Adjacent Basic Amino Acid Residues Recognized by the COP I Complex and Ubiquitination Govern Endoplasmic Reticulum to Cell Surface Trafficking of the Nicotinic Acetylcholine Receptor \( \alpha \)-Subunit*

Steven H. Keller‡§, Jon Lindstrom¶, Mark Ellisman‡, and Palmer Taylor‡

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The nicotinic acetylcholine receptor in muscle is a ligand-gated ion channel with an ordered subunit arrangement of \( \alpha \)-\( \gamma \)-\( \alpha \)-\( \delta \)-\( \beta \). The subunits are sequestered in the endoplasmic reticulum (ER) and assembled into the pentameric arrangement prior to their exit to the cell surface. Mutating the Arg313–Lys314 sequence in the large cytoplasmic loop of the \( \alpha \)-subunit to K314Q promotes the trafficking of the mutant unassembled \( \alpha \)-subunit from the ER to the Golgi in transfected HEK cells, identifying an important determinant that modulates the ER to Golgi trafficking of the subunit. The association of the K314Q \( \alpha \)-subunit with \( \gamma \)-COP, a component of COP I coats implicated in Golgi to ER anterograde transport, is diminished to a level comparable to that observed for wild-type \( \alpha \)-subunits when co-expressed with the \( \beta \)-, \( \delta \)-, and \( \gamma \)-subunits. This suggests that the Arg313–Lys314 sequence is masked when the subunits assemble, thereby enabling ER to Golgi trafficking of the \( \alpha \)-subunit. Although unassembled K314Q \( \alpha \)-subunits accumulate in the Golgi, they are not detected at the cell surface, suggesting that a second post-Golgi level of capture exists. Expressing the K314Q \( \alpha \)-subunit in the absence of the other subunits in ubiquitinating deficient cells (ts20) results in detecting this subunit at the cell surface, indicating that ubiquitination functions as a post-Golgi modulator of trafficking. Taken together, our findings support the hypothesis that subunit assembly sterically occludes the trafficking signals and ubiquitination at specific sites. Following the masking of these signals, the assembled ion channel expresses at the cell surface.

Multimeric transmembrane proteins, including complex ligand-gated ion channels represented by nicotinic acetylcholine receptors (nAchR), generally require subunit assembly to be transported beyond the endoplasmic reticulum (ER) into the secretory pathway leading to the cell surface (1–3). As integral membrane components of lipid trafficking vesicles, unassembled subunits are re-localized to the ER, stabilized by chaperones and either assembled with neighboring subunits or targeted for degradation by ubiquitination and cleavage in the proteasome (4, 5). Cellular mechanisms that distinguish whether a protein is folded and/or assembled and directed to the cell surface or misfolded and/or unassembled and shuttled into a degradative pathway are poorly understood, especially for the polytopic membrane proteins represented by ligand-gated ion channels. The importance for shedding light on this topic is borne out by several debilitating disorders associated with mutations that are believed to cause misfolding and inhibit the trafficking of physiologically important ion channels. Examples include inherited mutations in potassium channel subunits that increase the propensity to develop cardiac arrhythmias (6, 7) and amino acid substitutions in cystic fibrosis transmembrane conductance regulator that result in cystic fibrosis (8–10). In this study, we employ the nAChR \( \alpha \)-subunits as a model to identify factors that modulate the trafficking of ion channel proteins into the secretory pathway. We approached this question by identifying mechanisms that restrict the placement of unassembled \( \alpha \)-subunits at the cell surface and asked how subunit assembly abrogates these processes.

The nAChR in muscle is a ligand-gated ion channel composed of two \( \alpha \)-subunits and single \( \beta \)-, \( \gamma \)-, and \( \delta \)-subunits which surround a central cation channel pore. As determined by subcellular fractionation, the unassembled \( \alpha \)-subunits are confined primarily to the ER compartment (11). Insignificant amounts of unassembled \( \alpha \)-subunit are detected on the cell surface following transfection, transient expression in mammalian cells, and \( ^{125} \text{I}-\alpha \)-bungarotoxin exposure (12). In contrast, appreciable binding of \( ^{125} \text{I}-\alpha \)-bungarotoxin is detected on the cell surface when \( \alpha \)-subunits are co-expressed with the \( \beta \)-, \( \delta \)-, and \( \gamma \)-subunits, demonstrating that subunit assembly is a requirement for the cell surface expression (12, 13). As the nAChR assembles into a pentamer, assembled \( \alpha \)-\( \delta \) and \( \alpha \)-\( \gamma \) dimers and other intermediates are detected in intracellular pools of the cell (12–14). However, the \( \alpha \)-\( \delta \) and \( \alpha \)-\( \gamma \) dimers are also sequestered intracellularly, as evidenced by the lack of \( ^{125} \text{I}-\alpha \)-bungarotoxin binding to the surface of intact cells. (12, 13). Co-expression of the \( \beta \)-subunit with the \( \alpha \)-, \( \gamma \)-, and \( \delta \)-subunits is required to transport the assembled subunits to the cell surface.

