Concerted action of ENaC, Nedd4–2, and Sgk1 in transepithelial Na⁺ transport

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Kamynina, Elena, and Olivier Staub. Concerted action of ENaC, Nedd4–2, and Sgk1 in transepithelial Na⁺ transport. Am J Physiol Renal Physiol 283: F377–F387, 2002; 10.1152/ajprenal.00143.2002.—The epithelial Na⁺ channel (ENaC), located in the apical membrane of renal aldosterone-responsive epithelia, plays an essential role in controlling the Na⁺ balance of extracellular fluids and hence blood pressure. As of now, ENaC is the only Na⁺ transport protein for which genetic evidence exists for its involvement in the genesis of both hypertension (Liddle’s syndrome) and hypotension (pseudohypoaldosteronism type 1). The regulation of ENaC involves a variety of hormonal signals (aldosterone, vasopressin, insulin), but the molecular mechanisms behind this regulation are mostly unknown. Two regulatory proteins have gained interest in recent years: the ubiquitin-protein ligase neural precursor cell-expressed, developmentally downregulated gene isoform Nedd4–2, which negatively controls ENaC cell surface expression, and serum glucocorticoid-inducible kinase 1 (Sgk1), which is an aldosterone- and insulin-dependent, positive regulator of ENaC density at the plasma membrane. Here, we summarize present ideas about Sgk1 and Nedd4–2 and the lines of experimental evidence, suggesting that they act sequentially in the regulatory pathways governed by aldosterone and insulin and regulate ENaC number at the plasma membrane.

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ALDOSTERONE, THE PREDOMINANT human mineralocorticoid hormone, controls Na⁺ retention by enhancing Na⁺ reabsorption across “tight epithelia,” such as in the distal regions of the kidney or the colon (124, 129). It acts through binding with high affinity to the mineralocorticoid receptor and with lower affinity to the glucocorticoid receptor, causing translocation into the nucleus and governing the expression (and repression) of a network of proteins of mostly undefined function (105, 120). Consequently, transepithelial Na⁺ reabsorption is elevated through an increase in the apical epithelial Na⁺ channel (ENaC) and, to some extent, basolateral Na⁺-K⁺-ATPase activity. The precise mechanisms of this response are unknown, but they depend on protein synthesis, as they are prevented by inhibitors of both mRNA or protein synthesis. Three phases have been observed: 1) a latent period of 40–60 min, during which initial changes in transcription/translation of genes take place; 2) an “early response” of up to 3 h with a two- to fourfold increase in Na⁺ transport, mainly due to an increase in apical Na⁺ channel activity; and 3) a “late response” of up to 24 h with a further increase in the activity of Na⁺ channels and Na⁺-K⁺-ATPases. Whereas the late response is primarily the result of increased expression levels of ENaC and Na⁺-K⁺-ATPase subunits, the cellular events of the early response are not dependent on increased synthesis of these proteins but lead to an increase in apical ENaC activity that is not well understood at the molecular level. Recent studies have now provided substantial evidence for the involvement of aldosterone-induced, serum glucocorticoid-inducible kinase 1 (Sgk1), which causes an increase in ENaC.
channels at the cell surface, and the ubiquitin-protein ligase neural precursor cell-expressed, developmentally downregulated isoform Nedd4–2, a negative regulator of ENaC. This short review focuses on these recent developments and discusses the progress made in understanding the concerted action of ENaC, Sgk1, and Nedd4–2. For readers interested in more general questions related to aldosterone, a number of excellent reviews exist on this topic (124, 129).

**ENaC**

ENaC is located at the apical membrane of tight epithelia (i.e., epithelia with a high electrical resistance) that transport Na⁺. Such ENaC-expressing epithelia are found in the distal nephron, including the second part of the distal convoluted tubule (DCT2), the connecting tubule (CNT), and the cortical and the medullary collecting duct (CCD and MCD, respectively). ENaC-expressing epithelia are also present in the distal colon, airways, and ducts of exocrine glands (sali-

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cent reports of de novo mutations of ENaC in patients with Liddle’s syndrome who did not have family histories of hypertension (128, 135).

