Assembly of the Epithelial Na\(^+\) Channel Evaluated Using Sucrose Gradient Sedimentation Analysis*

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Three subunits, \(\alpha\), \(\beta\), and \(\gamma\), contribute to the formation of the epithelial Na\(^+\) channel. To investigate the oligomeric assembly of the channel complex, we used sucrose gradient sedimentation analysis to determine the sedimentation properties of individual subunits and heteromultimers comprised of multiple subunits. When the \(\alpha\) subunit was expressed alone, it first formed an oligomeric complex with a sedimentation coefficient of 11 S, and then generated a higher order multimer of 25 S. In contrast, individual \(\beta\) and \(\gamma\) subunits predominately assembled into 11 S complexes. We obtained similar results with expression in cells and in vitro. When we co-expressed \(\alpha\) with \(\beta\) or with \(\alpha\) plus \(\gamma\), the \(\beta\) subunit assembled into a 25 S complex. Glycosylation of the \(\alpha\) subunit was not required for assembly into a 25 S complex. We found that the \(\alpha\) subunit formed intra-chain disulfide bonds. Although such bonds were not required to generate an oligomeric complex, under nonreducing conditions the \(\alpha\) subunit formed a complex that migrated more homogeneously at 25 S. This suggests that intra-chain disulfide bonds may stabilize the complex. These data suggest that the epithelial Na\(^+\) channel subunits form high order oligomeric complexes and that the \(\alpha\) subunit contains the information that facilitates such formation. Interestingly, the ability of the \(\alpha\), but not the \(\beta\) or \(\gamma\), subunit to assemble into a 25 S homomeric complex correlates with the ability of these subunits to generate functional channels when expressed alone.

The epithelial Na\(^+\) channel (ENaC)\(^3\) plays a key role in Na\(^+\) absorption by kidney, lung, and gut epithelia and thus is crucial for the regulation of electrolyte homeostasis (1, 2). ENaC belongs to an expanding family of cation channels called the DEG/ENaC protein family (3–5). Members of this family are expressed in a wide variety of tissues and species and function in electrolyte transport, mechano-sensation, and neurotransmission. ENaC consists of three homologous subunits, \(\alpha\), \(\beta\), and \(\gamma\) (6–10). Compelling functional and biochemical evidence suggests that all three subunits contribute to the formation of the functional channel. For example, the expression of all three subunits is required to generate maximal Na\(^+\) currents (7, 10), and the three subunits associate with each other (11). Although \(\alpha\), \(\beta\), and \(\gamma\) ENaC share substantial sequence similarity, their functions are not identical. When expressed individually, only \(\alpha\) subunits can produce Na\(^+\) channel currents. \(\beta\) and \(\gamma\) subunits are not functional on their own, however, they can assemble with \(\alpha\) ENaC to form \(\alpha\beta\) and \(\alpha\gamma\) channels with new functional properties (12).

The oligomeric assembly of plasma membrane proteins is an important posttranslational modification which is crucial both to function and to intracellular trafficking (13). However, the mechanisms that underlie oligomeric assembly of ENaC subunits remain largely unknown. In preliminary studies, we examined the molecular mass of ENaC complexes using sucrose gradient sedimentation analysis and found that three family members, \(\alpha\) ENaC, BNC1, FaNaCh, and the combination of \(\alpha\), \(\beta\), and \(\gamma\) ENaC assemble into large molecular mass multimers (14). These results suggested that this family of channels may share a conserved feature in their oligomeric structure. In order to learn more about the oligomeric assembly of ENaC proteins, we studied further the homomultimerization of two other ENaC subunits \(\beta\) and \(\gamma\), as well as the heteromultimerization of different subunit combinations.

**EXPERIMENTAL PROCEDURES**

DNA Constructs—The cDNAs encoding human \(\alpha\), \(\beta\), and \(\gamma\) ENaC were cloned in pcDNA3, pCRII, or pMT3, as described previously (9, 10). Wild-type \(\alpha\) ENaC and \(\alpha\) ENaC(T273A), whose consensus glycosylation sites were mutated, were cloned in pSPORT1 as described previously (15). For in vitro translation, we used the cDNAs driven by the T7 promoter contained in pcDNA3, pCRII, or pSPORT1 plasmids. For expression in COS-7 cells, we used the \(\alpha\) ENaCs subcloned into the pMT3 plasmid. The flag epitope sequence was inserted at the C terminus before the stop codons of \(\alpha\), \(\beta\), and \(\gamma\) ENaC (\(\alpha\)\(\text{flag}\), \(\beta\)\(\text{flag}\), and \(\gamma\)\(\text{flag}\)) to facilitate immunoprecipitation. We previously showed that insertion of the flag epitope does not alter function of the channel (11).

