Regulation of Epithelial Sodium Channel Trafficking by Ubiquitination

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Amiloride-sensitive epithelial sodium (Na⁺) channels (ENaC) play a crucial role in Na⁺ transport and fluid reabsorption in the kidney, lung, and colon. The magnitude of ENaC-mediated Na⁺ transport in epithelial cells depends on the average open probability of the channels and the number of channels on the apical surface of epithelial cells. The number of channels in the apical membrane, in turn, depends upon a balance between the rate of ENaC insertion and the rate of removal from the apical membrane. ENaC is made up of three homologous subunits, α , β , and γ . The C-terminal domain of all three subunits is intracellular and contains a proline rich motif (PPxY). Mutations or deletion of this PPxY motif in the β and γ subunits prevent the binding of one isoform of a specific ubiquitin ligase, neural precursor cell expressed developmentally down-regulated protein (Nedd4-2) to the channel *in vitro* and in transfected cell systems, thereby impeding ubiquitin conjugation of the channel subunits. Ubiquitin conjugation would seem to imply that ENaC turnover is determined by the ubiquitin-proteasome system, but when MDCK cells are transfected with ENaC, ubiquitin conjugation apparently leads to lysosomal degradation. However, in untransfected epithelial cells (A6) expressing endogenous ENaC, ENaC appears to be degraded by the ubiquitin-proteasome system. Nonetheless, in both transfected and untransfected cells, the rate of ENaC degradation is apparently controlled by the rate of Nedd4-2-mediated ENaC ubiquitination. Controlling the rate of degradation is apparently important enough to have multiple, redundant pathways to control Nedd4-2 and ENaC ubiquitination.

Keywords: ENaC; degradation; trafficking; proteasome

The alveolar surface of the lungs is a unique epithelium, since it separates an internal vascular compartment (like most epithelia) from an air-filled compartment (unlike other epithelia). As such, the primary physiological purpose of the alveoli is to promote exchange of oxygen from the airspace into the blood and CO₂ out of the blood. However, efficient gas exchange in the lungs depends upon having a thin liquid layer on the air facing side of the alveolar epithelium. Proper gas exchange and alveolar function requires precise regulation of the amount of this luminal fluid. The amount of fluid on the airway surface represents a balance between the rate at which fluid is passively secreted from the vascular space and the rate at which it is actively reabsorbed. Fluid appears in the airways because of the passive movement of fluid driven by hydrostatic pressure out of the pulmonary capillaries across the airway epithelium. The amount secreted is relatively constant because, under normal physiological circumstances, the pulmonary capillary pressure and permeability of the epithelium are relatively constant; but, under pathological conditions, when either the pulmonary blood

pressure is abnormally elevated or the permeability of the epithelium is increased by inflammation or disease, the secretion may increase dramatically. Regardless, even under normal levels of secretion, the alveoli would rapidly fill with fluid were it not for fluid reabsorption by the alveolar epithelium. Since fluid absorption depends upon the rate of ion transport, regulation of ion transport provides the mechanism by which the amount of airway fluid is regulated.

In airway epithelium, fluid is transported from the airway lumen into the interstitial spaces, and this process can be substantially inhibited by the addition of amiloride, an epithelial Na⁺ channel (ENaC) inhibitor, to the alveolar space. While all parts of the airway have ENaC and absorb Na⁺, the alveolar epithelium covers more than 99% of the large internal surface area of the lung (~100–150 m² in humans), suggesting that alveolar epithelial cells are the major sites of Na⁺ transport and fluid absorption in the adult lung. The alveolar epithelium is composed of two distinct cell types, alveolar type I (T1) and type II (T2) cells. T2 cells, which cover only 2 to 5% of the internal surface area of the lung, are cuboidal cells that secrete pulmonary surfactant. T2 cells contain ion channels, including the amiloride-sensitive epithelial sodium channel (ENaC) and the cystic fibrosis transmembrane regulator (CFTR). T1 cells are large, squamous cells whose thin cytoplasmic extensions cover more than 95% of the internal surface area of the lung; and, although less is known about these cells, they also appear to have a full complement of ion channels, including ENaC, and contribute to alveolar Na⁺ absorption.

The ENaC protein found in lung, kidney, colon, sweat ducts, and salivary glands apparently consists of three homologous subunits, α , β , and γ , presumably arranged in a trimeric stoichiometry (1). Other subunits, the so-called δ and ϵ subunits, have been described which, when expressed in heterologous expression systems, can by themselves or when associated with β and γ (and possibly α) subunits produce cationic channels (2–6). The ϵ subunit has only been reported in *Xenopus* renal cells once (2), and bears no significant homology to any proteins other than those in *Xenopus* (where the homology is less than 30%). It has not been cloned from any genus other than *Xenopus*. Its properties are poorly described; therefore, we will not consider it in this review. The δ subunit appears to be primate specific (human and chimp), although there are predicted homologs in some other mammals that have as yet not been successfully cloned. Its channel-forming properties are similar to those of the α subunit when expressed in heterologous expression systems. The highest expression levels of δ ENaC mRNA are in brain, testis, ovary, and pancreas, suggesting that the primary function of δ ENaC may not involve epithelial tissue. This surmise is reinforced by the observation that δ ENaC cannot compensate for the loss of α ENaC in the lung or kidney in α ENaC knockout mice, which die shortly after birth from a failure to clear fluid from their lungs (7).

