Distinct Pools of Epithelial Sodium Channels Are Expressed at the Plasma Membrane*

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The epithelial Na⁺ channel (ENaC) is assembled in the endoplasmic reticulum from three structurally related subunits (α, β, and γ). Channel maturation within the biosynthetic pathway involves cleavage of the α and γ subunits by furin and processing of N-linked glycans on α, β, and γ to complex type. Both mature and immature subunits have been observed at the surface of stably transfected Madin-Darby canine kidney cells. We have examined whether channel maturation is an all-or-none event or whether heterogeneous processing of channel subunits occurs within an individual channel complex. Using an immobilized lectin to isolate proteins with complex type N-glycans, we found that individual channel complexes with mature subunits lack immature subunits. Furthermore, terminal processing of N-glycans on ENaC subunits was not dependent on cleavage of ENaC subunits, and proteolysis of channel subunits was not dependent on prior processing of N-glycans. Our results suggest that processing of subunits within an individual channel complex is an all-or-none event such that channels present on the cell surface contain either all mature or all immature subunits. The presence of immature channel complexes at the plasma membrane provides epithelial cells with a reserve of poorly functional channels that can be activated by proteases in post-Golgi compartments.

Epithelial Na⁺ channels (ENaCs) are expressed at the apical plasma membrane of high resistance Na⁺-transporting epithelia. These channels are composed of three structurally related subunits, termed α, β, and γ, that share a common structure, with two membrane-spanning domains separated by a large (~450 residue) ectodomain and cytosolic amino and carboxyl termini. ENaC subunits are thought to assemble in the endoplasmic reticulum (ER) where they also undergo N-linked glycosylation (1–3). The ectodomains of ENaC α and γ subunits have consensus sites for cleavage by furin (4). Furin is a member of the proprotein convertase family of serine proteases that is expressed primarily in the trans-Golgi network and participates in the biosynthetic processing of proteins (5). We recently observed that ENaC α and γ subunits are cleaved by furin (4), although it is likely that ENaC can be processed by other serine proteases, including prostatin and related enzymes (6–9).

ENaC likely transits through the Golgi complex during its biogenesis. Expression of active channels at the plasma membrane is blocked by treatment of cells with the fungal metabo-lite brefeldin A, which disrupts assembly of cytosolic coats that are required for intra-Golgi transport (10, 11). Most N-glycans on glycoproteins that transit the Golgi complex during biogenesis are modified from a high mannose type to a complex type by a series of enzymatic steps (12, 13). However, it is apparent that two distinct pools of ENaC subunits are expressed at the plasma membrane: (i) mature subunits that have N-glycans processed to complex type and cleaved α and γ subunits; and (ii) immature subunits that have high mannose type N-glycans on full-length (i.e. non-cleaved) subunits (see Fig. 1A) (14). These observations raise the question of whether individual channels at the plasma membrane are composed of subunits that either (i) have all undergone maturation in the biosynthetic pathway (i.e. complex type N-glycans and cleaved α and γ subunits) or (ii) have subunits that have all escaped maturation. Alternatively, a mix of both mature and immature subunits might be present within an individual channel complex. We have used a lectin that recognizes sialic acid to isolate ENaC subunits that have complex type N-glycans and demonstrate that biosynthetic processing of ENaC subunits within an individual channel complex is an all-or-none event.

EXPERIMENTAL PROCEDURES

Vectors, Antibodies, and Cell Culture—Preparation of mouse ENaC subunits with carboxyl-terminal epitope tags or mutant α and γ subunits lacking the sites for furin cleavage were described previously (4, 14). Antibodies and procedures for immunoprecipitation and immunoblotting from transfected MDCK cells were described previously (4, 14). Where indicated, kifunensine (5 μg/ml, Biomol International L.P., Plymouth Meeting, PA) was added to MDCK cells after transfection with ENaC.

