Potassium Channel $\alpha$ and $\beta$ Subunits Assemble in the Endoplasmic Reticulum*

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We have characterized the maturation of Shaker $K^+$ channel protein and the cellular site of assembly of pore-forming $\alpha$ and cytoplasmic $\beta$ subunits in a transfected mammalian cell line. Shaker protein is made as a partially glycosylated, immature precursor that is converted to a fully glycosylated, mature product. Shaker protein did not mature when transport from the endoplasmic reticulum (ER) to the Golgi apparatus was blocked. Consistent with this finding, only the immature form was sensitive to digestion with endoglycosidase H. These results indicate that the immature protein is core-glycosylated in the ER, whereas the oligosaccharides of the mature protein have been further processed in the Golgi compartment. After inhibiting ER-to-Golgi transport, the oligomeric state of Shaker subunits was assessed by cross-linking in intact cells or by solubilization and sucrose gradient sedimentation. The results indicate that Shaker subunits assemble with each other in the ER. When co-expressed, the Kv$\beta$ subunit also associated with Shaker in the ER. Assembly with the $\beta_2$ subunit did not increase the rate or extent of Shaker protein maturation. Our results indicate that the biogenesis of Shaker $K^+$ channels in vivo involves core glycosylation and subunit assembly in the ER, followed by efficient transfer to the Golgi apparatus where the oligosaccharides are modified.

Ion channels are multisubunit membrane proteins involved in action potential propagation, neurotransmitter release, and excitation-contraction coupling in excitable tissues. To achieve their correct quaternary structures, ion channels undergo a complex series of events during biogenesis, including co- and posttranslational folding, modification, and subunit assembly. Although many multisubunit membrane proteins fold and assemble in the endoplasmic reticulum (ER)1 (1), there are notable exceptions among channel-forming proteins (2).

Voltage-gated $K^+$ channels, which regulate the excitability of nerve and muscle, contain four $\alpha$ subunits that form the ion-conducting pore (3–6). Each $\alpha$ subunit has a hydrophobic core containing a pore-forming domain and six putative membrane-spanning segments, flanked by cytoplasmic amino and carboxyl termini (7). The $\alpha$ subunits in a $K^+$ channel may be identical or may be different members of a subfamily of closely related proteins (8–12). In neurons, the pore-forming $\alpha$ subunits may associate with cytoplasmic $\beta$ subunits in a one-to-one stoichiometry (13, 14). In expression systems, $\beta$ subunits have been shown to alter the functional properties, stability, and cell surface expression of $K^+$ channels (15–18).

Recently, some of the molecular determinants that control assembly of the Shaker channel and its mammalian homologues, the Kv1 subfamily, have been identified. A conserved amino-terminal domain mediates the recognition and assembly of $\alpha$ subunits (19–24). An overlapping region of the amino terminus is also important for the co-assembly of $\alpha$ and $\beta$ subunits (25, 26). Much less is known about other aspects of the biogenesis of $K^+$ channels in cells.

We have characterized the maturation and cellular site of assembly of the voltage-dependent Shaker $K^+$ channel in a transfected mammalian cell line. Our results demonstrate that maturation of Shaker protein requires ER-to-Golgi transport, with a corresponding change in the N-linked oligosaccharides from the high mannose type characteristic of the ER to the complex type characteristic of the Golgi apparatus. In addition, we find that Shaker channel $\alpha$ subunits assemble with each other and with cytoplasmic $\beta$ subunits in the ER, suggesting that only fully assembled channels are transported to the Golgi. Unlike the effect of $\beta$ subunits on some mammalian $\alpha$ subunits (18), the $\beta$ subunit does not act as a chaperone to facilitate the maturation of Shaker channels.

EXPERIMENTAL PROCEDURES

Cell Culture and Metabolic Labeling—Human embryonic kidney cells (HEK 293T) (27), kindly provided by Dr. R. B. DuBridge, were grown and transfected as described previously (28). For transfection experiments, the Shaker B cDNA (29) and a mutant construct, N259Q + N263Q, which eliminates N-linked glycosylation of Shaker protein (30), were transferred into the vector pcDNA1/AMP (Invitrogen). A subclone encoding the rat Kv$\beta$2 protein in the pRGB4 vector was the generous gift of Dr. James S. Trimmer, SUNY, Stony Brook (31). In co-transfection experiments, Shaker and Kv$\beta$2 DNAs were mixed in a 3:1 mass ratio. A total of 2 μg of DNA was used per 35-mm well of cells.

