Am J Physiol Renal Physiol 290: F1285–F1294, 2006;
doi:10.1152/ajprenal.00432.2005.

Invited Review

Regulation of epithelial sodium channels by the ubiquitin-proteasome proteolytic pathway

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Malik, B., S. R. Price, W. E. Mitch, Q. Yue, and D. C. Eaton. Regulation of epithelial sodium channels by the ubiquitin-proteasome proteolytic pathway. *Am J Physiol Renal Physiol* 290: F1285–F1294, 2006; doi:10.1152/ajprenal.00432.2005.—Amiloride-sensitive epithelial 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 on 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 COOH-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 downregulated 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 Madin-Darby canine kidney cells are transfected with ENaC, ubiquitin conjugation apparently leads to lysosomal degradation. However, in untransfected renal cells (A6) expressing endogenous ENaC, ENaC is indeed degraded by the ubiquitin-proteasome system. Nonetheless, in both transfected and untransfected cells, the rate of ENaC degradation is apparently controlled by Nedd4–2 activity. In this review, we discuss the role of the ubiquitin conjugation and the alternative degradative pathways (lysosomal or proteasomal) in regulating the rate of ENaC turnover in untransfected renal cells and compare this regulation to that of transfected cell systems.

Nedd4; Sgk; A6 cells; amiloride sensitive; membrane-spanning protein

THE EPITHELIAL SODIUM CHANNEL (ENaC) is a heteromultimeric protein usually formed from three homologous subunits: α, β, and γ. Although other combinations may exist (17, 42), channels composed of the three subunits 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 submicromolar concentrations, and is typically found in the apical membranes of sodium-transporting epithelial cells. The primary amino acid sequence derived from cloning studies suggests that each of the subunits consists of two transmembrane domains, one large extracellular domain and relatively short intracellular COOH- and NH_2-terminal regions (5). Normal ENaC function is critical because ENaC-mediated renal sodium transport is ultimately responsible for maintaining total body sodium balance and normal blood pressure (19, 20), whereas ENaC-mediated sodium transport in the lungs is responsible for normal fluid clearance from the alveolar space (41) and consequently for normal gas exchange in the lungs. Not surprisingly then, abnormalities in ENaC function have been linked to disorders of total body Na^+ homeostasis, blood volume, blood pressure, and lung fluid balance (20, 29). For example, a partial loss-of-function mutation of ENaC produces pseudohypoaldosteronism type I (6, 67) 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 (22, 23, 57, 74), 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 (22, 23, 57). 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 syndrome-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 comprise an inefficient process in polarized epithelia. The exact point in the trafficking pathway at which ENaC subunits are assembled into a functional channel is unclear as is the subunit stoichiometry of the assembled channel. 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 mech-

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anism by which the hormones vasopressin and, possibly, aldosterone alter sodium transport (39).

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 by the rate of retrieval and degradation of channels and the rate of recycling from intracellular pools. Hormones that alter 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 (discussed in detail in REGULATION OF ENAC DEGRADATION RATE IN UNTRANSFECTED CELLS). 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.

In this review, we discuss the role of the ubiquitin-proteasome proteolytic pathway in regulating the rate of ENaC turnover in untransfected renal cells expressing endogenous ENaC subunits and contrast this regulation to the lysosomal degradation occurring in transfected cell systems.

RETRIEVAL, DEGRADATION, AND RECYCLING OF MEMBRANE PROTEINS

Signal-transducing receptors, small-molecule transporters, and ion channels reside at the plasma membrane, where their function is tightly regulated. The activity of cell-surface proteins 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 the case of signaling receptors, this is part of the mechanism that allows a eukaryotic cell to return to an unstimulated, basal state after receiving and responding appropriately to an external signal. Internalized receptors can either be permanently inactivated by degradation or be recycled to the plasma membrane to function again. In general, the first step in receptor endocytosis is the association of the receptor with specialized structures on the membrane surface: clathrin-coated pits or caveolae. The process of 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 (56). The mechanism for retrieval from caveolae is not completely clear, except that it depends on tyrosine kinase activity and is independent of clathrin-mediated endocytosis (75). In endothelium, caveolar fission requires tyrosine phosphorylation of caveolin and dynamin-2 by Src (44). Caveolae-like lipid rafts are rich in cholesterol and sphingolipids, but unlike lipid rafts caveolae also contain the protein caveolin (47). Proteins associated with caveolae can be degraded by the proteasome (14), but it is unclear whether proteins retrieved in caveolae are degraded in the lysosomes.