Sucrose Gradient Sedimentation Analysis of ENaC Expressed in Vitro or in COS-7 Cells—cDNAs were transcribed and translated in vitro in the presence of canine pancreatic microsomal membranes, as described previously (14). After the reaction, membranes were collected by centrifugation at 213,000 \(\times\) g for 30 min and lysed with TBS (50 mM Tris, 150 mM NaCl) containing 1% digitonin or 1% CHAPS, as indicated. COS-7 cells were electroporated with 30 \(\mu\)g of each cDNA and cultured as described previously (11). The medium bathing cells transfected with all three subunits contained 10 \(\mu\)M amiloride. One or two days after transfection, cells were pulse-labeled with 100 \(\mu\)Ci/ml \(\[^{35}\text{S}\]methionine\) for 30 min at 37 °C and chased for 0–4 h at 37 °C. Cells were lysed with TBS containing 1% digitonin and protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 20 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml aprotinin, and 10 \(\mu\)g/ml pepstatin A). Lysates were cleared by centrifugation at 16,000 \(\times\) g for 20 min to remove insoluble material.

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The 10–30% and 10–45% sucrose gradients containing TBS and 0.1% digitonin or 0.1% CHAPS were generated by a Gradient Master (BioComp Instruments Inc., Fredericton, NB, Canada). Samples from in vitro translation or from COS-7 cells were layered onto the top of the gradient. The 10–30% gradient was centrifuged at 230,000 × g in a vertical tube rotor (VT65.1) for 2 h at 5 °C. The 10–45% gradient was centrifuged at 230,000 × g in a swing out rotor (SW40) for 17 h at 5 °C. Gradients were fractionated from top to bottom using a tube piercer (ISCO, Lincoln, NE). After sedimentation, fractions from in vitro translation were directly separated by SDS-PAGE. For COS-7 cells, fractions were immunoprecipitated with anti-flag M2 monoclonal antibody (WAYD) prior to SDS-PAGE. The labeled bands were quantitated by phosphorimaging (ImageQuant, Molecular Dynamics, Sunnyvale, CA). Protein markers were sedimented in the same rotor to estimate the molecular mass; they are: bovine serum albumin (70 kDa), aldolase (140 kDa), catalase (240 kDa), ferritin (440 kDa), thyroglobulin (690 kDa), and pentameric IgM (950 kDa).

The sedimentation coefficients of ENaC complexes were estimated using the method of McEwen (16). First, the solute concentration corresponding to extrapolation of a linear gradient distribution to a zero radius (Z₀) for rotor and gradient was obtained from the formula

\[ Z₀ = \frac{(Zₘ² - Zₚ²) rₚ}{r₂ - r₁} \]

(Eq. 1)

where Zᵢ is the minimum percent w/w of sucrose gradient, Zₘ is the maximum percent w/w of sucrose gradient, r₁ is minimum radial distance from centrifugal axis (cm), r₂ is the maximum radial distance from centrifugal axis (cm). Then, we used the Z₀ value and tables shown by McEwen (16) to obtain the time integral I₂ for sucrose at the meniscus of the gradient and I₁ at the separated zone for the particle. Finally, S was calculated from formula

\[ S = (I₂ - I₁) / ω² t \]

(Eq. 2)

where ω is the speed of rotor (2πω⁻¹) and t is the centrifugation time (s).

