

Some Assembly Required: Putting the Epithelial Sodium Channel Together*

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The epithelial sodium channel (ENaC)² constitutes the rate-limiting step in Na⁺ re-absorption in the apical membrane of epithelia, including kidney, lung, colon, salivary glands, and sweat glands (1). The channel is integral to homeostasis as knock-out of the α -subunit of ENaC is lethal in mice. Abnormal regulation of the channel that results in either gain or loss of function has been implicated in the pathogenesis of several disease states, including forms of salt-sensitive hypertension and pseudohypoaldosteronism type II, and may also contribute to the progression of pulmonary disease in cystic fibrosis (1–3).

ENaC is a highly Na⁺-selective amiloride-sensitive channel of relatively low conductance that appears to be constitutively active when present in cell membranes (1). The channel is a heterotrimeric protein, made up of three homologous subunits (α , β , and γ) with a likely stoichiometry of 1:1:1 (4, 5). A δ -subunit that can co-assemble with α -, β -, and γ -ENaC has also been described (6). Given its essential roles in fluid clearance and regulation of blood volume, it is not surprising that ENaC activity is under strict control. The apical membrane abundance and open probability of ENaC are altered by a wide variety of hormonal and cellular effectors (1, 7). Regulation of channel expression at the translational and post-translational levels is tissue-specific, and these differences are apparent for either whole channel levels or expression of individual subunits (8). For example, message for the individual subunits is differentially expressed not only in distinct tissues such as lung and kidney, but also temporally, so the message for some subunits is expressed constitutively, whereas that for others is induced hormonally (8). Differential translational efficiency of ENaC subunit message has also been noted. In endogenously expressing tissues and cell lines, it is clear that total cellular and apical membrane levels of some subunits remain unchanged, whereas others are either up- or down-regulated in response to factors that alter regulation of transport rate (9). This phenomenon has been referred to as non-coordinate regulation of ENaC, a term first applied by Farman and co-workers (10, 11) to the differential steroid regulation of ENaC subunit mRNA in lung, kidney, and colon. We have previously proposed three possible mech-

anisms to explain this phenomenon: 1) post-translational assembly of channels, 2) the existence of channels with alternative stoichiometry that may be differentially regulated, and 3) disassembly and recombination of subunits at some post-translational site (8). These mechanisms are not mutually exclusive, which highlights the likelihood that more than one pathway participates in the generation of the phenomenon broadly described as non-coordinate regulation. The purpose of this review is to consider the status of non-coordinate regulation of ENaC with respect to several recent developments in the field, in particular the appreciation of the probable trimeric stoichiometry of the channel and the accumulating evidence that subapical pools of ENaC subunits exist that can participate in recycling of the channel. Finally, we will discuss the impact that differing molecular mass forms of the ENaC subunits present at the apical membrane following proteolytic activation of the channel may have on our understanding of non-coordinate regulation.

ENaC Stoichiometry and Assembly

ENaC was originally identified and the primary structure of its three subunits elucidated through expression cloning (4). Initial studies of the channel in heterologous overexpression systems suggested that the channel oligomerizes co-translationally in a preferred stoichiometry that contains all three subunits (12). Although it is possible for channels containing only one or two subunits to reach the cell surface, expression of all three subunits results not only in greater membrane expression and activity, but also in stabilization of the channel (13, 14). The majority of channel subunits are rapidly degraded prior to reaching the cell surface (14, 15). Although several chaperones that participate in endoplasmic reticulum (ER)-associated degradation of ENaC have been identified, the details of this process remain unknown (16). The recent crystallographic demonstration that the chicken acid-sensing ion channel (a member of the ENaC/degenerin family of ion channels) is arranged as a heterotrimer has added a new dimension to our understanding of ENaC channel assembly (5).

The prevailing model posits that multisubunit channels form by assembly of newly synthesized proteins into heteromeric complexes of uniform stoichiometry in the ER. However, a considerable body of evidence suggests that this is not a requirement for the generation of functional Na⁺ channels. It is clear from studies in several model systems that channels comprising only one or two of the three subunits can assemble and traffic to the cell surface (12, 13). There are several reports that ENaC subunits or channels composed of one or two subunits can accumulate at intracellular sites and assemble into fully functional channels upon subsequent expression of the remaining subunit (17–20). Because the α -subunit seems to be required for channel activity (4, 12), it is possible for α -, $\alpha\gamma$ -, or $\alpha\beta$ -channels to function at the cell membrane (12, 13), whereas $\beta\gamma$ -dimers would represent precursors for channels containing all three subunits (17–20).

