Cell surface expression and biosynthesis of epithelial Na⁺ channels

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The epithelial Na⁺ channel (ENaC) complex is composed of three homologous subunits: α , β and γ . Mutations in ENaC subunits can increase the number of channels on the cell surface, causing a hereditary form of hypertension called Liddle's syndrome, or can decrease channel activity, causing pseudohypoaldosteronism type I, a salt-wasting disease of infancy. To investigate surface expression, we studied ENaC subunits expressed in COS-7 and HEK293 cells. Using surface biotinylation and protease sensitivity, we found that when individual ENaC subunits are expressed alone, they traffic to the cell surface. The

subunits are glycosylated with high-mannose oligosaccharides, but seem to have the carbohydrate removed before they reach the cell surface. Moreover, subunits form a complex that cannot be disrupted by several non-ionic detergents. The pattern of glycosylation and detergent solubility/insolubility persists when the N-terminal and C-terminal cytoplasmic regions of ENaC are removed. With co-expression of all three ENaC subunits, the insoluble complex is the predominant species. These results show that ENaC and its family members are unique in their trafficking, biochemical characteristics and post-translational modifications.

INTRODUCTION

The epithelial Na⁺ channel (ENaC) is present in the apical membrane of many Na⁺-absorbing epithelia, including kidney, intestine and airway [1–3]. In these epithelia it provides a pathway for Na⁺ absorption and a mechanism for regulating the rate of transepithelial Na⁺ transport. ENaC is composed of three subunits, α , β and γ [4–8]. The subunits have similar topologies, with two hydrophobic transmembrane domains, intracellular N-termini and C-termini and a large cysteine-rich extracellular domain that contains numerous sites for N-linked glycosylation. The expression of α ENaC in oocytes generates only very small currents, and the expression of either β ENaC or γ ENaC generates no current [5,7]. However, the co-expression of α ENaC, β ENaC and γ ENaC in heterologous cells generates large Na⁺ currents. This result provides functional evidence that the three ENaC subunits interact. Biochemical evidence of an interaction has come from the finding that ENaC subunits can be co-immunoprecipitated [9]. Specific mutations at the C-terminus of β ENaC and γ ENaC cause Liddle's syndrome, a genetic form of hypertension due to increased Na⁺ absorption in distal renal tubules [10,11]. Mutations associated with Liddle's syndrome increase the number of ENaC channels at the apical membrane, thereby increasing Na⁺ absorption [12,13]. Conversely, mutations that disrupt ENaC channel function cause pseudohypoaldosteronism type 1, a severe salt-wasting disease [14].

The goal of this work was to begin to understand the biosynthesis of ENaC, including the process of assembly and trafficking. As a starting point we examined whether individual ENaC subunits are able to traffic to the cell surface or whether they must first assemble into a heteromultimeric complex. On the basis of the finding that co-expression of all three subunits is required for generation of large Na⁺ currents, we initially hypothesized that ENaC would have a biosynthetic mechanism similar to that observed with the acetylcholine and T-cell receptors. In other words, we hypothesized that ENaC subunits would be retained in the endoplasmic reticulum (ER) until they formed a heteromultimeric complex, which could then traffic to

the cell surface. This speculation was also consistent with results in which binding of a radiolabelled antibody to epitope-tagged subunits on the cell surface seemed to require all three subunits [12]. Interestingly, our results suggest that individual subunits expressed alone are able to traffic to the cell surface.

MATERIALS AND METHODS

Cell culture and reagents

COS-7 and HEK-293 cells were obtained from the American Type Culture Collection (ATCC) and maintained in culture with Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum, and in Eagle's minimal essential medium with 10% (v/v) horse serum respectively. Both cell lines were incubated in a humidified atmosphere of air/CO₂ (19:1). For transfection of COS-7 cells, 10⁷ cells were electroporated with 30 μ g of plasmid DNA. The cells were plated and grown for 24–48 h before study. HEK-293 cells were seeded at 40% confluency. They were transfected 24 h later with 10 μ g of plasmid DNA per dish by using the calcium phosphate Profection kit (Promega, Madison, WI, U.S.A.) and studied 48 h later. All experiments were performed in COS-7 and repeated in 293 cells with the exception of those shown in Figures 5, 6 and 8.