Clathrin-mediated endocytosis from 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 5.9–6, 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 recycling 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 surface and the intravesicular pH is further reduced to 5–6. 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 (71). Alternatively, membrane proteins can be degraded by proteasome proteolysis rather than lysosomal degradation possibly by resorting to the *trans*-Golgi network (38). One such protein is the Met tyrosine kinase receptor (30). The downregulation of plasma membrane proteins is a crucial regulatory mechanism because a number of diseases result when these mechanisms go awry at the cellular level. For instance, as mentioned above, mutations that block the downregulation of epithelial Na^+ channels in human kidney cells result in an inherited form of hypertension, and cells that are unable to internalize activated receptors for epidermal growth factor develop a transformed phenotype, often leading to tumor development.

UBIQUITIN AND PROTEASOME-MEDIATED PROTEOLYSIS

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 multisubunit proteolytic complex known as the proteasome (25–27). Ubiquitin is covalently linked to substrate proteins via an isopeptide bond formed through its COOH-terminal glycine to the ϵ-amino group of lysine residues. Ubiquitination of proteins generally requires the 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 a 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. For polyubiquitination, this process is repeated until a chain of at least four or five ubiquitins are added using the terminal carboxylic group of glycine 76 on one ubiquitin and lysine 48 of another ubiquitin molecule (25). In contrast, for monoubiquitination, this step is repeated on different lysine residues on the target protein so that the final stoichiometry is one ubiquitin per lysine on multiple lysines on the target protein. The roles of poly- vs. monoubiquitination are discussed later in this review.

Membrane proteins are frequently degraded in lysosomes, but there are also examples of transport proteins being degraded by the 26S ubiquitin-proteasome system (8, 25, 30, 46). In fact, several different types of proteins with diverse functions, cellular localizations, and half-lives are degraded by this

pathway (46). Cytosolic proteins that are old or damaged, or proteins that undergo regulated destruction such as the cyclins and some transcription factors, are modified with a polyubiquitin chain and a hydrophobic region within ubiquitin, each consisting of Leu8, Ile44, and Val170 is then recognized by the regulatory cap of the 26S proteasome (25, 27). 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 13 Å. 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 (76). Ubiquitin chains are removed from the cleaved peptides by deubiquitinating enzymes and recycled (25).

UBIQUITIN AND LYSOSOMAL DEGRADATION

Besides the ubiquitin-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 the lysosomes (26, 27, 30). Interestingly, a number of mammalian receptors and ENaC undergo ubiquitination at the plasma membrane, and this modification is required for their degradation (30). While ubiquitination of cytoplasmic proteins invariably leads to proteasomal degradation, ubiquitination of membrane proteins can lead to proteasomal degradation, but can also act as a signal for internalization (27, 30, 72), thus promoting delivery to lysosomes. The pathway that proteins follow appears to depend on the difference between the addition of ubiquitin to form a single chain of ubiquitins (polyubiquitination), which directs proteins to the proteasome, or the addition of single ubiquitin molecules at one, two, or several different sites in the protein (monoubiquitination), which is a signal for endocytosis and lysosomal degradation (4, 43, 50, 55, 69, 72, 79). The role of polyubiquitination in degradation of the yeast membrane receptor Ste2 is well established (26); a mutation in the E2 enzyme causes a reduction in the internalization of Ste2 as well as a reduction in its ubiquitination (28). Similar reductions in ubiquitination and internalization are observed when the lysine in ubiquitin responsible for the formation of polyubiquitin is mutated to alanine (54). However, no effect on Ste2 degradation was seen when mutations were made that inactivated the proteasome (28), suggesting that, for Ste2, ubiquitination was required for internalization, but the proteasome was not important for degradation. Other membrane proteins conjugated to ubiquitin, subsequently endocytosed, and finally degraded in lysosomes are Ste6 (37), uracil permease (18), and the multidrug transporter Pdr5 (13). Some proteins can be degraded by both lysosomal or proteasomal proteolytic pathways depending on their cellular localization. CFTR is degraded by both lysosomal and proteasomal pathways, misfolded CFTR in the endoplasmic reticulum (ER) is polyubiquitinated, and then the polyubiquitinated molecules are degraded by the proteasome (26); however, membrane-resident CFTR is retrieved and degraded in lysosomes (49).

Direct ubiquitination of membrane proteins can promote internalization, but monoubiquitination of proteins closely associated with membrane proteins can also promote internalization. For example, monoubiquitination of the receptor-associated protein β-arrestin promotes internalization of G protein-coupled β_2-adrenergic receptor proteins, followed by receptor degradation (68). This is an integral part of β_2-adrenergic receptor downregulation by β-arrestin.

