Invited Review

Trafficking and cell surface stability of ENaC

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Rotin, Daniela, Voula Kanelis, and Laurent Schild. Trafficking and cell surface stability of ENaC. Am J Physiol Renal Physiol 281: F391–F399, 2001.—The epithelial Na+ channel (ENaC) plays a key role in the regulation of Na+ and water absorption in several epithelia, including those of the distal nephron, distal colon, and lung. Accordingly, mutations in ENaC leading to reduced or increased channel activity cause human diseases such as pseudohypoaldosteronism type I or Liddle’s syndrome, respectively. The gain of ENaC function in Liddle’s syndrome is associated with increased activity and stability of the channel at the plasma membrane. Thus understanding the regulation of channel processing and trafficking to and stability at the cell surface is of fundamental importance. This review describes some of the recent advances in our understanding of ENaC trafficking, including the role of glycosylation, ENaC solubility in nonionic detergent, targeting signal(s) and hormones. It also describes the regulation of ENaC stability at the cell surface and the roles of the ubiquitin ligase Nedd4 (and ubiquitination) and clathrin-mediated endocytosis in that regulation.

epithelia sodium channel; glycosylation; ubiquitin; Nedd4; endocytosis; WW domain; Liddle’s syndrome

THE AMILORIDE-SENSITIVE EPITHELIAL Na+ channel (ENaC) is an apically located channel expressed primarily in polarized epithelia in the distal nephron, lung, distal colon, and other organs (26). It is composed of three related subunits, α, β, and γ (9, 11, 47, 52, 53, 84), arranged in a stoichiometry of 2α:1β:1γ (23, 44; see, however, Ref. 74 for another view). Expression of all three ENaC subunits is needed for full channel activity, although expression of the α-subunit alone or αβ- or αγ-subunit combinations leads to the generation of small currents as well (9, 11, 24). Each ENaC chain is composed of two transmembrane domains, a large ectodomain separating them that contains numerous potential N-linked glycosylation sites and two short NH2 and COOH termini (10, 63, 75). The extracellular domain represents more than half of the mass of the ENaC subunits and contains two conserved cystein-rich domains. The COOH terminus of each subunit contains two proline-rich regions (65), the second of which also includes a highly conserved sequence now known as the PY motif (xPPxY; where x is any amino acid, P is proline, and Y is tyrosine) (70, 77).

ENaC plays a critical role in the regulation of Na+ and fluid absorption and, hence, in the regulation of blood volume and blood pressure. Accordingly, mutations in ENaC have been genetically linked to two disorders affecting fluid reabsorption in the distal nephron. The first is pseudohypoaldosteronism type I (PHA-1), a salt-wasting nephropathy caused by loss-of-function mutations in either α-, β-, or γ-ENaC that results from reduced channel opening (13, 29, 80). PHA-1-like disease is also recapitulated in knockout mice models lacking either β- or γ-ENaC or expressing a reduced dose of α-ENaC, all of which suffer from an effective reduction in ENaC activity (4, 37, 54), as seen in PHA-1 patients. The second, Liddle’s syndrome, is a hereditary (or sporadic) form of arterial hypertension (7) resulting from excessive ENaC activity. Liddle’s syndrome is caused by deletion or mutations of the PY motifs of β- or γ-ENaC (30, 31, 38, 72, 81, 87). These mutations lead to elevated activity of ENaC when it is expressed heterologously in Xenopus laevis oocytes (69), an eleva-
tion caused by an increase in both channel number and openings at the cell surface (24).

In this review, we will focus on recent advances in our understanding of ENaC trafficking and regulation of its stability and the implications they have on the functioning of this channel under physiological and pathological conditions.