Forty-eight h after transfection with Shaker alone, HEK 293T cells were incubated for 30 min in methionine- and cysteine-free DMEM (Mediatcheck), pulsed for 3 h with 200 μCi/ml $[^{35}$S]Met and cys and cys (Trans-AM-Label, ICN or EasyTag Express Protein Labeling Mix $[^{35}$S], DuPont NEN), and chased for 2 h in complete, nonradioactive medium. A crude membrane fraction was prepared and solubilized in the presence of protease inhibitors (30). Shaker protein was immuno-

Me$_2$SO, dimethyl sulfoxide; endo H, endoglycosidase H; HEK 293T, human embryonic kidney cells; NEM, N-ethylmaleimide.

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Maturation of Shaker Protein Is Blocked by Inhibiting ER-to-Golgi Transport—The glycosylation of Shaker $K^+$ channel protein has been characterized in several expression systems, including an insect cell line (Sf9), Xenopus laevis oocytes, and a mammalian cell line (HEK 293T) (28, 30). Asparagine-linked glycosylation, which occurs at Asn-259 and Asn-263 in the first extracellular loop of the protein, is not required for the assembly of functional channels or their transport to the cell surface (30). In oocytes and HEK 293T cells, glycosylation occurs in two stages, giving rise to a partially glycosylated, immature precursor that is efficiently converted to a fully glycosylated, mature product (Ref. 28; see Fig. 3).

This differential glycosylation suggests that the immature precursor has been core-glycosylated in the ER, whereas the mature product has been further modified in the Golgi apparatus (34). To determine whether maturation of Shaker protein requires transport from the ER to the Golgi, movement of newly synthesized protein between these compartments was arrested by two known inhibitors of intracellular transport in mammalian cells, low temperature incubation, and brefeldin A (BFA) treatment (35–39). The extent of maturation was then assayed by comparing the relative amounts of the immature and mature forms of the Shaker protein. At 37 °C, about 80% of the immature form is converted to the mature form within 1.5 h, and conversion is virtually complete within 2.5 to 3 h (Ref. 28; see Fig. 1D).

Incubation of mammalian cells at 15 °C prevents the transport of newly synthesized secretory and membrane proteins to the Golgi apparatus (35, 36). HEK 293T cells were metabolically labeled at 15 °C for 3 h and collected following a 2-h chase in nonradioactive medium at either 15 or 37 °C (Fig. 1A). Following pulse and chase at 15 °C, the extent of maturation was significantly reduced compared with that obtained after pulse and chase at 37 °C (see Fig. 1D). Of the total Shaker protein, nearly 90% was in the immature form after the 2-h chase at 15 °C. The blockade in maturation induced by incubation at 15 °C was reversible, however, as shown by the large increase in maturation following a 2-h chase at 37 °C (Fig. 1A).

In other mammalian cell lines, incubation at 20 °C does not block transport of membrane and secretory proteins to the Golgi compartment (35). However, similar to the results of incubation at 15 °C, we found that the extent of maturation was significantly but reversibly reduced following a 3-h pulse and a 2-h chase at 20 °C (Fig. 1B). These results suggest that ER-to-Golgi transport remains inhibited at 20 °C in HEK 293T cells. In contrast, incubation at 27 °C did not prevent maturation of the Shaker protein (Fig. 1C); the extent of maturation was similar to that seen after pulse and chase at 37 °C (Fig. 1D). Thus, the effects of low temperature incubation on the relative amounts of immature and mature Shaker protein suggest that ER-to-Golgi transport is required for maturation. Alternatively, ER-to-Golgi transport was blocked with BFA in either the presence or absence of nocodazole, a drug which makes BFA a more specific inhibitor of protein transport (37–39). HEK 293T cells were metabolically labeled during continued treatment with BFA, BFA and nocodazole, nocodazole, or MeSO4, the drug vehicle (Fig. 2). Following treatment with BFA, either alone or in combination with nocodazole, maturation of the Shaker protein was completely blocked. In contrast,
Maturation was not inhibited by treatment with nocodazole or Me$_2$SO alone. Thus, these results provide further evidence that ER-to-Golgi transport is required for maturation of the Shaker protein.