Because 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 as easy as for yeast proteins because most mutagenesis studies conducted so far did not differentiate between either the mode of ubiquitination (polyubiquitination vs. monoubiquitination) or the mode of degradation (proteasomal vs. lysosomal). Moreover, ENaC degradation might be different between cells expressing endogenous ENaC subunits compared with cells expressing 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 and moreover that Nedd4 is the ubiquitin ligase for ENaC subunits.

A SPECIFIC UBIQUITIN LIGASE, Nedd4, 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, neural precursor cell-expressed developmentally downregulated protein (Nedd4). Nedd4 contains a E6-AP COOH-terminal (Hect) domain that is homologous to other ubiquitin ligases, three WW domains in rat and mouse protein or four WW domains in human and *Xenopus laevis* protein, and a calcium/lipid binding domain (CaLB/C2) (65). 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 (24, 32). Nedd4 consists of 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 (24, 32).

Nedd4 has the same localization pattern as ENaC in renal cortical and outer medullary collecting duct principal cells and in airway epithelia (66). Nedd4 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 (63). Subsequently, Dinudom et al. (11) characterized the binding of Nedd4 to ENaC subunits using a far Western assay, in which they showed that there is direct binding of WW second and third domains to all three ENaC subunits. For human Nedd4, the first WW does not interact with ENaC subunits but the second, third, and fourth WW domains interact with all three ENaC subunits (25), and the third WW3 domain caused the largest reduction in ENaC function expressed in oocytes along with all three ENaC subunits (32). Interestingly, Nedd4–2 and not Nedd4–1 is the predominant ubiquitin ligase for ENaC subunits transfected in *X. laevis* oocytes (32). At a molecular level, the interaction of the Nedd4 WW domain with the PY motif of ENaC β-subunits involves contact between tyrosine 618 and leucine 621 and the WW domain of Nedd4 (33). Nedd4 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 (3). Other Nedd4-like proteins might also bind to and ubiquitinate ENaC subunits. WWP2 is one such example; McDonald et al. (41a) showed by Northern blot 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. In their work, the first WW1 domain of WWP2 protein did not bind to ENaC subunits, but the second and third WW domains bound to all three subunits and the fourth WW bound only to α- and β-subunits (41a). In contrast, all four Nedd4–2 WW domains bound to all ENaC subunits (41a).

Nedd4–2 binds to ENaC subunits in vitro but also can decrease ENaC function in systems transfected with ENaC or expressing ENaC RNA. When *X. laevis* 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 (1, 21). Also, more recently in untransfected renal cells (A6), Nedd4–2 was shown to coimmunoprecipitate with α- and β-ENaC and to associate with apical membrane proteins (40).

Despite the large amount of evidence for Nedd4–2 interaction with ENaC, several other members of the Nedd4 family of proteins also interact with ENaC. Therefore, although it has generally been assumed that Nedd4–2 is the ENaC ubiquitin ligase in cells expressing endogenous ENaC, there has been little direct evidence for this role of Nedd4–2. One study addressing this question directly was performed in untransfected renal cells (A6) expressing endogenous ENaC and showed that blocking Nedd4–2 synthesis with antisense oligonucleotides or small-interference RNA reduced the number of functional ENaC in the surface membrane and increased in the transepithelial current (40). Similar results were observed in FRT and H441 cells transfected with ENaC subunits and small-interference RNA for Nedd4–2 (61). 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); also, they imply 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.

