of rNedd4 (Figure 1B) encodes an open reading frame of 893 amino acids with a calculated M_r of 103 003. Both methionines at positions 3 and 7 conform to the consensus for translation initiation sites (Kozak, 1989). As there are no stop codons upstream of these methionines, we cannot exclude the possibility that we are still missing N-terminal sequences. The rNedd4 protein is 95.9% identical to the mouse (97.7% similarity, if conserved amino acids are taken in account) and 85.8% identical to the human (91.8% similarity) Nedd4 (Figure 1B). Both mouse and rat Nedd4, as well as the yeast homologue Rsp5, contain three copies

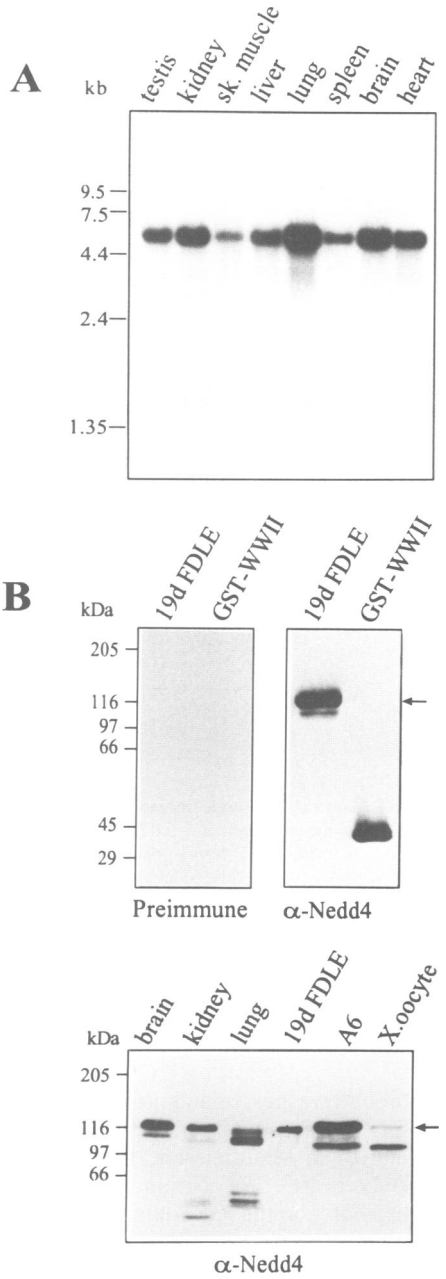

Fig. 3. Expression of Nedd4 in various tissues. (**A**) A rat multiple tissue Northern blot (MTN, Clontech) containing 2 μg of poly(A)$^+$ RNA from the indicated tissues was hybridized with partial Nedd4 clone 7.103 as a probe, as described in Materials and methods. Exposure time was 36 h. (**B**) 100 ng of GST-WWII fusion protein, ~50 μg protein/extract from adult rat whole brain, kidney, lung, primary cultures of rat fetal distal lung epithelial cells from 19-day gestation fetuses (19dFDLE), the *Xenopus*-derived A6 cells, and *Xenopus* oocyte (X. oocyte) were separated on 8% SDS–PAGE. Proteins were then transferred to nitrocellulose and immunoblotted with an antiserum (1:500) directed against GST-Nedd4-WWII fusion protein (α-Nedd4, right panel and lower panel), or with the corresponding preimmune serum (left panel), followed by horseradish peroxidase (HRP)-conjugated secondary antibody and ECL detection. The lower MW band seen in some of the samples (e.g. lung, X. oocyte) probably represents a degradation product.

(but four in the human) of the recently identified WW domain (André and Springael, 1994; Bork and Sudol, 1994; Hofmann and Boucher, 1995), a **Ca** lipid **b**inding (CaLB/C2) domain (Clarke *et al.*, 1991), and a Hect

Fig. 4. Interaction of rNedd4-WW domains with the βP2 region in the two-hybrid assay. Constructs encompassing the different rNedd4-WW domains and flanking sequences were generated as fusion proteins with the GAL4 activation domain for the yeast two-hybrid binding assays, as described in Materials and methods. Each clone was co-transformed with the βP2 sequence fused to the LexA DNA binding domain, grown on minimal plates lacking Trp and Leu, transferred to filter paper, and relative interaction analyzed by a filter β-galactosidase assay. ++++, very strong interaction; ++, intermediate interaction; +, weak interaction; –, no interaction.

(**h**omologous to the **E**6-AP **c**arboxyl **t**erminus) domain with homology to a ubiquitin ligase (E3) enzyme (Huibregtse *et al.*, 1995). Co-transformation in yeast of the βP2 bait together with either of the two clones we isolated showed very strong expression of β-galactosidase, a marker enzyme indicating an interaction between the two proteins. No β-galactosidase activity could be detected when either clone was transformed alone, or when either one was transformed together with the N-terminus of βrENaC (Figure 2). Northern blot analysis using either one of the isolated cDNA clones as a probe revealed a 6.5 kb mRNA species ubiquitously expressed in various tissues but with strongest expression in lung, kidney and brain (Figure 3A). An antibody raised against a GST fusion protein encoding the WWII domain (called αNedd4), but not pre-immune serum, recognized a protein with an apparent molecular weight of ~115 kDa in an immunoblot of rat whole brain, kidney, lung and primary fetal distal lung epithelial (FDLE) cells (Figure 3B), as well as in the kidney-derived MDCK cells (see below). Nedd4 was also detected by our antibodies in *Xenopus* oocytes and in the *Xenopus*-derived A6 cell line (Figure 3B).