A monoclonal antibody against the FLAG epitope was obtained from Kodak (Rochester, NY, U.S.A.). Brefeldin A was purchased from Calbiochem (La Jolla, CA, U.S.A.). Endoglycosidase H, N-glycanase, deoxymannojirimycin, castanospermine and swainsonine were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

DNA constructs

Construction of the cDNA species encoding α , β and γ human ENaC, brain Na⁺ channel (BNC1) and Phe-Met-Arg-Phe-NH₂ (FMRF)-activated Na⁺ channel (FaNaCh) (all in the pMT3

Abbreviations used: BNC1, brain Na⁺ channel; ENaC, epithelial Na⁺ channel; ER, endoplasmic reticulum; FaNaCh, Phe-Met-Arg-Phe-NH₂ (FMRF)-activated Na⁺ channel; NHS-LC-biotin, sulphosuccinimidyl 6-(biotinamido)hexanoate.

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expression vector) was as described elsewhere [7,15,16]. The FLAG epitope (DYKDDDDK) was introduced into full-length subunit cDNA, immediately upstream of the stop codon, by using the Muta-Gene phagemid *in vitro* mutagenesis kit (BioRad, Hercules, CA, U.S.A.). Epitope-tagged constructs were then cloned into pMT3 for expression. This did not alter the function of the subunit in *Xenopus* oocytes or epithelia or the ability to associate with other subunits [9]. Deletion of the N-terminal region of α ENaC ($\Delta 2$ –59) and β ENaC ($\Delta 2$ –79) was accomplished by PCR-driven mutagenesis. For the β ENaC Δ C-terminal construct, a stop codon was inserted after residue 563.

Immunoprecipitation, immunoblotting and deglycosylation

After transfection, cells were grown in medium containing 10 μ M amiloride to prevent cell swelling. For immunoprecipitation, cells were washed three times in ice-cold PBS containing 0.1 mM CaCl_2 and 1 mM MgCl_2 (PBS $\text{Ca}^{2+}/\text{Mg}^{2+}$) and lysed in Tris-buffered saline (TBS), pH 7.4, containing 1% (v/v) Triton X-100 (Pierce, Rockford, IL, U.S.A.) and the following protease inhibitors: 0.4 mM PMSF, 20 $\mu\text{g}/\text{ml}$ aprotinin, 20 $\mu\text{g}/\text{ml}$ leupeptin and 10 $\mu\text{g}/\text{ml}$ pepstatin A. Lysates were centrifuged at 16000 g at 4 °C; the Triton-soluble supernatant was incubated with 5 μg of anti-FLAG antibody. For immunoprecipitation of Triton-insoluble ENaC, the pellet generated by the 16000 g spin was solubilized in 100 μl of 2% (w/v) SDS/1% (v/v) 2-mercaptoethanol/50 mM Tris (pH 7.4)/1 mM EDTA by heating to 90 °C for 5 min. After solubilization, 1.0 ml of TBS with 1% (v/v) Triton X-100 was added and ENaC was immunoprecipitated with anti-FLAG antibody. Antigen-antibody complexes were precipitated with immobilized Protein A (Pierce) and precipitates were washed three times in TBS with 1% (v/v) Triton X-100 and eluted with sample buffer [4% (w/v) SDS/65 mM Tris (pH 6.8)/100 mM dithiothreitol/20% (v/v) glycerol/0.005% Bromophenol Blue]. Proteins were separated by SDS/PAGE [7% (w/v) gel] and transferred to nitrocellulose. Immunoblots were blocked with 5% (w/v) BSA, incubated first with primary antibody (2 $\mu\text{g}/\text{ml}$ anti-FLAG), then with a horseradish peroxidase-coupled secondary antibody (Amersham, Arlington Heights, IL, U.S.A.) at 1:20000 dilution. Bound antibody was detected by chemiluminescence (Pierce). For deglycosylation, the immunoprecipitates were incubated overnight with either endoglycosidase H or N-glycanase at 37 °C before elution off the Protein A-Sepharose beads and SDS/PAGE.

