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Regulation of the epithelial Na^+ channel by Nedd4 and ubiquitination

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Regulation of the endothelial Na^+ channel by Nedd4 and by ubiquitination. The epithelial Na^+ channel (ENaC) is comprised of three subunits, α, β and γ, and plays an essential role in Na^+ and fluid absorption in the kidney, colon and lung. We had identified proline-rich sequences at the C termini of αβγENaC, which include the sequence PPxY, the PY motif. This sequence in β or γENaC is deleted or mutated in Liddle's syndrome, a hereditary form of arterial hypertension. Our previous work demonstrated that these PY motifs bind to the WW domains of Nedd4, a ubiquitin protein ligase containing a C2 domain, three or four WW domains and a ubiquitin protein ligase Hect domain. Accordingly, we have recently demonstrated that Nedd4 regulates ENaC function by controlling the number of channels at the cell surface, that this regulation is impaired in ENaC bearing Liddle's syndrome mutations, and that ENaC stability and function are regulated by ubiquitination. The C2 domain is responsible for localizing Nedd4 to the plasma membrane in a Ca^{2+}-dependent manner, and in polarized epithelial MDCK cells this localization is primarily apical. In accordance, electrophysiological characterization of ENaC expressed in MDCK cells revealed inhibition of channel activity by elevated intracellular Ca^{2+} levels. Thus, in response to Ca^{2+}, Nedd4 may be mobilized to the apical membrane via its C2 domain, where it binds ENaC via Nedd4-WW:ENaC–PY motifs' interactions, leading to ubiquitination of the channel by the Nedd4-Hect domain and subsequent channel endocytosis and lysosomal degradation. This process may be at least partially impaired in Liddle's syndrome due to reduced Nedd4 binding, leading to increased retention of ENaC at the cell surface.

The apically located amiloride-sensitive epithelial Na^+ channel (ENaC) plays an essential role in Na^+ and fluid absorption in several epithelia, mainly in the lung, distal colon and distal nephron [reviewed in 1]. ENaC was originally cloned from rat colon using expression cloning in *Xenopus* oocytes [2–5] and subsequently from other species [6–12], and has been shown to be composed of three similar subunits, α, β and γ. These are assembled at a stoichiometry of 2α1β1 γ [13] to yield an active channel with a single channel characteristics of a highly Na^+ selective, low conductance (~5 pS) and high amiloride sensitivity (Ki ~10 to 100 nmol/L) [3, 14]. Each ENaC subunit is composed of two transmembrane (TM) domains, a large extracelluar domain and short intracellular N and C termini [15–17]. We have previously identified proline-rich sequences within the C termini of each ENaC subunit, including a highly conserved sequence now known as the "PY motif" (xPPxY; Fig. 1A) [18, 19].

Key words: ENaC channels, PY motif, WW domain, C2 domain, ubiquitin protein ligase, Liddle's syndrome.

ENaC AND LIDDLE'S SYNDROME

Precise regulation of ENaC is critical, as illustrated by the number of human diseases that have been linked to malfunction of or mutations in ENaC, including cystic fibrosis [20], pulmonary edema [21], pseudohypoaldosteronism type I (PHAI) [22, 23] and Liddle's syndrome. Liddle's syndrome is an autosomal dominant form of inherited human arterial hypertension, characterized by an early onset of severe hypertension, salt-sensitivity, hypokalemia, metabolic alkalosis, and low aldosterone and renin plasma concentrations [24, 25]. The disease has been linked genetically to mutations that delete or alter the PY motifs of either β- or γENaC [26–30]. Such deletions or mutations lead to elevated channel activity when expressed heterologously in *Xenopus* oocytes [19, 31]; this elevation is caused by an increase in channel numbers at the cell surface, as well as by increased channel opening [32]. Moreover, recent work by Kellenberger et al [33] has demonstrated loss of auto-inhibition by intracellular Na^+ [14, 34] in PY motif-deleted/mutated ENaC.