Identification of the region in rNedd4 responsible for binding to βrENaC in the two-hybrid system

Analysis of β-galactosidase activity in yeast transformed with the βP2 bait together with either clone 7.103 or 7.25 demonstrated no difference in activity, suggesting that the shorter of the two isolated clones (7.103) contained all the necessary binding information. As it only contains the three WW domains of rNedd4 and flanking sequences, but not the CaLB/C2 or the Hect domains, the latter two domains can be excluded as interacting partners to βP2. It was recently demonstrated that the WW domain of YAP65 interacts with proline-rich sequences with a suggested preliminary consensus of XPPXY (called the PY motif) (Chen and Sudol, 1995; Sudol *et al.*, 1995). The P2 regions of βrENaC (βP2) contains the sequence PPPNY, which conforms to that motif. We therefore tested whether

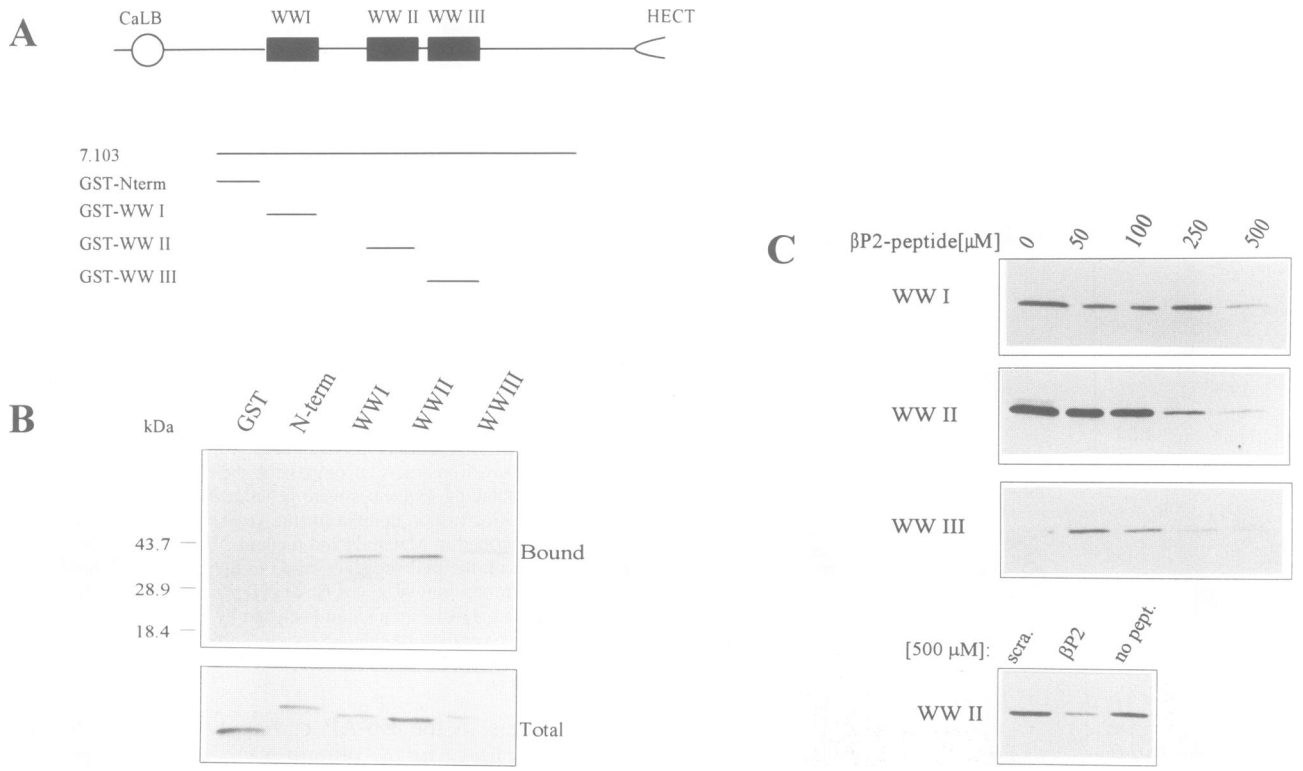

Fig. 5. *In vitro* binding of Nedd4-WW domains to βP2. (**A**) Schematic representation of the GST fusion proteins encompassing individual rNedd4-WW domains (GST-WWI, GST-WWII, GST-WWIII), or the N-terminal flanking sequence (GST-Nterm), generated in bacteria as described in the Materials and methods. (**B**) Soluble 0.3 μg of GST, GST rNEDD4-WWI, -WWII, -WWIII or GST-N terminus (N-term) fusion proteins were incubated with ~0.5 μg His-tagged βP2 immobilized on Ni–agarose beads. Following thorough washes of the beads, bound proteins were separated on 10% SDS–PAGE, transferred to nitrocellulose and blotted with GST antiserum (1:1000) followed by HRP-conjugated secondary antibody and ECL detection. Bound proteins represent those that bound to the βP2 region (upper panel). Lower panel represents the total amount of GST fusion proteins used for the binding assay. (**C**) Competition of a βP2-peptide (TLPIPGTPPPNYDSL) for binding of GST-rNedd4-WWI, WWII, or -WWIII to the βP2 region. Increasing peptide concentrations were added to the incubation reaction and the experiment proceeded exactly as described in (B) above. Lowest panel represents identical competition experiment only with 500 μM βP2 scrambled (scra.) peptide (PITDPLPLTYNPGSP) instead of the above βP2-peptide. Lack of a complete band representing WWIII binding to βP2 in the absence of competing peptide [in (C)] was caused by inappropriate protein transfer at the edge of the nitrocellulose membrane.

the rNedd4-WW domains serve as the actual binding partners to our βP2 bait. We thus generated a series of constructs, depicted in Figure 4, which encompass each WW domain alone, two or three WW domains together, or flanking sequences. Each of these constructs in pGAD10 vector was transformed into *Saccharomyces cerevisiae* reporter strain L40 together with the βP2 bait, to analyze its ability to interact with the bait in the two-hybrid system. Our results show (Figure 4) that the region comprising all three WW domains (WWI–III) was sufficient for full interaction with βP2, whereas no interaction could be detected with the N-terminal, C-terminal flanking regions of clone 7.103, or with a sequence in the middle (MID) between WWI and WWII. β-Galactosidase activity was reduced with the construct covering WWII+III, and was very weak with each single WW domain.