Biotinylation of protein at the cell surface

COS-7 or 293 cells expressing ENaC were washed three times with ice-cold PBS $\text{Ca}^{2+}/\text{Mg}^{2+}$ on ice, labelled with 0.5 mg/ml sulphosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) (Pierce) for 30 min on ice, washed again with PBS and lysed as above. The Triton-soluble and Triton-insoluble fractions were incubated with 100 μl of immobilized streptavidin (Pierce) for 1 h at 4 °C. The material not bound by the beads was immunoprecipitated with anti-FLAG antibody. The streptavidin beads were washed with TBS/1% (v/v) Triton X-100; biotinylated proteins were eluted by being heated to 95 °C in Laemmli sample buffer. The immunoprecipitated protein and the streptavidin-precipitated protein were separated by SDS/PAGE and immunoblotted with anti-FLAG antibody and detected by chemiluminescence.

Protease digestion of cell-surface proteins

COS-7 cells expressing ENaC subunits were gently scraped into ice-cold TBS containing 10 mM CaCl_2 (TBS_c) and centrifuged at

500 g at 4 °C. The cells were resuspended in TBS_c containing 100 $\mu\text{g}/\text{ml}$ proteinase K (Ambion, Austin, TX, U.S.A.) and digested for 5 min on ice. The reaction was stopped by washing the cells three times with ice-cold Ca^{2+} -free TBS containing 1 mM PMSF. The cells were then lysed and ENaC was immunoprecipitated as above.

RESULTS

Expression of ENaC at the cell surface

To evaluate biosynthesis, we expressed human ENaC subunits in COS-7 and 293 cells. Expression of membrane proteins in COS and 293 cells has provided critical insight into membrane protein biosynthesis, folding, assembly and trafficking. We immunoprecipitated individual epitope-tagged subunits. Consider first the Triton X-100-soluble α subunit (Figure 1, lane 1). Some of the protein migrated at approx. 75 kDa (solid arrowhead); this is consistent with its predicted molecular mass based on primary structure and the observed migration of unglycosylated α ENaC produced by translation *in vitro* [8]. A greater fraction of the Triton-soluble α subunit migrated at approx. 90 kDa (open arrowhead); this is consistent with the migration of glycosylated subunit produced by translation *in vitro* in the presence of microsomal membranes [8]. We found similar patterns with soluble β and γ subunits (Figure 1, lanes 3 and 5). However, for each subunit, much of the protein was Triton-insoluble. Figure 1 (lane 2) shows that much of the α subunit was Triton-insoluble and migrated at a rate that suggested that it was either not glycosylated or had only a small amount of attached carbohydrate (solid arrowhead). The β and γ subunits showed similar patterns (Figure 1, lanes 4 and 6). In addition, β ENaC, and to a smaller extent γ ENaC and α ENaC, showed bands of intermediate size that tended to be more prominent in the insoluble fractions; this migration suggests a partly glycosylated protein. We further examine solubility and glycosylation below.

To evaluate surface expression, we used two different methods: cell surface biotinylation and sensitivity to protease. For cell surface biotinylation, we studied intact cells expressing individual ENaC subunits. The cells were treated with NHS-LC-biotin to label surface proteins. Internalization of the biotinylation reagent was prevented by incubation at 4 °C [17]. The Triton-soluble and Triton-insoluble fractions were then incubated with immobilized

Figure 1 Expression of ENaC family members

Epitope-tagged ENaC subunits were expressed in COS-7 cells and studied 24–48 h after transfection. Cells were incubated for 4 h in methionine-free medium containing 50 μCi of [^{35}S]methionine. These cells were lysed in 1% (v/v) Triton X-100 and divided into Triton-soluble (S) and insoluble (I) fractions as described in the Materials and methods section. ENaC subunits were immunoprecipitated from each fraction and separated by SDS/PAGE. Solid arrowheads indicate the migration of unglycosylated subunits; open arrowheads indicate the migration of glycosylated subunits. The percentages (means $\pm$ S.D.) of total ENaC protein that were Triton-insoluble were calculated by phosphorimaging: α ENaC, $39 \pm 6\%$; β ENaC, $48 \pm 12\%$; γ ENaC, $34 \pm 3\%$ ($n = 2$ –5).

