KCNE1 Subunits Require Co-assembly with K\(^+\) Channels for Efficient Trafficking and Cell Surface Expression*  

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KCNE peptides are a class of type I transmembrane \(\beta\) subunits that assemble with and modulate the gating and ion conducting properties of a variety of voltage-gated K\(^+\) channels. Accordingly, mutations that disrupt the assembly and trafficking of KCNE-K\(^+\) channel complexes give rise to disease. The cellular mechanisms responsible for ensuring that KCNE peptides assemble with voltage-gated K\(^+\) channels have yet to be elucidated. Using enzymatic deglycosylation, immunofluorescence, and quantitative cell surface labeling experiments, we show that KCNE1 peptides are retained in the early stages of the secretory pathway until they co-assemble with specific K\(^+\) channel subunits; co-assembly mediates KCNE1 progression through the secretory pathway and results in cell surface expression. We also address an apparent discrepancy between our results and a previous study in human embryonic kidney cells, which showed wild type KCNE1 peptides can reach the plasma membrane without exogenously expressed K\(^+\) channel subunits. By comparing KCNE1 trafficking in three cell lines, our data suggest that the errant KCNE1 trafficking observed in human embryonic kidney cells may be due, in part, to the presence of endogenous voltage-gated K\(^+\) channels in these cells.

Voltage-gated K\(^+\) channels have diverse physiological roles ranging from repolarization of excitable tissues to salt and water homeostasis in epithelial cells. To achieve such functional diversity, many K\(^+\) channels function as membrane-embedded complexes made up of pore-forming \(\alpha\) subunits and tissue-specific regulatory \(\beta\) subunits. The KCNE type I transmembrane peptides are a class of small \(\beta\) subunits that assemble with and modulate the function of several types of voltage-gated K\(^+\) channels (1). KCNE1 (E1)\(^2\) is the founding member of the family and has been shown to form complexes with Kv3.1, Kv3.2, Kv4.2, Kv4.3, HERG, and KCNQ1 (Q1) K\(^+\) channels (2–7). When E1 assemblies with Q1, the complex produces the slowly activating cardiac \(I_{Ks}\) current and is the exclusive pathway for endolymphatic K\(^+\) recycling in apical membranes of strial marginal and vestibular dark cells (8, 9). Conversely, unpartnered Q1 channels are ill-equipped to perform either function because they open and close too quickly to maintain the rhythmicity of the heartbeat and are significantly inactivated and nonconducting at the resting membrane potential of inner ear cells (10). The biological importance of the proper Q1–E1 complex formation is further underscored by the inherited mutations in either Q1 or E1 that disrupt the assembly and/or trafficking of the complex and give rise to cardiac arrhythmias, most notably long QT syndrome (11). An autosomal recessive form of long QT syndrome also causes neural deafness (12).

Although proper K\(^+\) channel function requires completely assembled complexes at the plasma membrane, the cellular mechanisms that ensure K\(^+\) channel \(\alpha\) subunits combine with membrane-embedded \(\beta\) subunits are starting to be elucidated. For the ATP-sensitive K\(^+\) channels (Kir6.1 and 6.2), which assemble with sulfonylurea receptors (sulfonylurea receptors 1/2A/2B) in a 4:4 stoichiometry, unpartnered channel and receptor subunits are stringently held in the endoplasmic reticulum (ER)\(^3\) via a RXR retention signal (13). Once an octameric complex is formed, the K\(_{ATP}\)-sulfonylurea receptor complex is permitted to exit the ER and traffic to the cell surface.

In contrast, E1 subunits in complexes with voltage-gated K\(^+\) channels may traffic differently than their K\(_{ATP}\) counterparts. Two different studies suggest that Q1–E1 complex formation occurs at the plasma membrane, which would require both proteins to traffic through the secretory pathway independently (14, 15). More recently, it was proposed that Q1–E1 assembly occurs in the ER because mutant E1 proteins could retain Q1 channels there (16). However, unpartnered wild type E1 proteins freely exited the ER and migrated to the cell surface, an observation inconsistent with an ER-based assembly. A significant caveat with all of the above studies was that KCNE trafficking was studied in either *Xenopus* oocytes or human embryonic kidney (HEK) 293B cells, both of which possess endogenous transcripts of voltage-gated K\(^+\) channels that have been shown to assemble with KCNE subunits (7, 17).