Although other combinations may exist (8, 9), channels composed of the three subunits, α , β , and γ , have the following characteristic features: the channel is highly-selective for sodium over potassium, has a small single channel conductance of 4–6 pS; is inhibited by the diuretic, amiloride, at sub-micromolar

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concentrations; and is typically found in the apical membranes of sodium transporting epithelial cells. The primary amino acid sequence suggests that each of the subunits has two trans-membrane domains, one large extracellular domain, and relatively short intracellular C- and N-terminal regions (10). Normal ENaC function is critical, since ENaC-mediated renal sodium transport is ultimately responsible for maintaining total body sodium balance and normal blood pressure (11, 12), while ENaC-mediated sodium transport in the lungs is responsible for normal fluid clearance from the alveolar space (13) and consequently for normal gas exchange in the lungs. Not surprisingly, then, abnormalities of ENaC function have been linked to disorders of total body Na⁺ homeostasis, blood volume, blood pressure, and lung fluid balance (7, 12). For example, a partial loss-of-function mutation of ENaC produces pseudohypoaldosteronism type I (14), characterized by excessive fluid accumulation in the lung and mild salt-wasting diuresis. In contrast, a gain-of-function mutation leads to Liddle's syndrome (15–17), an inherited, autosomal-dominant, salt-sensitive form of hypertension associated with hypokalemia and metabolic alkalosis. Liddle's syndrome is associated with mutations in a PPxY motif within the β and γ subunits that is conserved across species (15, 16). Interestingly, the α subunit also contains a similar PPxY motif, but there are no reports of mutations in this motif in the α subunit producing Liddle's-like channel abnormalities.

The total number of ENaC subunits within epithelial cells is relatively large compared with the number in the apical membrane (that can functionally transport sodium into the cell). Therefore, it appears that assembly and trafficking of ENaC subunits out of the endoplasmic reticulum is an inefficient process in polarized epithelia. Estimates of the relative amounts in the ER versus the plasma membrane and other areas of epithelial cells vary enormously, probably at least partially based on the condition of the cells being studied. In transfected systems, only 1 or 2% of ENaC is outside the ER; in native cells, it appears to be much higher (10–30%) (18–22). The large variability in the native cells appears to depend upon the hormonal state of the cell (e.g., EGFR ligands can dramatically reduce ENaC outside the ER and also total cellular ENaC) (23). The exact point in the trafficking pathway at which ENaC subunits are assembled into a functional channel is unclear. Nonetheless, one of the mechanisms by which ENaC functional activity can be regulated is by altering the rate of delivery of assembled ENaC to the surface membrane. This mechanism has been suggested as the mechanism by which the hormones, vasopressin, and possibly aldosterone alter sodium transport (24).

Of course, the number of functional ENaC channels in the surface membrane of epithelial cells is determined not only by the rate of insertion of new channels, but also the rate of retrieval and degradation of channels and the rate of recycling from intracellular pools. Hormones that increase the number of functional channels on the apical surface of cells could just as easily reduce the retrieval and degradation rate as increase the insertion rate; in fact, hormones like aldosterone and vasopressin not only affect the rate of insertion, but also appear to alter the number of functional channels at the apical membrane by reducing the rate of ENaC retrieval and degradation. Therefore, it is important to understand the process by which membrane proteins in general, and ENaC in particular, are retrieved from the surface membrane and also the mechanisms for degradation or recycling.

RETRIEVAL, DEGRADATION, AND RECYCLING OF MEMBRANE PROTEINS

Signal-transducing receptors, small-molecule transporters, and ion channels reside at the plasma membrane where the activity

is often regulated by controlling the level of protein localized at the plasma membrane. To reduce activity, proteins can be removed quickly from their site of action at the cell surface by endocytosis into the cell. In general, the first step in membrane protein receptor endocytosis is the association of the receptor with specialized structures on the membrane surface, clathrin-coated pits or caveolae. The two types of structures tend to promote endocytosis from different regions of the surface membrane. Clathrin-coated pits form from areas of the membrane that include normal regions of the membrane; caveolae tend to form from specialized domains of the membrane known as lipid rafts. These are domains that contain high concentrations of inositol phospholipids and the enzymes that produce the lipids (25–27). Some fraction of ENaC is present in lipid rafts (28, 29) that contain high levels of inositol phospholipids and other charged lipids (30–33). Artificial disruption of lipid rafts reduces ENaC activity, but the prevalence of lipid rafts also depends upon hormonal factors (e.g., tumor necrosis factor- α [30], activators of protein kinase C, or PTEN tumor suppressor). Inositol phospholipids stabilize functional ENaC and are necessary for channel trafficking and channel gating as well (34–37). Overexpression of caveolin-1, a signature protein of caveolae, which promotes formation of caveolae and internalization, reduces the activity and membrane surface expression of ENaC (38). In general, membrane proteins in lipid rafts that are internalized via caveolae are likely to be degraded by the proteasome, while membrane proteins in nonraft areas of the membrane tend to be internalized via clathrin-coated pits and degraded in lysosomes. The mechanism for ENaC retrieval remains ambiguous; however, in oocytes at least, ENaC appears to be internalized by a dynamin-2-dependent process into either clathrin-coated pits or caveolae (39).

UBIQUITINATION AND PROTEIN DEGRADATION

Ubiquitin is a highly conserved 76-amino acid polypeptide with a role in a variety of cellular functions. Ubiquitin plays a major role in protein degradation because it serves as a tag for internalization of membrane proteins and, under appropriate conditions, as a tag for the recognition of proteins by the multi-subunit, proteolytic complex known as the proteasome (40–42). Ubiquitin is covalently linked to substrate proteins via an isopeptide bond formed through its C-terminal glycine to the ϵ -amino group of lysine residues. Ubiquitination of proteins generally requires three distinct enzymatic activities mediated by either two or three enzymes. First, a ubiquitin-activating enzyme (E1) activates ubiquitin by forming a high-energy thioester bond (E1-S~ubiquitin) in an ATP-requiring reaction. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), followed by the addition of ubiquitin to target proteins by a ubiquitin ligase (E3). For Hect domain E3 enzymes a third high-energy thioester bond is formed between ubiquitin and a Cys residue on the E3 before its transfer to the substrate. Additional ubiquitins can be added to the first using the terminal carboxylic group of glycine 76 on one ubiquitin and lysine 48 of another ubiquitin molecule, resulting in a chain of at least four or five ubiquitins (42), a process known as polyubiquitination. In contrast, single ubiquitin molecules can be added to different lysine residues on the target protein so that the final stoichiometry is one ubiquitin per lysine on multiple lysines on the target protein, a process known as monoubiquitination.