Isolation of Processed Channels—MDCK cells growing on 6-well plates were transfected with either no DNA (mock control) or the epitope-tagged combinations of αβγ ENaC noted in each figure legend as described previously (4, 14). After 24 h, each 35-mm well was extracted in 0.5 ml of lysis buffer (14) and centrifuged for 7 min at 14,000 rpm in a microcentrifuge to remove cell debris. Immobilized lectin from Sambucus nigra (SNA, 200 μl of slurry) from EY Laboratories, Inc. (San Mateo, CA) was added to the supernatants and incubated overnight at 4 °C on a rotating wheel. The beads were pelleted by centrifugation and washed in 0.1 M lactose at room temperature for 1 h. The beads were again pelleted, and the supernatant was recovered for immunoprecipitation with ENaC with anti-epitope tag antibodies and immunoblotting. Mouse anti-HA antibody from Covance Inc. (Princeton, NJ) was used for ENaC immunoprecipitations from cell extracts before SNA precipitation and immunoblotting; and goat anti-V5 antibody conjugated to agarose from Novus Biologicals (Littleton, CO) was used for ENaC immunoprecipitation from the SNA eluate.

Immunoblotting of Lamin—MDCK cells transfected, or not transfected, with ENaC were treated with sulfo-N-hydroxysuccinimidy-SS-
Biotin and extracted with detergent solution as described previously (14). Nuclear lamin was immunoprecipitated from cell extracts with mouse anti-lamin A/C IgM (clone XB10, Covance Inc.) using mouse anti-IgG conjugated to agarose from Sigma and incubated overnight at 4 °C on a rotating wheel. Lamin was eluted by boiling for 2 min, and 10% was retained for immunoblotting with mouse anti-lamin A/C antibody (clone 14) from Upstate (Waltham, MA). Any biotinylated lamin was recovered from the remainder of the immunoprecipitate (90%) using Immunopure immobilized streptavidin (Pierce) and analyzed by immunoblotting for lamin.

**RESULTS**

ENaC subunits with unique carboxyl-terminal epitope tags were transiently expressed in MDCK cells in various combinations as indicated in Fig. 1B, and cell surface proteins were labeled with biotin. For each combination of epitope-tagged α, β, and γ ENaC, the V5-tag was present on the subunit of interest. ENaC was recovered by immunoprecipitation with anti-V5 antibodies (10% of total), and biotinylated ENaC subunits expressed at the plasma membrane were recovered with streptavidin-conjugated beads from the anti-V5 immunoprecipitates (90%). Expression of ENaC subunits was analyzed by probing the precipitates on immunoblots using anti-V5 antibodies. For each subunit, two distinct bands were detected in immunoprecipitates from total cell lysates and at the plasma membrane (Fig. 1B). These correspond to bands that we previously observed and characterized as representing immature (or non-processed) subunits (95 kDa for α, 96 kDa for β, 93 kDa for γ) and mature (or fully processed) subunits (65 kDa for α, 110 kDa for β, 75 kDa for γ) (see Fig. 1A). We confirmed that biotin was specifically labeling plasma membrane proteins and did not label an intracellular protein (nuclear lamin, Fig. 1C). These data are in agreement with our previous observation that both mature and immature ENaC subunits are expressed at the plasma membrane.

We next examined whether all subunits within an individual channel complex undergo processing in the biosynthetic pathway or whether a mix of both mature and immature subunits is present within an individual channel complex. Mature subunits have complex type N-glycans, so we used the lectin SNA immobilized on agarose beads to precipitate proteins containing sialylated complex type N-glycans from solubilized MDCK cells expressing epitope-tagged ENaC subunits. Following elution from the SNA-agarose beads, selected ENaC subunits were immunoprecipitated and subjected to immunoblotting to detect co-immunoprecipitating subunits (Fig. 2). Only the mature forms of the β (110 kDa) and γ (75 kDa) subunits co-immunoprecipitated with α subunits that were isolated with SNA-agarose (see lanes 2 and 4); only mature forms of the α (65 kDa) and γ (75 kDa) subunits co-immunoprecipitated with β subunits isolated with SNA-agarose (see lanes 6 and 8); and only mature forms of the α (65 kDa) and β (110 kDa) subunits co-immunoprecipitated with γ subunits isolated with SNA-agarose precipitation (see lanes 10 and 12). Direct immunoprecipitation of α (lanes 5 and 9), β (lanes 1 and 11), or γ (lanes 3 and 7) from cell extracts before SNA-agarose precipitation indicated that both mature and immature forms of all three subunits were present in the initial cell extracts.

