Impact of Nedd4 Proteins and Serum and Glucocorticoid-Induced Kinases on Epithelial Na\(^+\) Transport in the Distal Nephron

Olivier Staub\(^*\) and François Verrey\(^†\)

\(^*\)Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland; and \(^†\)Institute of Physiology, University of Zurich, Zurich, Switzerland

The precise control of BP occurs via Na\(^+\) homeostasis and involves the precise regulation of the epithelial Na\(^+\) channel (ENaC) in the aldosterone-sensitive distal nephron (ASDN) and plays a major role in the regulation of whole-body Na\(^+\) homeostasis and consequently in the control of blood volume and pressure. ENaC is composed of three homologous subunits, \(\alpha\), \(\beta\), and \(\gamma\), each containing two transmembrane domains, an extracellular loop and intracellular N- and C-termini (1–3). In the kidney, ENaC is highly regulated by various hormonal pathways, such as those linked to aldosterone, vasopressin, and insulin (4). In recent years, considerable progress was made in understanding this regulation, but many of the molecular mechanisms involved remain unknown. Genetic evidence has given insight into the current view that ENaC is regulated via ubiquitylation and phosphorylation.

Liddle’s syndrome is a rare genetic form of human hypertension that was originally described in 1963 by Dr. Liddle and his collaborators (5) and presents with early onset of salt-sensitive hypertension, hypokalemia, metabolic alkalosis, and low circulating levels of renin and aldosterone. Patients can be treated with ENaC inhibitors (triamterene or amiloride) and low-Na\(^+\) diet (6). Linkage analysis linked Liddle’s syndrome to the epithelial Na\(^+\) channel (ENaC) localizes to the apical membrane of high-resistance epithelia of the aldosterone-sensitive distal nephron (ASDN) and plays a major role in the regulation of whole-body Na\(^+\) homeostasis and consequently in the control of blood volume and pressure. ENaC is composed of three homologous subunits, \(\alpha\), \(\beta\), and \(\gamma\), each containing two transmembrane domains, an extracellular loop and intracellular N- and C-termini (1–3). In the kidney, ENaC is highly regulated by various hormonal pathways, such as those linked to aldosterone, vasopressin, and insulin (4). In recent years, considerable progress was made in understanding this regulation, but many of the molecular mechanisms involved remain unknown. Genetic evidence has given insight into the current view that ENaC is regulated via ubiquitylation and phosphorylation.

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\[\text{ENaC} \rightarrow \text{ubiquitylation} \rightarrow \text{phosphorylation} \]

early findings were subsequently confirmed in other patients with Liddle’s syndrome, and mutations or deletions were found in the C-termini of \(\beta\) or \(\gamma\)-ENaC (8–20). To our knowledge, there is only one exception, in which a mutation was mapped to the extracellular loop of \(\gamma\)-ENaC (21). It is interesting that all of these mutations in the C-termini of \(\beta\)- or \(\gamma\)-ENaC are mutations that cause frame shifts, stop codons, or single amino acid changes and invariably delete or mutate a so-called proline-rich PY motif (22). Such PY motifs, which conform to a consensus sequence of Pro-Pro-Xaa-Tyr (Xaa: any amino acid), are found in many different proteins, often in plasma membrane proteins or ion channels (23), and are known to interact with WW (protein:protein interaction) domains (22,24). These sequences are present in the C-termini of all three ENaC subunits. When ENaC channels that bear Liddle’s mutations are expressed in heterologous expression systems, such as \textit{Xenopus laevis} oocytes or MDCK cells, they display higher ENaC activity at the plasma membrane (25–27). This increase is due to a rise in cell surface expression, elevated open probability (Po), and impairment of Na\(^+\)-dependant feedback inhibition of ENaC (i.e., inhibition of ENaC by intracellular Na\(^+\)) (25,28,29). A yeast two-hybrid analysis revealed that the ENaC PY motifs interact with the ubiquitin-protein ligase Nedd4, implicating ubiquitylation as a regulatory mechanism of ENaC activity (30).