DEGRADATION OF ENaC EXPRESSED IN HETEROLOGOUS SYSTEMS

In transfected cells, the WW domains of Nedd4–2 interact with the ENaC PPxY domain, and then the E6-AP COOH-terminal homologous domain (Hect) acts as a ubiquitin ligase to conjugate ubiquitin to the NH_2 termini of α- and γ-ENaC subunits (63). In Madin-Darby canine kidney (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 (64), although, Valentijn et al. (70) were unable to detect any ubiquitinated ENaC subunits in *X. laevis* oocytes injected with ENaC subunits and Nedd4–2. However, as mentioned before, coexpressing Nedd4–2 and ENaC subunits in *X. laevis* oocytes reduces ENaC protein at the cell surface and decreases whole cell sodium current (1, 64). Even though Nedd4 is associated with a reduction in both ENaC protein and function in transfected cells, however, the form of ENaC ubiquitination (mono- vs. polyubiquitination) and degradation (lysosomal vs. proteasomal) remains unclear. Because the form of ubiquitin conjugation, mono- vs. polyubiquitination, could determine the mode of protein degradation, one way to investigate the mechanism of ENaC degradation is to examine the type of ENaC ubiquitination (mono- vs. polyubiquitination). However, most studies to date have not differentiated between mono- and polyubiquitination because it is impossible to distinguish poly- from monoubiquitination based only on molecular weights of ubiquitinated ENaC as a chain of polyubiquitin attached to one lysine or single ubiquitin molecules coupled to several lysines will have similar molecular weights. Another method of distinguishing between proteasomal degradation (and presumptive polyubiquitination) and lysosomal degradation (monoubiquitination) uses inhibitors of these pathways. The ubiquitin-proteasomal proteolytic pathway can be inhibited by blocking proteasome activity with a variety of pharmacological agents such as lactacystin or MG-132, which block the chymotryptic activity of the proteasome (14, 15, 28, 35, 64), whereas 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. However, this approach to examining ENaC degradation has produced conflicting results. 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 (64). In contrast, another group reported that, in *X. laevis* oocytes, only lactacystin (but not chloroquine) increased the half-lives of individual ENaC subunits (70). The lactacystin-mediated increase in half-life depended on 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 (70).

ENaC internalization from the plasma membrane is dependent on dynamin-2. Previously, dynamin-2-dependent internal-

ization was only linked to endocytosis by clathrin-coated vesicles (56). 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 (14). 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.

Furthermore, because in previous work investigators were unable to detect any ubiquitinated ENaC subunits in *X. laevis* oocytes expressing ENaC subunits (even though, as mentioned before α- and γ-ENaC are ubiquitinated in transfected MDCK cells) but ENaC degradation in *X. laevis* oocytes is lactacystin sensitive, the investigators concluded that the unassembled ENaC subunits in the ER were degraded by the proteasome via a ubiquitin-independent pathway (70). One reason for the differences between these two studies could be the differences in cell lines used or different levels of ENaC protein expression. Moreover, the observed differences in response to lactacystin in oocytes expressing either one or all three ENaC subunits could also be explained by the differences in total expressed protein levels. This may not be surprising, because overexpressed proteins are often targeted to lysosomes regardless of the normal degradative pathway of the untransfected protein in cells constitutively expressing the protein. Another reason could be that the investigators used the lysosomal inhibitor chloroquine at relatively high concentrations, which can permeabilize mitochondrial membranes, therefore uncoupling the mitochondrial oxidative phosphorylation process and depleting cells of ATP (34). Because 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.

Another example of a membrane-spanning protein that is degraded by the proteasomal pathway is CFTR. The mechanism of CFTR degradation is well characterized: improperly folded CFTR channel molecules in the ER are ubiquitinated with subsequent degradation by the proteasome. However, properly folded CFTR at the apical plasma membrane is degraded by the lysosomal pathway. Because the mechanism of CFTR degradation is so well established, it is tempting to compare ENaC and CFTR degradation. It appears that ENaC degradation in transfected cells is similar to CFTR, where ER-resident, unassembled ENaC subunits are degraded by the proteasome, but the membrane-resident channel molecules are degraded in lysosomes (31, 38, 72, 73). However, there are some differences between ENaC in transfected systems and CFTR; ENaC expressed in *X. laevis* oocytes appear to be degraded by the proteasome in a ubiquitin-independent pathway, whereas CFTR is ubiquitinated before its degradation. ENaC degradation after transfection in MDCK cells is also different from CFTR, because when the CFTR internalization signal, YXX Φ (where Φ is any hydrophobic amino acid residue), which is linked to lysosomal degradation, is mutated, the whole cell levels of channel protein remain unchanged, but the membrane-resident CFTR levels increase (49). Unfortunately, similar mutational studies to determine the role of the lysosomal pathway have not been done for ENaC. Staruschenko et al. (62) mutated a serine residue within the internalization sequence of all three ENaC subunits and transfected each mutated subunit along with other two wild-type subunits in Chinese hamster ovary cells. They observed an increase in whole cell current but did not measure either whole cell or membrane-associated ENaC protein levels or functional ENaC density by patch-clamp analysis (62). In the absence of data showing a shift in either ENaC protein levels or number of channels at the apical membrane, the role of ENaC degradation in these mutants is difficult to assess. However, the studies with lysosomal inhibitors (chloroquine) can be compared with mutations in the CFTR internalization signal because they both inhibit the lysosomal pathway. When MDCK cells transfected with ENaC subunits were treated with chloroquine, both whole cell and membrane-resident ENaC protein levels increased, suggesting that ENaC transfected in MDCK cells follows a different degradative pathway than CFTR (64).