These results indicate that biosynthetic maturation of ENaC subunits (i.e. processing of N-linked glycans to complex type and cleavage of α and γ) within an individual channel complex is an all-or-none event. However, it is unclear whether (i) ENaC

![Fig. 1. Mature and immature ENaC subunits are expressed at the surface of transfected MDCK cells.](image-url)

**Fig. 1.** Mature and immature ENaC subunits are expressed at the surface of transfected MDCK cells. A, models of immature and mature α, β, and γ ENaC are illustrated. Immature subunits exhibit high mannose type N-glycans that are sensitive to treatment with Endo H, while mature subunits exhibit mostly complex type N-glycans with terminal sialic acid, making them neuraminidase-sensitive (4, 14). Mature α and γ are also cleaved. The estimated molecular weights of subunits and cleavage products based on their mobility on SDS-gels are noted in the gray ovals. B, MDCK cells were transiently transfected with either α(myec) (lane 1), α(V5), β(FLAG), γ(myec) (lane 2), α(FLAG), β(V5), γ(myec) (lane 3), or α(HA), β(FLAG), γ(V5) (lane 4). All subunits had carboxyl-terminal epitope tags. After 24 h, cell surface proteins were biotinylated on ice prior to incubation of cell extracts with anti-V5 antibodies. Ten percent of the resuspended immunoprecipitates (IP) was set aside (Total), and 90% was incubated with streptavidin-conjugated beads to recover any aberrantly biotinylated nuclear lamin (Biotinylated). Samples were analyzed by immunoblotting (IB) with anti-lamin antibodies. The arrow indicates the expected mobility of nuclear lamin (molecular mass: 75 kDa). Numbers beside the blots indicate mobility of Bio-Rad Precision Plus Protein Standards in kilodaltons (K).
N-linked glycan processing is dependent on the presence of cleaved α and γ, (ii) ENaC α and γ subunit cleavage is dependent on the presence of complex type N-linked glycans, or (iii) ENaC N-linked glycan processing and α and γ subunit cleavage are independent events. N-Glycan maturation occurs in the Golgi complex and trans-Golgi network, whereas ENaC proteolysis appears to be mediated by serine proteases, including furin and prostatasin, which are present in post-Golgi compartments (5, 6). Kifunensine is an inhibitor of the ER-localized α-mannosidase-I that is responsible for trimming mannose residues from immature N-linked glycans (15, 16). Inhibition of α-mannosidase-I prevents subsequent maturation of N-linked glycans. Whole cell and surface expression of ENaC subunits was examined in MDCK cells transiently expressing epitope-tagged ENaC subunits with and without overnight treatment with kifunensine (Fig. 3A). Cleavage of both the α and γ subunits was observed in both control and kifunensine-treated cells, indicating that α and γ subunit cleavage was not dependent on N-glycan processing. The mature (110 kDa) form of the β subunit was not seen in cells treated with kifunensine, consistent with a lack of processing of N-glycans, and thereby serving as an internal control. Furthermore, the mature α (65 kDa) and γ (75 kDa) subunits from kifunensine-treated cells migrated faster on SDS-gels, consistent with a lack of N-glycan processing. These faster migrating forms of the cleaved α and γ subunits were also completely sensitive to treatment with endoglycosidase H (Endo H), an enzyme that removes only high mannose type N-glycans from glycoproteins (data not shown).

We have previously shown that furin is a key protease responsible for cleaving α and γ subunits expressed in oocytes and CHO cells (4). Mutation of critical Arg (R) residues at consensus motifs for furin cleavage in the α and γ subunits prevented ENaC subunit cleavage when expressed in oocytes (triple mutant R205A, R208A, and R231A for α and R143A for γ) (4). These mutations also prevented α and γ subunit cleavage when expressed in MDCK cells (Fig. 3B). In the absence of ENaC cleavage, bands migrating slightly slower than the immature α (95 kDa) and γ (93 kDa) subunits were observed, consistent with processing of N-glycans. These slower migrating forms of the α and γ subunits were not observed when cells were treated with kifunensine, suggesting that these slower migrating forms had processed N-glycans. Finally, these slower migrating bands were partially resistant to Endo H indicating that these non-cleaved forms contain complex type N-glycans (data not shown).