We therefore hypothesized that trafficking signals posi-
tion in the α-subunit at the interface that assembles with the β-subunit are enclosed when the subunits assemble to form a circular pentamer. The trafficking signals then become sterically occluded from the cellular machinery that otherwise would retrieve proteins back to the ER. The major aim of this study was to identify the ER retrieval sequences in the α-subunit that inhibit the trafficking of the unassembled subunit beyond the ER. Our experimental findings demonstrate that the adjacent basic amino acid signal Arg131–Lys134 in the large cytoplasmic loop of the α-subunit regulates trafficking of the α-subunit from the ER to the Golgi. This is revealed in the trafficking characteristics of the α-subunit with the K314Q mutation that proceeds from the ER and accumulates in the Golgi when expressed in the absence of the other receptor subunits. Although the altered α-subunit proceeds to the Golgi, it does not express at the cell surface. Inhibition of ubiquitination results in detecting the α-subunit with the K314Q alteration at the cell surface. We therefore conclude that ubiquitination is a modulator for the Golgi to cell surface trafficking of the α-subunit. Thus, we disclose two mechanisms that regulate the trafficking of nAChR. One of these involves masking of ER retrieval sequences by assembly of the heterologous subunits that regulate the ER to Golgi trafficking of the α-subunit. The second mechanism regulates Golgi to cell surface trafficking and is modulated by ubiquitination. These two mechanisms may operate together to provide the “quality control” of the completed receptor that progresses to the cell surface.

MATERIALS AND METHODS

Antibodies—Western blotting and immunofluorescence protocols aimed at detecting the α-subunit employed the rat monoclonal antibody (mAb) 210, which recognizes an epitope in the extracellular domain (15). The antibody to γ-COP and β-COP (clone M3A5, Sigma) was added to the soluble fraction for 30 min, followed by addition of IgG-Sepharose beads for another 45 min. Equivalent volumes of sample were resolved on 10% SDS-polyacrylamide gels (NOVEX, San Diego, CA) and transferred to nitrocellulose. Western blots were developed with chemiluminescent techniques.

125I-α-Bungarotoxin Expression Assay—Cells were transfected with the indicated subunit combinations, grown for 48 h, and blocked first with 10 μM carbamylcholine followed by exposure to 10 nM 125I-α-bungarotoxin for 1 h, the resultant radioactive counts correspond to the residual nonspecific binding.

RESULTS

Altering the Adjacent Basic Signal Arg123–Lys124 into Arg123–Gln124 Promotes Trafficking of the α-Subunit to the Golgi—Acetylcholine receptor α-subunits are representative of the family of ligand-gated ion channels that display the general topology shown in Fig. 1A, consisting of one major extracellular domain, four transmembrane spans, and a large and small cytoplasmic loop (15, 17). The largest span of sequence is extracellular and is predicted to consist of the first 210 residues starting at the N terminus (17). The major cytoplasmic loop is thought to be located between residues 299 and 408 in the α-subunit and includes the trafficking signals examined in this study. Conservation in the adjacent basic sequences corresponding to positions 313–314 in the α-subunit is observed among most receptor subunits when alignments of the major cytoplasmic loops are examined (Fig. 1B), suggesting that this sequence may encode an important conservation of function. Therefore, an adjacent basic sequence at position 313–314 in the α-subunit (18) was selected as a candidate to be altered by site-directed mutagenesis from Arg131–Lys134 to Arg131–Gln134 (designated as the K314Q α-subunit) to examine whether subsequent changes occur in the trafficking characteristics. The potential trafficking signal RK was altered to RQ on the basis of its presence at an identical position in the β-subunit (Fig. 1B), the subunit that facilitates transport of the assembled receptor to the cell surface (12, 13).