**Maturation of Shaker Protein Involves Modification in the Medial Golgi**—To characterize the differential glycosylation of the immature and mature forms of Shaker protein, wild-type protein was expressed in HEK 293T cells and incubated in the presence ($+$) or absence ($-$) of endoglycosidase H (endo H) (Fig. 3). Conversion of a protein from an endo H-sensitive to an endo H-resistant form indicates that the oligosaccharide moieties have been processed in the medial Golgi compartment (40–42). The mature form of wild-type Shaker protein (Fig. 3, 108 kDa) showed no significant change in apparent molecular mass (107 kDa) following incubation with endo H, indicating that it is endo H-resistant. The immature form (80 kDa), however, decreased in apparent molecular mass by about 8 kDa following incubation with endo H (72 kDa), indicating that it is endo H-sensitive. The digested precursor comigrated with unglycosylated protein (72 kDa) of the mutant construct, N259Q N263Q, in which the two asparagine residues that are normally modified by the attachment of N-linked oligosaccharides have been replaced by glutamine residues (30). The differential sensitivity of immature and mature Shaker protein to endo H indicates that maturation involves the conversion of high mannose oligosaccharides to a complex form in the medial Golgi compartment.

**Oxidative Cross-linking in Situ Indicates that Shaker Subunits Assemble in the ER**—To determine whether Shaker subunits assemble in the ER, ER-to-Golgi transport was inhibited, and the state of assembly of Shaker protein was examined in intact cells by cross-linking with iodine. Iodine, an oxidizing reagent, catalyzes the formation of a specific disulfide bond between Cys-96 in the amino terminus and Cys-505 in the carboxyl terminus of adjacent Shaker subunits (6). Although the subunits are not normally linked by disulfide bonds (28), functional assays under oxidizing conditions indicate that the formation of these bonds does not disrupt the native structure or function of the channel (6).

HEK 293T cells were metabolically labeled at 15 or 37 °C and treated in the absence ($-$) or presence ($+$) of 1 mM iodine (Fig. 4). Under reducing conditions, only monomeric Shaker protein was observed; after incubation at 15 °C, the immature form was present (open arrow 1), whereas after incubation at 37 °C, primarily the mature form was present (filled arrow 1). Under nonreducing conditions, disulfide-bonded adducts of Shaker protein corresponding to a dimer (arrows 2), trimer (arrows 3), and two tetramers, one linear (arrows 4) and one circular (arrows 4c) were observed (6). After incubation at 15 °C, the adducts were composed of immature Shaker protein (open arrows), whereas after incubation at 37 °C, the adducts were primarily composed of mature protein (filled arrows). The fact that adducts were observed after blocking ER-to-Golgi transport by incubation at 15 °C suggests that assembly of the subunits occurs in the ER. The intensities of the tetrameric adducts indicate that the immature form of the Shaker protein...
standards are shown on the left. Incubation at 15 °C does not block Shaker channel assembly in HEK 293T cells. Following metabolic labeling and chase at either 15 or 37 °C, intact cells were incubated in the absence (--) or presence (+) of 1 mM iodine, which catalyzes the formation of disulfide bonds between Shaker subunits (6). Immunoprecipitated protein was subjected to electrophoresis under reducing or nonreducing conditions. Arrowheads (open, 15 °C; filled, 37 °C) indicate Shaker monomers (1) and their respective adducts: dimer (2), trimer (3), linear tetramer (4), and circular tetramer (4c) (Ref. 6). Bands migrating slightly above the disulfide-bonded dimers under both reducing and nonreducing conditions are likely to be noncovalently associated dimers of Shaker protein (6). Molecular mass standards are shown on the left.