Here, we examine the assembly and trafficking of wild type E1 subunits in the presence and absence of Q1 channels in Chinese hamster ovary (CHO) and African green monkey kid-
ney (COS-7) cells, both of which are revered for their “electrical tightness” or lack of measurable endogenous K⁺ currents. Using these cells, we find that the majority of E1 protein is localized to the ER when expressed alone. Co-expression with Q1 subunits promotes E1 protein trafficking through the secretory pathway, resulting in robust cell surface expression. Moreover, these results demonstrate that Q1-E1 complex formation occurs early in the secretory pathway and that unpartnered E1 subunits do not efficiently progress past the ER and cis-Golgi compartments until they assemble with Q1 channel subunits.

**EXPERIMENTAL PROCEDURES**

**Plasmids and cDNAs**—Human Q1 and E1 were subcloned into pcDNA3.1(−) (Invitrogen). Two hemagglutinin A (HA)-tagged versions of E1 were used. For cell surface immunofluorescence experiments, an extracellular HA tag (YPDVDPDYA) was incorporated in the E1 N terminus between residues 22 and 23 based on the work of Wang and Goldstein (18). For intracellular immunolocalization experiments, an HA tag was appended to the E1 C terminus via a SGSG linker. Biochemical experiments were performed with both HA constructs. For consistency, only the data from the extracellularly tagged version is shown.

**Cell Culture and Transfections**—Chinese Hamster Ovary-K1 (CHO) cells were cultured in F-12K nutrient mixture (Invitrogen). HEK cells and African green monkey kidney (COS-7) cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma). All of the media were supplemented with 10% fetal bovine serum (Hyclone) and 100 units/ml penicillin/streptomycin (Invitrogen). For biochemical analysis, the cells were plated at 60–75% confluency in 35-mm dishes. After 24 h, the cells were transiently transfected at room temperature with E1 alone, Q1-E1, or Q4-E1 at a ratio of 0.5 μg/1.5 μg with 8 μl of Lipofectamine 2000 (Invitrogen). Empty pcDNA 3.1(−) plasmid DNA (0.5 μg for CHO, 0.25 μg for COS-7, and 1.25 μg for HEK) was co-transfected in samples containing only E1 DNA to keep the total transfected DNA constant in all wells. To increase the amount of E1 protein with complex glycans in CHO cells, 0.75 μg of Q1 DNA was used in the co-transfection. For immunofluorescence studies, the cells were plated at 50% confluency onto sterile 12-mm glass coverslips (untreated for Co-immunoprecipitation—The cells were washed in TBS-T (four times for 5 min) and incubated with goat anti-rat horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc.) (1:2000) in Western blocking buffer (20 mM Tris HCl, pH 7.4, 140 mM NaCl, 10 mM KCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). All of the detergent buffers were supplemented with protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin, and aprotinin. For expression gels, CHO cells were lysed in 300 μl of 1% SDS buffer, and COS-7 cells were lysed in 250 μl of RIPKA buffer. Cell debris was pelleted in a microcentrifuge (16,100 × g for 10 min at room temperature). The supernatants were diluted with SDS-PAGE loading buffer containing 100 mM dithiothreitol, separated on a 15% SDS-polyacrylamide gel, and transferred to nitrocellulose (0.2 μ; Schleicher & Schuell). The membranes were blocked in Western blocking buffer (5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween 20 (TBS-T)) for 30 min at room temperature and then incubated with rat anti-HA (Roche Applied Science) (1:750) in Western blocking buffer overnight at 4 °C. The membranes were washed in TBS-T (four times for 5 min) and incubated with goat anti-rat horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc.) (1:2000) in Western blocking buffer for 45 min at room temperature. The membranes were subsequently washed with TBS-T (four times for 5 min). Horseradish peroxidase-bound proteins were detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce) and a Fujifilm LAS-3000 CCD camera.

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**Endo H and PNGase F Deglycosylation Analysis**—The cell lysates (30 μl) were diluted to 0.5% SDS with water and raised to 1% Nonidet P-40 (for PNGase F analysis only), 1% β-mercaptoethanol, 50 mM sodium citrate, pH 5.5 (for Endo H analysis) or 50 mM sodium phosphate, pH 7.5 (for PNGase F analysis) and then digested with Endo H (1 μl) or PNGase F (2 μl) (New England BioLabs, Inc.) for 30 min at 37 °C. The samples were then raised to 100 mM dithiothreitol and 3.5% SDS before resolving by SDS-PAGE (15% gel) and analyzed by Western blot.