Figure 2 Cell surface expression of ENaC

(a) Cell surface biotinylation of ENaC. HEK-293 cells were transfected with individual ENaC subunits 48 h before surface-labelling. Cells were incubated with 0.5 mg/ml NHS-LC-biotin for 30 min at 4 °C and then solubilized with 1% (v/v) Triton X-100. The Triton-soluble (S) and insoluble (I) fractions were then incubated with immobilized streptavidin. Biotinylated protein was eluted from the streptavidin and detected by immunoblotting in the lanes headed '+'. ENaC was immunoprecipitated from the material not bound by the streptavidin and is shown in the lanes headed '-'. The band marked with an asterisk was non-specific and unrelated to ENaC expression. Solid arrowheads indicate the migration of unglycosylated subunits and open arrowheads indicate the migration of glycosylated subunits. Although biotinylation of surface proteins was not likely to be stoichiometric, the following percentages (means $\pm$ S.D.) of insoluble ENaC were biotinylated: α ENaC, 47 $\pm$ 11%; β ENaC, 47%; γ ENaC, less than 5% ($n = 3$). (b) Protease digestion of cell-surface ENaC. COS-7 cells expressing ENaC subunits were metabolically labelled with [³⁵S]methionine for 4 h; 100 μ g/ml proteinase K was then applied for 5 min at 0 °C. The protease was removed by washing with 1 mM PMSF, the cells were solubilized and ENaC was immunoprecipitated from the Triton-soluble and Triton-insoluble fractions. Triton-insoluble ENaC degraded by proteinase K was: α ENaC, 63 $\pm$ 16%; β ENaC, 82 $\pm$ 13%; γ ENaC, 92 $\pm$ 8% ($n = 2$).

streptavidin. Protein bound to the streptavidin was eluted by boiling in SDS sample buffer, subjected to electrophoresis and detected by immunoblotting. Protein that did not bind to streptavidin was immunoprecipitated and then immunoblotted. Figure 2(a) shows that large amounts of α and β subunits were biotinylated (lanes 4 and 8), whereas a smaller amount of γ subunit (lane 12) was labelled. In contrast, little if any of the soluble α and β subunits were biotinylated (Figure 2a, lanes 3 and 7), suggesting that soluble subunits reside in an intracellular compartment. These results suggest that when they are expressed alone, individual subunits are present on the cell surface.

To assess sensitivity to extracellular proteases, we treated intact cells with proteinase K at 0 °C. Under these conditions, cell-surface proteins are exposed to protease and degraded, whereas intracellular proteins are protected [18]. Figure 2(b) shows that proteinase K degraded most of the insoluble α , β and γ subunits (lanes 4, 8 and 12), whereas the Triton-soluble protein remained intact (lanes 3, 7 and 11). The γ subunit was efficiently degraded (Figure 2b, lane 12), suggesting that the small amount of biotinylated γ subunit in Figure 2(a) (lane 12) was due to inefficient biotinylation rather than to an intracellular location. The sensitivity to protease and the biotinylation experiments

Figure 3 Digestion of ENaC with N-Glycanase and endoglycosidase H

ENaC subunits were immunoprecipitated from transfected COS-7 cells as described above and digested overnight at 37 °C with N-glycanase or endoglycosidase H. Immunoprecipitates were then immunoblotted as above. (a) Triton-soluble protein; (b) insoluble protein.

agree and suggest that when expressed alone, all three subunits were present on the cell surface, subunits in the plasma membrane were Triton-insoluble, and subunits on the surface migrated at a position consistent with protein with minimal glycosylation.