Immunofluorescence and Confocal Microscopy—Immunofluorescence methods were performed in Triton-permeabilized cells to detect intracellular protein or in non-permeabilized cells to detect α-subunits at the cell surface. Cells were fixed in 4% paraformaldehyde in PBS, rinsed, and quenched in PBS/glycine. Cells were then permeabilized and blocked in 0.1% Triton X-100, 1% fish gelatin (Sigma), and 1% bovine serum albumin in PBS. Exposure to primary antibody was for 1 h in the permeabilization solution diluted with an equivalent volume of PBS. Exposure to secondary antibody conjugated to fluorophores was also for 1 h in the same buffer. Non-permeabilized cells were processed in the same manner except Triton X-100 was omitted. Following exposure to antibodies, cells were preserved in gelvatol and stored in the dark under refrigeration. Confocal images were taken with a Bio-Rad MRC 1024 laser-scanning system attached to a Zeiss Axiovert microscope using a 40× oil NA 1.3 objective, and processed with Adobe Photoshop (San Jose, CA). The brightly fluorescent cells show evidence of gene transfection; other fainter appearing cells in the microscope field apparently do not express the transfected gene.
For control and comparison, transfected wild-type α-subunits expressed in ts20 cells (at 30 °C) generally display the reticulate-diffuse pattern reminiscent of proteins deposited in the ER (Fig. 2A, panels a and b, red) and appear to overlap in pattern with endogenous calnexin (Fig. 2A, panels a and c, blue), a diagnostic marker for the ER. Wild-type α-subunits expressed in ts20 cells also display minimal co-localization (Fig. 2A, panel a) with a GFP-Golgi protein marker (panel d, green, GFP linked to the Golgi localization signal of galactosyltransferase (19)), which was expressed in these cells by co-transfection with the plasmid DNA encoding the α-subunit. Since the GFP-Golgi protein marker was expressed from a transfected gene, only a subset of cells display its expression. Moreover, wild-type α-subunits expressed in HEK cells display minimal overlap with the endogenous medial-trans-Golgi marker α-mannosidase II (Fig. 2B, panel e), further substantiating that the unassembled wild-type α-subunits are sequestered primarily in the ER. In support of our experimental observations on the trafficking characteristics of unassembled wild-type α-subunits as revealed by transfection and confocal microscopy, subcellular separations employing sucrose gradients of α-subunits expressed in muscle cells from the endogenous gene also demonstrated that unassembled α-subunits are restricted to the ER (11). Under the numerous transfection and expression experiments employed over the course of our study, minimal overlap was observed between the unassembled wild-type α-subunits and endogenous α-mannosidase II. Thus, high levels of overexpression from the transfected gene did not appear to contribute to artificially induced trafficking patterns.

In contrast to the wild-type α-subunits expressed alone, cells co-expressing α-subunits with β, γ, and δ-subunits display appreciable co-localization between the α-subunits and α-mannosidase II (Fig. 3a), coinciding with the finding that co-transfection of plasmid DNAs encoding the entire complement of subunits in HEK cells results in detection of the receptor at the cell surface (13). Note that although significant co-localization between the α-subunits and α-mannosidase II is observed when the receptor subunits are co-expressed, a major fraction of α-subunits also display the reticulate-diffuse pattern suggestive of localization in the ER (Fig. 3, a and b); this likely reflects the fraction of α-subunits not assembled with the other subunits or assembled receptor protein that did not exit the ER. As a further note, α-subunits co-expressed with the other receptor subunits in HEK cells yield glycosylated α-subunits that traffic to the cell surface and are fully cleavable with Endo-H in whole cell lysates (20). Thus, Endo-H cleavage was not employed in this study as a tool to characterize α-subunit trafficking.

To examine whether cytoplasmically positioned adjacent basic amino acid sequences modulate the trafficking of nAChR α-subunits, the Arg313-Lys314 sequence was altered into Arg313-Gln314, with the aim of conserving side chain volume but neutralizing the charge of the lysine residue to remove the potential trafficking signal. HEK cells were transfected and processed for confocal microscopy in the same manner as the wild-type α-subunits displayed in Figs. 2B and 3. The confocal microscope image of cells expressing K314Q α-subunits is displayed in Fig. 4, with the α-subunits exhibited by FITC emission (Fig. 4b, green) and α-mannosidase II displayed by rhodamine emission (Fig. 4c, red). The yellow coloration corresponds to overlap between the α-subunits and α-mannosidase II (Fig. 4a). Evident co-localization is observed between the K314Q α-subunits and α-mannosidase II (Fig. 4a), suggesting spatial overlap in the cell and strongly indicating that the K314Q α-subunit traffics to and is retained in the Golgi compartment.