Binding of rNEDD4-WW domains to the βrENaC-P2 region in vitro

In order to show by an independent assay that the WW domains indeed mediate the interactions with the P2 region of βrENaC, we generated several GST fusion proteins corresponding to each Nedd4-WW domain alone or to the N-terminal (Nterm) region of clone 7.103 (Figure 5A). These soluble GST fusion proteins were then incubated with immobilized His-tagged βP2 fusion protein, and following thorough washes, bound proteins were separated on SDS–PAGE and immunoblotted with anti-GST antibodies, as detailed in Materials and methods. Figure 5B shows that only those GST fusion proteins containing WW domains, but not the GST-Nterm protein or GST alone, were able to bind to the βP2 protein. Because the βP2 protein also contains sequences flanking the actual P2 region, we wanted to test whether it is the P2 region itself that mediates the interactions with the WW domains. We therefore performed competition experiments with a peptide covering this βP2 area (called βP2-peptide), or with a corresponding scrambled peptide. Increasing concentrations (0–500 μM) of the βP2-peptide were added to the above binding reaction, to test for competition with WW binding to the immobilized βP2 fusion protein. Our results (Figure 5C) show that binding of all three WW domains could be competed with the βP2-peptide at a concentration ⩾250 μM. No inhibition in this range was seen with a scrambled βP2-peptide (Figure 5C, bottom panel). Thus, these competition experiments demonstrate that it is indeed the proline-rich βP2 region which mediates binding to the WW domains of rNedd4.

Mutation analysis of the βP2 PY (WW binding) motif

To identify key amino acids in the βP2 sequence involved in binding to rNedd4-WW domains, we mutated to Ala

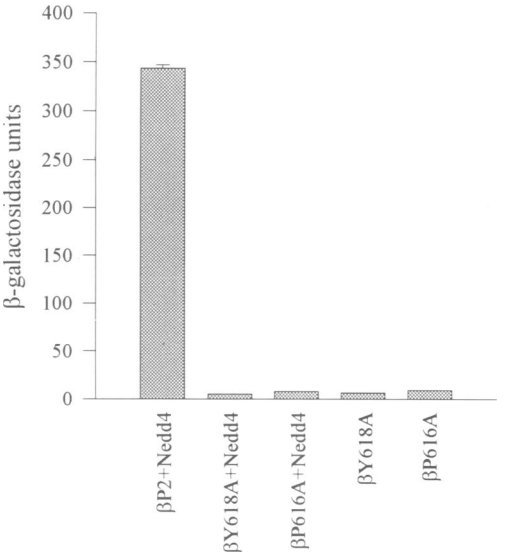

Fig. 6. Effect of point mutations within the βP2-PY motif on rNedd4-WW binding in the two-hybrid assay. Pro616 and Tyr618 of βrENaC, corresponding to the third proline and tyrosine in the PY motif of the βP2 region ($PPP_{616}NY_{618}$) were mutated to alanine (P616A and Y618A, respectively), and the mutants assayed in the two-hybrid system for interaction with rNedd4 (clone 7.103), as described in the legend to Figure 2. Interactions were quantified with the liquid β-galactosidase assay. Data represent mean ± standard errors of three separate measurements.

either the third Pro (Pro616) or the Tyr (Tyr618) in the PY motif of βrENaC (PPPNY to PPAPN or to PPPNA, respectively). These specific mutations were chosen because they disrupt the conserved amino acids of the PY motif (Chen and Sudol, 1995), and because recent studies have identified equivalent mutations in two Liddle's patients (Hansson *et al.*, 1995b; Tamura *et al.*, 1995). Thus, a βP2 construct bearing either one of the above mutations was transformed in yeast together with a clone (clone 7.103) encompassing the three WW domains of rNedd4, to test for interactions in the two-hybrid system. Our results demonstrate that mutation of either of the indicated Pro or Tyr abolished the interaction with rNedd4-WW domains (Figure 6).

Binding of rNedd4-WW domains to γ and αrENaC

Comparison of the P2 sequences of γrENaC or αrENaC (or γhENaC and αhENaC, as well as the *Xenopus* homologues) with that of βrENaC shows a high degree of conservation, especially around the PY motif (Figure 7A). Moreover, a recent study (Hansson *et al.*, 1995a) has identified a subset of Liddle's syndrome patients with a C-terminal truncation (including the P2 sequence) in γhENaC. We therefore focused our studies initially on γrENaC, and tested whether the P2 region of γrENaC can also bind to rNedd4. The C-terminus of γrENaC was co-transformed together with the three rNedd4-WW domains (clone 7.103) into yeast L40 cells, and β-galactosidase activity analyzed to determine interaction between the two proteins in the two-hybrid assay. The results (Figure 7B) show that, similar to βrENaC, γrENaC was also able to bind the rNedd4-WW domains. These interactions using the two-hybrid assays were subsequently confirmed by *in vitro* binding experiments. GST fusion proteins of each WW domain (WWI, WWII and WWIII) of rNedd4, or

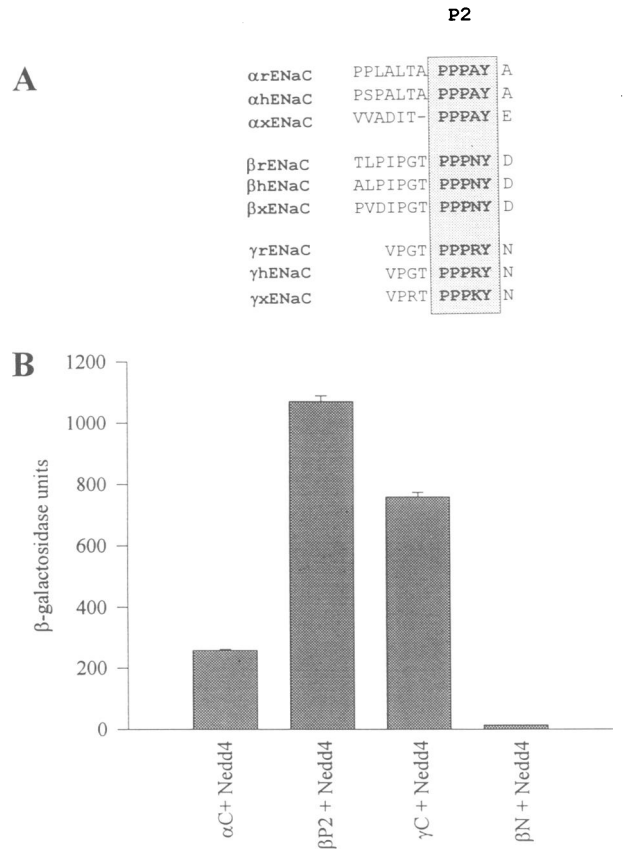

Fig. 7. Association of the proline-rich C termini of γrENaC (γC) and αrENaC (αC) with rNedd4-WW domains in the two-hybrid assay. (**A**) Sequence alignment of the PY motifs in the P2 regions of αβγ ENaC from rat (rENaC), human (hENaC) and *X.laevis* (xENaC). (**B**) Interactions of γC and αC regions with rNedd4 (clone 7.103) in the two-hybrid system quantified with the liquid β-galactosidase assay. Interaction of the βP2 (positive control) and βNterm (negative control) are also depicted. Bars represent mean ± standard errors of three separate assays.