BINDING OF Nedd4 TO ENaC

Because the presence of proline-rich regions in proteins often indicates their involvement in protein:protein interactions, and because loss of functional PY motifs in βENaC leads to an elevation of channel activity and to Liddle's syndrome, we embarked on a search for putative

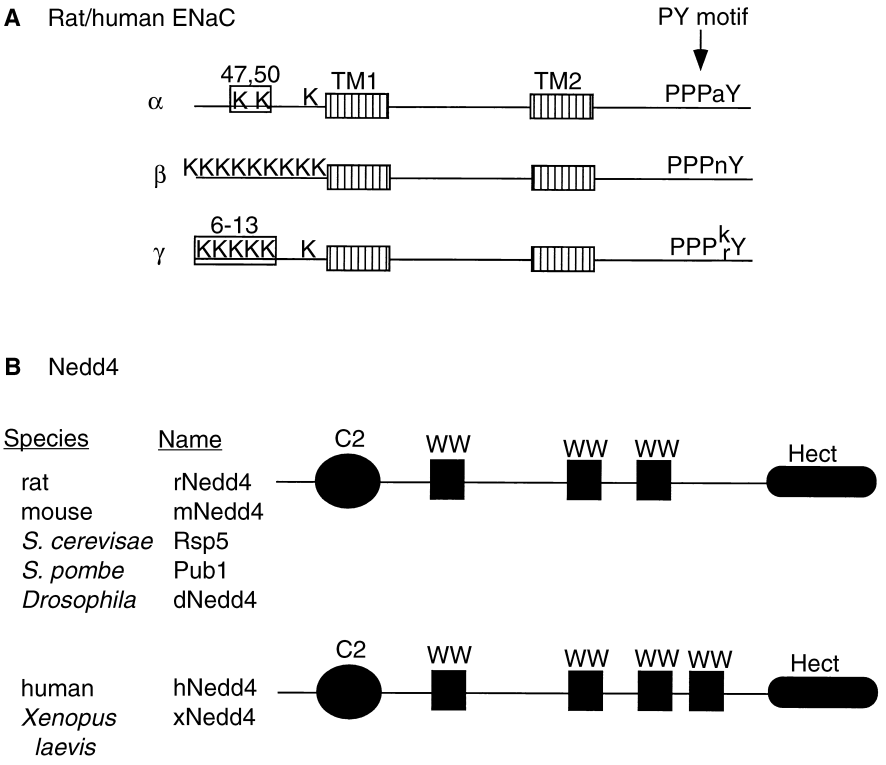

Fig. 1. Schematic representation of ENaC and Nedd4. (*A*) Schematic representation of αβγENaC depicting the conserved N terminal lysine (K) residues and C terminal PY (xPPxY) motifs (arrow). Boxed Lys residues are important for ENaC ubiquitination. Abbreviations are: TM, transmembrane domain; rENaC, rat epithelial Na^+ channel; hENaC, human ENaC. (*B*) Domain organization of Nedd4 isoforms from different species, depicting the C2 domain, three or four WW domains and the ubiquitin protein ligase Hect domain.

suppressor protein(s) that may interact with that region in ENaC and thus regulate channel function. Using a yeast 2 hybrid screen of rat lung library with a PY motif-containing sequence of the rat βENaC (identical to human ENaC at the PY motif) as a bait, we identified Nedd4 as a binding partner for ENaC [18].

Nedd4 (neuroneal precursor cells expressed developmentally down-regulated 4 [35]), is a ubiquitin protein ligase composed of an N terminal C2 domain (see below), three or four WW domains, an E2 binding region, and a C terminal ubiquitin protein ligase Hect domain (Fig. 1B). Both Nedd4 and ENaC show a similar expression pattern in selected tissues, such as the principal (but not intercalating) cells of the cortical and outer medulla collecting ducts in the distal nephron, and in airway and distal lung epithelia [36]. WW domains are protein:protein interaction modules ~40 amino acids in length [37–40] that bind PY motifs [18, 41, 42] or in some specific cases the PPLP motif [43, 44]. As shown in Figure 1A, a highly conserved PY motif is found in all three of the ENaC subunits. Using a series of experimental procedures, including quantitative yeast 2 hybrid binding (β-gal) assays, in vitro binding, in vitro peptide competition experiments with synthetic peptides encompassing the ENaC-PY motifs, and coprecipitation and coimmunoprecipitation from mammalian cells, we demonstrated that the WW domains of Nedd4 are the ones responsible for the association with the PY motifs of ENaC [18]. Moreover, we also showed that specific point mutations within the PY motif of βENaC, recently identified in Liddle's patients, led to an increase in ENaC activity [19] concomitant with an abrogation of binding to the Nedd4-WW domains [18]. More recent quantitative experiments using internal fluorescence has identified highest affinity of interactions ($kD < 20$ μmol/L) between the third WW domain of rNedd4 (Fig. 1B) and the PY motif of βENaC (Kanelis, Forman-Kay and Rotin, unpublished observations).