**KCNE1 Co-immunoprecipitation**—The cells were washed in ice-cold PBS (3 × 2 ml) and lysed at 4 °C in 250 μl of 1.5% digitonin buffer. The cell debris was pelleted in a microcentrifuge (16,100 × g for 15 min at room temperature). 30 μl of cell lysate was incubated with 5 μl of anti-Q1 antibody (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The samples were bound to protein G beads (Pierce) for 2 h at room temperature to separate out antibody-bound protein complexes from the cell lysate. The beads were washed (three times with 500 μl) in digitonin-free wash buffer (20 mM Tris HCl, pH 7.4, 140 mM NaCl, 10 mM KCl, 1 mM MgCl₂). Co-immunoprecipitated proteins were eluted from the beads using 1× SDS-PAGE loading buffer with 200 mM dithiothreitol for 15 min at room temperature. One-fifth of the total input used for the co-immunoprecipitation assay and eluted proteins were resolved by SDS-PAGE (15% gel) and analyzed by Western blot.

**Immunofluorescence**—For visualization of intact cells, the cells were first labeled with rat anti-HA antibody (1:200) in culture medium for 30 min at 37 °C. The cells were rinsed quickly in warm PBS (3 × 2 ml) before fixing in 4% paraformaldehyde (10 min at room temperature). The cells were then blocked in blocking solution (PBS containing 5% normal goat serum (Vector Laboratory, Inc.), 1% IgG-free bovine serum
albumin (Sigma)) for 30 min at room temperature. For permeabilized samples, the cells were first washed in PBS (3 × 2 ml) and then fixed in 4% paraformaldehyde (10 min at room temperature). The cells were blocked in Triton blocking buffer (blocking solution containing 0.2% Triton X-100) and then incubated with rat anti-HA antibody (1:500) in Triton blocking buffer for 1 h at room temperature. Nonpermeabilized and permeabilized samples were then washed with PBS (3 × 5 min) and incubated with goat anti-rat Alexa Fluor 594-conjugated antibody (Molecular Probes) (1:2000) in blocking solution or Triton blocking solution, respectively, for 45 min at room temperature. The cells were then washed with PBS (two times for 5 min; then for 1 h). The coverslips were dried at 37 °C for 45 min before mounting on slides with ProLong® Gold Antifade mounting solution (Molecular Probes). For co-staining with intracellular markers, the cells were fixed in 100% methanol at −20 °C for 8 min, rehydrated in PBS for 5 min, and blocked overnight in SeaBlock Blocking buffer (Pierce). The cells were first incubated with rabbit anti-calnexin (Stressgen) (1:500) or concentrated mouse serum from monoclonal antibody CM1A10 for β' coatomer protein (anti-β’ COP) (19, 20) (1:10) in SeaBlock Blocking buffer at room temperature for 90 min. The cells were then washed with PBS (3 × 10 min) and incubated with donkey anti-rabbit Alexa Fluor 488-conjugated antibody (1:500) or donkey anti-mouse Alexa Fluor 488-conjugated antibody (1:250) (Molecular Probes) in SeaBlock blocking buffer at room temperature for 90 min. The cells were washed in PBS (three times for 10 min) and labeled for E1 as described above with rat anti-HA (1:100) followed by goat anti-rat Alexa Fluor 594-conjugated antibody (1:500) in SeaBlock blocking buffer. After a final set of PBS washes (three times for 10 min), the coverslips were mounted on slides with Vectashield mounting medium (Vector Laboratories). All of the cells were visualized using a Zeiss Axiosvert 200M microscope with a 63 × 1.4 N.A. oil immersion objective. z stacks were generated by obtaining optical sections through the samples 0.4 μm apart along the z axis. Deconvolution of z stack images was performed with a constrained iterative algorithm on Slidebook 4.0 software (Intelligent Imaging Innovations) using a measured point spread function. The images are displayed as single 0.4-μm planes through the center of each cell.

Cell Surface Biotinylation—The cells were rinsed with ice-cold PBS 2+ buffer (four times with 2 ml PBS containing 1 mm MgCl₂, 0.1 mm CaCl₂) at 4 °C to arrest membrane internalization. The cells were then incubated with 1 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS 2+ buffer twice for 15 min at 4 °C. To quench the excess biotinylation reagent, the cells were washed quickly (three times with 2 ml) with quench solution (PBS 2+ containing 100 mM glycine) and then incubated with quench solution twice for 15 min at 4 °C. The cells were lysed in RIPA buffer for 30 min at 4 °C. Cell debris was removed by centrifugation (16,100 × g for 10 min at 4 °C). Total protein in each sample was quantitated by BCA analysis. Of these samples, 75 μg of total protein was separated by affinity chromatography on 25 μl of Immunopure® immobilized streptavidin beads (Pierce) overnight at 4 °C, whereas 15 μg was saved as an input control to determine the percentage of biotinylated proteins. The beads were washed (three times with 500 μl) in 0.1% SDS wash buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS). Biotinylated proteins were eluted from the beads using 2× SDS-PAGE loading buffer with 200 mM dithiothreitol for 15 min at 55 °C. The inputs and eluted proteins were resolved by SDS-PAGE (15% gel) and analyzed by Western blot. The images of nonsaturated bands were captured on a Fujifilm LAS-3000 CCD camera, and the band intensities were quantitated using MultiGauge V2.1 software (Fujifilm). An ER-resident protein, calnexin, was used as a control to determine the percentage of cell rupture that occurred during the labeling process. Background cell lysis was quantitated as a ratio of biotinylated calnexin protein to total input calnexin. The percentage of E1 protein on the cell surface was calculated from the ratio of avidin-bound protein to total input protein after background lysis subtraction.

