Assembly Intermediates of the Mouse Muscle Nicotinic Acetylcholine Receptor in Stably Transfected Fibroblasts

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Abstract. We have used fibroblast clones expressing muscle nicotinic acetylcholine receptor α and γ, and α and δ subunits to measure the kinetics of subunit assembly, and to study the properties of the partially assembled products that are formed. We demonstrate by coimmunoprecipitation that assembly intermediates in fibroblasts coexpressing α and δ subunits are formed in a time-dependent manner. The α and γ- and the α and δ-producing transfected cells form complexes that, when labeled with 125I-α-bungarotoxin, migrate in sucrose gradients at 6.3S, a value consistent with a hetero-dimer structure. An additional peak at 8.5S is formed from the δ subunit coexpressed in fibroblasts suggesting that δ may have more than one binding site for α subunit. The stability and specificity of formation of these partially assembled complexes suggests that they are normal intermediates in the assembly of acetylcholine receptor. Comparison of the binding of 125I-α-bungarotoxin to intact and detergent-extracted fibroblasts indicate that essentially all of the binding sites are retained in an intracellular pool. The fibroblast δ subunit has the electrophoretic mobility in SDS-PAGE of a precursor that does not contain complex carbohydrates. In addition, or7 and or6 complexes had lectin binding properties expected of subunits lacking complex oligosaccharides. Therefore, fibroblasts coexpressing α and δ or α and δ subunits produce discrete assembly intermediates that are retained in an intracellular compartment and are not processed by Golgi enzymes.

The normal expression of many cell surface receptors requires the assembly of heterooligomeric protein complexes. Surface expression of unassembled or partially assembled receptors, by uncoupling binding or the normal biological response, could have detrimental consequences to the organism. As a result, the correct assembly of these molecules must be relatively efficient, or surface expression must be regulated in order to edit incorrect events. One form of regulation common to several oligomeric proteins including the membrane-bound immunoglobulins, class I histocompatibility antigens, T cell receptors, and ligand-gated ion channels is the retention of unassembled, partially assembled, and incorrectly assembled complexes in an intracellular compartment (for reviews see Carlin and Merlie, 1986; Hurtley and Helenius, 1989; Klausner, 1989). The family of multi-subunit ligand-gated ion channels, including the muscle (Noda et al., 1983) and neuronal (Boulter et al., 1986; Goldman et al., 1987) nicotinic acetylcholine, GABA_A (Schofield et al., 1987), glutamate (Hollmann et al., 1989), and glycine (Grenningloh et al., 1987) receptors, have, in addition to the normal intricacies of assembly, the added complexity that alternate subunits may be substituted during development or in a cell-type specific manner. For example, the neuronal acetylcholine receptor forms a channel when any of three α subunits (α2, α3 or α4) are coexpressed with one other subunit (β2). Therefore, one mechanism of neuronal acetylcholine receptor diversity is assembly of the β2 with different α subunits expressed in different brain regions (Deneris et al., 1988). Similarly, switching from γ to ε subunit expression confers a change in acetylcholine receptor (AChR) channel properties during muscle development (Mishina et al., 1986). Finally, expression of different combinations of subunits can account for the type I and type II subtypes of the GABA_A receptor (Pritchett et al., 1989). Although alteration of subunit composition is likely to play an important role in the heterogeneity of ligand-gated ion channels, relatively little is known of how subunit switching contributes to the complex requirements for specific assembly and regulation of surface expression in this family of molecules.

The muscle-type AChR from the electric organ of Torpedo is the best characterized ligand-gated ion channel and remains the prototype for this family of molecules. The mammalian AChR is composed of five different but highly homologous transmembrane subunits (Noda et al., 1983; Mishina et al., 1984, 1986) in a stoichiometry of α2βγδ (Karlin, 1980), or α2βδε assuming a simple replacement of ε for γ (Mishina et al., 1986). The subunits are thought to be assem-

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Abbreviations used in this paper: AChR, acetylcholine receptor; BTX, α-bungarotoxin; PHA E, phytohemagglutinin; TCR, T cell receptor.