Sucrose Gradient Centrifugation Indicates that Shaker Subunits Assemble in the ER—The state of assembly of Shaker protein was also assessed by sucrose gradient centrifugation (Fig. 5). The detergent CHAPS has been shown to solubilize wild-type Shaker protein in an active, assembled form (43). Furthermore, Shaker homologues expressed in vitro and solubilized in CHAPS sediment with a velocity consistent with a tetrameric structure (20). In contrast, Shaker homologues solubilized in Zwittergent sediment as monomers (20). Following incubation at 37 °C (Fig. 5A) or treatment with Me2SO (Fig. 5C), Shaker protein solubilized in CHAPS sedimented with a velocity consistent with a multimeric state, whereas Shaker protein solubilized in Zwittergent sedimented with a velocity consistent with a monomeric state. To determine whether Shaker protein assembles in the ER, ER-to-Golgi transport was inhibited. Following incubation at 15 °C, immature Shaker protein solubilized in CHAPS sedimented to the denser region of the gradient, consistent with a multimeric state of assembly (Fig. 5B). Following Zwittergent solubilization, immature Shaker protein sedimented to a lighter region of the gradient, consistent with the monomeric state. Similar results were obtained after blocking ER-to-Golgi transport with BFA and nocodazole (Fig. 5D). Within experimental error, the sedimentation profiles of the mature and ER resident, immature forms of the protein overlapped in gradients containing CHAPS or in gradients containing Zwittergent. Thus, the results of sucrose gradient centrifugation, like those obtained from disulfide cross-linking, demonstrate that assembly of Shaker subunits occurs in the ER.

To determine whether the position of the Shaker protein on the CHAPS gradients corresponded to that of an authentic tetramer, we investigated the sedimentation properties of a covalently linked tetramer of Shaker subunits. This construct consists of four repeats of an inactivation-removed Shaker 29-4 monomer (44) connected by three 19-amino acid linkers (45) and has a predicted molecular mass of about 280 kDa (33). It provides an ideal marker for the hydrodynamic properties of the assembled channel (6). Similar to wild-type Shaker B protein expressed in HEK 293T cells, this covalent tetramer appears as an immature, core-glycosylated form in addition to the predominant, mature form (6).

The covalent tetramer was expressed and metabolically labeled in Xenopus oocytes, solubilized in CHAPS or Zwittergent, and subjected to sedimentation on sucrose gradients. As shown in Fig. 5E, covalent tetramer protein solubilized in CHAPS (filled circles) sedimented as one major peak to the denser half of the gradient. The position of this peak overlapped with that of the mature (Fig. 5, A and C) and immature (Fig. 5, B and D) forms of the protein in CHAPS. These results indicate that the multimers of mature and immature Shaker protein sediment...
with a velocity consistent with a tetrameric state following CHAPS solubilization. Thus, the results of sucrose gradient centrifugation confirm that Shaker subunits assemble into tetramers in the ER.

As expected, the covalent tetramer did not co-sediment with monomers after Zwittergent solubilization (open circles) but instead sedimented to the denser half of the gradient. This peak did not overlap with that seen in CHAPS, suggesting that Zwittergent weakens the interactions between the covalently linked subunits, which of course cannot be fully dissociated.

**β Subunit Assembles with Shaker Channel in the ER—**Cytoplasmic β subunits have previously been shown to associate with K⁺ channels, altering their functional properties, cell surface expression, or stability (14–18). Endogenous β subunits, which are present in some cell lines, have not been detected in HEK 293 cells, however (46). To determine whether β subunits assemble with Shaker subunits, the rat Kvβ2 protein was co-expressed with Shaker in HEK 293T cells. Kvβ2 is homologous to the Drosophila β subunit, Hyperkinetic, which is thought to associate with Shaker subunits in vivo (16). Specific association between the Shaker and Kvβ2 proteins in HEK 293T cells was demonstrated by reciprocal immunoprecipitation. Anti-Shaker antibodies precipitated Kvβ2 (~38 kDa), and anti-β antibodies precipitated Shaker protein (Fig. 6). Interestingly, the anti-β antibody precipitated both the immature and mature forms of Shaker protein, suggesting that Shaker and β subunits associate in the ER (Fig. 6, left panel). To test this idea further, the Shaker and Kvβ2 proteins were co-expressed and metabolically labeled during a 10-min pulse in the presence of BFA and nocodazole. Following blockade of ER-to-Golgi transport, both anti-β and anti-Shaker antibodies co-precipitated the immature form of Shaker with Kvβ2 (Fig. 6, middle panel), confirming that the α and β subunits assemble in the ER. Results from cells treated with Me₂SO alone were identical to those obtained from untreated cells (Fig. 6, right panel).