RESULTS
Active retention of unassembled membrane protein subunits is one mechanism by which a cell ensures that membrane-embedded K⁺ channel complexes are properly assembled before the proteins arrive at their final cellular destination (21). Distinguishing cellular markers for ER-resident proteins are immature N-linked glycans. The ER utilizes these high mannose oligosaccharide handles to promote protein folding, oligomerization, quality control, retention, and trafficking (22). Once N-linked glycoproteins reach the Golgi, however, they are no longer subject to the rigorous quality control systems of the ER and migrate through medial and trans-Golgi compartments relatively unabated while acquiring complex modifications to their N-linked glycans (22). Because KCNE peptides have two well conserved N-linked glycosylation consensus sites, we utilized this hallmark of ER residency to determine the cellular locales of E1 peptides expressed alone, with Q1, and with KCNQ4 (Q4), a K⁺ channel previously shown not to assemble with E1 (Fig. 1) (23). Transient expression of E1 alone in CHO cells gave rise to two strong bands at 16 and 22 kDa (though faint, higher molecular mass bands can be seen on this intentionally well developed, but not overexposed Western blot).
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When expressed with Q4, a similar banding pattern was observed with an additional band at 19 kDa. In contrast, expression with Q1 affords an additional, higher molecular mass band at ~40 kDa. The presence of this 40-kDa band was independent of the detergent lysis mixture used but only occurred in abundance when E1 was co-expressed with Q1. The abundance of the higher molecular mass form of E1 was dependent on the ratio of Q1-E1 DNA used for transient transfection. Limiting amounts of Q1 DNA yielded all three forms of E1 protein as shown in Fig. 1; optimizing the Q1-E1 DNA ratio (Experimental Procedures) afforded primarily the higher molecular mass species (Fig. 2; see also Fig. 4). We also observed the same gel banding patterns for E1 in COS-7 cells (Fig. 1).

The multiple bands and differences in gel shift mobility suggested that E1 peptides were differentially glycosylated depending on the presence of specific K⁺ channel subunits. We therefore determined whether the gel shift mobility difference that was observed with Q1-E1 co-expression was due to maturation of the glycoproteins on E1. The maturity of N-linked glycoproteins can be readily determined using two different glycosidases. Endo H is a glycosidase that removes the high mannose (immature) glycan found on ER and cis-Golgi proteins but is unable to process the complex (mature) glycoform once the protein reaches the medial Golgi in the biosynthetic pathway. In contrast, PNGase F is an endoglycosidase that removes all oligosaccharides. Because maturation of N-linked glycoproteins occurs in the medial and trans-Golgi, the presence of mature glycans in only the Q1-E1 sample shows that co-assembly with Q1 facilitates E1 peptide progression through the secretory pathway. Although the PNGase F-treated E1 protein was deglycosylated, several residual bands were observed (indicated by the asterisks). Prolonged treatment with PNGase F, the addition of more enzyme, varying the concentration of SDS in the gel loading buffer, or heat denaturation did not change the intensity or mobility of these bands (data not shown). The residual banding is not partial digestion of the individual N-linked glycans because PNGase F directly cleaves the oligosaccharide from the peptide backbone (24). It is possible that one of the two mature N-linked glycans on E1 is totally inaccessible to the enzyme; however, we have observed these similar bands with PNGase F-treated E1 mutants that possess only one N-linked glycosylation site (data not shown). These bands do not correspond to immature E1, because they have a slightly slower mobility that can be readily observed in either the E1 or the Q1-E1 PNGase F-treated samples. Moreover, PNGase F treatment of the E1 sample demonstrates that this enzyme cleaves both immature N-linked glycans efficiently. These additional bands were also resistant to a subsequent phosphatase or neuraminidase (to remove sialic acid residues from O-linked glycans) treatment (data not shown) and may be due to the presence of another type of post-translational modification.