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bled around a central ion channel (Karlin, 1980), and the two α subunits are not juxtaposed (Wise et al., 1981; Kistler et al., 1982; Zingheim et al., 1982; Bon et al., 1984). Several studies have indicated that the α subunits contribute domains for the binding of the snake venom α toxins and the small competitive antagonists and agonists (Hagerty and Froehner, 1981; Merlie and Lindstrom, 1983; Blount and Merlie, 1988; Kao et al., 1984; Dennis et al., 1989). Recent evidence has suggested that binding sites for small ligands may be at the interface of the αγ and αδ subunits (Pedersen and Cohen, 1990); thus, the γ and δ subunits may also contribute domains for ligand binding.

Although much is known about the structure of the AChR, many of the basic issues concerning the biogenesis of this molecule are still unresolved. For example, whether the structural homology between subunits leads to the transient formation of incorrect homo and/or heterooligomers is unknown; alternatively, the structural domains necessary for subunit association may be located in the variable portions of these molecules leading to the formation of only correct heterooligomers. One study in the mouse muscle cell line, BC3H-1, showed that the AChR subunits assembled into a pentameric complex in a time-dependent manner (Merlie and Lindstrom, 1983); maximal levels of assembled AChR were achieved 1 h or more after subunit synthesis. However, whether the step rate-limiting for AChR assembly is the coherent modification of newly synthesized subunits, the transient formation of incorrect subunit complexes, or if assembly is determined simply by mass action has yet to be resolved.

In this study we have used fibroblast clones coexpressing α and γ (αγ), or α and δ (αδ) subunits to study the kinetics of subunit assembly, and to study the properties of partially assembled complexes. We show that α subunits in contact with δ subunit in a time-dependent manner similar to AChR assembly in the BC3H-1 cell line. These data suggest αδ association may be a rate-limiting step for normal assembly. We also demonstrate that αγ and αδ complexes formed in fibroblasts are discrete structures that appear to be representative of normal assembly intermediates; large aggregates were not detected. The binding of α-bungarotoxin (BTX) to surface and intracellular binding sites suggests that essentially all of the unassembled α and αγ and αδ complexes are retained in an intracellular compartment. The electrophoretic mobility of δ subunit in SDS-PAGE and the lectin binding properties of αγ and αδ complexes suggest that complex oligosaccharides, normally present on the surface AChR, are absent. Therefore, we conclude that fibroblasts coexpressing the α and γ or the α and δ subunits produce normal and discrete αγ and αδ assembly intermediates that are retained in an intracellular compartment and are not processed by Golgi enzymes.

Materials and Methods

Materials

The protease inhibitor PMSF and Staphylococcus aureus cell walls were purchased from Bethesda Research Laboratories (Gaithersburg, MD); αγ-macroglobulin and swainsonine were purchased from Boehringer Mannheim Diagnostics (Indianapolis, IN); saponin and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Lectin gels were purchased from E. Y. Laboratories Inc. (San Mateo, CA). [35S]Methionine (>800 Ci/mmole) was purchased from Amersham Corp. (Arlington Heights, IL). BTX, antibodies to BTX, and the synthesis of [125I]-BTX have been previously described (Merlie and Lindstrom, 1983; Merlie and Sebbane, 1981).

Cell Growth and Labeling

Growth conditions for BC3H-1 cells (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983) and QT-6 cells (Moscovici et al., 1977; Blount and Merlie, 1988) as well as the transfection, selection, and maintenance of QT-6 clones expressing of the α (Blount and Merlie, 1988), α and γ, and the α and δ subunits (Blount and Merlie, 1989) of the mouse AChR have been described previously. Pulse labeling with [35S]methionine was performed at a final specific activity of ~800 Ci/mmol for 5-min pulses, 10 Ci/mmol for the 1-h pulses, or 6 Ci/mmol for longer pulses.