**ENaC Trafficking to the Cell Surface**

In recent years, several groups have focused their studies on characterizing ENaC processing and trafficking, primarily in A6 cells and in cells that express ENaC heterologously [e.g. *X. laevis* oocytes, Cos cells, HEK-293 cells, and Madin-Darby canine kidney (MDCK) epithelial cells]. In general, it appears that ENaC processing is inefficient, with ~1% of channels synthesized in the endoplasmic reticulum (ER) making it to the cell surface when they are expressed in *X. laevis* oocytes (82) and an estimated ~20% in A6 cells, which express *X. laevis* ENaC (xENaC) endogenously (86). Moreover, ENaC presence at the cell surface is almost undetectable in kidney tight epithelia that are not stimulated by aldosterone, as no channel activity is observed in cortical collecting ducts from rats with low plasma aldosterone levels (57), and ENaC subunits cannot be detected at the apical membrane of the distal nephron by immunohistochemistry under similar conditions (48, 50). An important determinant of the efficiency of ENaC maturation is the assembly of its chains in the ER. ENaC assembly likely occurs very early during its processing in the ER, as suggested by coimmunoprecipitation experiments that demonstrate association of ENaC chains in both glycosylated and unglycosylated forms (2). Although biochemical (2, 15, 24) and functional (11, 39) data have demonstrated that the expression of the α-subunit alone, or a combination of αβ- or αγ-subunits, can lead to the appearance of functional channels at the cell surface, the efficiency of maturation of such “partial” channels is extremely low. This coincides with enhanced ubiquitination and proteasomal degradation of ENaC chains when expressed individually (78, 82) (Fig. 1). Moreover, although ectopic expression of the isolated α-subunit leads to exclusive lactacystin-sensitive (proteasomal) degradation, expression of all three ENaC subunits results in the appearance of an ENaC pool that is sensitive to lysosomal inhibitors (78), suggesting that this is a more mature pool that enters the lysosomes, possibly after arrival at the plasma membrane and endocytosis. Thus assembly of all three ENaC chains leads to increased stability of the complex and likely increased efficiency of delivery to the plasma membrane, although, even with all three subunits assembled together, the maturation efficiency of this channel is quite low.

A consistent observation in several studies is the short intracellular half-life of the ENaC protein, even when all three subunits are coexpressed. Typically, the total cellular pool of ENaC turns over rapidly, with a reported half-life of ~40–120 min in cultured cells (51, 78, 86) and a somewhat longer half-life (3.5–4 h) in *X. laevis* oocytes (82); the latter apparent increased stability may reflect the lower temperature in which the oocytes were grown. This rapid turnover may have important implications for the regulation of ENaC function. The stability of ENaC at the cell surface, a more important factor, is discussed in a separate section below.
Glycosylation

A very useful tool in studying maturation and trafficking of transmembrane proteins from the ER to the Golgi (Fig. 1) is tracing their Asn (N)-linked glycosylation pattern. At the ER, transmembrane proteins acquire core glycosylation, which is sensitive to N-glycosidase-F (PNGaseF). This core glycosylation is then trimmed at the ER to become high mannose and, on transport to the Golgi apparatus and maturation, is replaced with complex glycosylation at the medial-Golgi compartment, which is now resistant to endoglycosidase H (Endo-H). The Endo-H-resistant pool is often used as a marker to follow the mature transmembrane protein. However, one of the major difficulties (and a source of great frustration) in studying ENaC trafficking and maturation is the apparent lack of a detectable Endo-H-resistant pool of this channel (10, 63, 75, 82; Hanwell D, Saleki R, Ishikawa T, and Rotin D, unpublished observations). This lack of a detectable Endo-H-resistant pool of cellular ENaC suggested that either this channel does not acquire complex glycosylation or that the very small fraction of ENaC that actually makes it to the cell surface is complex glycosylated but is below our detection limit. Our recent work, however, suggests that the cell surface pool of ENaC expressed in epithelial MDCK cells and analyzed by surface biotinylation is Endo-H sensitive (Hanwell D, Saleki R, Ishikawa T, and Rotin D, unpublished observations). This suggests that ENaC is able to traffic to the cell surface without acquiring complex N-linked sugars. This is in contrast to the ENaC relative FaNaCh, in which a large fraction of the synthesized channel become Endo-H resistant during maturation (16). Although uncommon, there have been several proteins reported to traffic normally to the cell surface without the acquisition of complex N-linked glycosylation. Noted examples include the anion exchanger AE1 (27), the Torpedo AChR (8), and the Shaker K channel (67), although, in the latter two, a lack of complex glycosylation was seen when they were expressed in X. laevis oocytes (which nevertheless resulted in the expression of functional channels at the cell surface). In contrast to previous reports suggesting that the mature ENaC is stripped of its N-linked sugars (60, 61), our work demonstrates that the surface pool of ENaC is glycosylated in a pattern similar to that for the ER pool and exhibits PNGaseF and Endo-H sensitivity (Hanwell D, Saleki R, Ishikawa T, and Rotin D, unpublished observations), hinting that, at least in epithelial MDCK cells, an immature pattern of glycosylation persists in ENaC during its trafficking to the cell surface.