the N-terminal region (Nterm, Figure 5A), were mixed with immobilized His-tagged γrENaC C-terminal fusion protein (γC). Following incubation and removal of unbound proteins by washes, bound proteins were separated on SDS–PAGE, and immunoblotted with anti-GST antibodies. Figure 8A and B shows that rNedd4 WWI and WWII domains, and to a lesser extent WWIII domain, were able to bind the proline-rich C-terminus of γrENaC. Moreover, this binding could be competed by a synthetic peptide encompassing the γP2 region (Figure 8B), suggesting that binding of rNedd4 WW domains is mediated by the P2 region of γrENaC.

Although so far there have not been any Liddle's syndrome patients reported with mutations/deletions within αhENaC, we have noticed the presence of a PY motif also in the P2 region of αrENaC/αhENaC (Figure 7A). Moreover, a recent study (Schild *et al.*, 1996, this issue) has demonstrated that mutations within this αrENaC PY motif led to hyperactivation of the channel when transfected into *Xenopus* oocytes, albeit at lower levels than the hyperactivation obtained with homologous mutations in the PY motifs of β or γrENaC. We therefore tested whether the PY-containing C-terminus of αrENaC could also bind rNedd4-WW domains in the two-hybrid binding assay. αrENaC C-terminus was therefore co-

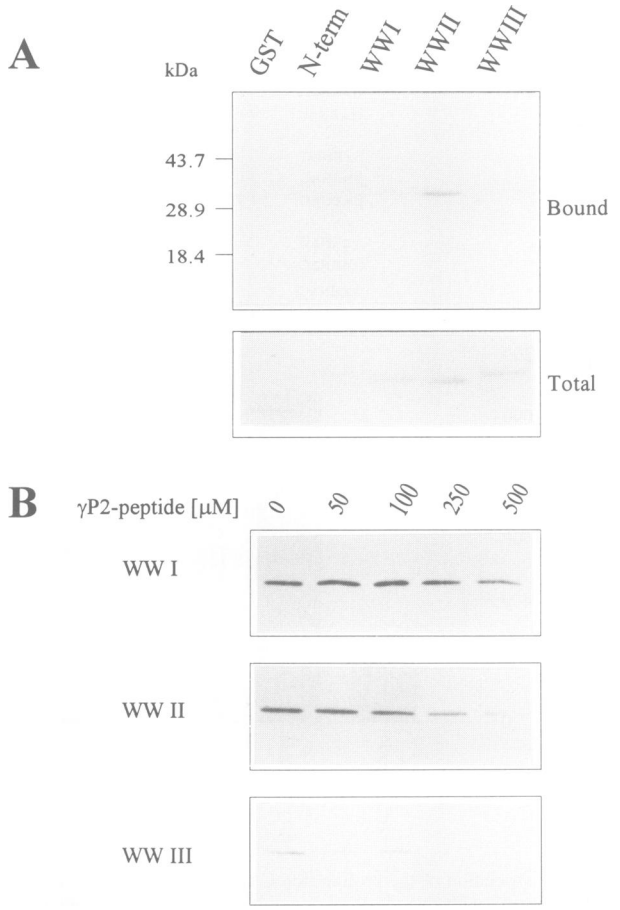

Fig. 8. Interaction of the C-terminus (γC) and the P2 region (γP2) of γrENaC with rNedd4-WW domains *in vitro*. (**A**) Soluble GST fusion proteins encompassing each rNedd4-WW domain, or N-terminal (N-term) sequences, were incubated with the His-tagged γC protein immobilized on Ni–agarose beads, washed and analyzed exactly as described in Figure 5B. (**B**) Competition of a γP2-peptide (STVPGTPPPRYNTLR) for binding of GST-rNEDD4-WWI, -WWII or -WWIII to the γP2 region. Increasing peptide concentrations were added to the incubation reaction and the experiment continued as described in Figure 5B.

transformed together with the three rNedd4-WW domains (clone 7.103) into yeast L40 cells, and β-galactosidase activity analyzed in the two-hybrid assay. The results (Figure 7B) show that, similar to β and γrENaC, αrENaC was also able to bind to the rNedd4-WW domains, but binding was weaker.

Recently, we have generated epitope (HA)-tagged αrENaC-transfected MDCK cells, which do not express endogenous ENaC, but express high levels of endogenous Nedd4 (Figure 9, lane 1). To test whether Nedd4 can interact with αrENaC *in vivo*, we performed reciprocal co-immunoprecipitation experiments (Figure 9). Thus, HA-αrENaC expressing MDCK cells were lysed and the lysate incubated with antibodies against rNedd4, HA or non-immune serum. The immunoprecipitates were then separated on SDS–PAGE, and immunoblotted with either anti-rNedd4 (Figure 9, left panel) or anti-HA (Figure 9, right panel) antibodies. Our results show that immunoprecipitation of αrENaC co-precipitated Nedd4 (Figure 9, lane 3), and vice versa: immunoprecipitation of Nedd4 led to co-precipitation of αrENaC (Figure 9, lane 8). No