Immunoprecipitations and Related Methods

Labeled cells were washed twice with PBS, 300 μM PMSF at 4°C, scraped from the plates, and pelleted for centrifugation for 10 s in a microtuge. The cell pellet from a single 10-cm dish was extracted with 1 ml PBS, 1-2 Triton X-100, 200 μM leupeptin, and 0.2 U/ml of α2 macroglobulin for 3-5 min, then the cells were centrifuged for 5 min in a microtuge, and the supernatant collected. Immunoprecipitations were performed as previously described (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983) with modifications (Blount and Merlie, 1988, 1989). The antibodies used were the α subunit-specific monoclonal antibodies mAb61 (Taarts et al., 1981) and mAb10 (Ratnam et al., 1986), a rabbit polyclonal anti-BTX antibody used to precipitate α subunit that was prebound to BTX (toxin antitoxin) (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983), the β subunit-specific mAb684 (Gullick and Lindstrom, 1983), and the δ subunit-specific mAb688 (Froehner et al., 1983). For reimmunoprecipitation, proteins were eluted from the Staphylococcus aureus pellets by incubation in 0.2% sodium dodecyl sulfate (SDS) at room temperature for 20 min, diluted 5 fold into PBS, 1% Triton X-100, and immunoprecipitated as above. After immunoprecipitation, S aureus pellets were resuspended in sample buffer and subjected to SDS polyacrylamide gel electrophoresis (PAGE) on a 10% acrylamide, 0.27% N,N'-bis-methyl acrylamide gel and buffer system (Laemmli, 1970). The gels were processed for fluorography using conditions such that band intensity was proportional to radioactivity and exposure time (Laskey and Mills, 1975). Quantitation was accomplished by measuring the fluorogram intensities with a LKB scanning densitometer.

Selective Labeling of Subunit Complexes, Sucrose Gradient Centrifugation and Lectin Binding

Confluent cell monolayers of α, αγ, or αδ cells on 60 mm plates were incubated in 10 mM phosphate buffer (pH = 7.5), 0.1% BSA, 10 mM EDTA, 0.5% saponin for 30 min. The cells were then rinsed with the same buffer and incubated for 2 h in the same buffer containing 20 nM BTX and either 10 nM curare or 100 µM carbamyloleine. The plates were washed again 3 times and incubated for 1 h in the same buffer containing 20 nM [125I]-BTX. The cells were then washed 3 times with buffer, scraped from the plates, and pelleted by centrifugation for 10 s in a microtuge.

Gradient centrifugation was performed on a Beckman SW 60 rotor at 60,000 rpm for a urt of 1.15 × 1011, fractionated from the bottom, and the fractions counted in a gamma counter (Beckman Instruments Inc., Palo Alto, CA). Sedimentation coefficients are estimates and were calculated using the 9.5S AChR (Boulter and Patrick, 1977; Merlie and Linstrom, 1983) and the 5S unassembled α subunit (Merlie and Lindstrom, 1983; Blount and Merlie, 1988) as standards.

Results

αδ Subunit Complexes Are Formed in Fibroblasts in a Time-dependent Manner

AChR assembly in the mouse muscle-like cell line, BC3H-1,
were combined into a single extract (c~ + /i). This mixed extract for 1 h with [35S]methionine. The cells were then harvested and the c~ or only the/i subunits of the mouse AChR were pulse labeled fibroblasts stably coexpressing the c~ and 5 (a/i) or expressing only subunits in stably transfected fibroblasts. In A, transfected QT-6 bodies mAb61, mAb210, and a polyclonal antibody against BTX for lysed by SDS-PAGE, and processed for fluorography as described and the extract from the c~/i cell line were immunoprecipitated, ana- precipitate the c~ subunit. The/i subunit-specific monoclonal anti- precipitating prebound toxin, were all used to specifically immuno-precipitate both the c~ and ~ subunit (Fig. 1 A, c~). Thus, the association of ot and 6 subunits, as measured by coimmuno- precipitation was dependent upon coexpression within the same cell, we next measured the kinetics of assembly of subunits in a6 fibroblasts by this method. Plates of a6 fibroblast cells were labeled with [35S]methionine for 5 min. Some plates were immediately harvested while others were further incubated with conditioned media supplemented with 1 mM unlabeled methionine (chased) for increasing times. As seen in Fig. 1 B, no delay was observed in the detection of the ot subunit as assayed by mAb61. In contrast, α subunit observed by its high affinity BTX binding (toxin antitoxin) increased from barely detectable to maximum levels over the first 40 min after the pulse. Although no delay was observed in the expression of the δ subunit, the appearance of α subunit coimmunoprecipitated with δ was slow, having a time course similar to acquisition of high