Glycosylation of ENaC

To evaluate whether the two bands of different sizes represented differences in glycosylation, immunoprecipitated protein was treated with either endoglycosidase H or N-glycanase. Triton-soluble α and β subunits were digested completely to a single lower-molecular-mass band with endoglycosidase H (Figure 3a, lanes 3 and 6). N-glycanase had a similar effect (Figure 3a, lanes 2 and 5). In contrast, endoglycosidase H and N-glycanase had a much smaller effect on the mobility of Triton-insoluble protein (Figure 3b). This result suggests that the Triton-insoluble protein does not possess a large amount of carbohydrate; the sharpening of the bands with enzyme treatment suggests a small amount of glycosylation. The effect of endoglycosidase H suggests that the glycosylated protein was located in the ER. However, subunits on the cell surface (i.e. the insoluble protein) showed little if any glycosylation. This pattern suggests that subunits are glycosylated in the ER and then carbohydrate is trimmed or removed before subunits reach the cell surface. Migration of the subunits after treatment with N-glycanase and endoglycosidase H also suggests that proteolysis of the protein was not responsible for the electrophoretic patterns shown in Figures 1 and 2.

We tested several inhibitors of ER glycosidases to see whether they could inhibit deglycosylation and cause the accumulation of glycosylated protein. Figure 4 shows that deoxymannojirimycin, an inhibitor of α -mannosidase I, did not alter the glycosylation pattern. We obtained similar results with swainsonine and castanospermine, inhibitors of mannosidase II and glucosidase I respectively (results not shown). The deglycosylation of ENaC

Figure 4 Effect of deoxymannojirimycin on Triton solubility and glycosylation pattern of β ENaC

HEK-293 cells were transfected with β ENaC, treated with the indicated concentrations of deoxymannojirimycin for 24 h and then immunoprecipitated from the Triton-soluble and Triton-insoluble fractions. Immunoprecipitates were analysed by immunoblotting. Abbreviations: S, soluble protein; I, insoluble protein.

therefore seems to involve complete or near-complete removal of carbohydrate that is not dependent on the action of trimming enzymes inhibited by these reagents.

Solubility of ENaC

Figure 1 shows that much of ENaC was insoluble. Although the relative amounts of soluble and insoluble ENaC detected by immunoblotting varied between experiments, Triton-insoluble ENaC was consistently predominant. Resistance to non-ionic detergent has been observed for caveolar proteins, cytoskeletal proteins and some oligomeric membrane proteins [19–21]; some of those proteins can be solubilized with other detergents at alkaline pH or at high ionic strength. We found that lysis with octylglucoside at pH 9.0 failed to solubilize ENaC (results not shown), suggesting that ENaC might not reside in caveolae [20]. Moreover we were unable to solubilize a greater portion of

Figure 5 Effect of tunicamycin (a) and brefeldin A (b) on Triton solubility and glycosylation pattern of β ENaC

(a) COS-7 cells were transfected with β ENaC 48 h before study. For 18 h before lysis, half of the cells were treated with tunicamycin (5 μ g/ml). β ENaC was immunoprecipitated and detected by immunoblotting. (b) COS-7 cells expressing epitope-tagged α ENaC were treated with 100 ng/ml brefeldin A (BFA) for 24 h before lysis. α ENaC was then immunoprecipitated and detected by immunoblotting. Longer exposures show the presence of soluble α ENaC, which did not change with treatment with tunicamycin or brefeldin A. Abbreviations: S, soluble protein; I, insoluble protein.

Figure 6 Glycosylation and Triton-solubility of cytoplasmic deletion constructs

cDNA species encoding $\alpha_{\Delta N\text{-term}}$ ENaC (a), $\beta_{\Delta N\text{-term}}$ ENaC (b) and $\beta_{\Delta C\text{-term}}$ ENaC (c) were constructed as described above. COS-7 cells were transfected with the indicated ENaC plasmid and studied 48 h later. Proteins were labelled metabolically with [35 S]methionine 4 h before lysis. ENaC was immunoprecipitated and analysed by SDS/PAGE. Abbreviations: S, soluble protein; I, insoluble protein.

ENaC with digitonin, CHAPS, Nonidet P40 or deoxycholate, even at salt concentrations as high as 2.5 M NaCl (results not shown). These findings were not cell-type-specific; we obtained similar results in COS-7 and 293 cells. Because previous work has shown that ENaC can associate with spectrin [22], it seemed possible that insolubility in Triton might be due to association with the cytoskeleton. However, solubilization with 2.5 M NaCl and treatment of cells with the cytoskeletal disrupters cytochalasin, nocodazole or colchicine failed to increase the fraction of soluble protein (results not shown).