The role of glycosylation of transmembrane proteins has received great attention, and it appears that in some cases it is involved in vectorial (e.g., apical) targeting, whereas in others it does not appear to be so (reviewed in Ref. 88). The role of ENaC glycosylation in ENaC trafficking is presently obscure. All three ENaC subunits possess numerous potential N-linked glycosylation sites at their ectodomains (10), and it is clear that all subunits are glycosylated in cells. All the glycosylation sites are utilized in α-ENaC, and mutating some or all of these sites does not seem to affect channel activity (10, 75), suggesting that at least sugar modification of that subunit does not affect proper trafficking to and insertion of the channel in the plasma membrane.

Solubility in Nonionic Detergent

Recent work in Cos and HEK-293 cells heterologously expressing ENaC has shown that the channel is transformed during its processing from a nonionic detergent (Triton X-100)-soluble form in the ER [or possibly in the cytosol (82)] to a Triton X-100-insoluble form during trafficking to the cell surface and has suggested that the mature ENaC is detergent insoluble (60, 61). The acquisition of such nonionic detergent insolubility is often proposed to be mediated by either an association with lipid rafts or with the cytoskeleton or by the formation of a macromolecular oligomeric complex. Lipid rafts are cholesterol- and sphingolipid-rich microdomains within membranes often associated with targeting (mainly apical) of proteins sequestered in them (73). Our recent floatation assays with ENaC suggest, however, that ENaC expressed in MDCK cells is not associated with lipid rafts (Hanwell D, Saleki R, Ishikawa T, and Rotin D, unpublished observations). This conclusion is also supported by the observation that lysis of ENaC-expressing cells in octylglucoside at high pH (treatment that solubilizes caveola-associated proteins) failed to solubilize ENaC (60). Thus it appears that ENaC is not utilizing lipid rafts to traffic to the cell surface.

Several reports have proposed interactions between ENaC and cytoskeletal proteins, including α-spectrin (65, 90) and actin (5, 40). However, although it is quite likely that cytoskeletal interactions are involved in stabilizing ENaC at the cell surface (as seen for numerous other transmembrane proteins) and possibly in regulating its activity, it is not known whether these interactions cause the detergent insolubility of this channel, as the addition of the cytoskeletal disrupters cytochalasin, nocodazole, or colchicine in one study failed to alter ENaC solubility (61). Thus so far it is not clear what factors are involved in mediating the acquisition of nonionic detergent insolubility during maturation of ENaC or what fraction of ENaC channels actually acquire such insolubility.