grown in conditioned media supplemented with 1 mM methionine (chased) for the times (in minutes) indicated (Chase Time). The cells were then harvested and extracted as described in Materials and Methods. The α subunit-specific monoclonal antibodies mAb661, mAb210, and a polyclonal antibody against BTX for precipitating prebound toxin, were all used to specifically immunoprecipitate the α subunit. The δ subunit-specific monoclonal anti-body, mAb88B, was used to specifically immunoprecipitate the δ subunit of the AChR. The NSI lane in each panel is a nonspecific control in which only the second antibody against the rat monoclonal antibodies and S. aureus was added. The NS2 lane in each panel is a precipitation with only the antitoxin antibody and S. aureus. Note that a nonspecific band that comigrates with the δ subunit is observed in these controls. The NS3 lane is an analogous nonspecific control for the mouse monoclonal antibody mAb88B. The α and δ subunits are labeled. In B, confluent 10-cm dishes of the a6 cell line were labeled with [35S]methionine for 5 min and further is a slow process; maximum levels of mature AChR are not observed until 1 h or more after subunit synthesis (Merlie and Lindstrom, 1983). The presence of all four subunits in the BC3H-1 cell line and the inherent complexity of a penta-meric structure has hampered our efforts to identify the step(s) rate-limiting for AChR assembly. The generation of stably transfected fibroblasts now allows us to characterize subreceptor complexes containing only two subunits, and to study the process of assembly of these simple complexes. Initially, we were concerned that the high expression levels in transfected fibroblast cell lines could lead to nonspecific association or aggregation of subunits subsequent to deter-gent extraction. Therefore, to test if nonspecific association of subunits occurred in cell extracts, we immunoprecipitated from a mixture of extracts derived from two stably transfected fibroblast cell lines metabolically labeled with [35S]methionine: one from a cell line producing only α subunit, the other from a cell line producing only δ subunit. The mixed extract was immunoprecipitated and the precipitates analyzed on SDS-PAGE and visualized by fluorography (Laskey and Mills, 1975). As seen in Fig. 1 A, α subunit–specific antibodies precipitated only α subunit; the δ subunit–specific antibody, mAb88B (Froehner et al., 1983), precipitated only δ subunit. In contrast, when an extract of the clonal fibroblast cell line expressing both α and δ subunits (a6) was immunoprecipitated with α subunit–specific monoclonal antibody mAb61 (Tzartos et al., 1981) or mAb-210 (Ratnam et al., 1986), both α and δ subunits were observed. Similarly, if BTX binding was allowed to occur, and the BTX-α subunit complex was immunoprecipitated with an anti BTX antibody (toxin antitoxin, Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983), both α and δ subunits were observed. The δ specific antibody also immunoprecipi-tated both the α and δ subunits (Fig. 1 A, a6). Thus, the association of α and δ subunits, as measured by coimmuno-precipitation, occurs within the cell; in mixed extracts, no subunit aggregation is observed.