To verify that the observed co-localization is due to spatial overlap in the three-dimensional field of the confocal image as opposed to artificial channel crossover caused by overly bright emission, wavelengths corresponding to FITC and rhodamine were individually blocked to examine whether images became apparent in the non-emitting channel. Since images were not observed in the non-emitting channel, crossover was excluded as the cause for the yellowish coloration observed in the merged images. Furthermore, since the steady state expression levels of the wild-type and K314Q α-subunits are similar (Fig. 5, lanes 3 and 4), overexpression and saturation of the quality control machinery of the cell can be excluded as the cause for the acquired trafficking characteristics of the K314Q α-subunit. Wild-type α-subunits, when expressed in the absence of other subunits, sediment as monomers upon sucrose gradient centrifugation (13). Since residues in the extracellular region govern subunit assembly, the K314Q mutation is unlikely to promote assembly of the mutant α-subunit into a more complex structure such as a homopentamer. The above results therefore suggest that altering the adjacent basic amino acid signal Arg313-Lys314 into Arg313-Gln314 directly promotes the trafficking of the substituted α-subunit from the ER to the Golgi. The Arg313-Lys314 sequence therefore appears to be a major determinant that modulates the ER to Golgi trafficking of the α-subunit. Since this sequence is largely conserved among mouse nAChR subunits (Fig. 1B), it can be postulated that it also plays a similar role in the trafficking of the other subunits.

**Interactions of the Adjacent Basic Residue Signal with the Cellular Trafficking Machinery—Proteins that have been retrieved back to the ER are incorporated into COP I vesicles that migrate in an anterograde direction from the intermediate compartment to the ER (1). Exposed adjacent basic amino acid sequences are known to interact with the COP I components that incorporate the retrieved proteins into the COP I vesicles (21–23). Therefore, in contrast to the isolated wild-type α-subunit, K314Q α-subunits as well as wild-type α-subunits coexpressed with the full complement of receptor subunits should...
be expected to display minimal interactions with the COP I components, since these proteins traffic to the Golgi. Pull-down experiments were performed in HEK cell lysates employing an antibody to the COP I component protein, α-COP. Cells were transfected to express K314Q α-subunits, wild-type α-subunits, or co-express wild-type subunits with the β-, γ-, and δ-subunits. Similar numbers of cells, volumes of buffer, and amounts of samples loaded into the gels were maintained in all steps. Proteins were subsequently resolved in 10% gels, transferred to nitrocellulose, and probed with an antibody to the α-subunit (mAb 210) and anti-α-mannosidase II (α-ManII) antibody.

FIG. 2. Wild-type α-subunits expressed alone in cells are deposited primarily in the ER, as evidenced by confocal microscopy. A, transfected wild-type α-subunits (a and b, red) expressed in ts20 cells (30 °C) overlap extensively with endogenous calnexin, a diagnostic marker for the ER (a and c, blue), but minimally with a co-transfected GFP-Golgi compartment marker protein (a and d, green, GFP linked to the Golgi localization signal of galactosyltransferase (19)). ts20 cells were co-transfected with plasmids encoding α-subunits and the GFP-Golgi marker. Cells were permeabilized and exposed to antibodies detecting the α-subunit (mAb 210) and calnexin (Stressgen, Victoria, British Columbia, Canada). B, transfected wild-type (wt) α-subunits expressed alone in HEK cells display the reticulate-diffuse pattern reminiscent of the ER (panels e and f, green) and display minimal co-localization with endogenous α-mannosidase II (α-ManII) (panels e and g, red), a diagnostic marker for the medial-trans-Golgi. Cells were transfected to express the α-subunit, permeabilized, and exposed to antibodies to the α-subunit (mAb 210) and α-mannosidase II.

FIG. 3. Wild-type α-subunits co-expressed with the β-, γ-, and δ-subunits co-localize with α-mannosidase II in permeabilized cells (a). HEK cells were co-transfected with plasmids encoding the α-, β-, γ-, and δ-subunits, permeabilized, and exposed to anti-α-subunit antibody (mAb 210, a, b, green) and anti-α-mannosidase II (α-ManII) antibody (a, c, red).