The characteristics of channels with Liddle’s mutations have been investigated primarily in *X. laevis* oocytes. Kellenberger and co-workers (66) reported that “Liddle’s channels” (missing the PY motif on β-ENaC) exhibit impaired Na+-feedback regulation (i.e., inhibition triggered by increased intracellular Na+), which may explain some of the observed higher activities for ENaC (66). The molecular mechanism of Na+-dependent downregulation involving the PY motifs is not entirely clear but may involve Nedd4–2 (27) (see NEDD4–2 SUPPRESSES ENaC CELL SURFACE EXPRESSION AND ACTIVITY). It was also suggested that ENaC bearing Liddle’s mutations are no longer sensitive to cAMP (11, 111).

Several laboratories have shown that the expression of Liddle’s channels yield higher channel activity (12, 107, 116) compared with wild-type channels. Snyder and collaborators (116) attributed this increase to elevated channel numbers at the cell surface, as determined by transient expression in Madin-Darby canine kidney (MDCK) cells, whereas Firsov et al. (35) provided evidence that both cell channel number and open probability were increased by expressing extracellularly FLAG-tagged ENaC constructs in *X. laevis* oocytes. With respect to open probability, it was suggested that the COOH termini of β- or γ-ENaC would be involved in gating and that in Liddle’s syndrome such gating would be impaired, causing increased open probability (57–59). Two mechanisms have been proposed to be perturbed and to be responsible for the observed increase in channel density: endocytosis or ubiquitination. The first, proposed by Shimkets et al. (109) and Snyder et al. (116), involves a defective tyrosine-based endocytosis signal (YXXΦ, where Y is tyrosine, X is any amino acid, and Φ is hydrophobic amino acid), which overlaps with the above-mentioned PY motif.

The evidence supporting this model is mostly indirect. It was shown that mutation of the leucine after the PY motif in β-ENaC (PPPNYDSL; the tyrosine motif is underlined) caused increased amiloride-sensitive Na+ currents (116). Although this may indeed suggest that endocytosis is impaired, it was not corroborated by showing that the number of channels increased with such a mutation. Moreover, this leucine is also involved in interaction with the WW domain of Nedd4, as recently demonstrated by resolving the structure of a complex between the third Nedd4 WW domain and a β-ENaC PY peptide (64). Hence, mutation of this leucine may also affect the interaction with Nedd4 (see NEDD4–2 SUPPRESSES ENaC CELL SURFACE EXPRESSION AND ACTIVITY). Further support for the endocytosis model came from experiments in *X. laevis* oocytes co-overexpressing ENaC with dominant-negative dynamin, a protein known to inhibit clathrin-mediated endocytosis (23). This caused an increase in ENaC activity, suggesting that ENaC is internalized via such a mechanism (109). It was also shown that brefeldin A, a drug interfering with the export of membrane proteins to the cell surface, causes a decrease in wild-type, but not mutated, ENaC activity, suggesting that endocytosis is impaired in Liddle’s channels (109).

Although it is conceivable that this tyrosine-based motif acts as an endocytosis signal for ENaC, it remains to be demonstrated that it interacts with the μ2-subunit of the endocytic adaptor protein-2 complex involved in this type of endocytic mechanism (70). In any case, the proposed model cannot entirely explain Liddle’s syndrome, as there are several Liddle’s mutations that change one of the two essential prolines in the PY motifs. Although such mutations are not part of the tyrosine-based sorting signal (41, 54), the patients concerned manifest Liddle’s disease.

As an alternative mechanism, it has been suggested by Rotin and collaborators (121) that ENaC cell surface expression is regulated by ubiquitination involving Nedd4 and that this mechanism is defective in Liddle’s syndrome (121). The proposed mechanism is not mutually exclusive of the previously described tyrosine-based endocytosis model, as the ubiquitination machinery has been shown to play a role in the internalization of many plasma membrane proteins (50, 106).