Regulation of Epithelial Na\(^+\) Channel by Nedd4/Nedd4-Like Ubiquitin Protein Ligases

Ubiquitylation (or ubiquitination) is a process that refers to the posttranslational modification of target proteins with ubiquitin, a 76 amino acid–long polypeptide (31). This modification
involves a cascade of enzymes, including a single E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases. The sequential action of these enzymes leads to the isopeptide formation of ubiquitin via its C-terminal glycine to the amino group of a lysine on the target protein. Internal lysines on ubiquitin are often used to form polyubiquitin chains. Hundreds of E3 enzymes participate in substrate recognition. These enzymes can be divided into two major protein families, namely the RING finger (31) and the HECT (homologous to E6-AP carboxyterminal) domain-containing proteins. Nedd4 is a member of the HECT domain ubiquitin-protein ligases (32). These proteins are characterized by a C-terminal, highly conserved 40-kD enzymatic HECT domain, and a variable, N-terminal region, involved in substrate recognition. The HECT domain contains a cysteine, which is essential for catalytic activity (32). Nedd4 is the founding member of a subfamily of HECT enzymes, which contains nine members (33). These enzymes are composed of an N-terminal (Ca\(^{2+}\)-dependent lipid binding) C2 domain (34), two to four WW domains (24), and the C-terminal HECT domain. They are involved in several different cellular functions (33,35,36). The isolation of the WW domain containing Nedd4–1 suggested that ENaC may interact with members of this family via its PY motifs. Upon interaction, Nedd4/Nedd4-like proteins would ubiquitylate one or several ENaC subunits, in analogy to many plasma membrane receptors (37), and cause their internalization and targeting to the endosomal multivesicular body pathway and ultimately degradation in the lysosome. In Liddle's syndrome, in which one of the ENaC PY motifs is missing, interaction with the Nedd4/Nedd4-like proteins would be impaired, leading to reduced ubiquitylation and internalization of ENaC (Figure 1). Many predictions of such a model have been confirmed since then.

**ENaC Interacts with Nedd4/Nedd4-Like Proteins via PY Motif/WW Domain Interaction**

Many investigators have shown by various in vitro and in vivo approaches that ENaC PY motifs and Nedd4/Nedd4-like WW domains interact. In particular, in vitro interaction of ENaC PY motif was shown with WW domains of Nedd4–1 (30,38–47), Nedd4–2 (47–49), WW1 (50), and WW2 (51). These interactions with ENaC were confirmed in intact cells by co-immunoprecipitation only with Nedd4–1 (30,52) and Nedd4–2 (52–55). Subsequently, evidence has accumulated that the PY motifs of \(\beta\)- or \(\gamma\)-ENaC interact with the WW domains 3 or 4 of human Nedd4–1 or Nedd4–2 (41,46,47,52,56).

**ENaC Is a Protein Complex That Has a Rapid Turnover and Becomes Ubiquitylated**

Several groups have demonstrated that ENaC is a protein with a rapid turnover of both the total and the cell surface pool (T\(_{1/2}\) = approximately 1 h) (57–61), although one report mentioned a half-life of the \(\alpha\)-ENaC at the plasma membrane of 24 to 30 h (62). However, in this report, \(\alpha\)-ENaC has an apparent molecular weight of 180 kD instead of the usual 90 kD; hence, the specificity of the used anti-\(\alpha\)-ENaC antibody remains to be proved. Rapid turnover is a hallmark of ubiquitylated proteins; in the case of ENaC, this rapid turnover depends on both proteasomal and lysosomal degradation (60,61,63). It has been shown using ENaC with cytoplasmic lysines mutated to arginine, hence with mutated putative ubiquitylation sites, that ubiquitylation of ENaC controls its cell surface expression but not its intrinsic activity (i.e., \(P_0\) or single channel conductance [60]). A direct demonstration that Nedd4/Nedd4-like proteins ubiquitylate ENaC is so far missing.

**ENaC Is Regulated by Nedd4/Nedd4-Like Proteins**

It has been demonstrated by different approaches that Nedd4/Nedd4-like proteins, in particular Nedd4, Nedd4–2, or WW2, can regulate ENaC. In mouse mandibular cells, it is possible to interfere with \(Na^+\) feedback regulation by adding Nedd4 antibodies or purified WW domains from Nedd4–1 or Nedd4–2 into the pipette of a whole-cell patch-clamp set-up and letting them diffuse into the cell (38,40,56,64). Such data have been interpreted as evidence that either Nedd4 or Nedd4–2 is involved in \(Na^+\) feedback regulation in mandibular cells. Strong support for an involvement of Nedd4/Nedd4-like proteins in ENaC regulation comes from experiments done in the *Xenopus laevis* oocytes system, in which regulation of ENaC by co-expressed Nedd4–1 (41,45,65), WW2 (51), and Nedd4–2 (52,53,66) has been demonstrated. The strongest effects are seen with Nedd4–2. Overexpression of Nedd4–2 leads to the nearly complete suppression of amiloride-sensitive \(Na^+\) channels, an effect that is dependant on the PY motifs of ENaC (52,53,66). Conversely, catalytically inactive Nedd4–2 mutants increase...
these currents, most likely by competing against endogenous Nedd4-2. Moreover, Nedd4-2 has been shown to control cell surface expression of ENaC (66).