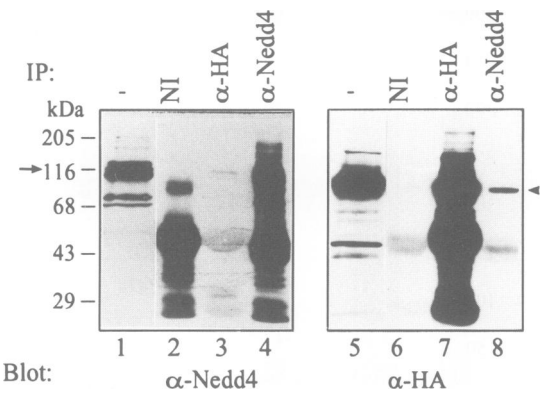

Fig. 9. Co-immunoprecipitation of Nedd4 with αrENaC. MDCK cells expressing endogenous Nedd4 and overexpressing transfected HA-tagged αrENaC (lanes 1 and 5) were lysed, and the lysate incubated with either non-immune serum (NI, lanes 2 and 6), anti-HA (α-HA, lanes 3 and 7) or anti-rNedd4 (α-Nedd4, lanes 4 and 8) antibodies. Following stringent washes (see Materials and methods), precipitated proteins were separated on 8% SDS–PAGE, transferred to nitrocellulose and blotted with either α-Nedd4 (left panel) or α-HA (right panel) antibodies. Lanes 1 and 5 represent loading of cell lysates to test for expression of Nedd4 and αrENaC, respectively. The arrow indicates Nedd4 in the left panel; the arrowhead indicates αrENaC in the right panel.

co-precipitation was observed with the non-immune serum (Figure 9, lanes 2 and 6). Our attempts to generate epitope-tagged β or γrENaC-transfected MDCK cells have not been successful. However, in a preliminary recent experiment, we were able to demonstrate co-immunoprecipitation of rNedd4 with α, β or γrENaC transfected into *Xenopus* oocytes (L.Schild, O.Staub, D.Rotin and B.Rossier, unpublished results). Taken together, these co-immunoprecipitation results demonstrate association of Nedd4 with rENaC in living cells.

Specificity of WW versus SH3 binding

Because the P2 regions of both β and γrENaC also conform to the consensus sequences (PXXP) for SH3 binding (PGTPPPNY and PGTPPPRY, respectively), we tested binding *in vitro* of GST fusion proteins from various SH3 domains (c-abl, p85, rasGAP, PLCγ, c-src and α-spectrin; kindly provided by M.Moran and V.P.Lehto) to immobilized βP2. Our results (Figure 10) show that, in contrast to the strong binding of the rNedd4-WWII domain (used as a positive control), there was no apparent binding of any of the tested SH3 domains to the βP2 sequence, suggesting that WW domains, and not SH3 domains, interact with the P2 region of βrENaC. Similar lack of binding by the above SH3 domains was also observed with the P2 region of γrENaC (data not shown).

Discussion

In this report we describe the isolation of rNedd4 by a yeast two-hybrid screen using the proline-rich P2 region of βrENaC as a bait, and demonstrate that the WW domains of rNedd4 are responsible for the interaction with the P2 regions of both β and γrENaC. We also demonstrate that rNedd4-WW domains can interact with the proline-rich C-terminus of αrENaC, and that αrENaC co-immunoprecipitates with Nedd4 in mammalian cells. The proline-rich C-terminal regions of the β and γ subunits of

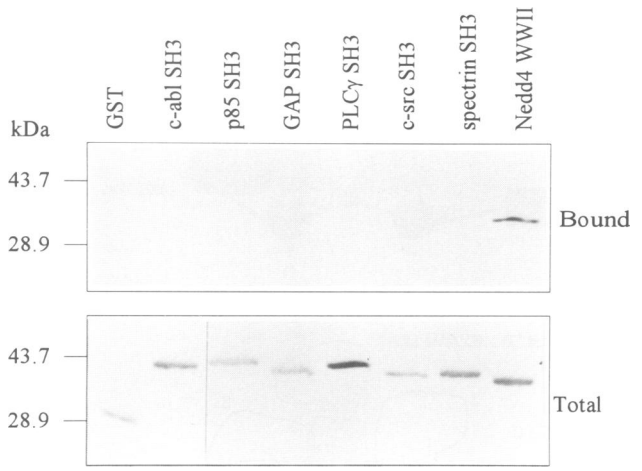

Fig. 10. Lack of binding of SH3 domains to βP2. Immobilized His-tagged βP2 was incubated with soluble GST fusion proteins from Nedd4-WWII (positive control), or with similar amounts of GST-SH3 domains from c-abl, p85, GAP, PLCγ, c-src and α-spectrin. Following washes, bound proteins were separated on 10% SDS–PAGE, transferred to nitrocellulose membrane and blotted with anti-GST antibodies, as described in the legend to Figure 5B.

the epithelial sodium channel are essential for proper channel regulation, and when deleted or mutated at specific residues lead to increased Na^+ channel activity and to Liddle's syndrome (Shimkets *et al.*, 1994; Hansson *et al.*, 1995a; Schild *et al.*, 1995, 1996). Deletion of the homologous proline-rich region in αrENaC was recently demonstrated also to hyperactivate the channel, although more mildly (Schild *et al.*, 1996); Liddle's patients with such mutations/deletions in the α subunit have not yet been identified.