Because Nedd4-WW domains clearly bind the ENaC-PY motifs, we proposed that such an interaction may facilitate ubiquitination of ENaC by the Nedd4-Hect domain, thus regulating channel stability and hence function. The obvious outstanding questions were then: (*1*) Is ENaC regulated by ubiquitination? (*2*) Does Nedd4 regulate ENaC function? and (*3*) Is this putative regulation impaired in Liddle's syndrome? In the following sections, we will describe our work aimed at answering these questions.

REGULATION OF ENaC BY UBIQUITINATION

Ubiquitination serves to tag proteins for rapid degradation [reviewed in 45]. It is carried out by the sequential transfer of the 76 amino acid ubiquitin (Ub) from a ubiquitin-activating enzyme (E1), to a ubiquitin-conjugating enzyme (E2) and usually to a ubiquitin protein ligase (E3) such as Nedd4. The E3 enzyme is thus responsible for the attachment of ubiquitin, or multiubiquitin

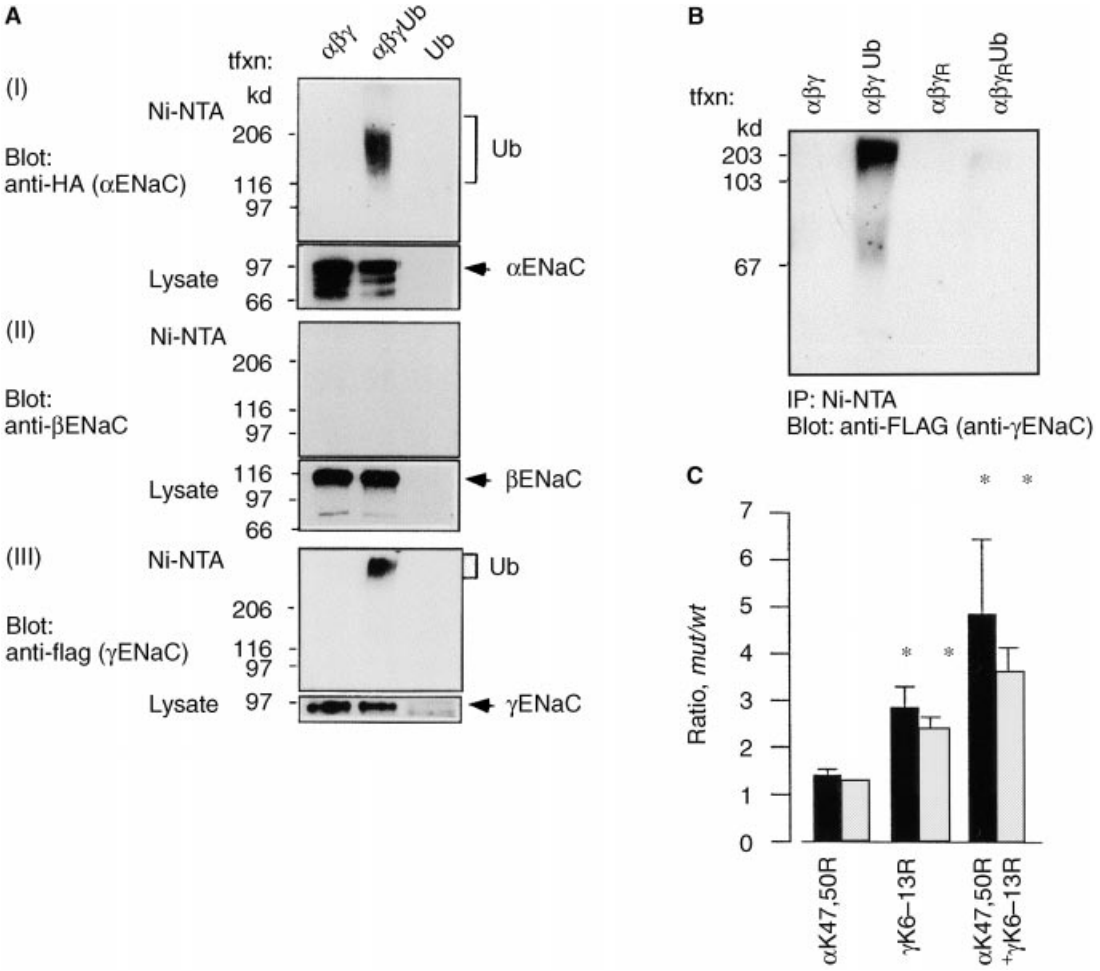

Fig. 2. Regulation of ENaC by ubiquitination. (*A*) Ubiquitination (ub) of γ and αENaC, but not βENaC, upon coexpression of αβγENaC in 293 cells together with His-tagged ubiquitin (Ub). (*B*) Loss of ubiquitination of the γK6–13R mutant ($\alpha\beta\gamma_R$). (*C*) An increase in channel numbers at the cell surface is responsible for the increase in ENaC activity in the ubiquitination-defective ENaC (γK6–13R mutant or γK6–13R plus α47,50R double mutant). Symbols are: (■) INa; (▩) Ab binding.

chains, onto lysine residues of target proteins. For most cytosolic or ER-associated proteins, such polyubiquitination targets them for degradation by the proteasome. Recently, however, it has become apparent that some transmembrane proteins also become ubiquitinated, and that this ubiquitination targets them for endocytosis and degradation by the lysosomes/vacuoles [46–48].

Many proteins regulated by ubiquitination have a very short half-life. Our work and that of others have shown that ENaC is a short-lived protein with $t_{1/2}$ of approximately one hour of its total cellular pool [49, 50] as well as a short half-life of the protein at the cell surface (Saleki and Rotin, unpublished observations) [49, 51]. Accordingly, we demonstrated that ENaC is ubiquitinated on the γ and α subunits in living cells (Fig. 2A) [49]. To identify the possible ubiquitination sites in ENaC, we searched