### RESULTS

#### Sedimentation Analysis of α, β, and γ Subunits Expressed Individually in Vitro—To produce ENaC subunits for sedimentation analysis, we used in vitro translation in the presence of microsomal membranes. This cell-free expression system, which mimics the endoplasmic reticulum environment, has been utilized to study protein folding and assembly in many cases (17, 18). To choose the optimal detergent for solubilization from membranes, we expressed ahENaC-solubilized proteins with either CHAPS or digitonin, and centrifuged the samples through 10–30% sucrose gradients followed by SDS-PAGE (Fig. 1). When CHAPS was used, both glycosylated and unglycosylated α sedimented diffusely throughout many fractions from near the top to the bottom of the gradient. We observed a similar sedimentation pattern when we used Triton X-100 (data not shown). Following digitonin solubilization, the unglycosylated protein sedimented predominantly at fraction 4–6; however, glycosylated proteins were concentrated at the bottom of the gradient. These data suggest that digitonin better preserves the tertiary structure of the high molecular weight complex than does CHAPS or Triton X-100. We also found that digitonin solubilized glycosylated ENaC proteins more efficiently than did CHAPS, consistent with our earlier observation that digitonin solubilizes ENaC proteins in COS-7 cells much more efficiently than does CHAPS (11). Therefore, in subsequent studies we used digitonin for solubilization. For better separation of large complexes, we optimized the centrifugation condition by using a 10–45% sucrose gradient.
ship between sedimentation coefficient and molecular mass of proteins: 

\[ M = (S/0.00242)^{1.49} \]

(19), the molecular masses of the 11 and 25 S complexes were estimated to be 280 and 950 kDa, respectively. Hence, these two independent methods produced consistent estimates of the molecular mass of the αENaC complexes. Given that the masses of unglycosylated and glycosylated αhENaC monomers are 75 and 87 kDa, respectively, these results suggest that both unglycosylated and glycosylated αhENaC assemble into multimers with glycosylated αhENaC assembling into larger multimers than unglycosylated αhENaC.

We asked whether β and γhENaC display the same sedimentation features as αhENaC when translated in vitro and processed in microsomal membranes. Fig. 2 shows that the unglycosylated forms of β and γ subunits sedimented predominantly in fractions 4 and 5, the position of the 240-kDa standard, and similar to the position of unglycosylated αhENaC. However, in contrast to glycosylated αhENaC, the majority of glycosylated β and γhENaC peaked in fractions 5 and 4, respectively; there was no distinct peak around fraction 9. Interestingly, while glycosylated αhENaC showed a sharp and homogenous peak at fraction 9, the peaks of β and γhENaC in fractions 5 and 4 displayed long tails toward the denser part of the gradient. Although these tails may suggest a tendency for β and γ to form higher order oligomers, these data suggest that β and γ preferentially form multimers of 11 S, smaller than the 25 S complex generated by αhENaC.

**Pulse-Chase and Sedimentation Analysis of α, β, and γ Subunits Expressed Individually in COS-7 Cells—**The in vitro expression system has the advantage that it minimizes the possibility that the observed molecular mass of the ENaC complex is artificially increased through its association with other cellular proteins. However, the reaction temperature is 30 °C rather than 37 °C, and the translocation and folding machinery may be less efficient than those in vivo. Therefore, we examined the oligomerization of ENaC subunits expressed individually in COS-7 cells. We pulse-labeled the cells for 30 min and chased for 4 h before solubilizing for sedimentation analysis. After sedimentation, proteins were detected by immunoprecipitation and SDS-PAGE. At the beginning of the chase period, most of both unglycosylated and glycosylated αhENaC sedimented at fractions 4 and 5 as 11 S complexes (Fig. 3A). However, a significant amount of the glycosylated form sedimented at fraction 6 and 7 (16–20 S) with long tails of sedimentation in denser fractions. At the end of the 4-h chase, the majority of glycosylated protein oligomerized into larger complexes with sedimentation peaking at fractions 8 and 9 as 25 S complexes. Moreover, there was little unglycosylated protein present in cell lysates. Thus, in COS-7 cells, αhENaC assembled into a 25 S complex, as it did in vitro. In addition, the 11 S and 16–17 S complexes may represent transient intermediates in the oligomerization process.