Nedd4 was originally isolated from a mouse brain library (Kumar *et al.*, 1992). The corresponding mouse or rat Nedd4 proteins contain three WW domains (but four WW domains in the human homologue), a CaLB/C2 domain and a ubiquitin ligase homology (Hect) domain. WW domains are newly described modules of 38 amino acids in length found in various unrelated proteins (André and Springael, 1994; Bork and Sudol, 1994; Hofmann and Boucher, 1995), which contain two highly conserved tryptophans and two conserved prolines, one invariant. The following lines of evidence from our experiments suggest that it is the proline-rich P2 sequences within β or γrENaC which are responsible for binding to the WW domains of rNedd4: (i) the two cDNA clones isolated in the two-hybrid screen contain the three WW domains, and the shorter clone (7.103) contains these domains only, with very little 5′ or 3′ flanking sequences; (ii) *in vitro*, each WW domain was able to bind to the P2 regions of β or γrENaC, as demonstrated by direct binding and by peptide competition experiments; (iii) analysis of interactions in the two-hybrid assays demonstrated association of the P2 region of βrENaC with WWII+III or with the three WW domains of rNedd4, but not with flanking regions; and (iv) mutating either Pro616 or Tyr618 within the βP2 sequence ($PPP_{616}NY_{618}$) to Ala leads to complete inhibition of binding to the rNedd4 WW domains in the two-hybrid binding assay. Recently, Chen and Sudol (1995) have screened an expression library using the WW domain of (the yes associated protein) YAP65 as a probe, and isolated two partial cDNA clones encoding the novel proteins WBP1 and WBP2. Both proteins contain proline-rich short sequences (PPPPY) which were then demonstrated to mediate binding to YAP65-WW domains *in vitro* (Chen and Sudol, 1995). Based on mutation analysis, these authors proposed a preliminary consensus for WW binding of XPPXY, called the PY motif. The proline-rich sequences within the P2 regions of β and γrENaC (PPPNY and PPPRY, respectively) conform to the proposed PY motif, and our peptide competition and mutation analysis indeed implicate these short sequences as the binding partners for rNedd4-WW domains. The PY motif is different from the PXXP consensus binding motif for SH3 domains (Feng *et al.*, 1994; Yu *et al.*, 1994). We, as well as Chen and Sudol (1995), were unable to detect binding of SH3 domains from numerous unrelated proteins to the P2 regions of β or γrENaC, or to the PY sequence of WBP1 (Chen and Sudol, 1995), nor did we isolate any SH3-containing proteins in our two-hybrid screen. These findings are interesting, since the proline-rich sequences in βrENaC (PGTPPPNY, Figure 7A), γrENaC (PGTPPPRY) and WBP1 (PGTPPPPY) all contain not only the PY motif, but also a typical SH3 binding (PXXP) consensus sequence, yet they all show clear preference towards WW and not SH3 binding. It is also interesting that the proline-rich (P2) region in the α subunit of the epithelial Na^+ channel (αENaC) was previously shown to bind to the SH3 domain of α-spectrin *in vitro*, and αrENaC co-immunoprecipitated with α-spectrin in living cells (Rotin *et al.*, 1994). The P2 region of αrENaC also contains the PY sequence (PPPAY), and indeed our studies indicate that it too can bind to Nedd4-WW domains in the two-hybrid binding assay, although more weakly than the P2 regions of β or γrENaC. Further binding studies are required to compare the affinity of interactions of αrENaC with rNedd4-WW domains and with the α-spectrin-SH3 domain. Although in our two-hybrid screen we isolated rNedd4 as the binding partner for our βP2 bait, and have demonstrated interactions of Nedd4 with rENaC in mammalian cells by co-immunoprecipitation, we cannot preclude the possibility that WW domains of other proteins can also interact with the channel.

Our *in vitro* binding studies have demonstrated interactions between the P2 regions of β or γrENaC with individual WW domains of rNedd4. In contrast, in the two-hybrid assay only a very weak interaction was observed with single WW domains, and at least two WW domains were necessary to detect significant association. Possible explanations for the difference may be the lower sensitivity of the two-hybrid assay relative to the *in vitro* binding assay, or that the association with the ligand is more stable when more than one WW domain is present.

The C-terminal, and in particular the P2 regions of β and γENaC, are important for the regulation of epithelial sodium channel activity. A direct linkage analysis has demonstrated that the P2 region is deleted in several Liddle's syndrome patients due to premature stop codons or frameshift mutations (Shimkets *et al.*, 1994). More recently, a linkage analysis has identified another subset of Liddle's syndrome individuals with a truncation in the C-terminus of γENaC (Hansson *et al.*, 1995a). Parallel measurements of Na^+ channel activity in *Xenopus* oocytes

microinjected with truncated β or γrENaC demonstrated excessive amiloride-sensitive Na^+ currents resulting from an increase in the number of active channels at the plasma membrane (Schild *et al.*, 1995). In this context, our identification of the WW domains of rNedd4 as binding partners for the P2 regions of both the β and γrENaC suggests that Nedd4 may be a regulator of the epithelial Na^+ channel. This is further supported by the recent identification of Liddle's patients with mutations in either the third Pro or Tyr (PPPNY) in the PY motif of βENaC (Hansson *et al.*, 1995b; Tamura *et al.*, 1995), two mutations shown by our two-hybrid binding assays to abrogate rNedd4-WW binding, and recently demonstrated to cause an increase in Na^+ currents when expressed in oocytes (Schild *et al.*, 1996). Moreover, recent careful mutation analysis of the amino acids in the PY motif of each of the channel subunits (α, β or γrENaC) expressed in oocytes identified these WW binding motifs as critical for regulating Na^+ channel activity (Schild *et al.*, 1996), in agreement with our identification of WW domains as binding partners for these regions. These results are further strengthened by our demonstration of endogenously expressed Nedd4 in *Xenopus* oocytes. In addition, our Northern and Western analyses demonstrated abundant expression of rNedd4 in kidney and lung epithelia, two important tissues for Na^+ channel function. Also, preliminary immunolocalization studies (O.Staub, S.Ernst and D.Rotin, unpublished results) show that rNedd4 is expressed primarily in cortical collecting ducts in the kidney, consistent with the known localization of the epithelial sodium channel (Duc *et al.*, 1994). These findings, and our αrENaC–Nedd4 co-immunoprecipitation experiments, provide strong evidence that by binding via its WW domains, Nedd4 may be a regulator of the epithelial Na^+ channel.

The mechanism(s) by which Nedd4 may modulate Na^+ channel function is(are) currently unknown and can only be speculated. In addition to the three WW domains, rNedd4 contains a CaLB/C2 domain and a ubiquitin ligase homology (Hect) domain. The C2 region, which includes the 43-amino acid CaLB domain, was originally identified in Ca^{2+}-dependent isoforms of PKC (Coussens *et al.*, 1986; Knopf *et al.*, 1986; Kaibuchi *et al.*, 1989) and other proteins including synaptotagmin I, PLA_2, rasGAP, PLC isoforms, rabphilin and others (Clark *et al.*, 1991). It was shown to function as a Ca^{2+}-dependent lipid binding module which is involved in translocation to and association with membranes, as well as in endocytosis/exocytosis pathways (Geppert *et al.*, 1994; Zhang *et al.*, 1995). These properties of the C2 region have been attributed to the CaLB domain (Clark *et al.*, 1991; Gawler *et al.*, 1995). The role of the CaLB domain in rNedd4 is not yet known.