**NEDD4–2 SUPPRESSES ENaC CELL SURFACE EXPRESSION AND ACTIVITY**

Ubiquitination of plasma membrane proteins, a post-translational modification involving the covalent linkage of 76-amino acid-long ubiquitin polypeptides on target proteins, has attracted considerable interest in recent years. It was found that such modification may either regulate the internalization/endocytosis or, alternatively, sorting, of these proteins [from the trans-Golgi network to the vacuole/lysosomes or to the plasma membrane; from the endosomal compartment to the multiple vesicular bodies (MVBs) in the lysosome] (for comprehensive reviews of these processes, see Refs. 50 and 106). Ubiquitination requires the sequential action of several enzymes. First, ubiquitin becomes activated in an ATP-dependent manner by forming a thioester with a cysteine on the ubiquitin-activating (or E1) enzyme. It is then transferred on the cysteine of a ubiquitin-conjugating (or E2) enzyme, which will interact with a ubiquitin-protein ligase (or E3 enzyme). These ubiquitin-protein ligases are thought to provide specificity by recognizing and binding to specific target proteins, while attaching ubiquitin via an isopeptide bond between its COOH terminus and the ε-amino group of a lysine residue (49).

Most plasma membrane proteins become either mono- (a single ubiquitin on a receptor lysine) or diubiquitinated (2 ubiquitins linked via an isopeptide bond between the ubiquitin COOH terminus of I molecule and the internal Lys63 of the other). On the other hand, most intracellular proteins are polyubiquitinated, involving Lys48 and the COOH terminus of ubiquitin, and are subsequently recognized by a cytosolic proteolytic complex, the proteasome, which rapidly degrades...
them. It is not yet understood how the ubiquitinated plasma membrane proteins are recognized. Originally, it was thought that ubiquitination of cell surface receptors and channels leads to internalization/endocytosis of these proteins, but this dogma is presently under debate, as there are examples, such as the epidermal growth factor receptor (EGFR), where activation-dependent ubiquitination is not necessary for internalization but seems to be required for import into lysosomal MVBs (83, 119).

The notion that ENaC may be regulated by ubiquitination first came from a two-hybrid screen using the COOH terminus of β-ENaC (including the PY motif) as bait. In this screen, Nedd4 was identified as a binding partner for the PY motifs in ENaC (121). Nedd4 was originally identified in a subtractive screen between mouse embryonic and adult brain (74). It is part of a protein family, which comprises 12–13 human proteins, the Nedd4/Nedd4-like family of ubiquitin-protein ligases (46, 106). These proteins are composed of one to four WW domains (serving protein-protein interaction; also known as WWP or rps5 domains) (4, 19, 51, 123), a domain homologous to the E6-AP-COOH-terminal domain (HECT), the catalytic portion of these proteins (52), and most contain an NH2-terminal C2 (Ca\textsuperscript{2+}-dependent lipid binding) domain (104). Two closely related Nedd4 isoforms (or paralogues) exist: Nedd4–1 (also named Nedd4, KIAA0093, or RFP1) and Nedd4–2 (also known as KIAA0439, Ldlr-1, Nedd4La, Nedd18, or Nedd4-L) (60, 61). Nedd4–1 is composed of one C2 domain, a HECT domain, and three to four WW domains. The rat and mouse species contain three WW domains, whereas in humans there are four WW domains. The difference in the number of WW domains may be due to alternative splicing, as there is evidence for multiple transcripts in human Nedd4–1 (62). Nedd4–2 contains four WW domains and a HECT domain. Only human and X. laevis Nedd4–2 comprise a C2 domain, whereas such a domain appears to be lacking in mouse Nedd4–2. Again, there is evidence for alternative splicing of this isoform as well (18, 62), and there may be isoforms that contain, and others that do not contain, a C2 domain.

The finding that ENaC interacts with a ubiquitin-protein ligase suggested that ubiquitination plays a role in the regulation of ENaC. We proposed a model in which Nedd4 interacts via its WW domains to the ENaC PY motifs and ubiquitiates one or several ENaC subunits. Such ubiquitination would cause a decrease in ENaC at the cell surface, likely by enhanced endocytosis (121), or, alternatively, by induced translocation of ENaC into the lysosomal MVBs, causing rapid ENaC degradation. In Liddle’s syndrome, where one of the PY motifs (and hence a binding site for Nedd4) is missing, proper binding (and, consequently, ubiquitination) of Nedd4 would be impaired, leading to increased Na\textsuperscript{+} channel density.