for highly conserved Lys residues in the ENaC sequence. A series of such lysines were identified at the N termini of each ENaC chain, and subsequently mutated individually or in clusters to Arg, to retain the positive charge while abolishing ubiquitin acceptor sites. These mutants were then expressed in *Xenopus* oocytes to test for ENaC activity. Using this approach, mutation of a cluster of lysine residues at the N terminus of γENaC to Arg (K6, 8, 10, 12, 13R, or γK6–13R) was found to cause a two- to threefold increase in channel activity, an elevation augmented (3- to 7-fold) by Lys to Arg mutation of lysines 47 plus 50 in αENaC (αK47,50R), which are otherwise ineffective on their own [49]. The increase in ENaC activity was caused entirely by an increase in channel numbers at the cell surface, as determined by binding of iodinated antibodies directed against a FLAG tag introduced into the ectodomain of each ENaC (wild-type or mutant) chain (Fig. 2C). Accordingly, the same Lys to Arg mutations in γ and αENaC result is a severe reduction of their ubiquitination (Fig. 2B), suggesting that this impaired ubiquitination was responsible for the increase in ENaC numbers [49]. In addition, using sev-

eral lysosomal and proteasomal inhibitors, we were able to demonstrate that the properly assembled αβγENaC is likely targeted for lysosomal degradation, whereas individually expressed ENaC chains are heavily ubiquitinated and rapidly degraded by the proteasome [49], as recently confirmed by others [51].

To determine whether ENaC ubiquitination plays a role in its stability at the cell surface, we treated the oocytes with Brefeldin A (BFA) to block ER to Golgi transport, and compared amiloride-sensitive channel activity of the WT and the Lys to Arg double mutant (αK47,50R plus γK6–13R). Our work showed that while most of the WT channel disappeared from the cell surface by eight hours after the addition of BFA, about half of the Lys to Arg mutant appeared to be stuck at the plasma membrane [49]. This suggests that ubiquitination likely plays a role in regulating ENaC endocytosis/degradation and hence stability at the plasma membrane. This places ENaC in a class of a growing number of transmembrane yeast and mammalian proteins in which ubiquitination and endocytosis/lysosomal (vacuolar) degradation are somehow linked [46, 52–54].