The Nedd4 protein from mouse, rat, human and yeast (Rsp5) all contain an ~100 amino acid C-terminal ubiquitin ligase homology (Hect) domain, which shares sequence homology with the C-termini of several unrelated proteins (Huibregtse *et al.*, 1995), including E6-AP [hence the name **h**omology to the **E**6-AP **c**arboxy **t**erminus (Hect); Scheffner *et al.*, 1993]. Ubiquitination usually functions to tag proteins destined for regulated degradation by proteosomes, and ubiquitin ligase (E3) mediates the final step (ligation of the ubiquitin group) in the ubiquitination pathway (Ciechanover, 1994). The Hect domain of Rsp5,

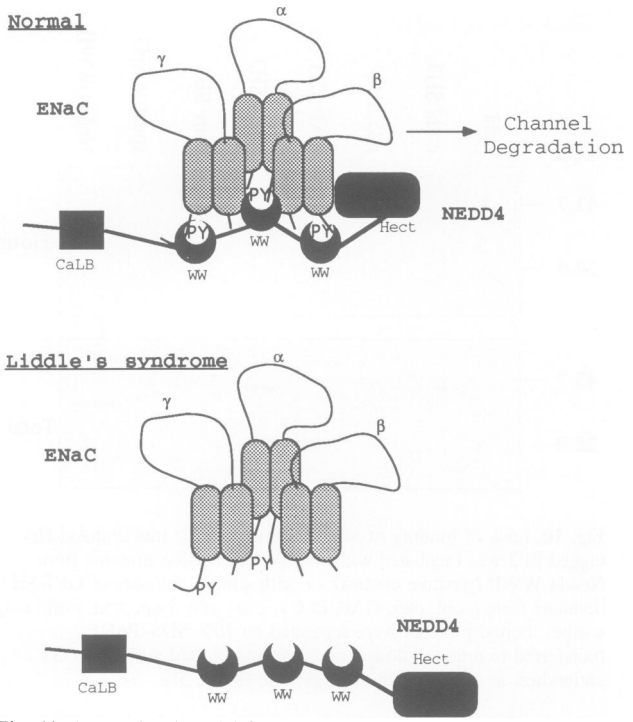

Fig. 11. A postulated model for ENaC–Nedd4 interactions, and implications for Liddle's syndrome. The three WW domains of Nedd4 bind to the PY motifs in the C-termini of α, β and γ ENaC, thereby bringing the ubiquitin ligase (Hect) domain in close proximity to ENaC. This allows the ubiquitination and subsequent degradation of the channel by proteosomes, with the resultant decrease in channel numbers. In Liddle's syndrome, deletions/mutations within the PY motifs of βENaC (or γ ENaC) leads to abrogation of Nedd4-WW binding, resulting in a lack of the putative suppressive effect of Nedd4, which may explain the increase in channel activity associated with the Liddle phenotype. The role of the CaLB (C2) domain is not known, but it may be associated with channel mobilization from the apical membrane.

the yeast homologue of Nedd4, was shown recently to form a high-energy thioester bond with ubiquitin (Huibregtse *et al.*, 1995), suggesting that it functions as a ubiquitin ligase. It is likely therefore that the other Nedd4 proteins will prove to be functional ubiquitin ligases as well. A speculative model (Figure 11) for Nedd4 function could be that Nedd4 binds to the C-termini of the epithelial Na^+ channel (ENaC) via its WW domains by protein–protein interactions. This association with Nedd4 may then bring the CaLB/C2 domain and ubiquitin ligase domain in close proximity to the channel. The CaLB/C2 domain may be involved in membrane association and possibly ENaC mobilization (e.g. endocytotic degradative pathway), possibly removing the channel from the apical membrane. The Hect domain may then ubiquitinate ENaC, thereby tagging it for degradation. The end result is a decrease in the number of channels due to channel degradation, and hence the putative suppressive effect Nedd4. Removal of the WW binding (PY motif) sequences from ENaC in Liddle's patients prevents proper Nedd4 binding and channel degradation, with consequent excessive Na^+ channel activity. This model assumes that Nedd4 exerts its effect by binding to all PY motifs, since deletion of individual PY motifs from individual channel subunit is sufficient to cause the Liddle phenotype. This speculative scenario however, does not include the alternative possibility that Nedd4 may affect channel gating directly.

Future analysis of Nedd4 effect on Na^+ channel function, as well as structure–function analysis of Nedd4, should help unravel its putative role as a regulator of the epithelial Na^+ channel.

Materials and methods

Constructs for the two-hybrid system

The C-terminal 49 amino acids of βrENaC encompassing the P2 region and downstream sequences (nt 1840–1989; called βP2), the C-terminus of γrENaC (nt 1922–2051, called γC), the C-terminus of αrENaC (nt 1910–2173) and the N-terminal region of βrENaC (nt 73–210, called βN) were amplified by PCR using Vent DNA polymerase (Biolabs), tagged with an HA epitope and cloned into pBTM116, a plasmid carrying the TRP1 selection marker and the LexA protein (Vojtek *et al.*, 1993). These constructs were transfected into the *S.cerevisiae* reporter strain L40 cells (*MATa his3 LYS2:LexA-His3 URA3::lexA-lacZ*; Vojtek *et al.*, 1993) using the lithium acetate method (Schiestl and Gietz, 1989) to give Trp prototrophs. Transformants were tested for expression of the LexA-HA fusion protein by Western blotting of a total cell lysate using an anti-HA antibody (Babco). WWI (rNedd4 nt 718–925), WWII (rNedd4 nt 1184–1386), WWIII (rNedd4 nt 1362–1543), Nterm (rNedd4 nt 463–753), the domain between WWI and WWII (rNedd4 nt 875–1220, called MID), and the C-terminus (rNedd4 nt 1527–2355) were amplified by PCR using primers which contain either *Xho*I or *Eco*RI sites. The amplified products were then digested with *Xho*I and *Eco*RI and subcloned into pGAD10. Pro616→Ala and Tyr618→Ala substitutes in pBTM116HAβP2 were carried out by PCR, as previously described (Nelson and Long, 1989). All constructs were verified by sequencing.