More studies with other lysosomal inhibitors such as leupeptin, which is a general cysteine and serine protease inhibitor, might further elucidate the mechanism of ENaC degradation in transfected cells. Alternatively, direct measurement of proteasomal protein degradation in the presence of putative lysosomal inhibitors could determine the specificity of the inhibitor; moreover, studies with mutations in proteins involved in lysosomal or proteasomal pathways might further clarify the mechanism of ENaC degradation.

ENaC DEGRADATION IN UNTRANSFECTED EPITHELIAL CELLS EXPRESSING ENDOGENOUS ENaC

Based on studies using proteasome and lysosome inhibitors as well as other biochemical analysis, degradation of endogenous ENaC in untransfected renal A6 cells has some similarities to, 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 (39, 64). Also, the half-lives of all subunits in the total cellular pool is relatively short (1–3 h) and increases severalfold after proteasome inhibition in both transfected and untransfected cells (39, 64). 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 (39). In untransfected cells, all three ENaC subunits are at least polyubiquitinated and proteasome inhibition increases polyubiquitination of ENaC (40), but in transfected MDCK cells only α- and γ-subunits are coupled to ubiquitin, and whether this coupling is mono- or polyubiquitinated remains unresolved.

Proteasome inhibition also affected plasma membrane-resident ENaC subunits; in one report, the half-lives of membrane-associated (biotinylated) β- and γ-ENaC subunits increased: for the β-subunit from 8.3 ± 0.96 h in untreated cells to 64.6 ± 0.86 h in MG-132-treated cells and for the γ-subunit from 16.5 ± 3.4 h in untreated cells to 95.3 ± 1.9 h in cells treated with MG-132 (40). The half-life of the α-subunit may also increase after proteasome inhibition, but the normal half-life for α-subunits at the surface of untransfected cells is at least 42 h (36) and, therefore, an additional increase after proteasome inhibition is difficult to measure (40). However, another study in untransfected cells A6 cells reported an increase in ENaC protein levels in response to the lysosomal inhibitor chloroquine (70). The differences between the two studies could be due to differences in the concentration of inhibitors used, cell lysis conditions, or the antibodies used to measure protein levels (40). Besides the difference in lysis methods, the difference in inhibitor concentrations by each group might be the most significant factor that contributed to the observed differences in results. Rosa et al. (2) used the lysosomal inhibitor chloroquine at 100 μM concentration, whereas Malik et al. (39) used chloroquine (0.01 μM), methylamine (10 μM), and leupeptin (10 μM). As mentioned before, chloroquine especially at higher concentrations can deplete cells of ATP, which is required for proteasomal degradation, and hence, decrease degradation by the proteasomal pathway. However, it is also possible that some other protein that is required for ENaC endocytosis is degraded by the lysosomal pathway. In the absence of this protein, there would be an increase in membrane-associated ENaC subunits.

DIFFERENCES IN ENaC DEGRADATION BETWEEN TRANSFECTED AND UNTRANSFECTED CELLS

It seems reasonable to attribute these differences in handling of ENaC to the differences in expression between untransfected and transfected cells. The most obvious difference is the magnitude of expression: in untransfected cells the surface expression of ENaC channels ranges from ~40 channels/cell in mouse cortical collecting duct cells (M1) to as low as 4 channels/cell in the *X. laevis* renal cell line (A6), 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 an 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 fact, inhibition of lysosomes does increase the amount of ENaC at the surface of transfected cells (64). As mentioned above, that membrane ENaC in transfected cells is degraded via lysosomes is probably not surprising because the increased expression of protein in transfected cells is often associated with increased lysosomal activity to degrade the excess protein. In addition, the large excess of ENaC protein may limit the extent of ubiquitination, thus promoting monoubiquitination (and lysosomal degradation) rather than polyubiquitination (and proteosomal 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 (39). The alternative degradative pathway appears consistent with the longer half-life of all surface subunits in untransfected cells, which may reflect the additional time necessary to form a polyubiquitin chain. A schematic diagram of ENaC degradation in cells transfected with ENaC subunits and cells expressing endogenous ENaC subunits is shown in Fig. 1.

ENaC HALF-LIVES

The large 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, because 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. It is possible that the α-β-γ complex is disassembled and the β- and γ-subunits sorted to the proteosome, whereas 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 et al. (78).