REGULATION OF ENaC FUNCTION BY Nedd4: ROLE IN LIDDLE'S SYNDROME

To determine whether Nedd4 regulates ENaC function, we used the *Xenopus* oocyte system to study the effect of overexpressed WT or catalytically inactive (bearing a C to S mutation at the Hect domain) Nedd4 on ENaC activity. The *Xenopus* Nedd4 (xNedd4; Fig. 1B) and rat ENaC (rENaC) were used in these studies. Our work showed that overexpression of WT-Nedd4 resulted in an almost complete inhibition of ENaC activity, an effect that was dose-dependent. Conversely, overexpression of a catalytically inactive Nedd4 (Nedd4-CS) enhanced ENaC activity two- to fivefold, presumably by acting in a dominant negative fashion to compete off binding of endogenous Nedd4 (Fig. 3) [55]. Moreover, using ENaC chains expressing a FLAG tag at the ectodomain to quantitate channel numbers at the cell surface and directly compare it to channel activity, we then demonstrated that the suppressive effect of WT-Nedd4 and the stimulatory effect of Nedd4-CS could be attributed entirely to changes in channel numbers at the plasma membrane [55]. This suggests that the effect of Nedd4 on ENaC is manifested at the cell surface, as also demonstrated visually using immunofluorescence staining [55].

As indicated above, we had previously demonstrated binding of Nedd4-WW domains to ENaC-PY motif and abrogation of such binding by mutations in the conserved proline or tyrosine of the PY motif [18]. Because such mutations within the PY motif, or its deletion, cause Liddle's syndrome [26–30], we investigated the effect of Nedd4 on the function of ENaC bearing mutations in these motifs. Our studies showed that mutating the conserved tyrosines in all three PY motifs of ENaC rendered the channel completely unresponsive to WT-Nedd4 or Nedd4-CS, demonstrating that binding via the Nedd4-WW domains is essential for regulation of the channel by Nedd4. When the effect of Nedd4 was tested on ENaC lacking only one PY motif (βR564stop), to mimic the original Liddle's syndrome truncation [26], such "Liddle's" channel was only partially responsive to WT-Nedd4 or Nedd4-CS (Fig. 3) [55]. This suggests that the regulation of ENaC by Nedd4 is impaired in Liddle's syndrome, and that the more intact PY motifs present in ENaC, the tighter the control Nedd4 exerts on the channel.

Recently, Shimkets, Lifton and Canessa have proposed that the PY motifs of βENaC or γENaC serve as internalization signals that are responsible for clathrin-mediated endocytosis of the channel, and hence their deletion/mutation in Liddle's syndrome may lead to an accumulation of channels at the cell surface due to defective internalization [56]. Our work, however, suggests that Nedd4 and channel ubiquitination likely regulate ENaC stability at the cell surface, and that such regulation may be impaired in Liddle's syndrome due to reduced binding of Nedd4 to the channel. Who is right, then? We believe that both hypotheses may be correct and complementary. As described above, numerous recent reports have demonstrated a link between ubiquitination and endocytosis of transmembrane proteins, and it is quite likely that ENaC also belongs to this group of proteins.

THE C2 DOMAIN OF Nedd4 MEDIATES Ca^{2+}–DEPENDENT MEMBRANE TARGETING

In addition to WW and Hect domains, Nedd4 also contains a C2 domain. C2 (CaLB) domains in other proteins have been demonstrated to bind membrane and phospholipids in a Ca^{2+}-dependent fashion [reviewed in 57, 58]. We have therefore investigated the role of the Nedd4-C2 domain. Our work demonstrated that Nedd4, endogenously expressed in MDCK cells, redistributes from the soluble to the particulate fraction following treatment of cells with Ca^{2+} plus ionomycin, which leads to elevation of intracellular Ca^{2+} levels. Accordingly, immunolocalization of endogenous Nedd4 in polarized MDCK cells revealed a rapid (within 5 min) Ca^{2+}-dependent translocation of the protein to the plasma membrane, mainly the apical membrane [59]. This plasma membrane association was mediated by the C2 domain, because deletion of this domain led to abrogation of targeting to the cell surface. Moreover, the Nedd4-C2 domain alone, generated as a glutathione-S-transferase (GST) fusion protein, was able to associate with membranes and purified phospholipids in vitro in response to Ca^{2+} [59]. Despite the ability of the C2 domain on its own to bind phospholipids, we believe that another