Fig. 3, B and C, shows the sedimentation profiles of β and γhENaC expressed in COS-7 cells. Both subunits sedimented predominantly at fraction 4 as 11 S complexes at the beginning of the chase, although small peaks were present at fractions 6 and 7. In sharp contrast to the α subunit, β and γ subunits migrated at the same gradient position after the 4-h chase period. Even after 7 h of chase, γ subunit sedimentation still peaked at fraction 4 (data not shown). These results suggest

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3 ENaC subunits have a very fast turnover rate (24). Therefore, to increase the opportunity for subunit oligomerization in the endoplasmic reticulum, we also chased cells expressing γ at 15 °C for 7 h to inhibit transport from the endoplasmic reticulum to the Golgi apparatus. Despite this, γ expressed alone did not generate a 25 S complex (not shown).
that, unlike α subunits, β and γ subunits are incapable of multimerizing into higher order 25 S oligomeric complexes. These in vivo observations parallel the findings obtained with the in vitro expression system. We also observed that about half of β and γ remained unglycosylated at 4 h. In contrast, most of α was glycosylated at 4 h. We speculate that β and γ may progress through biosynthesis at a slower rate than α. However, sedimentation at least some of the unglycosylated β and γ, at the same position as the multimeric glycosylated forms, suggests that unglycosylated subunits can form multimers. This is consistent with earlier work showing that subunits can interact before glycosylation (11).

Oligomerization of α and β Subunits Co-expressed in COS-7 Cells—As the functional ENaC complex is composed of more than one type of subunit, we asked how two subunits with different oligomeric features co-assemble. We chose to co-express α and β subunits in COS-7 cells for two reasons. First, α and β subunits can form functional channels (7). Second, glycosylated β subunits can be co-immunoprecipitated with α subunits and thereby separated by their different migration on SDS-PAGE (11), whereas γ subunits cannot be separated from α subunits due to their nearly identical electrophoretic mobilities. We co-transfected COS-7 cells with αflag and β subunits and performed pulse-chase experiments as described above. After sedimentation, αflag subunits were immunoprecipitated with anti-flag antibody and β subunits were co-immunoprecipitated. Fig. 4A shows that, at the end of chase, glycosylated α subunits sedimented predominantly at fraction 8, 9, and 10 as a 25 S complex. Glycosylated β subunits, which were co-immunoprecipitated with α, sedimented in the same fractions. In contrast, when β was expressed alone it sedimented at fraction 4 as an 11 S complex (Fig. 3B). These results suggest that β subunits multimerized with α subunits into high order oligomers. By selecting β co-immunoprecipitated with α, the experiment in Fig. 4A told us only about the oligomerization features of β that associated with α. To investigate total β subunits, we co-expressed βflag and α in COS-7 cells and immunoprecipitated with βflag anti-flag antibody after sedimentation. Fig. 4B shows that the majority of the glycosylated β still sedimented at fractions 4 and 5 as an 11 S complex, although a substantial amount of β sedimented as heavier complexes. α subunits did not co-immunoprecipitate with β subunits in fractions 4 and 5, therefore, the 11 S complex contained only β subunits. α subunits that co-immunoprecipitated with βflag sedimented as a peak around fractions 8–10. We concluded that all the ENaC multimerized into 25 S complexes at the end of the chase period, either by itself or in association with βENaC. In contrast, only those β subunits that associated with α assembled into 25 S complexes; a substantial amount of β remained unassociated with α and stayed in a low order oligomeric state 4 h after protein synthesis. This result could be due to an inefficient association between α and β subunits. It is also possible that some α and β subunits are expressed in different cells; however, this would not influence our interpretation.
We also compared the sedimentation pattern of the α subunit when it was associated with β to the sedimentation of the α subunit expressed alone (Fig. 4C). α subunits seemed to sediment in a similar fashion with and without β subunits, although in the presence of β subunits, α appeared to sediment consistently as a slightly sharper peak around fraction 9. On the other hand, significantly more β protein sedimented as a 25 S complex compared with β expressed alone (Fig. 4D). These results support the notion that co-expression of α subunits facilitates the multimORIZATION of β subunits into the 25 S complex.

Oligomerization of α, β, and γhENaC Heteromultimers—Because the fully functional ENaC channel is composed of all three subunits, we examined the oligomeric assembly of ENaC complexes consisting of α<sub>ααα</sub>β, γ, and β subunits. We previously showed that α, β, and γ can co-assemble into 25 S complexes at 15 °C (14). Here, we pulsed the cells co-expressing α<sub>ααα</sub>β, and γ for 30 min, then chased the cells at 37 °C for 2 or 4 h. Immediately after the pulse, glycosylated α subunits sedimented mainly at fraction 4 as an 11 S complex; insufficient β was co-immunoprecipitated for accurate quantitation (Fig. 5A).