Having established that the subunit association we ob-served by communoprecipitation was dependent upon coexpression within the same cell, we next measured the kinetics of assembly of subunits in a6 fibroblasts by this method. Plates of a6 fibroblast cells were labeled with [35S]methionine for 5 min. Some plates were immediately harvested while others were further incubated with conditioned media supplemented with 1 mM unlabeled methionine (chased) for increasing times. As seen in Fig. 1 B, no delay was observed in the detection of the α subunit as assayed by mAb61. In contrast, α subunit observed by its high affinity BTX binding (toxin antitoxin) increased from barely detectable to maximum levels over the first 40 min after the pulse. Although no delay was observed in the expression of the δ subunit, the appearance of α subunit coimmunoprecipitated with δ was slow, having a time course similar to acquisition of high

**Figure 1.** Specificity and kinetics of association of the α and δ subunits in stably transfected fibroblasts. In A, transfected QT-6 fibroblasts stably coexpressing the α and δ (aδ) or expressing only the α or only the δ subunits of the mouse AChR were pulse labeled for 1 h with [35S]methionine. The cells were then harvested and extracted with Triton X-100. Extracts from the α and the δ cell lines were combined into a single extract (α + δ). This mixed extract and the extract from the αδ cell line were immunoprecipitated, analyzed by SDS-PAGE, and processed for fluorography as described in Materials and Methods. The α subunit-specific monoclonal antibodies mAb661, mAb210, and a polyclonal antibody against BTX for precipitating prebound toxin, were all used to specifically immunoprecipitate the α subunit. The δ subunit-specific monoclonal anti-body, mAb88B, was used to specifically immunoprecipitate the δ subunit of the AChR. The NSI lane in each panel is a nonspecific control in which only the second antibody against the rat monoclonal antibodies and S. aureus was added. The NS2 lane in each panel is a precipitation with only the antitoxin antibody and S. aureus. Note that a nonspecific band that comigrates with the δ subunit is observed in these controls. The NS3 lane is an analogous nonspecific control for the mouse monoclonal antibody mAb88B. The α and δ subunits are labeled. In B, confluent 10-cm dishes of the αδ cell line were labeled with [35S]methionine for 5 min and further
I\[--a

The identity of the peaks was confirmed by before 125I-BTX labeling. The concentration of high affinity BTX binding, and the association of \( \alpha \) with the \( \delta \) subunit occurs in stably transfected fibroblasts in a time-dependent manner. In addition, the formation of \( \alpha \delta \) complexes correlates with a dramatic change in degradation rate constant.

**\( \alpha \gamma \) and \( \alpha \delta \) Subunit Complexes Are Discrete Heterooligomers**

The homology between subunits of ligand-gated channels and the potential for substitution of alternate subunits suggests that subunit interactions may be promiscuous and that incorrect heterooligomers may be transiently formed during normal assembly. To determine whether the heterooligomeric subunit complexes formed in the \( \alpha \gamma \) and \( \alpha \delta \)-producing fibroblast cell lines were discrete complexes or larger aggregates, we analyzed the 125I-BTX binding sites formed in the \( \alpha \gamma \) and \( \alpha \delta \) cell line by velocity centrifugation in sucrose gradients. As markers for 5 and 9.5S, extracts from the \( \alpha \) cell line and the surface labeled BC3H-I cells were combined and overlayed on a single gradient (Fig. 2). Several studies have demonstrated previously that the unassembled \( \alpha \) subunit and the AChR migrate as 5 and 9.5S, respectively (Boulter and Patrick, 1977; Merlie and Lindstrom, 1983; Blount and Merlie, 1988). The position of free 125I-BTX is also shown (Fig. 2 A, filled diamonds). A gradient containing the 125I-BTX binding sites extracted from \( \alpha \delta \) fibroblasts revealed one major peak at 6.3S; a shoulder was also present at 5S (Fig. 2 B, open squares). The efficient formation of the 6.3S complex in the \( \alpha \delta \) cell line may be due to the overproduction of the \( \delta \) subunit and/or the increased stability of the \( \alpha \delta \) complex (see Fig. 1 B). In contrast, for the \( \alpha \gamma \) cell line the predominate 125I peak was at 5S, a long shoulder in this profile suggested complexes at higher sedimentation coefficients (Fig. 2 C, open circles).