The experimental observations indicate that wild-type α-subunits expressed alone have the most pronounced association with γ-COP (Figs. 5, A and B, lane 1), whereas the K314Q α-subunit (Fig. 5A, lane 2) and wild-type α-subunits co-expressed with the β-, γ-, and δ-subunits (Fig. 5B, lane 2) display minimal associations. Note also that in the sample that displays the α-subunits co-expressed with the β-, γ-, and δ-subunits (Fig. 5B, lane 2), a minor interaction of the α-subunits with γ-COP is apparent; this likely reflects the population of unassembled α-subunits present in these cells (13, 24).

In contrast to the interaction of the wild-type α-subunits with γ-COP, association with β-COP, another component of COP I coats (21–23), was not detected. By employing the same buffer and conditions for immunoprecipitation, the β-COP antibody did not co-immunoprecipitate the α-subunits, although α-subunits were abundant in these cells, and β-COP was immunoprecipitated in the samples (data not shown). Taken to-
FIG. 4. The αK314Q α-subunit expressed in the absence of the other acetylcholine receptor subunits in HEK cells co-localize with α-mannosidase II (a), demonstrating that altering the signal Arg293-Lys314 to Arg293-Gln314 promotes the trafficking of the mutant α-subunit from the ER to the Golgi. Plasmid encoding the mutant α-subunit was transfected into cells, which were permeabilized and exposed to mAb 210 (b, green) and antibody to α-mannosidase II (α-ManII) (c, red). The merged image displays co-localization between the α-subunits and α-mannosidase II, as evidenced by the yellowish coloration (a).

FIG. 5. The K314Q mutation in the α-subunit, as well as subunit assembly, diminish the interaction of the α-subunit with γ-COP, a component of the COP-I-mediated ER retrieval machinery. Separate experiments are displayed in A and B. Wild-type (wt) α-subunits expressed alone show an evident association with γ-COP (A and B, lanes 1), whereas K314Q α-subunits expressed alone (A, lane 2), and wild-type α-subunits co-expressed with β-, γ-, and δ-subunits (B, lane 2) display minimal associations. Cells were transfected as indicated, and samples were immunoprecipitated with antibody to γ-COP (A and B, lanes 1 and 2). Equivalent volumes of lysates removed before immunoprecipitation were also diluted in Laemmli sample buffer and resolved in gels (A and B, lanes 3 and 4). Similar numbers of cells, amounts of plasmids, and sample volumes were employed in the experimental work-up for each lane.

FIG. 6. The K314Q α-subunit maintains the capacity to assemble into the receptor complex and express at the cell surface, as evidenced by carboxymethylcholine protection of 125I-α-bungarotoxin-binding sites on the surface of cells. Similar numbers of HEK cells were co-transfected with plasmids encoding the wild-type (wt) α-subunit with the β-, γ-, and δ-subunits or the K314Q α-subunit with the β-, γ-, and δ-subunits and exposed or unexposed to carboxymethylcholine (Carb.) prior to the addition of 125I-α-bungarotoxin. Each histogram bar represents a single sample tube, with the percent of maximum counts calculated among the tubes.

together, the experimental findings suggest that recognition of unassembled α-subunits by the COP I complex is mediated through the Arg293-Lys314 signal, which potentially directly interacts with γ-COP. Subunit assembly diminishes the interaction between the α-subunits and the COP I complex, which is reflected in the trafficking of the receptor beyond the ER compartment.