Because maturation of N-linked glycans on E1 requires co-expression with Q1, we determined whether cell surface expression of E1 was also dependent on expression with specific K⁺ channel subunits. Cell surface expression of E1 protein was visualized by immunofluorescence with permeabilized and nonpermeabilized cells using an extracellularly HA-tagged version of E1 (18). In nonpermeabilized cells, no cell surface labeling of E1 was observed when E1 was expressed alone or with Q4 (Fig. 3, E1 and Q4/E1 panels). Co-transfection with green fluorescent protein DNA verified that the absence of cell surface staining was not a result of untransfected cells. Permeabilization of the cells confirmed that the majority of the E1 protein was intracellular, as evidenced by the punctate staining. In contrast, co-expression with Q1 resulted in striking cell surface staining of E1 in intact and permeabilized cells (Fig. 3, Q1/E1 panels).

The plasma membrane protein levels of E1 were then quantitated by cell surface biotinylation. To minimize labeling of intracellular proteins and membrane recycling, cells expressing E1 with or without Q1 were labeled with a membrane-impermeant, amine-reactive biotin derivative at 4 °C. When co-expressed with Q1, E1 shows robust cell surface expression (Fig. 4A). Moreover, no immature E1 was ever observed on the plasma membrane, which is consistent with the enzymatic deglycosylation assays (Fig. 2). Little to no protein was observed at the cell surface when E1 was expressed alone. To account for cell rupture during biotinylation, the ER-resident protein cal-
Because E1 does not reach the plasma membrane when it is expressed alone, we determined its intracellular localization. Results from enzymatic deglycosylation (Fig. 2) narrow the spectrum of intracellular locales to the ER and/or cis-Golgi because the glycans on E1 were predominately immature. Initial examination of the intracellularly localized E1 protein by immunofluorescence afforded weak overall staining. The strongest signals were punctate in nature and not reminiscent of ER staining, as can be seen in the E1 permeabilized cells in Fig. 3. Accordingly, E1 did not co-stain with ER markers (data not shown). These observations did not correlate with the strong protein expression seen in Western blots (Fig. 1). We wondered whether antibody accessibility to the E1 HA tag was being prevented by luminal proteins binding to the E1 N terminus because this extracellular tag is positioned between the two N-linked glycosylation consensus sequences (18). Thus, we repositioned the HA tag to the intracellular C terminus of E1. This C-terminal construct behaved similarly to the extracellularly tagged construct in all aspects; it assembles with Q1 channel subunits to afford the cardiac I_{Ks} current and required co-expression with Q1 to reach the cell surface with mature N-linked glycans (data not shown). In contrast to the extracellularly tagged E1 construct, visualization of the C-terminally HA-tagged E1 protein by immunofluorescence gave strong perinuclear staining (Fig. 5A). Counterstaining with calnexin and overlaying the images showed that the majority of E1 protein co-stains with this ER marker, although some E1 protein did not. E1 also co-stained with the cis-Golgi marker, B’COP (20); however, the majority of the E1 protein stained in a pattern similar to calnexin. Little to no co-staining was observed when E1 was expressed with EYFP-Golgi, which localizes to the trans-Golgi network.

The intracellular staining of E1 showed that the majority of the protein resides in the ER when it is expressed alone. We determined whether Q1 subunits could co-assemble with the immature glycoforms of E1 found in the ER. Fig. 5B shows that all glycoforms, mature, immature, and unglycosylated, co-immunoprecipitate with Q1. Immunoprecipitation of E1 required co-expression with Q1 because no bands were observed when E1 was expressed alone.

To verify that there are inherent differences in cellular trafficking of E1 in HEK cells versus CHO and COS-7 cells and not variability in experimental design, reagents, or constructs, we also examined E1 protein expression and trafficking in HEK cells. Unlike in CHO and COS-7 cells, expression of E1 in HEK cells primarily afforded the higher molecular mass species

nexitin was used as an internal control in all experiments. The percentage of E1 protein at the cell surface was quantitated as a ratio of biotinylated proteins over the total protein input shown in Fig. 4B. For Q1-E1, ~35% of E1 protein was at the cell surface after background subtraction. For E1 alone, the percentage of protein detected on the cell surface by biotinylation was within error of background calnexin labeling.