The effect of Nedd4/Nedd4-like proteins was also investigated in ENaC transfected thyroid cells (FRT cells) that were grown on permeable supports; co-transfection of either Nedd4 or Nedd4-2 was found to diminish transepithelial amiloride-sensitive Na⁺ currents (45,49,67). In the same system, it was demonstrated by RNA interference that Nedd4-2 controls ENaC, as interference with endogenous Nedd4-2 but not Nedd4-1 increased transepithelial Na⁺ transport (68).

**Genetic Evidence Linking Nedd4-2 to BP Variation**

There are also some genetic features that seem to be compatible with the hypothesis that Nedd4-2 plays a role in the control of Na⁺ reabsorption and BP. The Nedd4-2 gene localizes to chromosome 18q21-22, extends over 400 kb, and is composed of at least 40 exons. Numerous splice variants are expressed in a tissue- or cell-specific manner and encode proteins that do or do not include the C2 domain, have various numbers of WW domains, or lack certain phosphorylation sites (69–71). The functional role of these alternatively spliced isoforms is not yet entirely clear. The C2 domain of Nedd4-2 has been shown to be required for the translocation of this enzyme to the plasma membrane upon elevation of intracellular Ca²⁺ (72), similar to the C2 domain of Nedd4-1 (73,74). In *Xenopus* oocytes, the C2 domain is not required for the downregulation of ENaC (52,72), whereas a splice variant that lacks both the C2 domain and the WW domains 2 and 3 is inactive (72).

Genetic studies have indicated a link between markers in the chromosomal region that contains the Nedd4-2 gene and different BP phenotypes (75–79). Although a number of polymorphisms have been identified in the Nedd4-2 gene (55,69), no linkage between specific Nedd4-2 variants and BP phenotypes have been reported so far.

**Nedd4-2 Binding or Modifying Proteins**

Not surprising, there are a number of proteins that interact with or modify Nedd4-2 action on ENaC. As outlined above, ubiquitylation involves a cascade of enzymes, including E1, E2, and an E3 enzyme (Nedd4-2). An E2 enzyme that acts in concert with Nedd4-2 has been identified by a two-hybrid screen using the HECT domain as bait. Further investigations have shown that this enzyme, referred to as UBE2E3, co-immunoprecipitates with Nedd4-2, that its inactive mutant interferes with Nedd4-2-dependent regulation of ENaC, and that when it is overexpressed in mpkCCD cells (a mouse cortical collecting duct [CCD] cell line [80]), it inhibits transepithelial Na⁺ current (81). Another protein that interacts with Nedd4-1 and Nedd4-2 is N4WPB5a, a transmembrane protein expressed in the CCD (82). This protein interacts via PY motif/WW domain interaction with Nedd4-1 and Nedd4-2, and when co-expressed in *Xenopus* oocytes, it prevents Na⁺ feedback regulation. It was proposed that this protein localizes to intracellular organelles and binds Nedd4-2, thereby segregating it away from ENaC and thus preventing ENaC downregulation (82). Several kinases also have been suggested to influence regulation of ENaC by Nedd4-2. Garty *et al.* (83) reported that Thr613 on β-ENaC and Thr623 on γ-ENaC just upstream of the PY motifs can be phosphorylated by extracellular signal-regulated kinase and that this phosphorylation stimulates interaction with Nedd4-2. Indeed, mutation of these phosphorylation sites leads to increased channel activity. Grr2 is another kinase that seems to phosphorylate the C-terminus of β-ENaC and renders the channel insensitive to Na⁺ feedback regulation and inhibition by Nedd4-2 (84). The physiologic significance of the regulation by these kinases remains to be determined. Another kinase, serum and glucocorticoid induced kinase 1 (Sgk1), has received considerable attention in recent years, as its expression is rapidly induced by aldosterone and appears to regulate ENaC.