The K314Q α-Subunit Maintains the Capacity to Fold and Assemble, as Detected with 125I-α-bungarotoxin—To examine whether the K314Q α-subunit maintains the capacity to fold and assemble into the mature receptor pentamer, ligand binding assays were performed by protecting receptor-binding sites with carboxymethylcholine from 125I-α-bungarotoxin. Carboxymethylcholine recognizes and binds to the α-subunits associated with the γ- or δ-subunits but does not bind to unassembled α-subunits in the concentration range employed (25). In contrast, 125I-α-bungarotoxin recognizes both unassembled and assembled α-subunits (25). Therefore, prior exposure of cells to carboxymethylcholine followed by the later addition of 125I-α-bungarotoxin provides a means to distinguish whether the α-subunits are assembled. Since the addition of the β-subunit in the transfection results in transport of the associated α-, δ-, and γ-subunits to the cell surface, detection of the receptor by carboxymethylcholine inhibition of 125I-α-bungarotoxin binding demonstrates complete assembly of the subunits and expression of the receptor at the cell surface. Equivalent numbers of cells were transfected with plasmid DNAs encoding wild-type α-subunits with δ-, γ-, and β-subunits, or K314Q α-subunits with δ-, γ-, and β-subunits, and cells were subjected to the carboxymethylcholine protection assay. As displayed in Fig. 6, K314Q α-subunits are assembled and expressed at the cell surface, since virtually all 125I-α-bungarotoxin counts are blockable by carboxymethylcholine. Although the apparent expression of the assembled wild-type α-subunits is approximately twice that of the assembled K314Q α-subunits, this assay suggests that the K314Q α-subunit maintains a sufficient capacity to fold, assemble, and traffic to the cell surface. However, the lower expression observed for the K314Q α-subunit suggests that the cationic lysine 314 residue in the cytoplasmic loop might facilitate interactions that mediate the assembly of the subunits. Moreover, escape of the K314Q subunit from the ER prior to subunit assembly might decrease the pool of fully assembled receptor at the cell surface.
The mutated Golgi in HEK cells, it does not appear to be sufficient to bring the unassembled subunit at the cell surface. This suggests that additional mechanisms other than COP I-mediated retrieval modulate the trafficking of the K314Q α-subunit beyond the Golgi compartment. Two pathways for the post-Golgi sorting of the unassembled K314Q α-subunit can be postulated. First, the subunit might traffic beyond the trans-Golgi network to the lysosomes and subsequently be degraded. Second, the subunit could reach the cell surface but undergo rapid endocytosis. As with the ER retrieval sequences, the assembly of the subunit might occlude other targeting signals that potentially intercept and sort proteins away from the cell surface. Our earlier study demonstrates that α-subunits are ubiquitinated (5), and ubiquitination has been shown to modulate trafficking in post-Golgi compartments by targeting proteins to the lysosome (26–29). We therefore examined whether ubiquitination potentially functions as a signal that further interferes with the expression of the unassembled subunit at the cell surface.

To determine whether ubiquitination modulates post-Golgi trafficking in the context of expressing unassembled protein, α-subunits were expressed transiently by gene transfection in the hamster fibroblast cell line ts20, which encodes for a mutant ubiquitin-activating enzyme. This enzyme is inactive when cells are grown at 40 °C (16). To detect expression of the α-subunits at the cell surface, non-permeabilized cells were exposed to an antibody that recognizes the extracellular domain of the α-subunit (mAb 210). Other plates of cells, transfected with aliquots of the same transfection mixture, were permeabilized to detect the α-subunits in intracellular locations. Confocal microscope images are shown in Fig. 8 displaying images that exhibit the approximate frequencies of cells showing evidence for expression of the transfected gene. Two images from a plate are shown to emphasize the representative expression patterns for the fraction of cells exhibiting a positive immunoreactivity; significantly bright emission relative to background was interpreted to display receptor subunit expression. As shown in Fig. 8, wild-type α-subunits were not found to show expression on the surface of ts20 cells under conditions that repress ubiquitination. Fluorescent emission distinguishable from background was not observed on the surface of cells expressing wild-type α-subunits, indicating that suppressing ubiquitination is not sufficient to promote the trafficking of this unassembled subunit to the cell surface. By contrast, K314Q α-subunits, expressed alone, are observed on the cell surface when ubiquitination is suppressed at a frequency approximately comparable to that detected in the permeabilized cells (Figs. 8, a and b), suggesting that the mutated α-subunit traffics to the cell surface. The observed immunoreactivity on the surface of cells expressing the K314Q α-subunits was clearly discernible over that detected for the wild-type α-subunits in.
multiple fields; exact quantitative estimates would simply show the number of cells transfected and demonstrating expression relative to those showing no expression, respectively. By comparing the trafficking characteristics of the K314Q α-subunit relative to the wild-type α-subunit, these data strongly suggest that ubiquitination directly influences the sorting of the subunit into post-Golgi compartments, in contrast to modulating expression through degradation.