It has been previously reported that Kvβ2 acts as a chaperone for mammalian K⁺ channel α subunits (18). To determine whether this is a general property of Kvβ2, we investigated the effect of co-expression on the maturation of Shaker protein. The presence of Kvβ2 did not increase the rate or extent of Shaker protein maturation (data not shown). In fact, co-expression slowed the rate of Shaker maturation (data not shown). This is likely to be a nonspecific effect, caused by competition for the cellular factors needed to over-express two exogenous proteins simultaneously. Similar effects have been seen upon co-expression of other proteins.²

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² R. M. Jiménez and D. M. Papazian, unpublished observations.

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**DISCUSSION**

We have characterized three important features of Shaker K⁺ channel biogenesis in a mammalian cell line. First, maturation of the Shaker protein requires transport from the ER to the Golgi apparatus. Second, the immature form of the Shaker protein contains high mannose oligosaccharides characteristic of the ER, whereas the mature form contains complex oligosaccharides characteristic of modification in the Golgi apparatus. Third, Shaker subunits assemble with each other and with β subunits in the ER. Thus, it is now possible to assign some of the steps in Shaker channel biogenesis to specific intracellular compartments.

**Shaker K⁺ Channels Assemble in the ER—**Our conclusion that Shaker channels assemble in the ER is consistent with biochemical and electrophysiological analysis of K⁺ channel protein translated in vitro. Co-immunoprecipitation experiments suggest that Shaker homologues form heteromultimers within 15 min of the start of translation in vitro (47). Shaker protein translated in vitro in the presence of microsomal membranes can be reconstituted into lipid bilayers to produce functional channels (48), consistent with the idea that the core-glycosylated, ER form of the protein is not only assembled but functionally competent.

For many oligomeric proteins, assembly occurs in the ER and is required for transport to the Golgi apparatus and subsequently to the cell surface (1, 49). Among channel-forming proteins, however, there are exceptions. Whereas subunits of the nicotinic acetylcholine receptor appear to be transported out of the ER as assembled pentamers (50–52), gap junction hemichannels and voltage-dependent Na⁺ channels assemble, at least in part, after leaving the ER (53, 54). Connexin43, a subunit of gap junctions, assembles into hexameric connexons in a post-ER compartment, most likely the trans-Golgi network (53). Similarly, in rat brain neurons, association of the α and β subunits of the Na⁺ channel occurs in the Golgi apparatus late during biogenesis (54). In contrast, our data indicate that both the pore-forming α and cytoplasmic β subunits of voltage-dependent K⁺ channels assemble in the ER.

Shi et al. (18) have recently reported that Kvβ2 increases the glycosylation, stability, and cell surface expression of a mammalian Shaker homologue, Kv1.2, expressed in a cell line. They proposed that the β subunit acts as a chaperone to promote the proper folding and assembly of pore-forming α subunits. In contrast, co-expression of Kvβ2 with Shaker does not increase the rate or extent of Shaker maturation. Therefore, the chaperone action of the β subunit depends on the specific α subunit with which it is expressed. One key difference between the Shaker and Kv1.2 proteins is their efficiency of glycosylation in cell culture. When expressed in the absence of Kvβ2, Shaker undergoes efficient, virtually complete glycosylation and transfer to the Golgi apparatus (28), whereas the Kv1.2 protein is poorly glycosylated and is primarily found in the ER (18). These results suggest that the Drosophila α subunit is more robust than its mammalian counterpart, able to mature efficiently in expression systems without the need for chaperone action by a β subunit.

**Shaker Protein Is Subject to the Quality Control System of the ER—**We have previously shown that maturation provides a consistent and reliable indication that the Shaker protein is in a native conformation (55).³ For wild-type Shaker protein expressed in HEK 293T cells, maturation is very efficient, occurring with an apparent t½ of 45 min and reaching virtual completion within 2.5 h (28). However, some site-directed

³ S. K. Tiwari-Woodruff, C. T. Schulteis, A. F. Mock, and D. M. Papazian, submitted for publication.
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