Experimental evidence in support of this model has accumulated during recent years. It was shown that the total pool of ENaC subunits has a rapid turnover, a hallmark for ubiquitinated proteins, with a half-life of \(\sim 1\) h (42, 85, 87, 122). Moreover, the half-life of ENaC at the cell surface is on the same order of magnitude (42). It was also demonstrated that both inhibitors of the proteasome or lysosome increase the stability of ENaC subunits, either in transfected MDCK cells (122) or in A6 cells expressing endogenous ENaC (85). The inhibition by the proteasome could interfere at different levels: it may inhibit the endoplasmic reticulum degradation machinery, known to degrade misfolded proteins of the endoplasmic reticulum in a ubiquitination- and proteasome-dependent fashion (118); such a mechanism was put forward by us, as we found that proteasome inhibition halted the turnover of α-ENaC efficiently only when the subunit was transfected alone into MDCK cells (and therefore not expected to translocate to the plasma membrane) but with considerably less efficiency when all three ENaC subunits were transfected (allowing efficient sorting of ENaC to the plasma membrane) (56, 122). Alternatively, proteasome inhibition interferes with the degradation of ubiquitinated proteins and hence recycling of ubiquitin; the resulting depletion of the available pool of free ubiquitin would be sufficient to impair the ubiquitination reaction (94). On the other hand, it is also possible that proteasome activity is required to translocate ENaC into the lysosomal MVB, as has been shown for the EGFR (83). The inhibition of proteasome may interfere with this process and enhance recycling of ENaC back to the cell surface. It was also shown that both α- and γ-, but not β-, ENaC subunits become ubiquitiated on lysine clusters situated in the NH\(_2\) terminus of either subunit and that mutation of these lysines to arginine reduces ubiquitination and elevates channel density at the plasma membrane. This suggests that impaired ubiquitination causes the accumulation of ENaC at the cell surface (122).

The most compelling evidence for an involvement of Nedd4 proteins comes from expression studies done in X. laevis oocytes, as it has now been shown by several laboratories that overexpression of Nedd4 with ENaC decreases ENaC activity (1, 32, 38, 60, 114). This inhibition depends on intact PY motifs and on the catalytic activity of Nedd4 and involves a decrease in cell surface expression (1, 38, 60). Additional support for the involvement of Nedd4 in ENaC regulation came from another approach applied by Cook and co-workers (27, 43, 44). These investigators studied Na\textsuperscript{+}-dependent feedback regulation in mouse mandibular duct cells, using whole cell patch-clamp techniques. With this system, they provided indirect evidence that Na\textsuperscript{+} feedback regulation is dependent on Nedd4 proteins by including either glutathione-S-transferase fusion proteins comprising Nedd4 WW domains or Nedd4 antibodies in the patching pipette, which interfere with feedback inhibition.

Much recent data suggest that it is primarily Nedd4–2 which regulates ENaC (1, 24, 43, 60, 62, 115), despite the original identification of the Nedd4–1 isoform as a binding protein of ENaC (121). Nedd4–2 is considerably more efficient in suppressing ENaC activity compared with Nedd4–1 (60, 62). Some reports
from other laboratories, showing an effect of Nedd4–1 on ENaC in X. laevis oocytes, may be explained by the fact that Nedd4–1 lacking a functional C2 domain was used (32), because we (62) and others (114) have recently shown that the C2 domain renders Nedd4–1 inefficient in ENaC regulation. However, it is plausible that alternative splicing (62), or the recently described proteolytic cleavage of Nedd4–1 during apoptosis (45), may generate C2-lacking Nedd4–1 proteins, although such proteins have not yet been described in the kidney. In contrast, the C2 domain in Nedd4–2 does not affect the ability of this protein to regulate ENaC (62).