After a 2-h chase, more than half of the α subunit assembled into larger multimers and emerged as a sharp peak at fraction 9, while the rest of the α subunits remained at fractions 4 and 5 (Fig. 5B). β subunits co-precipitated with α showed the same sedimentation pattern, with one peak concentrated around fractions 4 and 5, and the other at fraction 9. This result suggests that prior to assembling into the final oligomeric complex, an 11 S (~280 kDa) heteromultimer was assembled that contained at least both α and β subunits. This result is also important because it confirms our conclusion that the complex sedimenting at fractions 4 and 5 is multicentric.

After a 4-h chase, we found that α, as well as β, co-preципitated with α, homogeneously sedimented at fraction 9, suggesting that they co-assembled into 25 S complexes (Fig. 5C). Because glycosylated γhENaC migrated at the same position as αhENaC on SDS-PAGE, we were unable to directly detect γhENaC. However, comparison of Figs. 4A and 5C shows that, in the presence of γ, a greater percentage of α and β sedimented at fraction 9, and the peak was sharper than in the absence of γ. This indirectly suggested that γ subunits were present in the 25 S complex. This conclusion is also consistent with the observation that expression of all three subunits is required to generate maximal amiloride-sensitive Na<sup>+</sup> currents (7, 10) and earlier work showing that γ subunits can be co-immunoprecipitated with α or β (11).

Glycosylation Is Not Essential for Homo- and Heteromultimerization—Since it was the glycosylated subunits that assembled into high order oligomers, we asked whether glycosylation is required for oligomerization. We first asked whether glycosylation is necessary for homomultimerization of α subunits. The six glycosylation site asparagines in αhENaC were mutated to glutamine (αhENaC<sub>Cααα</sub>Q) (15). αhENaC<sub>Cααα</sub>Q was expressed in vitro and sediemented on a 10–45% sucrose gradient. Fig. 6A shows that αhENaC<sub>Cααα</sub>Q sedimented as two peaks, one at fraction 4, the other one at fraction 9. This result indicates that a significant amount of αhENaC<sub>Cααα</sub>Q oligomerized into a 25 S complex despite the lack of glycosylation. This result is consistent with functional studies showing that αhENaC<sub>Cααα</sub>Q produces channels similar to wild-type αhENaC (15).

We then asked whether elimination of αhENaC glycosylation affects its ability to heteromultimerize with β subunits. We co-expressed αhENaC<sub>Cααα</sub>Q with the β<sub>ααα</sub> subunit in COS-7 cells. The sedimentation pattern of β subunits showed that more α subunits sedimented as 25 S complexes than when α subunits were expressed alone (Fig. 6B). This result suggests that αhENaC<sub>Cααα</sub>Q can assemble with β subunits into 25 S heteromultimers.

Formation of Intra-chain Disulfide Bonds—ENaC subunits have several conserved cysteine-rich motifs in their extracellular domains (6–10). We hypothesized that either intra- or interchain disulfide bonds may exist between these cysteines. To test this hypothesis, we expressed αhENaC in vitro and immediately electrophoresed αhENaC under nonreducing and reducing conditions. Electrophoresis under nonreducing conditions allows detection of disulfide bonds, because monomeric molecules with intra-chain disulfide bonds may exist between these cysteines. We then expressed αhENaC in vitro and immediately electrophoresed αhENaC under nonreducing and reducing conditions. Electrophoresis under nonreducing conditions allows detection of disulfide bonds, because monomeric molecules with intra-chain disulfide bonds may exist between these cysteines. We then expressed αhENaC in vitro and immediately electrophoresed αhENaC under nonreducing and reducing conditions. Electrophoresis under nonreducing conditions allows detection of disulfide bonds, because monomeric molecules with intra-chain disulfide bonds may exist between these cysteines.
Interestingly, we found that inter-chain disulfide bonds formed between subunits if the samples were kept in detergent at 4 °C overnight and then electrophoresed under nonreducing conditions (data not shown). This result is consistent with what has been reported with the FMRFamide-gated Na\(^+\) channel (FaNaCh) (20). We cannot rule out the possibility that inter-chain disulfide bonds may form \textit{in vivo}, or at some point during the processing and trafficking of the ENaC complex. Nevertheless, our data indicate that inter-chain disulfide bonds are not a prerequisite for oligomerization \textit{in vitro}, because before inter-chain disulfide bonds formed, \(a\)hENaC had already multimerized into the 25 S complex.