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² The abbreviations used are: ENaC, epithelial sodium channel(s); ER, endoplasmic reticulum.

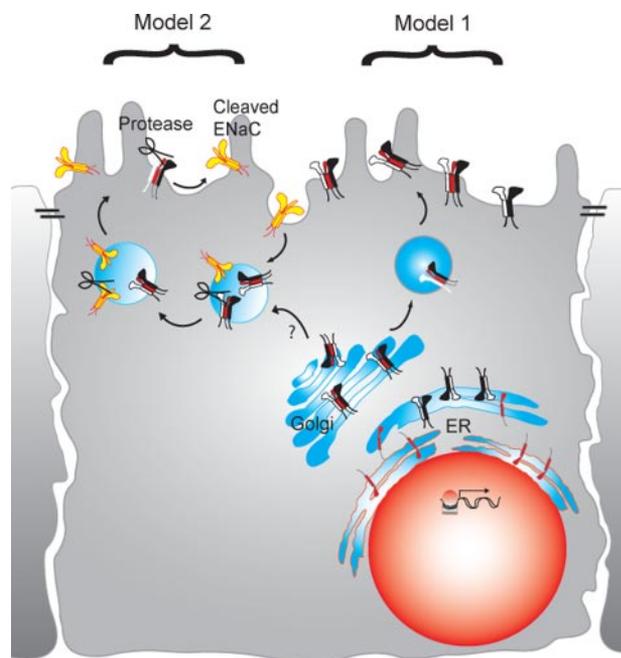


FIGURE 1. Models of non-coordinate regulation of ENaC by post-ER assembly and proteolysis. In *Model 1*, assembly of ENaC heterotrimeric channels is not co-translational: partial ENaC subunits or partial channels in post-ER compartments can combine with induced subunits to form fully active channels that traffic to cell surface. In *Model 2*, physiological stimuli cause uncleaved channels at or near the apical membrane to be either cleaved or replaced by fully mature cleaved channels (yellow). Evidence for both of these models is presented in the text.

Most of the early studies of channels formed from one or two subunits were performed using *Xenopus* oocytes or other heterologous expression models, but there are examples of alternative channel assemblies *in vivo*. Randrianarison *et al.* (21) examined fluid clearance in newborn mice with a disruption in the β -ENaC gene locus. The reduction in β -ENaC levels resulted in compensatory up-regulation of α - and γ -ENaC and, surprisingly, led only to a modest impairment in ENaC function. This study concluded that functional channels with alternative stoichiometries may traffic to the apical membrane of epithelial cells *in vivo*, consistent with the previous heterologous expression system studies.

A selective increase in the synthesis of one or two ENaC subunits has also been previously noted (8). This would suggest that a pool of "partial" ENaC channels exists within these cells and that production of a limiting third subunit can facilitate the formation of a heterotrimeric channel (Fig. 1, *Model 1*). The most dramatic example of this is in rat kidney, where intracellular pools of β - and γ -subunits can be visualized in salt-loaded animals when ENaC activity at the apical membrane is minimal (17, 18, 20). Following stimuli for Na^+ re-absorption, these subunits migrate to the apical membrane along with newly synthesized α -ENaC (17–20). A similar situation may occur in the lung, where Boucher and co-workers (2) demonstrated that ENaC activity may be enhanced by up-regulating expression of β -ENaC, but not by α - or γ -ENaC overexpression.

Based on these examples, selective expression of a single ENaC subunit in response to physiological cues could modulate ENaC surface expression and activity. Studies have suggested that modulation of mRNA translational efficiency can also con-

tribute to differential subunit expression. It appears that β - and γ -ENaC are longer lived than the α -subunit. In a study investigating ENaC mRNA abundance and translational efficiencies in fetal lung, Otulakowski *et al.* (22) demonstrated that there was an overabundance of α -ENaC mRNA and that this mRNA was not as long lived as β - and γ -ENaC mRNAs. A follow-up study demonstrated that α -ENaC mRNA translation in the fetal lung epithelium could be modulated by both alterations in oxygen tension and glucocorticoid stimulation (23). Other external factors such as hypotonicity can alter the longevity of α -ENaC mRNA, potentially via a mitogen-activated protein (MAPK) pathway (24). This diversity of mRNA expression and/or translational efficiency could therefore lead to the formation of different combinations of subunits forming channels of differing biophysical properties depending on regulatory inputs. Such a phenomenon has been proposed for lung tissue as a mechanism to provide flexibility to the regulation of Na^+ re-absorption (25–28).