In further support of the role of Nedd4–2 in ENaC regulation is the demonstration that Nedd4–2 interacts in vivo (i.e., coimmunoprecipitates) with ENaC (60, 62). Potential interaction between ENaC and Nedd4–1 appears to be much weaker; until this point, no one has been able to demonstrate conclusively the interaction of these proteins in vivo. This may explain why Nedd4–1 is not efficiently inhibiting ENaC. Of interest is a large body of important studies that has dealt with in vitro interaction between Nedd4–1 WW domains and the PY motifs of ENaC (32, 43, 44, 63, 64, 84, 114, 121). Although they may not directly represent the situation in the cell, they are instructive for an understanding of Nedd4–2–ENaC interaction, because the WW domains of Nedd4–1 and Nedd4–2 are highly similar. Globally, these studies indicate that the first WW domain has the weakest affinity for the PY motifs of ENaC, a finding that is also supported by our functional studies with Nedd4–2 (60, 62). The other two Nedd4–1 WW domains (or 3 in the case of humans) have considerably higher affinities, with dissociation constants in the micromolar range (5, 64, 84). The highest affinity has been found between the additional (third) human WW domain and human β-ENaC PY motif (11.1 μM) (84) and between the third domain of rat Nedd4–1 and rat β-ENaC PY peptide (21 μM) (64). The solution structure of the latter complex has been determined recently, and it was found that not only is the above-defined PY motif important for the interaction with WW domains but also the leucine in a tyrosine-based sorting motif just after the PY motif (PPPNYDSL) (64). In the future, it will be important to determine the relative affinities between Nedd4–2 WW domains and ENaC PY motifs and to resolve the structure of such complexes. Such experiments will likely provide explanations as to why no intracellular interactions are detectable between Nedd4–1 and ENaC. Other factors may also play a role, such as the arrangement and the distance between the WW domains, creating possible sterical hindrance, different posttranslational modifications, or interaction with cofactors (e.g., E2 enzyme). Furthermore, it will be necessary to develop techniques that allow the direct measurement of the interaction between the entire ENaC complex and full-length Nedd4–2.

Efforts have been made to link blood pressure variations to the genes encoding each Nedd4 protein. hNedd4–1 is located on chromosome 15, and so far no strong linkage has been reported. On the other hand, hNedd4–2 is situated on chromosome 18q21, where it is positioned in a region of linkage for two related blood pressure disorders: 1) hypertension triggered by a postural challenge (standing) (92) and 2) familial orthostatic hypotensive disorder (which manifests itself as a marked decrease in systolic blood pressure triggered by standing) (18, 26). This and the role in ENaC regulation make hNedd4–2 a candidate gene for these disorders.

**ALDOSTERONE-INDUCED Sgk1 KINASE INCREASES ENaC CELL SURFACE EXPRESSION AND ACTIVITY**

Sgk1 is another regulatory protein that has gained considerable interest in recent years with respect to ENaC regulation. Sgk1 was originally identified in a differential screen for glucocorticoid-inducible transcripts in a rat mammary tumor cell line (133). It is a member of the “AGC” family of protein kinases, which include protein kinases A, G, and C. Two other isoforms of Sgk1 have been identified, referred to as Sgk2 and Sgk3 (or CISK), which share 80% of sequence identity with Sgk1 (72, 80). The three isoforms also share homology with protein kinase B/Akt kinase. Sgk kinases are highly conserved and are found in all eukaryotes, from yeast to mammals. In yeast, there are two proteins that are encoded by the YPK1 and YPK2 genes. Deletion of these two genes is lethal but can be rescued by Sgk1 (16, 126). Interestingly, Ypk1 and Ypk2 seem to regulate the endocytosis of receptor proteins (25). Sgk1 is expressed in a number of tissues in a serum- or glucocorticoid-dependent fashion and has been demonstrated to affect various cellular functions such as cell volume control, regulation of apoptosis, activation of K+ channels, and others. Whereas we are focusing on the effect on epithelial Na+ transport and Nedd4–2, the interested reader is referred to several recent and detailed reviews on Sgk1 (76, 81, 91, 95).