To prove the existence of intra-chain disulfide bonds more explicitly, we synchronized disulfide bond formation using methods described by Brodsky (18). During the first hour of \textit{in vitro} translation of \(a\)hENaC, the reducing agent DTT was included in the reaction to inhibit formation of disulfide bonds. Cycloheximide was added to stop protein synthesis. Then we added the oxidizing reagent GSSG to raise the redox potential and initiate disulfide bond formation. We removed an aliquot of the sample at various times after addition of GSSG, stopped disulfide bond formation by adding sulfhydryl reactive compound N-ethylmaleimide, and analyzed samples on a nonreducing gel. The results are shown in Fig. 7B; note that as shown in Fig. 2, \textit{in vitro} translated glycosylated and unglycosylated \(a\)hENaC migrate as doublets on SDS-PAGE. With time after addition of GSSG, a faster migrating band appeared on the nonreducing gel. There was no such shift on reducing gels. The faster migrating band represents subunits with intra-chain disulfide bonds and may originate from either unglycosylated or glycosylated proteins. We also observed that at the later time points less protein was present on the nonreducing gel. With time, protein may form aberrant inter-chain disulfide bonds and aggregate; in fact, at the later time points of this experiment, we observed high molecular weight aggregates at the top of nonreducing resolving gel, but not the reducing gel (not shown).

We asked whether intra-chain disulfide bond formation is required for oligomerization of \(a\)hENaC. In the experiment shown above, we used 1 mM DTT to stop disulfide bond formation during the 1st h of translation. To ensure complete elimination of disulfide bond formation, we translated \(a\)hENaC in the presence of 10 mM DTT and then performed sedimentation analysis. Fig. 8 shows that a substantial amount of glycosylated protein sedimented at fractions 8–10, suggesting a complex of 25 S was still a major oligomeric product under this condition. However, the 25 S complex was less dominant compared with \(a\)hENaC synthesized in the absence of DTT. Moreover, the \(a\)hENaC synthesized under reducing conditions appeared to spread out along the gradient, indicating that complexes of heterogeneous sizes were produced. These results suggest that formation of intra-chain disulfide bonds are not essential to oligomerization. However, \(a\)hENaC without intra-chain disulfide bonds may not achieve a stable oligomeric structure, resulting in the formation of heterogeneous sized multimers.

**DISCUSSION**

Using sucrose gradient sedimentation analysis, we found that when each ENaC subunit is expressed by itself, only
\( \alpha \text{hENaC} \) can efficiently multimerize into a large complex of 25 S; \( \beta \) and \( \gamma \text{hENaC} \) form smaller 11 S complexes. However, when the other subunits are co-expressed with \( \alpha \), they assemble into a 25 S complex. We conclude that the 25 S complex is the favorable oligomeric structure of the ENaC channel complex.

A proposed process of oligomeric assembly of ENaC subunits is shown in Fig. 9. When \( \alpha \text{hENaC} \) was expressed alone, the newly synthesized unglycosylated proteins assembled into an 11 S complex prior to glycosylation. Following glycosylation of the 11 S complex, the glycosylated intermediates then oligomerized into the final complex of 25 S. Although \( \beta \) and \( \gamma \) were able to generate an 11 S glycosylated complex when expressed individually, they did not efficiently oligomerize further. These results suggest that the information required for oligomerization into an 11 S complex is contained in each subunit, but the information required for oligomerization into a 25 S complex is probably encoded by the \( \alpha \) subunit.

We found that when \( \alpha \), \( \beta \), and \( \gamma \) were co-expressed, they followed a similar sequence of oligomerization to produce the 25 S complex; first an 11 S, and then a 25 S complex was generated. During oligomerization the \( \alpha \) subunit may associate with \( \beta \) and \( \gamma \) subunits, and by co-assembly may carry them into higher order complexes. In earlier work we showed that assembly of the subunits occurs co-translationally; it does not occur afterward (11). Moreover, the 11 and 25 S complexes contained both \( \alpha \) and \( \beta \), and most likely \( \gamma \) subunits. These results suggest that intermediate sized heteromultimers form first and then assemble to produce the larger 25 S heteromultimers.