Given the differences in mRNA abundance and longevity, it is likely that the abundance of each ENaC subunit would not be comparable at all times in the cell. Under steady-state conditions, it is possible to detect one or two of the subunits in the absence of the third subunit, indicating that the subunits that are synthesized are not immediately degraded by the quality control machinery. Longer lived subunits could therefore reside in the cells with the ability to reassemble upon induction of additional subunit(s) by external cues. The site of this assembly is not clear. The most likely location would be in the ER before forward transport to the Golgi and exit to the apical membrane. As the abundance of the three subunits becomes comparable, the population of channels containing one or two subunits will be replaced by heterotrimeric channels.

Studies in cultured renal cell models have not fully replicated the regulation of ENaC assembly observed *in vivo*. Although steroids preferentially increase production of α -ENaC expression in the kidney, adenovirus-mediated overexpression of α -ENaC in M1 collecting duct cells did not increase sodium currents, even though the overexpressed subunits were trafficked out of the ER (29). Overexpression of γ -ENaC had a larger impact on base-line current, but only when all three subunits were overexpressed did the measured short-circuit current increase in a manner similar to that seen with the administration of dexamethasone to stimulate ENaC production. These findings suggest that if one or two of the subunits are limiting, no amount of overproduction of a third subunit will increase ENaC transport. It is possible that cultured cells lack key mechanisms described above to modulate ENaC subunit abundance, assembly, or trafficking.

ENaC Recycling and Remodeling

A corollary of the idea that channels may not be co-translationally assembled is that subunits could remodel into channels of alternative stoichiometries after initial assembly. Post-synthetic remodeling of heteromeric proteins, although unusual, has been demonstrated (8). Non-coordinate trafficking of subunits of the T cell antigen receptor CD3 complex and of the individual chains of the trimeric interleukin-2 receptor has been described (30, 31). More recently, it was demonstrated

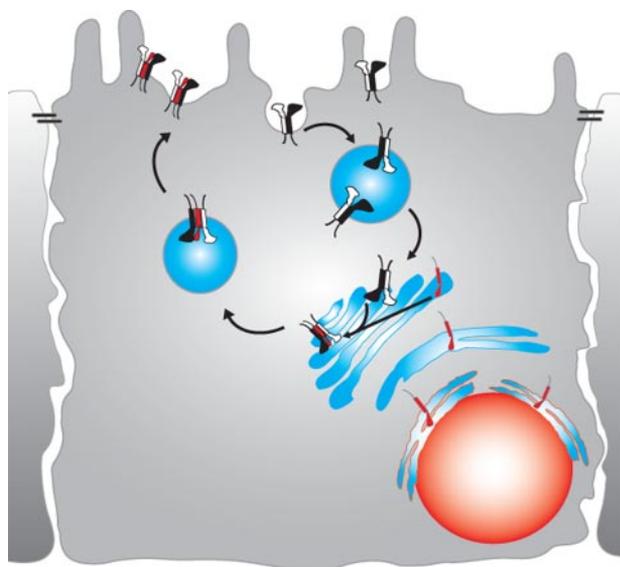


FIGURE 2. Model for non-coordinate regulation by subunit remodeling. In this model, channels composed of two subunits exist and are capable of remodeling at the membrane or in endocytic compartments. Alternatively, non-trimeric channels are trafficked back to the Golgi (potentially via the retromer complex), where they would disassemble to recombine with newly synthesized subunits. Newly assembled, fully active heterotrimeric channels would then traffic to the apical membrane. Evidence for this speculative model is discussed in the text.