Ubiquitination of membrane proteins promotes internalization; but once internalized, the degradative pathway that proteins follow appears to depend upon the difference between the addition of ubiquitin to form a single chain of ubiquitins

(polyubiquitination) that directs proteins to the proteasome or addition of single ubiquitin molecules at one, two, or several different sites in the protein (monoubiquitination or multi-ubiquitination), which is a signal for endocytosis and lysosomal degradation (43–49).

PROTEASOMAL PROTEIN DEGRADATION

Proteins that are degraded by the proteasome (42, 50, 51) are usually modified with a polyubiquitin chain. Proteins that are degraded by the proteasome tend to be associated with lipid rafts and internalized via caveolae. The mechanism for retrieval from caveolae is not completely clear except that it depends upon tyrosine kinase activity and is independent of clathrin-mediated endocytosis (52). In endothelium, caveolar fission requires tyrosine phosphorylation of caveolin and dynamin-2 by Src (53). Once internalized, a hydrophobic region within protein-conjugated ubiquitin consisting of Leu8, Ile44, and Val170 is recognized by the regulatory cap of the 26S proteasome (40, 42). Subsequently, the targeted protein is unfolded, threaded into the interior of the proteasome, and reduced to small peptides by active proteolytic activity within the proteasome. The proteasome complex is large and consists of a core proteinase, the 20S proteasome and a pair of 19S regulatory proteins. The 20S proteasome is made up of four rings stacked together forming a cylindrical structure with a pore of 13Å. The protease activity exists inside this cylindrical core; each ring consists of seven subunits, with proteasome α subunits forming the outer two heptameric rings, and the proteasome β subunits the inner ring. The regulatory, 19S protein at the pore opening recognizes polyubiquitin chains and unfolds the ubiquitin-conjugated protein and, in the process, hydrolyzes one ATP molecule. As a polypeptide chain passes through the pore, catalytic groups with tryptic, chymotryptic, and peptidylglutamyl peptidase activity on the proteasome β subunit cleave the protein into smaller polypeptides (54). Ubiquitin chains are removed from the cleaved peptides by deubiquitinating enzymes and recycled (42). Proteins associated with caveolae can be degraded by the proteasome (55), but it is unclear if proteins retrieved in caveolae are also degraded in lysosomes.

LYSOSOMAL PROTEIN DEGRADATION

Besides the proteasomal proteolytic pathway, the lysosomal proteolytic pathway is another major degradative pathway in most cells. Many membrane proteins are internalized and then degraded by proteases in lysosomes (40, 41, 56). Ubiquitination is not a prerequisite for internalization of some membrane proteins that are subsequently degraded in the lysosome. However, a number of mammalian receptors and ENaC undergo ubiquitination at the plasma membrane, and this modification is required for their degradation (56). While ubiquitination of cytoplasmic proteins invariably leads to proteasomal degradation, ubiquitination of membrane proteins can lead to proteasomal degradation, or can also act only as a signal for internalization (40, 48) via clathrin-mediated endocytosis. Internalization via clathrin-coated pits usually leads through several endosomal compartments to lysosomes. The first vesicle formed is an early endosome (also referred to as recycling endosome) which, like caveolae, requires dynamin protein to form. The intravesicular environment has a low pH of 5.9 to 6, which can release a ligand from its receptor but does not denature the internalized protein, allowing proteins from this compartment to be recycled back to the plasma membrane. Several plasma proteins, such as channels, transporters, and permeases, can enter this specialized compartment, the recy-

cling endosome, from which they are returned to the membrane. If early endosomes are not recycled back to the plasma membrane, then they become late endosomes by acquiring rab7-GTP protein on their surfaces and the intravesicular pH is further reduced to 5 to 6. Membranes of late endosomes invaginate and pinch off into the lumen of the organelle, generating endosomes containing small vesicles with internalized plasma membrane protein. These endosomes, consisting of small vesicles, subsequently fuse with lysosomes that contain proteolytic enzymes that degrade internalized plasma membrane proteins (57).

Since the two types of ubiquitin coupling appear to lead to different proteolytic pathways (proteasomal after polyubiquitination or lysosomal after monoubiquitination), the next obvious question for ENaC subunits is: what is the mode of ENaC ubiquitination and what is the pathway for ENaC degradation? The answer for ENaC subunits is not easy, since most studies have not distinguished between either the mode of ubiquitination (polyubiquitination versus monoubiquitination) or the mode of degradation (proteasomal versus lysosomal). Moreover, ENaC degradation might be different between cells expressing endogenous ENaC subunits compared with cells overexpressing transfected ENaC subunits. However, it is clear that ubiquitin conjugation of ENaC plays a pivotal role in the channel degradation in both transfected and untransfected cells. A schematic diagram of ENaC degradation is shown in Figure 1.