**Aldosterone-Induced Sgk1 Regulates ENaC Activity and Cell Surface Expression**

Sgk1 was originally identified as a kinase that is inducible by serum and glucocorticoids (85). It is a member of the AGC family of Ser/Thr kinases and shares approximately 50% identity in its catalytic domain with other AGC kinases such as protein kinase A, Akt, or protein kinase C (85). There are two close homologues, Sgk2 and Sgk3 (or CISK), that share approximately 80% sequence identity with Sgk1 (86,87). They also share a high degree of similarity with Akt/protein kinase B. Sgk1 was one of the earliest aldosterone-induced gene products in renal epithelial cells (88–94). Interest in Sgk1 rose considerably when it was found that Sgk1 stimulates ENaC activity in oocytes (49,54,88–91,95–100). This increase of ENaC activity is mostly due to elevated cell surface expression (54,91,95,98), although one report also observed an effect on PO (97). Further evidence for a role in epithelial Na⁺ transport came from transduction experiments in renal epithelial cells, which demonstrated that expression of Sgk1 in various cell lines increased transepithelial Na⁺ currents (101–104). Thereby, Alvarez De La Rosa *et al.* (104) observed in A6 cells that were transduced with constitutive active Sgk1 a 4-fold effect on ENaC cell surface expression as well as an increase of 43% in PO. It is not clear how Sgk1 stimulates the PO of ENaC, as the Sgk1-dependent phosphorylation site on α-ENaC, described by Diakov *et al.* (105) below, is not conserved in *Xenopus laevis* α-ENaC. Sgk1 is known to be regulated by the phosphatidylinositol-3-kine pathway and needs to be phosphorylated by PDK1, demonstrating that its function depends on the activity of this pathway. Indeed, evidence was provided from several groups that stimulation of Na⁺ transport by aldosterone and/or Sgk1 is inhibited by phosphatidylinositol-3-kine inhibitors (99,106,107). Antisense (103), RNA interference (94), or overexpression of catalytically inactive Sgk1 (101–103) further corroborates the role of Sgk1 in positive regulation of transepithelial Na⁺ transport. The strongest evidence for such a function comes from the Sgk1 knockout mice, which present a pseudohypoaldosteronism type 1 phenotype when kept under low-Na⁺ diet (108).

**Mechanisms of Sgk1 Action**

Several mechanisms have been proposed for Sgk1-dependent regulation of ENaC. Wang *et al.* (99) showed that Sgk1 directly interacts with the C-terminus of β-ENaC. Diakov *et al.* (103)
identified a consensus phosphorylation site on the C-terminus of α-ENaC (S621) and found by the outside-out patch-clamp technique and inclusion of recombinant Sgk1 in the pipette that this site may be important for ENaC stimulation. However, mutation of this site to alanine (S321A) does not impair the stimulatory effect of Sgk1 (as well as of Sgk2 and Sgk3) on amiloride-sensitive Na⁺ currents in Xenopus laevis oocytes (109). Demonstrating that α-ENaC indeed becomes phosphorylated by Sgk1 will be required to shed some light on this mechanism. An alternative model proposes that Neddd4-2 is a substrate of Sgk1, as Neddd4-2 contains three consensus phosphorylation sites for Sgk1 (49,54), and Sgk1 itself contains a PY motif, rendering it possible that Sgk1 and Neddd4-2 interact via PY motif/WW domain (Figure 1). In support, it was demonstrated that Sgk1 does interact with Neddd4-2 (48,49) and that the PY motif on Sgk1 is partly required for Sgk1-dependent stimulation of ENaC in Xenopus laevis oocytes (54). In oocytes, it was also shown that Sgk1 induces phosphorylation of Neddd4-2 on two sites, primarily on Ser444 but also on Ser338. Moreover, mutation of these sites interfered with Sgk1-dependent phosphorylation of Neddd4-2 and stimulation of ENaC (54). It was further demonstrated that Sgk1 reduced the interaction between Neddd4-2 and ENaC, indicating that Sgk1 impairs ENaC ubiquitylation and subsequent internalization. Taken together, these data suggest that one of the mechanisms by which aldosterone regulates ENaC function is by transcriptionally inducing Sgk1 that in turn phosphorylates Neddd4-2. As expected from such a mechanism, the phosphorylation of Neddd4-2 on Ser444 is increased upon aldosterone treatment in mpkCCD14 cells and in adrenalectomized rats (107). Demonstrating that α-ENaC indeed becomes phosphorylated by Sgk1 will be required to shed some light on this mechanism. An alternative model proposes that Neddd4-2 is a substrate of Sgk1, as Neddd4-2 contains three consensus phosphorylation sites for Sgk1 (49,54), and Sgk1 itself contains a PY motif, rendering it possible that Sgk1 and Neddd4-2 interact via PY motif/WW domain (Figure 1). In support, it was demonstrated that Sgk1 does interact with Neddd4-2 (48,49) and that the PY motif on Sgk1 is partly required for Sgk1-dependent stimulation of ENaC in Xenopus laevis oocytes (54). In oocytes, it was also shown that Sgk1 induces phosphorylation of Neddd4-2 on two sites, primarily on Ser444 but also on Ser338. Moreover, mutation of these sites interfered with Sgk1-dependent phosphorylation of Neddd4-2 and stimulation of ENaC (54). It was further demonstrated that Sgk1 reduced the interaction between Neddd4-2 and ENaC, indicating that Sgk1 impairs ENaC ubiquitylation and subsequent internalization. Taken together, these data suggest that one of the mechanisms by which aldosterone regulates ENaC function is by transcriptionally inducing Sgk1 that in turn phosphorylates Neddd4-2. As expected from such a mechanism, the phosphorylation of Neddd4-2 on Ser444 is increased upon aldosterone treatment in mpkCCD14 cells and in adrenalectomized rats (107). When phosphorylated, Neddd4-2-Ser444 is part of a consensus binding site for 14-3-3 proteins, suggesting that it is the binding of 14-3-3 proteins to Neddd4-2 that sterically interferes with the interaction of Neddd4-2 with ENaC and prevents ENaC ubiquitylation (96).