that the α - and β -subunits of the Na^+/K^+ -ATPase, which assembled in the ER, could dissociate in the plasma membrane and undergo differential degradation (32). The possibility of ENaC remodeling would likely depend on the ability of the channel to recycle. We demonstrated previously that ENaC is internalized from the apical surface via an epsin- and clathrin-dependent pathway (7, 33). The post-endocytic itinerary of the channel is only beginning to be described. Upon ubiquitin-dependent retrieval, ENaC localizes to EEA1 (early endosome antigen 1)-positive early endosomes (33, 34), where it encounters the proteins of the ESCRT (endosomal sorting complex required for transport) machinery (34).³ Some channels are apparently targeted for lysosomal degradation, whereas others are deubiquitinated to enable recycling. At least two deubiquitinating enzymes that regulate ENaC expression have been described, one of which is clearly localized and enriched in EEA1-positive endosomes (35, 36). When ENaC enters the tubular endosomal network, possibilities exist for degradation, fast or slow recycling via the EHD/Rab11 pathway (34), or retrieval to the *trans*-Golgi network via the retromer complex (37). The latter possibilities would provide a potential mechanism for channel remodeling (Fig. 2). Several cargo proteins have been identified that undergo transport between endosomes and *trans*-Golgi compartments. One of these, furin, is known to cleave ENaC and could represent a pathway for selective generation of cleaved activated channels (see below).

Several studies of ENaC trafficking and degradation have suggested the possibility that individual subunits have different lifetimes after reaching the cell surface (8, 9, 38). Because ENaC is not an abundant protein in natively expressing tissues and only a minority of synthesized subunits reach the cell mem-

brane, it has been difficult to reach a consensus regarding the half-life of functional channels or individual subunits. Half-lives in endogenously expressing cell lines such as A6 have varied from 15–30 min for channels that reach the apical membrane to 5–6 h for β -ENaC with longer half-lives for α - and γ -subunits (7, 14). In mouse cortical collecting duct cells, the half-life of functional channels appears to be 4 h, although apical residence time may be shorter because the channels appear to recycle in these cells (7, 39). These differences in ENaC subunit half-lives may reflect altered or selective trafficking of individual ENaC subunits or subunit combinations in response to physiological stimuli. However, another compelling explanation is provided by the recent appreciation that ENaC can exist in varying molecular mass forms as a result of proteolytic processing (see below).

Proteolytic Processing of ENaC

Activation of ENaC by serine proteases was first described by Vallet *et al.* (40) in oocyte experiments, and activation of near-silent channels in cell membranes by proteolytic cleavage was shown by Caldwell *et al.* (41, 42). A correlation between ENaC activation by aldosterone in rat kidney and the appearance of a lower molecular mass (70 kDa) form of γ -ENaC was subsequently reported by Masilamani *et al.* (19). These findings suggested that ENaC may be activated by proteolytic cleavage, a suggestion that was confirmed and extended in a series of elegant studies by Hughey *et al.* (43–46). These studies demonstrated the furin-dependent cleavage of both α - and γ -ENaC into lower molecular mass forms and established that cleaved channels have a greater open probability than channels composed of uncleaved subunits. Glycans on cleaved subunits are terminally processed, confirming that the subunits have traversed the Golgi apparatus *en route* to the apical membrane. A distinct subset of uncleaved channels lacking complex glycans also proceeds to the apical membrane, possibly bypassing the Golgi (45). These “unprocessed” subunits might exist in alternative conformations or stoichiometries, although this has not been directly examined. It is also apparent that there is more than one site for cleavage in both α - and γ -ENaC and that multiple proteases can act on these subunits, so intermediate stages of activation can be noted (43). Channels composed of uncleaved or partially cleaved subunits at the apical membrane could thus be activated by cell-surface proteases, by prolongation of apical residence time, or by traffic of uncleaved ENaC through intracellular compartments, where they may interact with proteases. (Fig. 1, Model 2).

There are two potentially related mechanisms by which proteolytic activation of channel subunits could contribute to the phenomenon of non-coordinate regulation. First, antibodies to ENaC subunits that recognize only full-length α - and γ -ENaC would not necessarily detect selective alterations in cleaved, more active forms of these subunits. For example, we reported that up-regulation of ENaC activity by either long-term (18 h) incubation with aldosterone or short-term exposure to vasopressin is associated with selective increases in apical membrane β -ENaC without significant changes in the apical membrane unprocessed forms of α - or γ -ENaC in A6 cells (9). In addition, stimulation of ENaC exocytosis by vasopressin leads

³ M. B. Butterworth, O. A. Weisz, and J. P. Johnson, unpublished data.