To learn whether the acquisition of carbohydrate after translation was essential for the Triton-insolubility of ENaC, COS-7 cells transfected with β ENaC were treated with tunicamycin. Figure 5(a) shows that tunicamycin did not prevent the protein from becoming insoluble. We also examined whether subunits become insoluble before reaching the plasma membrane: we used brefeldin A to block anterograde transport to the plasma membrane [23]. Figure 5(b) shows that brefeldin A did not decrease the amount of Triton-insoluble ENaC or increase the amount of soluble protein. These results indicate that insolubility in Triton occurs before the protein reaches the cell surface.

To test the hypothesis that the cytoplasmic portions of ENaC subunits are responsible for the insolubility, we deleted these regions and examined solubility. Figure 6 shows that removal of the cytoplasmic regions of ENaC did not affect the distribution between Triton-soluble and Triton-insoluble fractions, suggesting that potential interactions involving these regions are not essential for insolubility in Triton.

Figure 7 Effect of co-expression of ENaC subunits on Triton-solubility of βENaC

(a) HEK-293 cells were transfected with epitope-tagged βENaC alone (lanes 1 and 2), with αENaC (lanes 3 and 4), with γENaC (lanes 5 and 6), or with both αENaC and γENaC (lanes 7 and 8). After lysis, βENaC was immunoprecipitated and detected by immunoblotting. Percentages (means ± S.D.) of total βENaC solubilized with Triton X-100 were: β, 21 ± 4%; αβ, 14 ± 8%; αβγ, 8 ± 5% ($n = 2$). (b) Effect of co-expression of ENaC subunits on surface expression. COS-7 cells expressing only epitope-tagged βENaC or epitope-tagged βENaC with αENaC and γENaC (without epitopes) were treated for 5 min with 100 μg/ml proteinase K at 0 °C. Cells were washed with 1 mM PMSF and solubilized; βENaC was immunoprecipitated and then immunoblotted. Abbreviations: S, soluble protein; I, insoluble protein.

Figure 8 Glycosylation pattern and Triton-solubility of BNC1 and FaNaCh

COS-7 cells were transfected with epitope-tagged BNC1 or FaNaCh and solubilized as above. Protein was immunoprecipitated and then detected by immunoblotting. Abbreviations: S, soluble protein; I, insoluble protein.

We also examined the effect of co-expressing ENaC subunits. Figure 7(a) shows that the amount of βENaC in the Triton-soluble fraction decreased as other subunits were co-expressed. The glycosylation pattern was unchanged. This result suggests that the Triton-soluble glycosylated form of βENaC, which might represent protein in the ER, might traffic either to the cell surface or some other compartment more efficiently in the presence of other subunits. However, digestion with proteinase K suggested that there was not a large increase in the amount of βENaC present at the cell surface with co-expression of all three subunits (Figure 7b).

ENaC is a member of the DEG/ENaC protein family [24,25]. To learn whether this pattern of solubility is unique to ENaC members of the family, we studied two other DEG/ENaC proteins, BNC1 (also called BNaCl and MDEG) [16,26,27] and FaNaCh [28]. We found a pattern similar to that obtained with ENaC (Figure 8); much of the protein was found in the Triton-insoluble fraction.

DISCUSSION

Biosynthesis of ENaC

ENaC shows several novel features in its biosynthesis. When expressed alone, single subunits are produced in the ER and glycosylated. In this same time frame the protein forms a homo-oligomer, as demonstrated in previous studies [9]. Most of the carbohydrate is removed either in the ER or another intracellular compartment. At some point along the biosynthetic pathway, individual subunits become Triton-insoluble and are expressed on the cell surface. With co-expression, the three subunits assemble in the ER to form a hetero-multimeric complex [9] and the biosynthetic process is qualitatively similar. Association of the subunits decreases steady-state levels of soluble βENaC, perhaps by influencing the rate at which they leave the ER. One limitation of our study is that it does not examine apical compared with basolateral targeting.