Thus it appears that in transfected cells the surface expression of ENaC is dependent on the rate of trafficking from the ER and insertion into the plasma membrane, whereas 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 on the rate of internalization and proteasomal degradation with a 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 ENaC DEGRADATION RATE IN UNTRANSFECTED CELLS

So far we have discussed mechanisms by which ENaC is internalized and degraded. Another important question besides the mechanism of ENaC degradation is, why is ENaC degradation so complex, involving several steps and proteins? Ob-

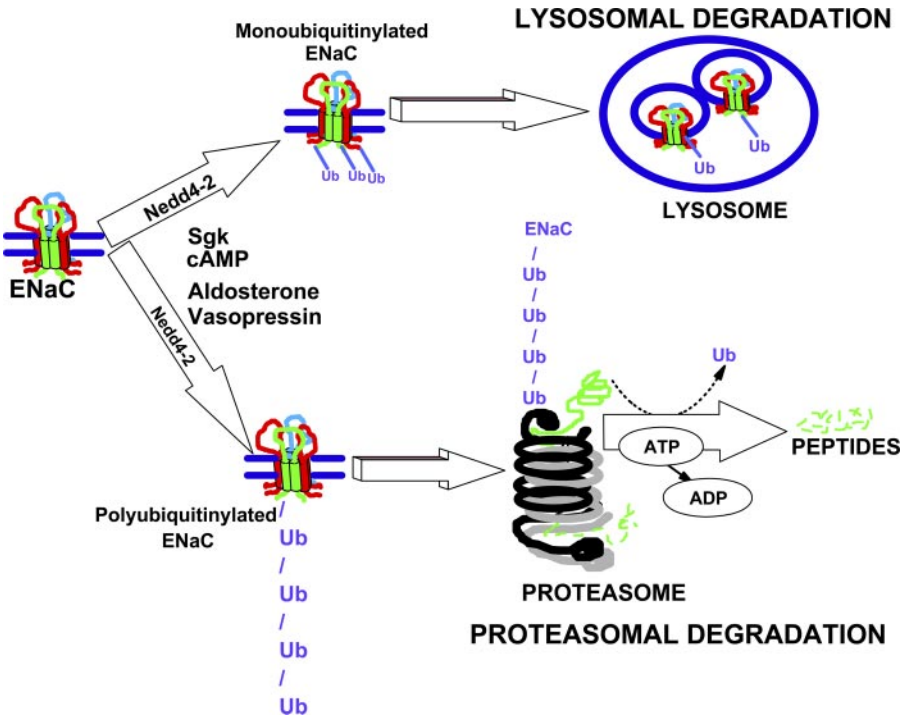

Fig. 1. Schematic diagram of epithelial Na^+ channel (ENaC) degradation. The first step involves either mono- or polyubiquitination of ENaC. This step can be modulated by regulation of the Nedd4 enzyme by Sgk, cAMP, aldosterone, or vasopressin. In the second step, monoubiquitin-conjugated ENaC is trafficked to and degraded in the lysosomes, whereas polyubiquitin-conjugated ENaC is recognized and degraded by the proteasome. The data indicate that in cells expressing endogenous ENaC subunits, the polyubiquitin-conjugated channel is degraded by proteasomes; in contrast, in cells transfected with ENaC subunits, monoubiquitin-conjugated ENaC molecules are degraded in lysosomes. Ub, ubiquitin.

viously, this allows the cells to exquisitely regulate ENaC protein levels, and hence, channel function. By manipulating these steps (by inhibiting proteasomal activity or reducing the expression of Nedd4–2, for instance), it is possible to alter the degradation of ENaC and, thereby, alter the surface membrane pool of functional ENaC. The question remains as to whether there is a physiological mechanism by which any of the steps in the degradative pathway can be altered. In transfected cells, the amount of ENaC is so large that the degradation rate appears more or less constant, but 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 (12, 53). This appears to be a generalized response to cell stress, and extended exposure to glucocorticoids and other stressors can lead to proteasome-dependent apoptosis (10, 45). Second, related to the increase in proteasomal activity, glucocorticoids also promote the increased expression of ubiquitin (10, 53). 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 renal cells, one effect of aldosterone and glucocorticoids is to increase the amount and activity of serum- and glucocorticoid-dependent kinase (Sgk) (2, 7, 48). One target of this kinase is Nedd4–2, which, when phosphorylated, has a lower affinity for binding to the PPxY motif in ENaC (9, 60). Moreover, Sgk degradation is regulated by Nedd4–2 (80). Thus aldosterone, a hormone that increases Na^+ transport, accomplishes the increase, at least in part, by reducing the degradation rate of ENaC and allowing an increase in the surface membrane pool of functional ENaC. Over and above the potential for aldosterone-mediated increases in functional ENaC, the inhibition of Nedd4–2 guarantees that in epithelial cells, the glucocorticoid-mediated increases in proteasomal activity and ENaC degradation will be balanced by a Nedd4–2-mediated decrease in ubiquitination and ENaC degradation. Which effect is more important will only be determined by additional experiments. In transfected cells, Nedd4–2 function is inhibited by increases in cAMP via Nedd4–2 phosphorylation by a cAMP-dependent protein kinase (PKA) (58). Vasopressin, another hormone that stimulates ENaC activity by stimulation of cAMP, therefore, might also control ENaC function by regulating Nedd4–2 function (58).