Targeting Signals

The determinants that control apical targeting of ENaC are not yet known. Earlier work has demonstrated retention of the COOH terminus of α-ENaC, when it is expressed as a glutathione-S-transferase fusion protein, at the apical membrane of polarized alveolar epithelial cells (65). However, because removal of the COOH termini of each or all three subunits of ENaC does not impede channel activity, it is likely that the COOH terminus of α-ENaC is not necessary for apical targeting but may instead help stabi-
lize the channel at that membrane, at least in alveolar epithelial cells. Unlike neuronal precursor cell developmentally downregulated (Nedd4), which utilizes lipid rafts for apical targeting, ENaC does not seem to exploit this mode of transport to the apical surface. Nedd4 itself is unlikely to be involved in targeting and transport of ENaC to the cell surface, because channels bearing mutations in all their PY motifs, and thus incapable of binding Nedd4 (see below), display enhanced, not decreased, channel activity at the cell surface (1, 28, 77). A protein implicated recently in regulating ENaC transport to the cell surface is syntaxin 1A (62, 68). This soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein directly binds ENaC, and its ectopic expression decreases the number of channel at the cell surface (however, see Ref. 68). In contrast, the SNARE protein syntaxin 3 was found to enhance ENaC activity (68). These observations suggest that ENaC transport utilizes the SNARE machinery for vesicular transport to the cell surface, although the regulation of this process is not known.

Other Regulatory Hormones and Proteins

Vasopressin (antidiuretic hormone; ADH) increases ENaC activity by binding to a V2 receptor and activating adenylate cyclase. The vasopressin effect is mediated by cAMP and activation of protein kinase A; the nature of this phosphorylation has not yet been elucidated. A number of observations suggest that cAMP acts by translocating ENaC from a cytoplasmic pool into the apical membrane, but this issue still remains controversial (20, 43). Similar to aldosterone (see below), vasopressin also increases the abundance of ENaC, but to a lesser extent (17, 19). The stimulation of ENaC activity at the cell surface by vasopressin seems different from that of aldosterone (see below) because both effects are synergistic.

Patch-clamp experiments in rat renal cortical collecting ducts indicated that variations in plasma aldosterone levels, induced by changes in dietary salt intake, modify the abundance of active ENaC in the apical plasma membrane (57). No apical channel activity could be detected in animals that had low plasma aldosterone and were fed a high-salt diet, whereas increasing plasma aldosterone levels by restriction of Na+ intake dramatically increased the number of functional apical channels. The physiological relevance of this regulation has been quantitatively assessed recently by measurements of the amiloride-sensitive Na+ currents recorded in cortical collecting tubule cells of rats that were subjected to short-term salt deprivation and had their urinary Na+ excretion measured (25). This study showed that the activation of ENaC current in response to a two- to threefold increase in plasma aldosterone levels can account for the reduced Na+ excretion under these conditions. This regulation of ENaC activity in the distal nephron is likely important for the day-to-day variations in urinary Na+ excretion. In situ immunohistochemical studies in rodent kidney clearly showed that changes in plasma levels of aldosterone affect the intracellular distribution of ENaC subunits (48, 50). An increase in plasma aldosterone level associated with dietary salt restriction causes a large increase in intracellular abundance of α-ENaC and a redistribution of ENaC subunits from the cytoplasm to the apical membrane. The induction of α-ENaC expression and its shift from a cytoplasmic to an apical membrane pool in the distal nephron can be observed as early as 2 h after aldosterone injection (49). The molecular and cellular mechanisms underlying these effects remain to be elucidated. Besides the increase in α-ENaC mRNA levels, aldosterone induces or represses the synthesis of many other transcripts in principal cells of the distal nephron (aldosterone-induced and aldosterone-repressed transcripts; see Ref. 64). These aldosterone-induced transcriptional events are likely to play an important role in ENaC regulation. For instance, the expression of the serum and glucocorticoid-induced kinase (sgk) (85) rapidly increases in the distal nephron in response to aldosterone activity (14, 49, 55). The sgk protein is expressed in the cytoplasm of the principal cells expressing ENaC (49). When coexpressed with ENaC in X. laevis oocytes, sgk stimulates ENaC activity (14, 55) by increasing the number of ENaC channels at the cell surface. It has been proposed that this increase is mediated by an augmented insertion of channels at the plasma membrane rather than by a reduction in endocytosis. (3). Taken together, the effects of sgk on ENaC in X. laevis oocytes and the in vivo expression of sgk in the distal nephron suggest that sgk is involved in the regulation of cell surface expression of ENaC during the early phase (<4 h) of aldosterone stimulation (49).