Glycosylation of ENaC

Our results suggest that ENaC subunits at the cell surface have minimal amounts of attached carbohydrate. This could occur in at least two ways. ENaC might be glycosylated in the ER but then carbohydrate would be removed before ENaC reached the cell surface. Alternatively, there might be two pools of ENaC, one that receives little if any glycosylation and moves directly to the cell surface and one that is glycosylated but does not reach the cell surface. We favour the first alternative for its simplicity. In addition, we previously showed that human and rat ENaC subunits produced by translation *in vitro* in the presence of microsomes are glycosylated [7,8,29]. We found that three inhibitors of ER glycosidases, deoxymannojirimycin, swainsonine and castanospermine, failed to prevent deglycosylation. Moreover, ENaC did not seem to acquire endoglycosidase H-resistant glycosylation in the *cis*-Golgi. These observations suggest that a novel ER glycosidase might have been responsible for deglycosylation. There is a precedent for the deglycosylation of proteins along the biosynthetic pathway. The opsin protein in *Drosophila* acquires high-mannose glycosylation in the ER and loses this modification by N-glycanase activity somewhere along the biosynthetic pathway [30]. In addition, a mammalian N-glycanase has recently been purified from rat liver ER that completely removes endoglycosidase H-sensitive carbohydrates from glycoproteins [31]. We speculate that it or a related enzyme might be responsible for the enzymic deglycosylation of ENaC subunits. However, we are unable to test this hypothesis because there is no known inhibitor of this N-glycanase for use in cultured cells.

Solubility of ENaC

Before ENaC arrives at the cell surface it becomes insoluble to non-ionic detergents. There are several potential explanations for the insolubility. Although we used conditions that solubilize some caveolar and cytoskeleton-associated proteins, it is possible that such associations could have persisted. ENaC subunits might themselves form high-molecular-mass oligomers that are resistant to detergent extraction. That is true of p63, which forms insoluble homomultimers in the ER [21]. Additionally, ENaC might associate with other proteins that cause insolubility. Although such an interaction does not seem to involve the cytoplasmic domains, there is no information yet on the function of the large extracellular domains of ENaC/DEG family members. These regions contain multiple cysteine-rich repeats [4,5] that might associate with each other or with other extracellular proteins.

Presence of ENaC subunits on the cell surface

Because the production of large amiloride-sensitive Na^+ currents requires the expression of all three ENaC subunits, we initially hypothesized that the assembly of all three subunits would be required for cell surface expression. This would be similar to assembly and trafficking of the T-cell receptor and the MHC class I complex [32,33]. However, our results, with two different techniques and two different mammalian cell lines, indicate that individual subunits, probably assembled as a homomultimer [9], can traffic to the cell surface.

Another study suggested that only channels composed of the α , β and γ subunits of rat ENaC are expressed at the cell surface [12]. We think it unlikely that the difference is due to the use of human compared with rat ENaC. Reasons for the difference in conclusions might have to do with the methods used. The earlier study relied on the binding of a radiolabelled antibody to an extracellular epitope that was inserted in the extracellular region of each subunit. It is possible that antibody might not have detected the epitope unless all three subunits were co-assembled, and all epitopes might not have been equally accessible. Perhaps a more likely explanation for the difference is that the previous study [12] was performed in *Xenopus* oocytes incubated at 20–25 °C, whereas our studies were performed in two different mammalian cell lines at 37 °C; these differences might influence biosynthesis and trafficking.

It is interesting to consider the physiological consequences of the cell surface expression of individual types of subunit. For the heteromultimeric T cell receptor, allowing individual subunits to travel to the cell surface could pose a biological risk of abnormal stimulation of the immune system by antigen-presenting cells. In contrast, allowing individual β and γ subunits to reside on the cell surface would seem to pose little risk in terms of increasing Na^+ permeability, because βENaC and γENaC do not form ion channels on their own [5,7,34]. If some αENaC were present on the cell surface, the resulting Na^+ permeability would appear very small. This speculation might also apply to other members of the DEG/ENaC family. For members that function as homomultimers, a checkpoint that monitors subunit composition is not required. In contrast, members that function as heteromultimers would not require a checkpoint based on subunit composition if incorrectly assembled complexes lacked function.

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