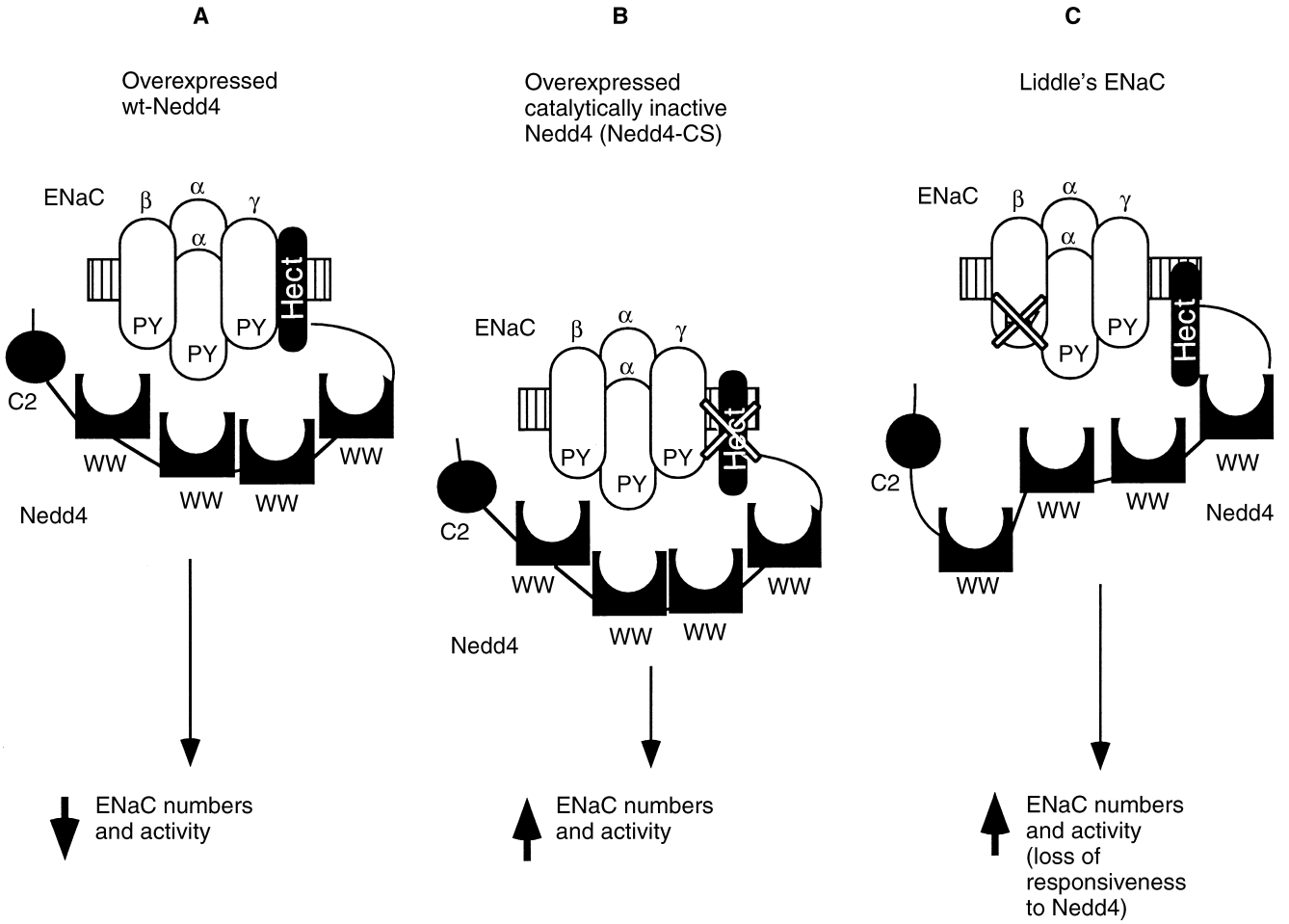

Fig. 3. Regulation of ENaC by Nedd4 and impaired regulation in Liddle's syndrome. (*A*) Overexpression of wild-type (wt) xNedd4 together with αβγrENaC in *Xenopus* oocytes leads to inhibition of channel activity due to excessive channel degradation. (*B*) Overexpression of catalytically inactive (Nedd4-CS) xNedd4 together with αβγrENaC leads to increased ENaC numbers at the cell surface and hence increased activity due to competition of this dominant negative Nedd4 with endogenous Nedd4. (*C*) Loss of proper response to Nedd4 of ENaC channels lacking one (as in Liddle's syndrome) or more PY motifs, which leads to impairment of binding to Nedd4-WW domains and hence reduced ubiquitination of ENaC and increased channel numbers at the plasma membrane.

factor(s)/protein(s) may be involved in the polarized distribution of Nedd4 in MDCK cells. Our preliminary work has indeed identified a putative Nedd4-C2 interacting protein, Annexin 13b, which is a protein previously demonstrated to be enriched in apical rafts found in MDCK and other epithelial cells [60]. Annexin 13b may therefore facilitate transport of Nedd4, via interactions with the Nedd4-C2 domain, to the apical membrane.