Sgk1 is the earliest known protein that is induced by aldosterone and appears to be a primary mediator of aldosterone action not only in A6 cells but also in primary rabbit cells derived from the CCD. In these cells, it was shown that aldosterone induces the expression of Sgk1 mRNA and protein within 30 min and that the induction of the Sgk1 transcript is not prevented by cycloheximide, suggesting that this depends on a direct effect of aldosterone on gene expression (20, 90). This stimulated expression is also found in vivo, as demonstrated in adrenalecтомized animals injected with aldosterone (10, 20, 82). It was also shown that Sgk1 simulates ENaC activity, when coexpressed in X. laevis oocytes (20, 90), and evidence was presented that the increased activity is due to higher channel density at the plasma membrane (3, 24, 82, 130). Importantly, these findings in X. laevis oocytes have now been confirmed in the renal A6 cell line, by showing that transfected Sgk1 enhances transepithelial amiloride-sensitive Na+ transport (30). Moreover, Sgk1 knockout mice exhibit a PHA-I phenotype when kept on a low-Na+ diet, corroborating the role of Sgk1 in the regulation of Na+ balance (134). As mentioned earlier, transepithe-
lial Na⁺ transport in principal cells of the CCD is also regulated by insulin, which is thought to induce a rapid increase in apical Na⁺ channel conductance, by increasing the number of channels into the membrane (101). The molecular events for this regulation are not well understood, but Sgk1 may also represent the mediator in this context, as it is phosphorylated and activated via phosphatidylinositol 3-kinase (PI 3-kinase) through phosphatidylinositol 3,4,5-triphosphate-dependent kinase 1 or 2 (8, 71, 93). PI 3-kinases play an important role in insulin signaling, and it was indeed shown that insulin-dependent stimulation of transepithelial Na⁺ transport can be inhibited by inhibitors of PI 3-kinase (100). Moreover, it was demonstrated that PI 3-kinase inhibitors inhibit the early action of aldosterone and phosphorylation of Sgk1 (131), suggesting that Sgk1 may be an integrator of both the aldosterone and insulin pathways with respect to ENaC regulation (9).

Despite the establishment of Sgk1 as a mediator of hormonal regulation (aldosterone, insulin), which requires its kinase activity (3, 24, 82), its substrate(s) relevant to ENaC regulation has remained elusive until recently. Although it was shown in vitro pulldown assays that the COOH termini of α- and β-ENaC can interact with Sgk1 (131), this was not further corroborated by in vivo demonstration of the interaction (e.g., coimmunoprecipitation) or by provision of evidence that Sgk1 phosphorylates ENaC. A number of laboratories have looked for such evidence, but the generally held opinion is that ENaC is not a substrate of Sgk1 (21, 95). Recently, we and others have proposed a novel mechanism in which Sgk1 regulates the interaction between Nedd4–2 and ENaC (24, 115).

**THE CONVERGENCE POINT: Sgk1 PHOSPHORYLATES NEDD4–2 AND INTERFERES WITH NEDD4–2–ENAC INTERACTION**

The observation that both Sgk1 and Nedd4–2 control ENaC density at the cell surface (although in opposite directions) suggested that the two proteins may act on the same regulatory pathway. The recent identification of the consensus phosphorylation motif for Sgk1 \[(RXRXX(S/T))] (71, 93) and the presence of a conserved PY motif (hence a putative binding site for the WW domains of Nedd4–2) on Sgk1 prompted us to look for Sgk1 phosphorylation sites on Nedd4–2. Indeed, two motifs were identified (24), and a third, present only on certain spliced forms of human Nedd4–2, was described by Snyder et al. (115). We showed in *X. laevis* oocytes that Nedd4–2 (but not Nedd4–1, which does...
not contain such consensus sites) becomes phosphorylated by Sgk1 and that this phosphorylation is impaired when the Sgk1 PY motif is mutated. We further demonstrated that mutation of the Nedd4–2 phosphorylation sites impairs the ability of Sgk1 to increase ENaC activity at the cell surface and provided evidence (by communoprecipitation) that Sgk1-dependent phosphorylation of Nedd4–2 reduces its ability to interact with ENaC (24). Certain of these aspects were confirmed by Snyder and collaborators (115), who in addition demonstrated, using an in vitro pulldown assay, that in vitro translated Sgk1 can interact with Nedd4–2 in a PY motif-dependent manner. Of note is that there is some controversy over the involvement of the ENaC PY motifs in Sgk1-dependent regulation of ENaC. While we and Snyder et al. observed that Sgk1 failed to upregulate ENaC channels devoid of functional PY motifs, other groups found that Sgk1 still stimulated such mutant channels (3, 21, 130). The reasons for these differences are not clear, but they may be explained by the fact that in one study only one PY motif was mutated (21) and heterologous (mouse) Sgk1 was used in X. laevis oocytes (3, 21, 130). Alternatively, Sgk1 may influence other PY motif-independent mechanisms as well.