FIGURE 3. Cell surface expression of KCNE1 requires co-expression with K^+ channel subunits that assemble with KCNE peptides. Cell surface immunostaining of E1 expressed alone or with Q4 or Q1 in CHO cells is shown. The cells were fixed and left intact to visualize cell surface staining or permeabilized to visualize total cellular staining. The images shown are single 0.4-μm planes through the center of each cell. Differential interference contrast (DIC) images show both untransfected and transfected cells. Green fluorescent protein (GFP) images identify transfected cells. Alexa 594 images show E1 protein staining with Alexa 594-conjugated antibodies. The fluorescent images were captured using the same exposure time and rendered identically to qualitatively compare the fluorescent intensities between panels. Note the strong cell surface staining for the Q1-E1 panels (nonpermeabilized and permeabilized), whereas the E1 and Q4-E1 permeabilized panels show mostly punctate staining, indicative of intracellular distribution.

FIGURE 4. Quantification of the KCNE1 plasma membrane protein by cell surface biotinylation. A, a representative immunoblot of E1 expressed in CHO cells with and without Q1 channel subunits. Transfected cells were labeled with a membrane-impermeant biotin reagent and subsequently lysed, and the biotinylated proteins were isolated from 75 μg of total protein. Lanes identified as one-fifth of the total input are 15 μg of each sample lysate that was set aside to quantify the total amount of biotinylated proteins. Biotinylated lanes are the streptavidin-bound proteins that were eluted from the beads and separated by SDS-PAGE (15%). The calnexin (CNX) immunoblot shows that the majority of the cells remained intact during the biotinylation procedure. B, quantification of the biotinylated E1 proteins. The percentage of biotinylated protein was calculated by dividing the band intensities in the biotinylated lane by the band intensities in the one-fifth total input lane, which were multiplied by five. There was no significant difference between background calnexin labeling and cell surface labeling of E1 when the peptide was expressed alone. Co-expression with Q1 results in 34 ± 11% E1 protein on the cell surface after subtracting for background cell lysis (calnexin control). The error bars are standard error measurement (S.E.) from three immunoblots. The dotted line indicates background biotinylation, which was calculated from calnexin staining (4 ± 1%).
when it was expressed alone or with Q1 (Fig. 6). Treatment with Endo H and PNGase F confirmed that this species contained mature N-linked glycosylation. As we observed with CHO and COS-7 cells, PNGase F digestion of E1 peptides from HEK cell lysates resulted in bands (indicated by the asterisks) that migrated slower than both immature and unglycosylated protein. Nonetheless, these results show that the N-linked glycans on E1 peptides in HEK cells mature without transiently expressed K+ channel subunits.

Given that E1 peptides in HEK cells exit the ER and traffic through the Golgi without co-expressed K+ channel subunits, we determined whether these peptides were reaching the cell surface after calnexin subtraction; co-expression with Q1 increased the E1 cell surface protein to 22 ± 3%.

The combination of weak cell surface expression and the overwhelming maturation of the N-linked glycans on E1 when it is expressed in HEK cells prompted us to examine its intracellular distribution. The C-terminally HA-tagged version of E1 was expressed in HEK cells and overlaid with a panel of intracellular markers (Fig. 7B). Negligible co-staining was observed with the ER marker, calnexin. Modest co-staining was observed with β-COP, but a substantial amount of E1 co-localized with EYFP-Golgi in the trans-Golgi network. However, in all merged images, there was some E1 protein that did not co-stain with any of the markers used. In total, these results in HEK cells demonstrate that the E1 protein traffics through the ER and to the Golgi apparatus without an exogenously expressed K+ channel.

**DISCUSSION**

We studied the trafficking of E1 peptides through the biosynthetic pathway by following the maturation of the oligosaccharides on E1 because N-linked glycan processing is intimately tied to cellular compartmentalization (25). By expressing E1 alone, with Q1, or with Q4 (a K+ channel that does not assemble with E1), we determined that E1 glycoproteins do not mature unless they are co-expressed with K+ channel subunits that assemble with KCNE peptides. In addition, cell surface expression of E1 was also co-dependent on Q1 subunit expression, which was demonstrated in both qualitative and quantitative cell surface labeling experiments. Moreover, only the mature form of E1 was ever observed at the cell surface, indicating that the immature E1 protein bands observed at ~20 kDa do not correspond to E1 protein in functioning Q1-E1 complexes at the plasma membrane. Our results in CHO and COS-7 cells recapitulate what has been observed in *Caenorhabditis elegans* where a KCNE homolog requires its corresponding K+ channel subunits to reach the cell surface (26).