The existence of different isoforms of Nedd4 and different affinities of the WW domains for ENaC subunits suggests a more complex and regulated process for ENaC degradation by Nedd4 than the current simple picture. Moreover, different isoforms of Nedd4 might also have different cellular localizations and, hence, regulate ENaC at different steps in ENaC assembly and trafficking. Therefore, the entire process of ENaC regulation by Nedd4 or Nedd4-like proteins might be different in various cells. In A6 cells and MDCK cells, Nedd4 is associated with the plasma membrane via the C2 domain (51), presumably causing ubiquitin coupling of membrane-associated ENaC subunits and then ENaC degradation (40). However, in *X. laevis* oocytes, the C2 domain of Nedd4 is not required for Nedd4 to decrease ENaC function (32, 59), suggesting that there is no increase in membrane-associated ENaC subunit degradation, but rather that ENaC in the cytosol (ER or endosomes) is degraded at a faster rate, with the result that fewer ENaC subunits reach the plasma membrane. Moreover, it is also possible that different isoforms of Nedd4 are responsible for mono- or polyubiquitination. It will be interesting to resolve the roles of the various members of the Nedd4 family in controlling ENaC trafficking and degradation.

That the rate of protein degradation for each ENaC subunit is different suggests that ENaC subunit protein degradation could be noncoordinated, similar to its protein synthesis.

Therefore, it is possible that ENaC can be regulated in a noncoordinated manner at both the ENaC protein synthesis and protein degradation levels. Although the mechanism of such regulation at both the protein synthesis and protein degradation levels is unknown, the physiological relevance of such regulation would be important in almost all tissues expressing ENaC protein that have been studied so far (78). The regulation of individual ENaC subunit degradation in a noncoordinated manner could be due to separation of the subunits, with different rates of degradation for each subunit, and would fit in with any of the three possible pathways proposed for noncoordinated trafficking by Weisz and Johnson (77).

SUMMARY

In humans, several inheritable disorders of epithelial cell sodium transport are linked to mutations in ENaC protein. One such disorder is Liddle's disease, an autosomal dominant form of salt-sensitive hypertension (22, 23, 57). Liddle's disease is caused by mutation or deletion of the proline-rich (PPxY) region of ENaC β- or γ-subunits and because Nedd4–2 binds to this region to regulate ENaC degradation, this disorder is related to a dysfunction in the degradation pathway of the ENaC protein. This reduced binding to Nedd4–2 ultimately leads to an increase in number of membrane-resident ENaC molecules and ENaC hyperactivity, which can be mimicked by inhibitors of proteasome activity. Also, the reduction in affinity of Nedd4–2 (because of mutations in the binding motif) are likely to mimic the effect of aldosterone-mediated Nedd4–2 phosphorylation. Indeed, cells expressing ENaC with Liddle's mutations are relatively unresponsive to aldosterone (52).

However, ENaC protein degradation is different in untransfected renal cells expressing endogenous ENaC compared with transfected cells systems. Nedd4–2 appears to be the ubiquitin ligase responsible for ubiquitin conjugation of ENaC, thereby serving as a signal for ENaC degradation in both untransfected and transfected cell systems. However, once ubiquitinylated, ENaC degradation seems to follow different pathways in untransfected cells (via proteasomes) and transfected cells (via lysosomes), but more work in different systems is needed to properly understand the role of both of these degradative pathways in regulating ENaC function. Therefore, the ubiquitin-proteasomal proteolytic pathway plays a significant role in the regulation of ENaC activity in untransfected renal cells expressing endogenous ENaC and, to a lesser extent, in transfected cells systems. Shown in Fig. 2. is a schematic summary of possible steps in ENaC degradation and trafficking.