ENaC Stability at the Cell Surface

Regulation of cell surface stability of ENaC has very important implications for the control of channel function under physiological and pathological (e.g., Liddle’s syndrome) conditions and has received much attention in recent years. The half-life of ENaC at the plasma membrane has been estimated from either its activity (amiloride-sensitive Na+ currents) in X. laevis oocytes after brefeldin A (BFA) treatment, which inhibits ER-to-Golgi transport of proteins, or from direct measurement of cell surface ENaC protein using cell surface biotinylation, also after the addition of BFA or blockers of protein synthesis. In either case, the fraction of ENaCs that is recycled to the cell surface after internalization has not been determined. The half-life of (heterologously expressed) ENaC at the cell surface of X. laevis oocytes after BFA treatment was reported to be ~2–3.5 h (71, 78). In mammalian MDCK cells, this value was between 1 and 2 h (Hanwell D, Saleki R, Ishikawa T, and Rotin D, unpublished observations), which is quite comparable considering the higher temperature in which these cells are grown relative to that for X. laevis oocytes. A recent study using A6 cells proposed a much longer half-life of xENaC and suggested that cell surface stability of α- and γ-ENaC (at
least 24 h) is much greater than that of β-ENaC (6 h) (86). It is presently difficult to understand, however, the discordant internalization of the ENaC chains and the suggestion that they may traffic to and from the plasma membrane independently, considering the various studies demonstrating that ENaC chains assemble very early on in the ER. Thus most studies suggest that ENaC at the cell surface is turned over quite rapidly. This rapid turnover is greatly inhibited, however, in β- or γ-ENaC chains bearing Liddle’s syndrome mutations in their PY motifs, with several-fold increased stability of the channel at the plasma membrane (24, 70, 71, 76). Presently, there are two models, not mutually exclusive, that attempt to explain this increased stability: 1) decreased binding of ENaC to the ubiquitin ligase Nedd4 and 2) loss of an internalization signal. These models are discussed below in some detail.

**Nedd4 and Its Regulation of Cell Surface Stability of ENaC**

Nedd4 is a ubiquitin protein ligase composed of a C2 domain, three or four WW domains, and a ubiquitin ligase (E3) Hect domain (45). Ubiquitination of proteins serves to tag them for degradation, usually by the proteasome (34). In some cases, however, especially of transmembrane proteins, ubiquitination appears to tag proteins for endocytosis and lysosomal degradation (35, 66). The E3 Nedd4 catalyzes the third and final step in the ubiquitination cascade and is responsible for attaching ubiquitin moieties onto lysine residues of target proteins.

The C2 domain of Nedd4 has been demonstrated to bind to membranes in a calcium-dependent manner and to localize Nedd4 to the apical membrane in polarized MDCK cells (59). This apical membrane targeting is achieved by a calcium-dependent association of the Nedd4-C2 domain with annexin XIIIb (58), a protein enriched in apical rafts (46), which thus recruits Nedd4 to these rafts and to the apical membrane.

WW domains are protein-protein interaction modules that bind proline-rich sequences, and in the case of Nedd4, seem to prefer binding to PY motifs (xPPxY). Earlier work has demonstrated that the Nedd4-WW domains bind to the PY motifs of ENaC and that mutations in the PY motif of β-ENaC, which cause Liddle’s syndrome (31, 38, 72, 81) and lead to elevation of channel activity (70), also abrogate binding to the Nedd4-WW domains (77). More recently, the solution structure of the third WW domain of Nedd4 in a complex with the PY motif of β-ENaC was solved by NMR (42) (Fig. 2). It reveals several interesting features that help explain the failure of the WW domain to bind the Liddle’s syndrome mutations. The actual region in ENaC that binds the WW domain includes not only the PY motif itself but also COOH-terminal residues and thus encompasses the sequence pPnYdsL, where P (proline), Y (tyrosine), and L (leucine) indicate the residues that contact the domain. The sequence pP-PxYxxL is highly conserved in all ENaC subunits in all