A SPECIFIC UBIQUITIN LIGASE ISOFORM, Nedd4-2, IS RESPONSIBLE FOR UBIQUITINATION OF MEMBRANE ENaC

Ubiquitin conjugation is a prerequisite for ENaC internalization and subsequent degradation, regardless of the subsequent degradative pathway. Ubiquitin conjugation of ENaC subunits in both untransfected and transfected cell systems requires a specific ubiquitin ligase, Nedd4 (neural precursor cell expressed developmentally down-regulated protein). Nedd4 contains a E6-AP carboxyl terminus (Hect) domain that is homologous to other ubiquitin ligases, three WW domains in the rat and mouse protein or four WW domains in human and *Xenopus* protein, and a calcium/lipid binding domain (CaLB/C2) (58). The Nedd4/Nedd4-like family consists of five subgroups: (1) Rsp5, Pub1, CAB16903, and CAB91803; (2) WWP1, WWP2, and AIP4; (3) SMURF; (4) KIAA0322; and (5) Nedd4 (59, 60). Nedd4 has two known isoforms, Nedd4-1 (hNedd4-1, mNedd4-1, and rNedd4-1) and Nedd4-2 (hNedd4-2a, hNedd4-2b, mNedd4-2, and xNedd4). Most Nedd4-1 proteins contain a C2 domain, with hNedd4-1 containing four WW domains and mNedd4-1 and rNedd4-1 containing three WW domains. All Nedd4-2 isoforms except for xNedd4-2 lack a C2 domain and contain four WW domains, except for hNedd4-2b, which is a splice variant of hNedd4-2a (59, 60). Nedd4-2 appears to be the isoform responsible for ubiquitination of ENaC in all mammalian epithelial cells (61). The localization, properties, and structures of all members of the Nedd4 family has been recently thoroughly reviewed and tabulated by Rotin and Kumar (62).

Nedd4-2 has the same localization pattern as ENaC in renal cortical and outer medulla collecting duct principal cells, and in airway epithelia (63). Nedd4-2 apparently requires direct association with ENaC to produce ubiquitin conjugation. The initial description of Nedd4 association with ENaC used a yeast two-hybrid screen to demonstrate an interaction between Nedd4 and the highly conserved PPxY domains found in all of the three ENaC subunits (64). Subsequently, Dinudom and

colleagues (65) characterized the binding of Nedd4 to ENaC subunits using a Far Western assay in which they showed that there is direct binding of the second and third WW domains to all three ENaC subunits. For human Nedd4-2, the first WW does not interact with ENaC subunits but the second, third, and fourth WW domains interact with all three ENaC subunits (25), but the third WW3 domain caused the largest reduction in ENaC function expressed in oocytes along with all three ENaC subunits (59). At a molecular level, the interaction of Nedd4-2 WW domain with PY motif of ENaC β subunits involves contact between tyrosine 618 and leucine 621 and WW domain of Nedd4-2 (66). Nedd4-2 WW domain binding to ENaC β and γ subunits is practically irreversible because this binding has a very slow dissociation rate when determined by surface plasmon resonance (67).

Nedd4-2 binds to ENaC subunits *in vitro*, but also can decrease ENaC function in systems transfected with ENaC or expressing ENaC RNA. When *Xenopus* oocytes were injected with ENaC subunit and Nedd4-2 mRNA together, there was a reduction in whole cell current and the amount of ENaC subunit protein at the cell surface compared with oocytes injected with ENaC mRNA alone. Oocytes expressing ENaC mutants lacking the PPxY motif did not exhibit this reduced current or reduced cell surface expression of ENaC (68, 69). Also, more recently in untransfected renal cells (A6), Nedd4-2 was shown to co-immunoprecipitate with α and β ENaC and to associate with apical membrane proteins (70).

Despite the large amount of evidence for Nedd4-2 interaction with ENaC, several other members of Nedd4 family of proteins also interact with ENaC. WWP2 is one such example; McDonald and coworkers showed by Northern analysis that WWP2 protein is expressed in human adult and embryonic epithelial tissues and that the transepithelial current is inhibited when coexpressed with ENaC subunits in Fischer rat thyroid epithelial cells (71). Therefore, although it has generally been assumed that Nedd4-2 is the ENaC ubiquitin ligase in cells expressing endogenous ENaC, there has only recently been direct evidence for this role of Nedd4-2 (70, 72, 73). These results imply that Nedd4-2 is the ubiquitin ligase for ENaC and that Nedd4-2 is associated with surface membrane ENaC molecules (although these results do not rule out the possibility that Nedd4-2 might also be associated with ENaC in other parts of the cell), and also that Nedd4-2 is the ubiquitin ligase responsible for ubiquitination of ENaC at the surface membrane of both untransfected renal cells expressing native ENaC subunits and cells transfected with ENaC subunits.

CONTRASTING PATHWAYS FOR DEGRADATION OF ENDOGENOUS AND OVER-EXPRESSED ENaC

Whether ENaC is endogenously expressed in sodium-transporting epithelial cells or over-expressed in a variety of heterologous expression systems, the WW domains of Nedd4-2 interact with the ENaC PPxY domain, and then the E6-AP carboxy terminus homologous domain (Hect) acts as a ubiquitin ligase to conjugate ubiquitin to the amino termini of, at least, the α and γ ENaC subunits (64). In MDCK cells transfected with all three ENaC subunits, ubiquitin couples to ENaC α subunit at lysine amino acid 47 and 50, and to the γ subunit at lysines from 6 to 13 (74), although Valentijn and colleagues were unable to detect any ubiquitinated ENaC subunits in *Xenopus* oocytes injected with ENaC subunits and Nedd4-2 (75). However, as mentioned before, co-expressing Nedd4-2 and ENaC subunits in *Xenopus* oocytes reduces ENaC protein at the cell surface and decreases

whole cell sodium current (68, 74). However, the fate of ubiquitinated ENaC subunits appears to be different depending upon whether ENaC is endogenously expressed in native cells or overexpressed in cell models; endogenous ENaC appears to be degraded by the proteasome, while heterologously expressed ENaC appears to be degraded in lysosomes or by a combination of lysosomal and proteasomal degradation. Proteasomal degradation (and presumptive polyubiquitination) and lysosomal degradation (monoubiquitination) can be distinguished by using inhibitors of these pathways. The proteasomal pathway can be inhibited by blocking proteasome activity with agents such as lactacystin or MG-132, which block the chymotryptic activity of the proteasome; while the lysosomal pathway can be blocked either by increasing the lysosomal pH with cell permeable weak bases like methylamine, ammonium chloride, or chloroquine or by using cysteine, aspartic, and serine proteases inhibitors such as leupeptin (74, 76, 77).