Regulation of ENaC by Sgk2 and Sgk3

As outlined above, there are two Sgk paralogues, referred to as Sgk2 and Sgk3 (110). These two kinases share the same consensus phosphorylation sites as Sgk1 [RXRXX(S/T)], and they are both expressed in renal epithelial cells. In contrast to Sgk1, their expression is not regulated by glucocorticoids or by aldosterone (86,111,112). A possible role in ENaC regulation has been studied in the Xenopus laevis system, and it has been found that Sgk2 and Sgk3 are as efficient in ENaC regulation as Sgk1 (109). However, an Sgk3 knockout does not show any discernable phenotype with respect to Na⁺ handling, suggesting that Sgk3 does not play a major role in ENaC regulation (113).

Perspectives

The past decade has provided us with a novel and at the time unexpected mode of regulation of ENaC, involving regulated ubiquitylation of this channel complex by Neddd4/Neddd4-like proteins (see Figure 1). Many of the aspects of such a model have been demonstrated experimentally, such as the PY motif/WW domain interaction, the rapid turnover of ENaC sub-units related to ubiquitylation, and the control of cell surface expression of ENaC by members of this E3 family. Nevertheless, many questions remain open, in particular that of the identity of the physiologically relevant ubiquitin-protein ligase(s). Functional, biochemical, and genetic evidence point at Neddd4-2 as the primary regulator, but to date, we cannot exclude the involvement of other family members of the Neddd4/Neddd4-like proteins. It is possible that several of these proteins are implicated, as has been shown recently for the ubiquitylation and degradation of a membrane protein in melanocytes, Melan-A, which seems to be ubiquitylated by Neddd4–1, whereas its degradation seems to be controlled by Itch (a Neddd4/Neddd4-like protein) (114). Direct genetic linkage of BP variation with mutations in the Neddd4-2 gene or knockout mice will be required to answer these questions definitively. That Neddd4-2 can be phosphorylated by Sgk1, an aldosterone-induced protein in the CCD, suggests its potential physiologic importance. Recently, it was shown that aldosterone stimulates phosphorylation of Neddd4-2, one of the predictions of such a mechanism. This phosphorylation of Neddd4-2 on Ser444 induces the binding of the 14-3-3 protein, likely causing steric hindrance of the interaction of Neddd4-2 with ENaC. Although this mechanism is attractive, it probably does not explain entirely the effect of Sgk1 on ENaC and certainly not the complexity of aldosterone action on ENaC function. It is more than likely that other molecular events play a role. Furthermore, ubiquitylation of ENaC may be reversible and involve de-ubiquitylating enzymes that are under the control of hormones such as aldosterone, vasopressin, or insulin. It is clear that the regulatory pathways involving members of the Neddd4/Neddd4-like family and Sgk1 play a central role in ENaC regulation.

Note added in proof: Since submission of this article, Bhalla et al. showed that 14-3-3 proteins bind to phosphorylated Neddd4-2 and interfere with Neddd4-2/ENaC interaction and ENaC ubiquitylation (Mol Endocrinol 2005, in press).

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