**DISCUSSION**

The findings of this study reveal that at least two distinct and sequential mechanisms target nAChR α-subunits to the cell surface, as diagrammed schematically in Fig. 9. The first mechanism regulates trafficking from the ER to the Golgi and is modulated through recognition of the ER retrieval sequence Arg<sup>113</sup>-Lys<sup>114</sup> by the COP I protein complex. Altering this sequence reduces the interaction of the mutant α-subunit with the COP I complex and corresponds to the trafficking of the mutant α-subunit from the ER to the Golgi; this is labeled path I in Fig. 9. However, removal of the ER retrieval sequence is not sufficient to place the mutant α-subunit at the cell surface in detectable levels. Sorting beyond the Golgi is modulated by ubiquitination. Trafficking from the Golgi to the cell surface is evident by detection of the K314Q α-subunit on the cell surface when ubiquitination is inhibited, which is labeled as path II in Fig. 9. The experimental observations therefore demonstrate that simultaneous inhibition of both γ-COP recognition and ubiquitination are sufficient to place unassembled α-subunits on the cell surface. The requirement for full assembly of the subunits of the receptor for expression at the cell surface is likely due to the masking of the trafficking signals and potential ubiquitination sites exposed in the unassembled subunit.

As the subunits are oriented and assembled into dimers and larger complexes, exposure of adjacent basic amino acids at an unassembled interface to the COP I machinery should continue to sequester the subunits in the ER and facilitate the assembly of additional subunits into the ion channel configuration. Introduction of the adjacent basic sequences might therefore be an evolutionary adaptation that facilitates the ordered assembly of the subunits into a completely enclosed and functional pentameric ion channel receptor. The roles for basic amino acid sequences in the maturation and trafficking of potassium channel subunits have also been observed (30), suggesting that their eventual occlusion during folding and assembly might be a general phenomenon in the processing of multisubunit channel proteins. Other factors, such as chaperones, might assist in the maturation process by promoting folding, stabilizing the intermediates from degradation or by contributing to the retention of unassembled subunits prior to their assembly (1, 31). However, our experimental observations suggest that the basic amino acid trafficking signal is a major factor that governs the trafficking of the nAChR α-subunit to proceed beyond the ER compartment.

Studies of type 1 transmembrane proteins have revealed that ER retrieval signals modulate protein trafficking from the ER to the cell surface and that addition of an adjacent basic amino acid retrieval sequence to a protein that otherwise traffics constitutively to the surface restrict this protein to the ER (23, 32). The polytopic configuration of acetylcholine receptor subunits or the presence of additional signals such as those that recruit the ubiquitination machinery add additional levels of complexity to events that modulate the post-Golgi sorting of the receptor. An unassembled subunit that proceeds beyond the Golgi might follow a pathway that sorts the protein from the trans-Golgi network to the lysosomes when specific sites are ubiquitinated. A similar trafficking route has been identified in yeast (28), and it can be expected that related pathways are manifested in mammalian cells. In the absence of ubiquitination, as in the case for nAChR subunits expressed in ts20 cells at the non-permissive temperature, the subunit might proceed from the Golgi and deposit at the cell surface.

Rapid endocytosis signaled by ubiquitination is another plausible pathway that could interfere with the placement of the unassembled subunit at the cell surface. The precedence for this mechanism comes from the down-regulation of surface receptors by ubiquitination and subsequent endocytosis (25, 29, 33, 34). Expression of growth hormone receptor in ts20 cells has shown that endocytosis of this protein is blocked at the non-permissive temperature for ubiquitination, suggesting that ubiquitin is the tag that promotes internalization of the receptor (35, 36). Moreover, the ubiquitin moiety itself has recently been demonstrated to contain an endocytosis signal (37). However, our preliminary data indicate that the K314Q α-subunit expressed in the absence of the other subunits does not follow a route of transient placement at the cell surface and rapid endocytosis, since the K314Q α-subunit does not co-localize with fluorescently labeled transferrin, a protein that follows an endocytotic pathway (data not shown). Therefore, a plausible route for the post-Golgi sorting of ubiquitinated K314Q α-subunits might involve interception at the trans-Golgi network and subsequent targeting to the lysosomes. Subunit assembly presumably occludes the key ubiquitination sites and allows the subunit to sort into an alternative pathway that leads to the cell surface. Although beyond the scope of this study, further investigations examining whether ubiquitination modulates the targeting of the subunit from the trans-Golgi network to the lysosomes should be revealing.

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