We have recently performed an extensive electrophysiologic characterization of αβγrENaC heterologously expressed in epithelial MDCK cells, using patch clamp analysis [14]. This work has demonstrated a biphasic (rapid and slow) inhibition of ENaC activity in response to elevation of intracellular Ca^{2+}, in agreement with previous studies demonstrating Ca^{2+}-mediated inhibition of Na^+ channels in native epithelia [reviewed in 1]. Although speculative, it is possible that the slow response (>5 min) may be mediated by a decrease in channel numbers, possibly by Nedd4, which is mobilized to the apical membrane by Ca^{2+}.

SUMMARY

Our work summarized here provides strong evidence that Nedd4, which binds ENaC, regulates ENaC function by controlling the number of channels at the cell surface, and accordingly, that channel numbers at the plasma membrane are controlled by ubiquitination. Although we have not yet demonstrated direct ubquitination of ENaC by Nedd4, we believe ENaC ubiquitination is carried out, at least in part, by Nedd4, because a catalytically inactive Nedd4 has lost its ability to regulate ENaC, and channel defective for Nedd4 binding (lacking PY motifs) no longer responds to Nedd4 regulation. We propose the following model for regulation of ENaC by Nedd4: In epithelial cells, Nedd4 is translocated to the

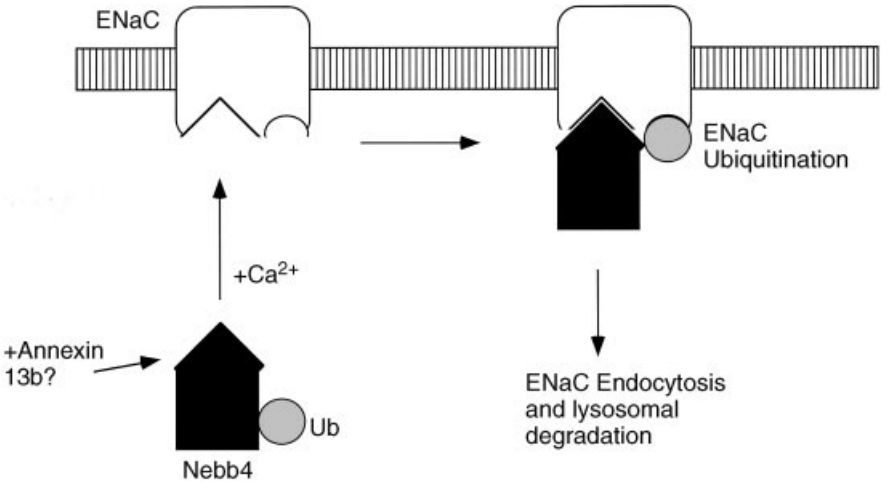

Fig. 4. Summary: Role of Nedd4 and ubiquitination in ENaC function. Nedd4 is a cytosolic protein. Upon a rise in cytosolic [Ca^{2+}], it is mobilized to the apical membrane via its C2 domain, possibly with the aid of Annexin 13b. Once there, it binds the ENaC-PY motifs via its WW domains, allowing ubiquitination of the channel by the Hect domain, which facilitates channel endocytosis and lysosomal degradation. Thus, Nedd4 is involved in regulating ENaC stability at the cell surface.

apical membrane in response to elevated intracellular Ca^{2+}, to the same cellular compartment where ENaC is located. This then allows binding of the Nedd4-WW domains to the ENaC-PY motifs, thus bringing the ubiquitin protein ligase Hect domain in close proximity to the channel, leading to channel ubiquitination, endocytosis and lysosomal degradation (Fig. 4). This process is likely impaired in Liddle's syndrome due to defective binding of Nedd4 to the channel (Fig. 3). Whether Nedd4 also participates in the inhibition of ENaC by elevated intracellular Na^+, recently demonstrated to be impaired in Liddle's syndrome [33, 61], remains to be investigated.

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