Degradation of endogenous ENaC in untransfected epithelial cells has some similarities, but also some major differences from ENaC degradation in transfected cell systems expressing exogenous ENaC subunits. In both situations, proteasome inhibition increases the total cellular amount of all ENaC subunits (24, 74). Also, the half-lives of all subunits in the total cellular pool is relatively short (1–4 h) and increases several fold after proteasome inhibition in both transfected and untransfected cells (24, 74). However, in MDCK cells transfected with ENaC subunits, lysosomal and proteasomal inhibition increased half-lives of cellular ENaC subunits, but in untransfected renal cells expressing endogenous ENaC, lysosomal inhibition produced no change in the total cellular amount of ENaC subunits. Moreover, in untransfected renal cells, proteasome inhibition, but not lysosomal inhibition, caused an increase in amiloride-sensitive, transepithelial current. The increase in amiloride-sensitive, transepithelial current induced by inhibiting proteasome activity is associated with an increase in the density of apical sodium channels measured by patch clamp methods, and an increase in the number of ENaC subunits that can be surface labeled with biotin (24). In untransfected cells, all three ENaC subunits are at least polyubiquitinated and proteasome inhibition increases polyubiquitination of ENaC (70), but in transfected MDCK cells only α and γ subunits are coupled to ubiquitin. In COS-7 cells overexpressing ENaC subunits, subunits located at the cell surface are modified with multiple mono-ubiquitins (multi-ubiquitination), and Nedd4-2 modulates this ubiquitination. Under these conditions, ENaC is associated with the μ 2 subunit of the AP-2 (adaptor protein 2) clathrin adaptor (78). Mono- or multi-ubiquitination of other membrane proteins is a signal for their internalization by clathrin-mediated endocytosis, suggesting that in this system ubiquitin and clathrin adaptor binding sites act in concert to remove ENaC from the cell surface, presumably to the lysosome, although the degradative pathway was not explicitly tested.

In heterologous systems, one group of investigators observed an increase in α and γ ENaC half-lives in response to both chloroquine and lactacystin in MDCK cells transfected with all three ENaC subunits, and the increase in half-lives induced by both inhibitors was comparable, suggesting that each pathway contributed equally to subunit degradation (74). In contrast, another group reported that, in *Xenopus* oocytes, only lactacystin (but not chloroquine) increased the half-lives of individual ENaC subunits (75). The lactacystin-mediated increase in half-life depended upon which ENaC subunits were expressed in the oocytes: the maximum increase occurred when only the β subunit was expressed, and decreased when all three ENaC subunits were expressed together (75).

ENaC internalization from the plasma membrane is dependent on dynamin-2. Previously, dynamin-2-dependent internalization was only linked to endocytosis by clathrin-coated vesicles (39). Proteins endocytosed by clathrin-coated vesicles are generally degraded in the lysosomes and not by the proteasome. Therefore, because ENaC endocytosis is dynamin-2 dependent but ENaC degradation is lactacystin sensitive, it appeared that unassembled subunits were degraded by the proteasome, but that the properly assembled plasma membrane-resident ENaC was degraded in lysosomes (55). However, recent studies suggest that for some proteins, dynamin-2 can also mediate endocytosis through caveolar-dependent internalization (44). Proteins internalized in caveolae can be degraded by proteasomal proteolysis so that dynamin-2 dependence does not necessarily imply lysosomal degradation.

One reason for the differences between untransfected and transfected cells could be different levels of ENaC protein expression: in untransfected cells the surface expression of ENaC channels ranges from about 40 channels per cell in mouse cortical collecting duct cells (M1) to as low as 4 per cell in the *Xenopus* renal cell line (A6) (with alveolar type 2 cells somewhere in between), whereas in transfected cells, surface expression levels may exceed several thousand per cell. Considering this difference, the simplest explanation consistent with all of these observations is a model in which trafficking of ENaC out of the ER is extremely inefficient, so that most of the cellular pool of ENaC resides in the ER. Proteasomes are responsible for the degradation of most proteins that are not rapidly trafficked out of the ER compartment. Therefore, inhibition of proteasomal degradation should cause an accumulation of ENaC subunits in the ER and an increase in the total cellular amount of all ENaC subunits. Untransfected cells and transfected cells share this common mechanism; however, the accumulation of subunits in transfected cells would be much greater than in untransfected cells. If even a small fraction of these extra subunits in transfected cells traffic to the cell surface, there would be a large increase in the number of channels in the plasma membrane. Once in the membrane, large numbers of the channels promote clathrin-mediated endocytosis and are degraded via a lysosomal pathway at a rate that is independent of proteasome activity. In addition, the large excess of ENaC protein may limit the extent of ubiquitination, thus promoting monoubiquitination (and lysosomal degradation) rather than polyubiquitination (and proteasomal degradation). The situation in untransfected cells expressing endogenous ENaC is different; ENaC subunits do accumulate in the ER (but not to

the extent that they do in transfected cells) and some may escape to reach the surface membrane, but once in the membrane their fate appears quite different. Unlike transfected cells, the rate of membrane ENaC degradation is reduced by proteasomal inhibitors, implying that proteasomes play a role in the degradation of membrane-associated ENaC. In contrast, lysosomal inhibitors have no detectable effect on the amount of ENaC expressed in the surface membrane (24). Another reason for the observed differences could be that the investigators often used the lysosomal inhibitor, chloroquine, at relatively high concentrations that can permeabilize mitochondrial membranes, therefore uncoupling the mitochondrial oxidative phosphorylation process and depleting cells of ATP (79). Since proteasomal proteolysis is ATP dependent, it is possible that these lysosomal inhibitors, especially at high concentrations, could affect both lysosomal proteolysis directly and proteasomal proteolysis by depleting ATP.