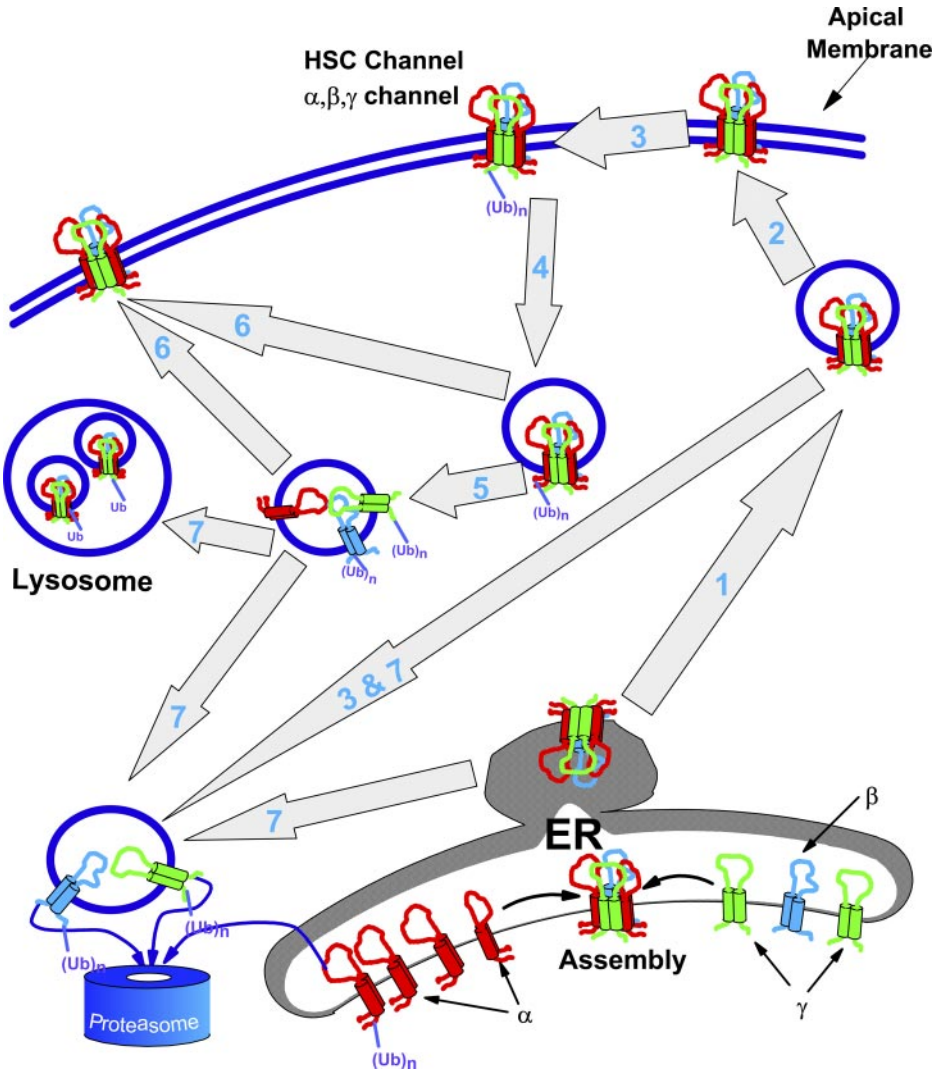

Fig. 2. ENaC trafficking. Shown is a schematic of possible steps in ENaC trafficking. ENaC are translated and assembled in the endoplasmic reticulum (ER), and assembled ENaC are trafficked to the apical plasma membrane via the Golgi apparatus, whereas mistranslated or misassembled ENaC subunits are coupled to ubiquitin and degraded by the proteasomal complex. ENaC reach the apical membrane via trafficking from the Golgi apparatus and are internalized from the apical membrane subsequent to their coupling to ubiquitin. The internalized ENaC are either resorted and recycled back to the apical membrane or degraded. The pathway for degradation depends on the extent of ubiquitination. Monoubiquitination of ENaC (usually in transfected cells) targets ENaC to the lysosome; polyubiquitination (more prevalent in native cells) targets ENaC to the proteasome. Here, we show a simplistic model for ENaC trafficking without any reference to noncoordinated regulation of ENaC. However, differences in rates of degradation of different ENaC subunits from any of the intracellular locations shown here could account for noncoordinated regulation of ENaC degradation. For possible pathways involved in noncoordinated regulation see Ref. 77, and any of the 3 different pathways for noncoordinated regulation could explain the results presented in this review. Arrows represent the following processes: *1*) ENaC protein trafficking and processing, *2*) ENaC insertion into the apical membrane, *3*) ENaC ubiquination by Nedd4–2 in the apical membrane, *4*) ENaC retrieval from apical membrane, *5*) ENaC resorting, *6*) ENaC recycling back to the apical membrane, and *7*) ENaC degradation. HSC, highly selective channel.

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