**DISCUSSION**

We have shown that two distinct pools of ENaC subunits are expressed at the plasma membrane: (i) mature subunits that have N-glycans processed to complex type and cleaved α and γ subunits and (ii) full-length subunits that have immature, high mannose type N-glycans (Fig. 1B) (4). The processing of N-glycans on ENaC subunits was not dependent on cleavage of ENaC subunits, and proteolysis of ENaC subunits was not dependent on prior terminal processing of N-glycans (Fig. 3). Furthermore, our data demonstrate that there are distinct pools of heterologeric ENaC complexes at the plasma membrane: (i) channel complexes that contain only mature subunits and (ii) channel complexes that lack mature subunits (Fig. 2). Thus, biosynthetic processing of ENaC subunits within an individual channel complex, as determined by the processing of N-glycans and cleavage of the α and γ subunits, appears to be an all-or-none event.

Integral membrane proteins expressed at the plasma membrane are synthesized in the ER where folding is completed and post-translational processing of N-glycans begins. Subsequent transit through the Golgi complex and post-Golgi compartments can result in further processing prior to delivery to the plasma membrane. This paradigm predicts that integral mem-
brane proteins expressed at the plasma membrane will be subjected to processing by enzymes that are localized to either the Golgi complex or post-Golgi compartments during transit from the ER to the plasma membrane. Thus, it was surprising to find that a pool of ENaCs was expressed at the plasma membrane that appears to have bypassed key processing events known to occur in Golgi (N-glycan maturation) or post-Golgi (cleavage) compartments.

There are several possible explanations for these findings. First, it is possible that one distinct population of channel complexes exiting the ER transits through Golgi and post-Golgi compartments where subunits are processed, whereas a second population of channels exiting the ER bypasses Golgi and post-Golgi compartments and are delivered directly to the plasma membrane with immature subunits. Previous studies suggest that several proteins, including the cystic fibrosis transmembrane conductance regulator (17), a membrane type-I metalloproteinase (18), the protein phosphatase CD45 (19), and a complex containing F3/contactin and caspr/paranodin (20), traffic to the plasma membrane by non-conventional pathways that bypass the Golgi complex. A second possible explanation for our findings is that all ENaC subunits traffic to the plasma membrane via conventional pathways, but a population of channels is resistant to post-translational processing by enzymes present in Golgi and post-Golgi compartments.

At present, there is no evidence for specific signals that direct channels exiting the ER to be processed in post-ER compartments or to escape post-ER processing. Perhaps some channels exiting the ER exhibit distinctive conformational features, or different subunit stoichiometries, that dictate their fate regarding post-ER processing. In this regard, the results of several biophysical studies based on channel activity indicate that ENaC has a 2α1β1γ subunit stoichiometry (21, 22). Other groups using combined biophysical and biochemical approaches have proposed a more complex subunit stoichiometry, raising the possibility that channels with different subunit compositions and/or stoichiometries exit the ER and perhaps have different fates (23–25).

Both mature and immature subunits have been found on the plasma membrane of epithelial cells in vitro. Weisz et al. (26) observed a doublet of the β subunit of Xenopus ENaC on the surface of A6 cells consistent with the mature and immature forms of the β subunit, and Alvarez de la Rosa et al. (27) found both mature and immature forms of both the α and β subunits on the surface of A6 cells. Planes et al. (28) found only the smaller (i.e. 65 kDa) of two forms of the α subunit on the surface of rat lung alveolar epithelial type II cells. Interestingly, Masilamani et al. (29) observed a partial shift in the size of the γ subunit from 85 to 70 kDa in kidneys obtained from rats following 10 days on a sodium-restricted diet. Cumulative results (4, 7, 14, 30–34) suggest that channels must be cleaved to exhibit significant activity, as non-cleaved channels apparently have a very low open probability. Therefore, we propose that the presence of two distinct pools of channels at the plasma membrane provides epithelial cells with a reserve pool of immature, poorly functional channels that can be activated by proteases in post-Golgi compartments.

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