ENaC HALF-LIVES

The reported values for the half-life of ENaC in the plasma membrane vary widely, from as short as 15 minutes (5) to over 24 hours (32). This large variability almost surely has to do with exactly what form of ENaC is being measured and in which cellular system. Several studies have suggested that a mature and functional ENaC channel must undergo several post-translational modifications, which include formation of intra-subunit disulfide bonds (6, 25), transforming the N-glycan linked on ENaC each subunit from endoglycosidase H (endo-H)-sensitive to endo-H-insensitive forms (11, 13), as well as proteolytic cleavage of α and γ subunits. However, not every ENaC subunit expressed in the apical membrane is fully processed post-translationally; both mature and immature forms of the sodium channels are co-existence in the plasma membrane (21). It has also been reported that the immature channels expressed in the plasma membrane have very low channel activity (4). Since both mature and immature channels have been described in the plasma membrane, perhaps the various different molecular weights for ENaC subunits that have been reported (using similar Western blot techniques) are due to the differences in subunit maturity. Because there is uncertainty about the precise molecular weight of each subunit using standard Western blotting techniques, some of the following issues related to ENaC are difficult to discern: (1) how post-translational modifications or proteolytic cleavage can affect apparent subunit weight; (2) the precise stoichiometry of the subunits necessary

Figure 2. A schematic of ENaC trafficking and regulation by Nedd4-2-mediated ubiquitination. $\alpha\beta\gamma$ ENaC heterotrimers are assembled, exit the ER through the Golgi. They then traffic through a post-Golgi compartment (*step 1*) before entering the apical membrane in phosphatidyl-inositol-rich lipid rafts (*step 2*). These channels are functional and responsible for most, if not all, Na^+ transport (*step 3*). The functional heterotrimers in lipid rafts are associated with and ubiquitinated by an ENaC-specific ubiquitin ligase, Nedd4. Ubiquitination promotes internalization of the $\alpha\beta\gamma$ trimers into a subapical, sorting compartment (*step 5*). ENaC can suffer two fates within this compartment. One fate is to be de-ubiquitinated by the enzyme, UCH-L3, and returned to a post-Golgi recycling compartment (*step 9*) from which they can return to the surface membrane (*step 10*). The other fate is to be disassembled into individual, ubiquitinated subunits (*step 6*). Some of the ubiquitinated subunits will be recognized by the 26S proteasome and degraded (*step 7*), while others will be de-ubiquitinated by ubiquitin-specific proteases (like USP2-45) and trafficked back to the Golgi (*step 8*). Once disassembled, the fate of the subunits is apparently different: β and γ are more likely to be degraded by the proteasome (a few hours after internalization) while α is more likely to be trafficked back to the Golgi and, therefore, remain undegraded for long periods (tens of hours). Besides the removal of ubiquitin by de-ubiquitinating enzymes, Nedd4-2-mediated ubiquitination can be regulated in several ways by. Phosphorylation of ENaC plays a role in altering Nedd4 ubiquitination (*step 11*). Mitogen-activated protein kinase (MAPK) phosphorylation of ENaC promotes Nedd4-2 binding and enhanced retrieval and degradation. In contrast, casein kinase 2 (CK II) phosphorylates ENaC and prevents interaction with Nedd4, thereby reducing ubiquitination, retrieval, and degradation. Phosphorylation of Nedd4-2 itself plays a role in altering ENaC ubiquitination (*step 12*). Serum and glucocorticoid-dependent kinase (SGK) can phosphorylate Nedd4-2, which when phosphorylated has a lower affinity for binding to the PPxY motif in ENaC. This effect is apparently caused by the phosphorylated form of Nedd4-2 interacting strongly with the scaffold protein, 14-3-3 β and ϵ , that prevents Nedd4-2 interaction with ENaC. G protein receptor kinase 2 (GRK2) also phosphorylates Nedd4-2 to reduce interaction with ENaC and prevent ubiquitination and degradation and, finally, Nedd4-2 is phosphorylated and inhibited by cAMP-dependent protein kinase A.

to form a functional channel; and finally, (3) the stability of membrane surface ENaC. Another reason it is difficult to correlate the biochemical data to the functional stability of ENaC is that there are considerable data suggesting that changes in α -, β -, and γ -ENaC subunit levels in the cell surface are not coordinately regulated (21, 31, 32) meaning that the half-life of each subunit may differ from one another. Therefore, it is difficult to accurately correlate the quantitative changes in the expression levels of ENaC (determined using biochemical approaches) to the actual functional stability of ENaC. However, determining the functional stability of ENaC is the most relevant physiological parameter determining lung sodium reabsorption. In attempts to estimate the physiological contribution of the channels and to overcome the technical limitations of biochemical assays, a few groups have measured the decrease of amiloride-sensitive whole cell current in oocytes and MDCK cells (20, 26) under experimental conditions that prevent newly synthesized channels from being delivered to the plasma membrane. Since post-translational modifications and trafficking of ENaC in heterologous expression systems sometimes have characteristics that differ from channels found endogenously in native epithelial cells (10, 32), the stability of ENaC in cells overexpressing ENaC may differ significantly from the sodium channels found in native cells. Furthermore, amiloride may inhibit transporters and channels other than ENaC (8, 9), and therefore, the half-life of ENaC observed based solely on the decline of amiloride-sensitive current may not actually reflect the half-life of individual ENaC. In addition, protein biochemical data suggest that the half-life of apically expressed β -ENaC is much shorter than apically expressed α and γ ENaC subunits (32). The noncoordinated regulation of ENaC subunits may produce functional channels composed of α alone, or α and γ channels. Therefore, examination of the half-life of functional channels using single-channel (ENaC) activity probably provides the most reliable measure of the lifetime of channels. When measured in this way, the half-life of ENaC channels is approximately 3.5 hours (80).