to depletion of full-length β -ENaC (but not α - or γ -ENaC) from endosomal populations. Our results are consistent with the possibility that channels inserted in the apical membrane and/or depleted from endosomal populations under these conditions are primarily composed of cleaved forms of α - and γ -ENaC along with full-length β -ENaC; however, the appearance of cleaved forms of ENaC was not investigated in these studies. It has recently been demonstrated that brief treatment with aldosterone results in the appearance of cleaved ENaC subunits, although these changes tended to be associated with concomitant decreases in the total cellular levels of full-length subunits (47). These results indicate that changes in processing and maturation of channels occur rapidly enough to regulate ENaC activity in native kidney. In a follow-up to this study, Frindt *et al.* (48) employed a novel biotinylation approach in whole rat kidney that demonstrated that aldosterone or salt depletion led to an increase in surface expression of full-length β -ENaC, but primarily the cleaved form of γ -ENaC. Neither full-length nor cleaved α -ENaC was clearly demonstrated by this technique, but membrane fractionation studies suggested an increase in both cleaved and full-length α -ENaC. These results, similar to our findings in A6 cells (9), are consistent with the notion that aldosterone leads to increased surface expression primarily of cleaved fully mature channels in rat kidney and do not imply a reorganization of ENaC stoichiometry. Interestingly, in rat kidney, stimulation of endogenous aldosterone production by salt restriction led to cleavage of both α - and γ -ENaC, whereas stimulation via K^+ loading led to the appearance of cleaved γ -ENaC, but not α -ENaC (47). Maturation and cleavage may therefore not necessarily involve all subunits of the channel. Such a phenomenon would have to be invoked if proteolysis were to explain the observation by Planès *et al.* (49) that hypoxia selectively reduces and β_2 -agonists selectively increase the apical membrane amounts of full-length β - and γ -ENaC in alveolar epithelial cells without affecting the surface expression of full-length α -ENaC or the whole cell amounts of any of the three subunits.

The above observations also suggest the second mechanism by which proteolysis may contribute to the phenomenon of non-coordinate regulation: the possibility that differences exist in trafficking, particularly following hormonal stimulation, between channels composed of cleaved *versus* uncleaved subunits. Little is known at present about whether channels made up of different combinations of subunits traffic differently. We have shown a significant difference in the rate of current decay of $\alpha\gamma$ -channels compared with channels containing all three subunits after cycloheximide addition (38), but the effect of proteolytic activation on trafficking is not clear. Indirect evidence in an overexpression system suggests, however, that the rate of ENaC internalization is not altered by proteolytic cleavage (50). The N- and C-terminal fragments of cleaved subunits are known to remain associated (44), and cleaved channels are retrieved in a Nedd4-2-dependent manner (50). As it has been demonstrated that prolonged half-life leads to an increase in cleaved ENaC subunits (51) and fully mature subunits have been shown to be enriched in clathrin-coated vesicles (33), the possibility exists that cleaved subunits may traffic differently from unprocessed channels *in vivo*. Even if differential appear-

ance of selected cleaved forms of α - and/or γ -ENaC could explain the aspects of non-coordinate regulation suggested by distinct changes in expression levels of full-length subunits, other aspects, including the differing half-lives of subunits (9), the presence of cellular pools of individual subunits (β and γ) that traffic to the apical membrane following induction of α -ENaC (17, 18), and the differential regulation of message for the subunits, remain to be understood.

Conclusion

Since our initial review of this subject, multiple examples of non-coordinate regulation of ENaC subunit protein or message have continued to appear in the literature (22, 23, 52–54). It seems likely that more than one mechanism is required to fully explain this phenomenon. Physiological control of this channel is even more complex than we previously appreciated. On the other hand, many aspects of non-coordinate regulation can now be explained by these new data.

Critical questions include the location and mechanism of ENaC assembly and the state of the subunits not present in trimeric channels. In addition, new awareness of the requirement for proteolytic cleavage for ENaC activity necessitates re-examination of biochemical studies aimed at elucidating the trafficking of ENaC subunits under normal conditions and in response to physiological stimuli. The biochemical measurements of ENaC subunit levels do not necessarily correlate with physiologically relevant changes in functional channels or with observed changes in activity. Future studies will need to monitor changes in ENaC levels, processing, and activity to generate a comprehensive model to describe the regulation of this channel. Channel remodeling is the most speculative explanation for non-coordinate regulation and the one for which the least evidence exists at present. It remains to be determined if ENaC subunits that have reached the plasma membrane can be retrieved to the *trans*-Golgi network for remodeling and return to the apical membrane during the course of regulated transport. These intriguing questions will need to be explored further to gain a full appreciation of the physiologically relevant processes underlying ENaC control *in vivo*.

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