We obtained similar sized complexes using two different expression systems, \textit{in vitro} translation with endoplasmic reticulum-like microsomal membranes and \textit{in vivo} production in COS-7 cells. The results suggest that the complex forms without the requirement for other co-assembled proteins. In our pulse-chase studies in COS-7 cells, we did not consistently detect other proteins co-precipitating with ENaC and co-migrating on sucrose gradients. Therefore, if the 25 S complex does contain other proteins, they may have a slow turnover rate and therefore are not labeled efficiently during the pulse period. Moreover, it would be required that such proteins are present in the \textit{in vitro} expression system, because results were similar \textit{in vitro} and \textit{in vivo}. Nevertheless, we cannot exclude the possibility that other proteins associate with ENaC to form a 25 S complex. The size of the 25 S complex is also consistent with electrophysiological data suggesting that ENaC channels are composed of at least nine subunits (14), although some data suggest that an ENaC complex may be composed of four subunits (21, 22).

Our results differ from a recent report on another DEG/ENaC protein, FaNaCh. Coscoy \textit{et al.} (20) found that FaNaCh

![Fig. 7. Detection of intra-chain disulfide bonds in \( \alpha \text{hENaC} \). A, \( \alpha \text{hENaC} \) was \textit{in vitro} translated in the presence of microsomal membranes. Then the samples were analyzed either by nonreducing (NR) and reducing (R) 6% SDS-PAGE. B, \( \alpha \text{hENaC} \) was \textit{in vitro} translated as described above except that 1 mM DTT was included in the reaction mixture. After 1 h, protein synthesis was stopped by cycloheximide. Oxidation was initiated by adding 4 mM GSSG and then stopped at the indicated time points by addition of 20 mM \( N \)-ethylmaleimide. Samples were analyzed on reducing and nonreducing SDS-PAGE as indicated. The asterisk on the nonreducing gel indicates the faster migrating bands, suggesting the existence of intra-chain disulfide bonds.](image)

![Fig. 8. Elimination of intra-chain disulfide bonds decreases the efficiency of \( \alpha \text{hENaC} \) oligomerization. \( \alpha \text{hENaC} \) was \textit{in vitro} translated in the presence of microsomal membranes and the reducing reagent, 10 mM DTT. Microsomal membranes were lysed and sedimented through a 10–45% sucrose gradient, and resolved on SDS-PAGE. Glycosylated \( \alpha \text{hENaC} \) was quantitated and compared with the quantitation of \( \alpha \text{hENaC} \) produced in the absence of DTT.](image)

![Fig. 9. Proposed model of assembly of ENaC subunits. The model may be valid independent of actual number of subunits that contribute to a channel. See text for details.](image)
formed a 9 S complex, instead of the 25 S complex we find. There are several potential explanations for the difference. It seems unlikely that use of FaNaCh expressed in human embryonic kidney cells gives different-sized complexes than ENaC expressed in COS cells or in vitro, because we found an approximately 25 S complex with FaNaCh in vitro (14). A more likely explanation relates to the detergents used. Coscoy et al. used CHAPS and Triton X-100, whereas we used digitonin. Solubilization with CHAPS and Triton X-100 may have disrupted interactions between subunits as suggested in our Fig. 1. Retention of a multimeric complex in Triton requires relatively stronger interactions between subunits than are required in digitonin. For example, the Na,K-ATPase subunits. The results show that intra-subunit disulfide bonds form in the α subunit, but inhibiting their formation does not prevent oligomerization. However, when disulfide bonds are allowed to form, the subunit migrates as a sharper, more homogenous peak on a sucrose gradient. This suggests that intra-chain disulfide bonds may stabilize the oligomeric structure. Although we cannot exclude the possibility that disulfide bonds form between subunits, the data suggest that such disulfide bonds are not required for oligomerization of the channel.

It is interesting to note that the ability of single subunits to multimerize into the 25 S complex when expressed alone correlates with their ability to form functional channels. αhENaC, brain Na+- channel 1, and FaNaCh can all form functional channels and all generate a 25 S complex when expressed alone (14). In contrast, β and γENaC are able to do neither. We speculate that formation of a 25 S complex is a key step in the biosynthesis of this family of channels. Perhaps the inability of some members of the DEG/ENaC family to form a high order oligomer may account for their inability to generate functional homomultimeric channels.

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