The presence of immature N-linked glycans on E1 indicates that these proteins have yet to traffic beyond the cis-Golgi in the
biosynthetic pathway and strongly suggests that unpartnered E1 peptides are actively prevented from trafficking past these early compartments in the secretory pathway. Co-localization experiments using a panel of intracellular markers place the majority of the E1 protein in the ER and a small fraction in the cis-Golgi. Although these results cannot rule out Q1-E1 complex assembly in the cis-Golgi, the sheer abundance of unpartnered E1 peptides in the ER and co-immunoprecipitation of the immature and unglycosylated E1 with Q1 strongly suggest that Q1-E1 complex assembly occurs in the ER. An ER-based assembly for Q1-E1 complexes directly contradicts two previous functional studies where it was proposed that complex assembly occurs at the plasma membrane (14, 15). The basis for this proposal was that *Xenopus* oocytes expressing unpartnered Q1 K<sup>+</sup> currents could be converted to KCNE-modulated currents within 24 h by a subsequent injection of KCNE mRNA. However, this functional change was the only evidence in support of this hypothesis. The cell surface and ER lifetimes of Q1 protein were not measured, nor was it directly shown that previously unpartnered, plasma membrane Q1 channels acquired newly synthesized KCNE peptides. The fact that the majority of the unassembled E1 peptides reside in the ER, possess immature N-linked glycans, and do not reach the cell surface in the absence of Q1 channel subunits eliminates a plasma membrane-based assembly mechanism for Q1-E1 complexes.

Enzymatic deglycosylation assays also revealed that E1 peptides potentially possess another post-translational modification, because we routinely observed digestion products that migrated slower than unglycosylated E1 (Figs. 2 and 5) in all three cell lines: CHO, COS-7, and HEK. These bands appeared whether the E1 peptide possessed either or both N-linked glycosylation sites. In addition, changing the detergent composition used for cell lysis or the concentration of SDS in the loading buffer did not change the migration or intensity of these bands. The modification may be specific to E1 because PNGase F treatment of KCNE3 peptides co-expressed with Q1 channels in *Xenopus* oocytes (27), and CHO cells<sup>4</sup> are completely digested to unglycosylated peptides. The retarded migration is not due to phosphorylation because treatment with phosphatase had no effect on the mobility of these bands. These residual bands were also resistant to a subsequent neuraminidase treatment, suggesting that if O-linked glycans are present on E1, the removal of the sialic acids does not change the mobility of the protein on a denaturing gel. Neuraminidase treatment of the N-linked glycans on Q1-E1, however, did show a subtle redistribution of the heterogenous E1 mature bands consistent with the removal of sialic acid residues (data not shown).

Previous studies of E1 trafficking in HEK cells observed that wild type E1 peptides could readily exit the ER with or without a co-expressed K<sup>+</sup> channel (16). In light of our contrasting results using CHO and COS-7 cells, we re-examined the cellular processing of E1 protein in HEK cells. As was previously reported, E1 peptides exited the ER without the exogenous expression of K<sup>+</sup> channels subunits. Using immunofluorescence, E1 protein could be detected at the plasma membrane in some cells; however, quantitative analysis of the cell surface population by biotin labeling revealed that the expression of E1 alone was barely detectable after background subtraction (4 ± 2%) and was considerably lower when compared with co-expression with Q1 (22 ± 3%). Visualization of the intracellular distribution of E1 in HEK cells by immunofluorescence showed that the majority of the protein has trafficked past both the ER and cis-Golgi. Some E1 protein is localized to the trans-Golgi network; however, a fair amount of E1 does not co-stain with any of the secretory pathway markers used. The presence of E1 protein in the ER and cis- and trans-Golgi network; however, a fair amount of E1 does not co-stain with any of the secretory pathway markers used. The presence of E1 protein in the ER and cis- and trans-Golgi leaves open the possibility that Q1-E1 complex assembly in HEK cells may not be confined to the early compartments of the secretory pathway. This series of experiments suggests that the majority of the E1 protein in HEK cells exits the ER but is trapped in the trans-Golgi or is trafficked to another compartment in the cell. Redistribution of E1 peptides to intracellular vesicles may be an ancillary mechanism that occurs in HEK cells to redirect unassembled, mature E1 peptides to recycling and degradation compartments. These contrasting results suggest that there are inherent differences in KCNE trafficking in HEK versus CHO or COS-7

<sup>4</sup>S.D. Gage and W. R. Kobertz, unpublished results.
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cells. Traditionally, the biochemical investigation of K+ channel α and β subunits has been performed in HEK cells because copious amounts of protein can be generated; however, these cells have been avoided for electrophysiological studies because endogenous voltage-gated K+ currents can be measured (17). It is now known that some of these K+ channels can assemble with KCNE peptides, including E1 (2). The presence of native K+ channels in HEK cells could explain the enigmatic trafficking of E1. In HEK cells, E1 peptides do not reach the plasma membrane unaccompanied but assemble with endogenous K+ channel subunits in the secretory pathway. This inference harkens back to the initial discovery of E1 by expression cloning in *Xenopus* oocytes, where exogenously introduced E1 peptides assembled with endogenous Q1 channel subunits and trafficked to the plasma membrane producing the cardiac *I*_κ current (28, 29). The low cell surface expression of E1 supports the idea that it forms complexes with limiting amounts of endogenous K+ channel subunits. However, the lack of immature E1 observed in our immunoblots suggests that protein overexpression may also contribute to the artificial ER exit of E1 in HEK cells. Whether cell surface expression of E1 is mediated by endogenous K+ channels or by overwhelming the retention/retrieval machineries of the cell, studying the assembly and trafficking of KCNE peptides in HEK cells should be avoided.