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**Fig. 2.** Surface representation of Nedd4-WWIII in complex with the “extended” PY motif of β-ENaC. Molecular surfaces of the WW domains are represented by white (hydrophobic surfaces), red (negative), and blue (positive) electrostatic potentials. The backbone of the extended PY motif (residues P614-L621) is in green and side chains in yellow. Bold letters indicate residues of the extended PY motif that contact the WW domain (modified from Ref. 42).
species. The two prolines that precede the tyrosine form a polyproline type II helix and are accommodated in an XP groove in the WW domain (42) (Fig. 2), as seen for other WW domains (36, 83) and for SH3 domains (89). What was unexpected and unique (so far) to the Nedd4-WW:ENaC-PY motif complex is the sharp turn formed by the YxxL region (Fig. 2) and the strong interactions of both tyrosine and leucine with the WW domain (42). In accordance, Snyder et al. (76) had demonstrated that mutation of leucine, similar to mutation of the prolines and tyrosine of the PY motif (70, 76), leads to elevation of ENaC activity. Thus these studies suggest that our definition of the PY motif should be extended to PPyYxxL, which includes, in the case of β-ENaC, COOH-terminal sequences to the original “core” PY motif (PPxY). They also suggest the existence of Liddle’s syndrome mutations in the leucine of the extended PY motif, which have not yet been described but should be searched for. Moreover, these studies have some important biological implications regarding endocytosis of ENaC, as described below.

The interaction between Nedd4-WW domains and ENaC-PY motifs prompted the obvious investigations into the physiological relevance of such an association. Moreover, Nedd4 is expressed in numerous tissues and cell types that express ENaC, such as the principal cells of the cortical and outer medullary collecting ducts in the distal nephron and lung airway and distal epithelia (18, 21, 79). Several recent reports have indeed demonstrated that Nedd4 is a suppressor of ENaC that regulates the number of channels at the cell surface (1, 22, 28, 33). This function of Nedd4 requires the presence of intact PY motifs, to allow binding to Nedd4-WW domains, and accordingly, mutations in the PY motif that cause Liddle’s syndrome also impair the suppressive effect of Nedd4 on ENaC (1, 28). Moreover, suppression by Nedd4 also required its Hect domain to be active, indicating that ENaC ubiquitination by this E3 ligase is involved (1). Ubiquitination of ENaC was previously demonstrated to regulate its cell surface stability (78), and mutation (to arginine) of key conserved lysine residues located at the NH2 termini of γ- and α-ENaC led to both impaired channel ubiquitination and increased stability of the channel at the plasma membrane (78). Thus, like several other transmembrane proteins, including numerous yeast receptors and permeases (which are regulated by the Nedd4 ortholog Rsp5p; reviewed in Ref. 66), ENaC stability at the cell surface is regulated by ubiquitination. Of the Nedd4 isoforms that affect ENaC, it appears that those isoforms (or Nedd4-like proteins) possessing four WW domains have a stronger suppressive effect on ENaC than those possessing three WW domains (1, 32, 41). This could be due to increased affinity and avidity of interactions between Nedd4 and ENaC because of the presence of the “extra” WW domain. Taken together, it appears that, in response to elevated calcium levels, Nedd4 translocates to the apical membrane by association of its C2 domain with annexin XIIIb. It then binds the PY motifs of ENaC by its WW domains, thus allowing ubiquitination of the channel by the Hect domain, leading to endocytosis and degradation of ENaC. This process is at least partially impaired in Liddle’s syndrome, causing increased retention of ENaC at the cell surface and, hence, increased Na+ (and fluid) absorption in the distal nephron, resulting in hypertension.