However, these results do not reconcile the significant difference measured biochemically. The short half-lives measured by Alvarez (81) are likely due to selection of a limited population of ENaC channels by using a cold Triton lysis buffer which will look only at a limited population of channels in the membrane (not in lipid rafts). Other than this one report of very short half-lives, other investigators have observed half-lives consistent with the patch clamp results of 3 to 10 hours (24, 70, 82, 83), but in general, the half-lives for the different subunits are different, with the lifetime of the α subunit being longest. The difference in the half-lives for biotinylated β and γ ENaC subunits compared with α subunits is interesting. Measuring the lifetime of biotinylated subunits is not the same as measuring the lifetime in the membrane, since some of the biotinylated subunits could have been internalized but not degraded. The large difference in the half-lives of α subunits compared with β and γ subunits implies that they are handled differently once they are internalized. Since the proteasome cannot handle multimeric proteins; the α - β - γ complex must be disassembled, after which the β and γ subunits could be sorted to the proteasome while the α subunits are recycled to the membrane. The differential ENaC degradation would provide a parallel between ENaC degradation and the noncoordinated regulation of ENaC by differential ENaC subunit protein synthesis previously reported by Weisz and coworkers (84, 85).

Thus, it appears that in transfected cells the surface expression of ENaC is dependent upon the rate of trafficking from the ER and insertion into the plasma membrane, while the rate of removal from the membrane and degradation is constant and

dependent on lysosomal activity. In contrast, in untransfected cells, the surface expression of ENaC is dependent upon the rate of internalization and proteasomal degradation with relatively constant insertion rate. Therefore, in untransfected renal cells the proteasome plays an important role in ENaC degradation, whereas in transfected cell systems, both proteasomal and lysosomal pathways play a significant role in ENaC degradation.

REGULATION OF Nedd4-2 ACTIVITY AND ENaC DEGRADATION RATE

In untransfected cells, the degradation rate of ENaC by proteasomes appears to be regulated in several ways. First, the synthesis of proteasomal proteins and the assembly of proteasomes can be regulated by glucocorticoids (86, 87). This appears to be a generalized response to cell stress, and extended exposure to glucocorticoids and other stressors can lead to proteasome-dependent apoptosis (88, 89). Second, related to the increase in proteasomal activity, glucocorticoids also promote the increased expression of ubiquitin (87, 88). Both of these events are general cellular responses that are not specific to ENaC: increased glucocorticoids would increase the degradation rate of any protein degraded by proteasomes. However, glucocorticoids and aldosterone can apparently more specifically alter ENaC degradation. In A6 epithelial cells, one effect of aldosterone and glucocorticoids is to increase the amount and activity of serum and glucocorticoid-dependent kinase (Sgk) (90–92). Lung epithelial cells contain both mineralocorticoid and glucocorticoid receptors (93–95), but since circulating levels of aldosterone are usually low compared with glucocorticoids, it is likely that the major steroids regulating lung ENaC are glucocorticoids. However, alveolar type II cells do contain 11- β -hydroxysteroid dehydrogenase 2 (11 β -HSD2) (96, 97), which is often considered a hallmark of aldosterone target cells (98), and there are some reports of an effect of aldosterone concentrations on alveolar fluid reabsorption (99) and ENaC activity in type 2 cells (100). Regardless, both aldosterone and glucocorticoids regulate sodium absorption by similar short- and long-term processes: an initial phase that increases transport four- to sixfold in the first 2 to 6 hours, and a late phase that requires 12 to 48 hours and increases transport another three- to fourfold (reviewed in Reference 101). Aldosterone or a glucocorticoid, like other steroid hormones, enters target cells and binds to cytosolic, MR, or GR receptor complexes. After some rearrangement, the steroid-bound receptor acts as a DNA-binding protein that targets steroid response elements on genetic DNA. Binding to the response elements alters gene expression. Increases in Na^+ transport can be measured within 1 hour of exposure to aldosterone or glucocorticoids, and this increase is dependent on gene transcription and translation with the gene products generically referred to as steroid-induced proteins (SIPs) (37, 101). In the short term, aldosterone or glucocorticoids regulate sodium transport by inducing expression of the small G protein, K-Ras2A, and by subsequent K-Ras2A-induced activation of phosphatidylinositol phosphate-5-kinase (PIP-5-K) and phosphatidylinositol-3-kinase (PI-3-K) to produce phosphatidylinositol-3,4,5-phosphate that ultimately increases the activity of individual ENaC channels (102–104). In the long term, steroids regulate sodium transport by inducing SIPs that alter trafficking, assembly, and degradation of ENaC (105), and thereby change the number of ENaC in the surface membrane.

Expression of steroid receptors and steroid-induced gene products are developmentally regulated (106). In humans, expression of corticosteroid-induced genes in renal cortex in-

creases at term. In contrast, in lung expression of MR and GR mRNAs were greater at 100 days to term than postnatally, and 11 β -HSD1 peaked at 145 days. The corticosteroid-induced genes also increased prenatally: Sgk-1 and ENaC α increased by 120 days, peaking at 145 days, and Na,K-ATPase α 1 was greatest at 130 days. The expression of high levels of MR and 11 β -HSD1 in preterm fetal lung suggest that low endogenous fetal cortisol may exert actions at the high affinity MR *in vivo*, leading to increases in expression of sodium channels important in the regulation of lung liquid secretion and reabsorption.