The ER has two well known mechanisms for retaining misfolded and unassembled protein subunits: recognition of ER retention/retrieval signal sequences and chaperone-mediated retention of N-linked glycoproteins. The best studied ER retention signal to date is the RXR motif, which has been shown to regulate the ER exit of *K*_ATP channel subunits and sulfonlurea receptors (13). ER retention through this motif is a stringently controlled mechanism because neither protein can escape the ER with this signal sequence exposed. E1 peptides do not possess such a motif nor are they exquisitely retained in the ER when overexpressed in CHO or COS-7 cells, because faint bands that corresponded to mature glycoprotein were always observed with solitary expression of E1 (Figs. 1 and 2). Because we also observe some E1 protein in the cis-Golgi, it is possible that E1 is subject to ER retrieval. E1 does possess a KK motif found with the extracellularly HA-tagged E1 construct, which necessitated repositioning of the tag. If E1 peptides are retained in the ER by calnexin or calreticulin, this mechanism would be applicable to all KCNE peptides because the N-linked consensus site closest to the N terminus is conserved throughout the family. It is also feasible that multiple retention/retrieval machineries are acting on unassembled E1 peptides or that Q1–E1 co-assemble promotes active export of the complex from the ER.

The goal in preventing unassembled K+ channel α and β subunits from leaving the ER is to ensure the formation of fully assembled K+ channel complexes because unpartnered K+ channels would have inappropriate gating and ion conducting properties. Although our results show that unpartnered E1 peptides reside primarily in the ER, homotetrameric Q1 channels have been shown to exit the ER and function at the plasma membrane in several different ion channel expression systems, including CHO and COS-7 cells (10, 32, 33). In E1 knock-out mice, however, Q1 channels do not traffic to the apical membranes of vestibular dark cells, where they would normally be found with E1 in wild type cells (34). In addition, RNA interference experiments in *C. elegans* suggest that the stability of the K+ channel complex is dependent on the presence of both pore-forming and KCNE-like subunits (26). The discordant observation of unpartnered Q1 channels at the cell surface in expression systems may be due to Q1 channels leaking from the ER as a result of overexpression. Consistent with swapping the ER with overexpressed protein is the fact that the majority of the Q1 protein in expression systems is intracellular (35). Moreover, Q1 expressed in *Xenopus* oocytes or CHO cells is not N-linked glycosylated and thus not subject to glycosylation-dependent ER retention. An alternative explanation for the ER exit of unpartnered Q1 channels is that E1 protein synthesis always precedes Q1 subunit expression in native tissues. With this temporal control, the ER is always chock full of E1 peptides that can readily assemble with newly synthesized K+ channel subunits. This mechanism alleviates the need to retain unpartnered Q1 channels as long as the rate of co-assembly with E1 is faster than ER exit of the homotetrameric channel. There is some *in vivo* evidence in the developing ear to support this notion, because E1 protein is expressed in embryonic mouse vestibular dark cells 24 h before the Q1 protein (34).

Many studies have shown that modulation of voltage-gated K+ channels by KCNE peptides provides the functional diversity required for these complexes to work in the nervous system, muscle, colon, ear, and heart; however, for proper biological function these complexes must reach the plasma membrane fully assembled. Our results show that E1 peptides cannot advance past the cis-Golgi until they assemble with Q1 channel subunits, which is the first step in the assembly and trafficking of KCNE–K+ channel complexes. Given that mutations in KCNE peptides affect the assembly and trafficking of the entire K+ channel complex and cause long QT syndrome, identifying the cellular machineries that directly ensure the proper assembly and trafficking of healthy KCNE–K+ channel complexes will be critical for following and interpreting the aberrant assembly and trafficking of diseased complexes.

Acknowledgments—We are indebted to Dr. Haley Melikian for the use of the wide field microscope. We thank Dr. Geoffrey Abbott for providing us the HA-tagged E1 clone, Graham Warren for CM1A10 concentrated mouse serum, and David Clapham for EYFP-Golgi plasmid.

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