Library screening

The general description of two-hybrid screens was detailed elsewhere (Chien *et al.*, 1991). For our screen, a single colony of L40 cells transformed with pBTM116HAPβ2 was grown in YPD (Ausubel, 1994) and transformed with a rat lung cDNA library (MATCHMAKER, Clontech) constructed into the plasmid pGAD10. A total of 4×10^6 independent transformants was selected on medium lacking Trp, Leu and His, but containing 25 mM 3-AT (Sigma). After incubation at 30°C for 5 days, replica filters were made, cells permeabilized by freezing in liquid nitrogen (30 s) and thawing at room temperature. Filters were transferred onto Whatmann 3MM paper saturated with XGal solution (Breeden and Nasmyth, 1985) and incubated at 30°C. β-Galactosidase-positive colonies were patched on agar plates containing SD-Broth (Ausubel, 1994) lacking Trp and Leu, and used for further analysis. Single colonies were grown in SD-Broth lacking Leu, and total DNA isolated and transformed into *Escherichia coli* MH6 cells, as described (Ausubel, 1994). Transformed colonies were then transferred to M9 minimal plates lacking Leu and selected for expression of LEU2 gene on pGAD10. To check for true positives, isolated plasmids were transfected into L40 cells either alone, or with pBTM116HAβP2, or with pBTM116HAβN (βrENaC nt 73–210) encoding the N-terminus of βrENaC, and tested for β-galactosidase activity. Clones were then sequenced using the dideoxy method and T7 DNA polymerase (Pharmacia). The library was rescreened using clone 7.25 as a random primed probe. Several partial clones encoding the C-terminus (including the Hect domain) were isolated.

β-Galactosidase assays

Filter lift assays were carried out as described in the library screening section earlier. Liquid culture assays were performed as detailed previously (Guarente, 1983). Briefly, 4 ml cultures of transformed L40 cells were grown in the appropriate selection media to OD_{600} of 1.0. 100 μl of cells were then prepared and permeabilized as described earlier. For quantitation using *o*-nitrophenyl-β-D-galactoside (ONPG), standard conditions were used. The results are expressed as units, as defined by Miller (1972).

Construction and generation of fusion proteins

To generate GST fusion proteins from the rNedd4 WWI, WWII and WWIII domains, these regions were amplified by PCR with primers which also contain either a *Bam*HI or an *Eco*RI site. The PCR products were digested with *Bam*HI and *Eco*RI and subcloned into pGEX-4T1 (Pharmacia). The N-terminal domain (Nterm, nt 463–674) was subcloned from an exonuclease III deleted clone into pGEX-2TK (Pharmacia). The constructed plasmids were transformed into HB101 and protein expression induced for 5 h with 0.2 mM IPTG. Proteins were then affinity-purified with glutathione–agarose beads and eluted with reduced glutathione, as described (Ausubel, 1994). The concentration of the eluted proteins was estimated by measuring OD_{280}, Bio-Rad assay and by Coomassie blue staining on SDS–PAGE. To generate His-tagged fusion proteins from the C-termini of β or γrENaC, the P2 region (nt 1840–1989) of βrENaC (called βP2) and the C-terminus of γrENaC (nt 1922–2051, called γC) were amplified by PCR and subcloned into pQE-30 vector containing a 6×His tag (Qiagen). Protein expression was induced with 1 mM IPTG and proteins immobilized and affinity-purified on Ni–agarose beads, according to kit instructions (Qiagen).

In vitro binding (mixing) experiments

For binding assays, 300 ng of eluted GST fusion proteins (GST alone, GST-WWI, GST-WWII, GST-WWIII or GST-N terminal control sequences, see Figure 5A) were incubated with ~0.5 μg His-tagged βP2 or γC fusion proteins immobilized on Ni–agarose beads in 0.5 ml HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) for 2 h at 4°C. Beads were then washed (2×) with high salt (500 mM) HNTG and twice more with HNTG to remove unbound GST fusion proteins. Bound proteins were then eluted from beads with sample buffer, separated on 10% SDS–PAGE, transferred to nitrocellulose membrane and blotted with anti-GST antiserum (1:1000) which we had previously generated, followed by HRP-conjugated secondary antibody (1:20 000) and ECL detection (Amersham). Peptide competition experiments were performed exactly as the mixing experiments above, except that binding of the GST fusion proteins to the immobilized βP2 or γC proteins was carried out in the presence of the indicated concentrations of either a 15mer βP2 peptide (LPIPGTPPPNYDSL) or the same peptide but scrambled (PITDPLPLTYNPGSP), or with a 16mer P2 peptide (STVPGTPPPRYNTLR), corresponding to the second proline-rich region in γrENaC.

Northern blot analysis

A commercially prepared blot loaded with mRNA from various rat tissues (Clontech) was hybridized overnight with random primed labelled cDNA corresponding to clone 7.103 (Figure 1) as a probe (Ausubel, 1994). After washing (0.1× SSC/50°C) the blot was exposed for 36 h at −80°C.

Western (immunoblot) analysis

Rat tissues were homogenized as a 25% homogenate in STKM (0.25 M sucrose, 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, PMSF) with a polytron. FDLE, A6 cells and *Xenopus* oocytes were lysed as described below. Approximately 50 μg of whole tissue homogenate/cell lysate or 100 ng of GST-WWII protein were separated on 8% SDS–PAGE, transferred to nitrocellulose and probed with a polyclonal antibody raised against GST-Nedd4-WWII or the corresponding pre-immune serum (each at 1:500 dilution). Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibody and visualized using a chemiluminescence technique (ECL, Amersham).

Co-immunoprecipitation experiments

MDCK cells stably transfected with αrENaC tagged with an HA epitope at the C-terminal end in pLK-neo (Hirt *et al.*, 1990; T.Ishikawa and D.Rotin, unpublished results) were induced for 18 h with 1 μM dexamethasone, 2 mM butyrate and 1 μM amiloride. Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin). A polyclonal antibody raised against GST-Nedd4-WWII or monoclonal anti-HA, or a non-immune serum was added and incubated for 4 h. The immunocomplexes were collected with protein A–Sepharose (polyclonal antibodies) or protein G–Sepharose (monoclonal anti-HA antibodies) beads, washed with lysis buffer, then with high salt HNTG. Proteins were separated on 8% SDS–PAGE, transferred on nitrocellulose membrane and analyzed by immunoblotting with either anti-HA or anti-Nedd4 antibodies.

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