Endocytosis of ENaC

An alternative explanation for the increased cell surface retention of ENaC in Liddle’s syndrome was put forward by Shimkets and colleagues (71) and Snyder and colleagues (76), proposing that the PY motifs of β- or γ-ENaC serve as endocytic motifs recognized by the clathrin-AP-2 complex and that their deletion/mutation in Liddle’s syndrome impedes endocytosis, leading to increased retention of ENaC at the plasma membrane. This model does not preclude the involvement of Nedd4/ubiquitination in regulating ENaC endocytosis, especially as it is now clearly recognized that the two processes are somehow connected. The relationship between binding of the Nedd4-WW domain to the same region in ENaC that may bind the clathrin endocytic machinery brings up some interesting questions. Does ENaC bind μ2 of AP-2 [which is part of the clathrin complex that binds YxxL motifs (6)] and if so, does it bind μ2 via its YxxL sequence? According to the modeling prediction by Shimkets and colleagues (71), the pPPxYxxL sequence of β-ENaC should adopt a β-turn conformation. Our experimental results using NMR, however, demonstrate that this region is extended. It only forms a helical turn when complexed to the WW domain of Nedd4 (42). Moreover, according to the recently solved three-dimensional crystal structure of μ2 when complexed to a YxxL motif of the epidermal growth factor receptor, the YxxL motif binds μ2 in an extended conformation, not in a β-turn (56). Thus, if the YxxL sequence of ENaC binds μ2, it can only do so when extended, not when bound to the WW domain of Nedd4 (unless it binds a different surface of μ2 than that reported by Owen and Evans). This suggests a possible scenario in which Nedd4 binds ENaC first, ubiquitinates it, and is then released from the ubiquitinated channel, allowing the pPPxYxxL motif to become extended and free to now bind μ2 of AP-2 and clathrin-mediated endocytosis to proceed. Solving the structure of the pPPxYxxL region of ENaC in complex with μ2 of AP-2 should shed some light on this issue. Alternatively, the Nedd4-ENaC complex may present itself to some other components of the endocytic machinery and internalize together. Indeed, Nedd4 was recently detected in endosomes (58). Regardless of the mechanism, how ubiquitination of ENaC is involved in its internalization is still not understood.

Endocytosis of ENaC may not rely solely on its extended PY motif, and other sequences may be involved as well. A recent report has suggested the presence of an endocytic region in the NH2 terminus of α-ENaC, which includes residues 47–67 (12). Curiously, this region includes Lys47 and Lys50, previously demonstrated to be targets for ENaC ubiquitination (78).
However, point mutation of these lysines to arginines was only effective in stabilization of ENaC at the cell surface in the context of Lys→Arg mutations of a critical cluster of lysine residues (K6, 8, 10, 12, 13) in $\gamma$-ENaC (78). Thus it is not presently clear whether the role of residues 47–67 of $\alpha$-ENaC (78). Thus it is not presently clear whether the role of residues 47–67 of $\alpha$-ENaC in endocytosis involves channel ubiquitination.

**CONCLUDING REMARKS**

Despite difficulties in studying ENaC trafficking due to the low abundance of this protein in native tissues and the lack of an apparent Endo-H-resistant pool of the channel, recent published and unpublished work is beginning to shed some light on how this channel is processed and travels to the cell surface, although, clearly, our understanding of these processes is rudimentary. Similarly, our understanding of ENaC stability at the cell surface is very basic. However, the interaction of the channel with the ubiquitin ligase Nedd4 both structurally and functionally, as well as the described role of clathrin-mediated endocytosis in ENaC internalization, provides insight into at least some of the processes involved in regulation of channel stability at the plasma membrane. This knowledge is important for understanding both the physiological regulation of ENaC and its impaired regulation in diseases such as Liddle’s syndrome.

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