One target of the steroid-induced kinase SGK-1 is Nedd4-2, which when phosphorylated has a lower affinity for binding to the PPxY motif in ENaC (107, 108). This effect is apparently caused by the phosphorylated form of Nedd4-2 interacting strongly with the scaffold protein, 14-3-3 β and ϵ , that prevents Nedd4-2 interaction with ENaC (109). Thus, aldosterone or glucocorticoids, hormones that increase Na⁺ transport, produce the increase in activity by a coordinated response that starts by rapidly increasing the activity of existing channels (110), followed by an SGK-mediated reduction in Nedd4-2 activity that reduces internalization and degradation (90–92), allowing an increase in the surface membrane pool of functional ENaC. Finally, after several hours, ENaC transcription and translation is increased (81). All of these processes represent multiple redundant mechanisms that progressively increase ENaC activity to respond to increasing demands for sodium transport. Interestingly, the system contains its own brake: in the presence of large concentrations of steroid after a long period of time (hours or days), steroid stimulates mitogen-activated protein kinase (MAPK) activity. MAPK phosphorylates ENaC and promotes Nedd4-2 binding, and thus increases ubiquitination and ENaC degradation (111). This effect partially reverses the effect of SGK inhibition of Nedd4-2 and presumably prevents steroids from increasing ENaC transport activity for long periods of time or to a level that might be deleterious to the sodium-transporting epithelial tissue.

Phosphorylation is a common theme in regulating ENaC ubiquitination and degradation (112). G protein receptor kinase 2 (GRK2) phosphorylates Nedd4-2 to reduce interaction with ENaC and prevent ubiquitination and degradation (113). Nedd4-2 function is also inhibited by phosphorylation by cAMP-dependent protein kinase A (PKA) (114). Transmitter agents that increase cAMP (vasopressin in the kidney, β -adrenergic agents in the lung) stimulate ENaC activity. The stimulation has been widely attributed to PKA's ability to promote ENaC insertion, but clearly increases of cAMP might also control ENaC function by regulating Nedd4-2 function. This implies that there is again a concerted response with one action of the transmitter agents promoting addition of channels to the membrane and the other action preventing their removal.

Although it has yet to be demonstrated for α , β , or γ ENaC, ubiquitination of the δ subunit can be promoted by interactions of the COMMD1 (formerly MURR1) protein with Nedd4-2 (5, 6). COMMD1 plays numerous other roles in cellular regulation, so the significance of its regulation of the δ subunit remains to be determined (as does the role of the δ subunit itself in epithelial function).

Phosphorylation of ENaC itself plays a role in altering Nedd4 ubiquitination. As mentioned above, MAPK phosphorylation of ENaC promotes Nedd4-2 binding. Adenosine monophosphate-activated kinase (AMPK) promotes Nedd4-2 retrieval of ENaC, and AMPK knockout mice have excess ENaC activity (115). Whether AMPK has its effect by phosphorylating ENaC or Nedd4 remains to be determined. In contrast, casein kinase 2 phosphorylates ENaC and prevents interaction with Nedd4 (116).

As mentioned above, overexpression of caveolin-1 (Cav-1) down-regulates the activity and membrane surface expression of ENaC, and ENaC and Cav-1 can be co-immunoprecipitated (38). In addition, the effect of Cav-1 on ENaC requires Nedd4-2. This suggests that in the absence of ubiquitination, even in the presence of caveolae, ENaC will not be degraded. This does not necessarily mean that ENaC is not internalized, but that internalization alone is not sufficient to promote ENaC degradation and that absent ubiquitination, internalized ENaC must be recycled to the membrane.

All of the mechanisms mentioned above involve changes in the affinity of Nedd4-2 for ENaC with the consequent change in the rate of ubiquitination and the rate of internalization. If ENaC subunits remain ubiquitinated, they will be degraded by the proteasome, but one final level of control involves the regulated removal of ubiquitin from ENaC at various places in trafficking pathways after internalization. In early endosomes, the deubiquitinating enzyme, UCH-L3, appear to remove approximately half of the ubiquitin from internalized subunits (117). At some later point in the endosomal progression, another de-ubiquitinating enzyme, USP2-45, can be induced by steroids and promote recycling of ENaC to the surface membrane (118). Thus, steroids not only reduce the rate of ubiquitination and internalization, but once ENaC is internalized, they also promote the removal of ubiquitin to allow functional ENaC to return to the surface membrane.

CONCLUSIONS

The importance of ENaC ubiquitination is underscored by Liddle's disease, an autosomal dominant form of salt-sensitive hypertension (15, 16, 119), which is characterized by reduced ENaC ubiquitination and internalization. But what is more surprising is the large diversity of mechanisms that in some way or other alter the extent of ENaC ubiquitination and, thereby, determine the number of functional channels that are at the surface of the cell. Shown in Figure 2 is a schematic summary of possible steps in ENaC degradation and trafficking. The prospects for pharmacological interventions to alter abnormal lung fluid balance (e.g., acute respiratory distress syndrome, cystic fibrosis) by manipulating ENaC function are good. Compounds that destabilize cell surface ENaC, or enhance Nedd4-2 activity in the lung, could potentially serve as drug targets for cystic fibrosis. Inhibiting Nedd4-2 or reducing proteasomal degradation offer the possibility of reducing life-threatening lung fluid edema. In addition, recent discovery of regulation of activation of ENaC by proteases such as furin, prostatic, and elastase, which cleave the extracellular domain of this channel leading to its activation, provides further avenues for drug targeting of ENaC and the control of lung fluid balance.

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