Hence, on the basis of our data, we propose the following model for ENaC activity regulation, involving Nedd4–2, Sgk1, aldosterone, and insulin (Fig. 1). ENaC cell surface expression is routinely regulated by the balance between the insertion of more channels from vesicular stocks and their retrieval via Nedd4–2-mediated ubiquitination. Aldosterone, on binding to the mineralocorticoid and glucocorticoid receptors and mediated ubiquitination. Aldosterone, on binding to the mineralocorticoid and glucocorticoid receptors and translocation into the nucleus, induces the expression of Sgk1 (and a number of other genes). On the other hand, insulin stimulates PI 3-kinase-dependent Sgk1 phosphorylation, leading to the activation of Sgk1. Sgk1 will bind via its PY motif to a WW domain of Nedd4–2 and phosphorylate it primarily on Ser444 but also on Ser338. Such phosphorylation weakens the interaction between ENaC and Nedd4–2 and reduces ENaC ubiquitination, resulting in the accumulation of ENaC at the cell surface and increased Na+ reabsorption.

PERSPECTIVES

Although it is now well established that Nedd4–2 and Sgk1 are regulators of ENaC cell surface expression, there are a number of open questions. Despite the demonstration that Nedd4–2 negatively controls ENaC density at the plasma membrane of X. laevis oocytes, this remains to be shown in epithelial cells derived from the distal nephron or in transgenic/knockout mouse models. Nedd4–2 mutations may be found in patients suffering blood pressure variations. As mentioned earlier, the hNedd4–2 gene is located in a chromosomal region genetically linked to autosomal dominant orthostatic hypotensive disorder (18), but as yet no Nedd4–2 mutations have been described for this disease.

From a mechanistic point of view, it is necessary to demonstrate that Nedd4–2 directly ubiquinates ENaC, which may be achieved by reconstitution of an in vitro ubiquitination assay. The identification of the ubiquitin-conjugating (E2) enzyme acting in concert with Nedd4–2 would be of outstanding interest, as this enzyme may represent another susceptibility gene for hypertension. Moreover, a number of Nedd4–2-interacting proteins have been described (47, 89, 96, 98, 99), some of which may be Nedd4–2 substrates and others involved in either Nedd4–2 activity modulation or Nedd4–2 targeting or localization. Other points of interest concern the cellular localization of ENaC-Nedd4–2 interaction and the effect of ENaC ubiquitination. Does it enhance endocytosis as originally suggested for many plasma membrane proteins (106)? does it regulate sorting at the Golgi level, as shown for the Gap1 permease in yeast (48, 117); or does it control translocation into the lysosomal MVBs, as shown, for example, for the EGF and other receptors (83)?

Our work has suggested a potential mechanism for the Sgk1 regulation of Na+ transport by showing that Nedd4–2 is a target of this kinase. However, it remains to be shown in epithelial cells of the distal nephron or in vivo that Sgk1 phosphorylates Nedd4–2 and that such phosphorylation is enhanced by insulin and/or aldosterone. Despite the fact that all the evidence points at Sgk1 as a mediator of aldosterone-dependent stimulation of epithelial Na+ transport, there are also a number of open questions. It was shown by Loffing and co-workers (82) that treatment of adrenalectomized rats with aldosterone for 2 h stimulates the expression of Sgk1 all along the aldosterone-sensitive distal nephron, including the second part of the DCT, the CNT, and the CCD and MCD. Conversely, this treatment causes translocation of ENaC to the apical membrane only in the DCT and the CNT, suggesting that other cell-specific factors are also important for ENaC stimulation (82). It will be interesting to determine the expression and the phosphorylation levels of Nedd4–2 in these different segments. It is also puzzling that a recently generated knockout model of Sgk1 presents a relatively mild phenotype, compared with that in either ENaC (6, 88) or mineralocorticoid receptor (7) knockout mice, suggesting that, beside Sgk1 and Nedd4–2-dependent mechanisms, there are other regulatory pathways controlling ENaC and Na+ transport that also likely depend on aldosterone.

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