Because complexes of \( \alpha \gamma \) and \( \alpha \delta \) have different association rates for BTX binding and possess binding properties for agonists and competitive antagonists not observed in the un-

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**Table I. Selection of \( \alpha \gamma \) and \( \alpha \delta \) Complexes by Differential Drug Binding: Percent BTX Binding Sites Remaining after Treatment**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Carb</th>
<th>Curare</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>( \alpha \delta )</td>
<td>71*</td>
<td>53</td>
</tr>
<tr>
<td>( \alpha \gamma )</td>
<td>30</td>
<td>45*</td>
</tr>
</tbody>
</table>

Three 60-mm plates of \( \alpha \), \( \alpha \gamma \), and \( \alpha \delta \)-producing fibroblast cell lines were treated with a 10 mM phosphate buffer with 2 mg/ml BSA and 0.5% saponin for 10 min. The control plate for each was incubated in the same buffer with 20 nM 125I-BTX for 1 h. The other plates were preincubated in this saponin containing buffer with 20 nM BTX and either 100 \( \mu \)M carbamylcholine (Carb) or 10 \( \mu \)M d-tubocurarine (Curare) for 1 h, rinsed, and then labeled with 20 nM 125I-BTX for 1 h. The cells were washed extensively, harvested, and counted in a gamma counter (Beckman Instruments Inc.). The values shown are expressed as a percent of total counts.

* The values show the BTX binding remaining for the treatments used in subsequent experiments.

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**Figure 2.** Gradient analysis of BC3H-1 surface AChR, unassembled \( \alpha \), and \( \alpha \gamma \) and \( \alpha \delta \) complexes. Surface AChRs in BC3H-1 cells were labeled by incubating in 5 nM of 125I-BTX for 4 h. Cells from an \( \alpha \)-producing fibroblast cell line were treated with a 10 mM phosphate buffer with 2 mg/ml BSA and 0.5% saponin for 10 min. This buffer was replaced with the same buffer containing 20 nM 125I-BTX. Extracts of the \( \alpha \) cells and surface-labeled BC3H-1 cells were then combined and fractionated on a 5-20% linear sucrose gradient. Fractons were collected from the bottom and counted in a gamma counter (Beckman Instruments Inc.). Points are plotted as percent cpm of the greatest fraction. A shows the combined extracts of BC3H-1 and \( \alpha \) cell lines (filled triangles) where the AChR and the \( \alpha \) subunit peaks have been labeled (100% = 9,000 cpm). 125I-BTX that was diluted in extraction buffer and spun in an adjacent gradient is also shown (filled diamonds, BTX peak) (100% = 1240 cpm). In B, cells from the \( \alpha \delta \) cell line that were treated in a similar manner to the \( \alpha \) cell line (open squares) are shown (100% = 8,600 cpm). As in Table I, some \( \alpha \delta \) cells were enriched for complexes by preincubating in the same buffer with 100 \( \mu \)M carbamylcholine and 20 nM unlabeled BTX and washing extensively before 125I-BTX labeling (filled squares) (100% = 6,530 cpm). C shows results from the \( \alpha \gamma \) cell line that was similarly pretreated (filled circles) (100% = 2,500 cpm) or not (open circles) (100% = 1,900 cpm) with 10 \( \mu \)M d-tubocurarine and 20 nM unlabeled BTX before labeling. The identity of the peaks was confirmed by immunoprecipitation; \( \alpha \)-specific mAb210 specifically immunoprecipitated >68% of the 125I from pooled 5S peak fractions in A, and >85% of the 125I from the 6.3S peak fractions in B; but <2% of the 125I from the BTX peak fractions in A. The \( \delta \)-specific mAb88B specifically immunoprecipitated >65% of 125I from the 6.3S peak in B but <2% of the 125I from the BTX peak A.
Table II. Surface and Intracellular BTX Binding Sites in aγ and aδ Cell Lines

<table>
<thead>
<tr>
<th>Binding</th>
<th>aγ</th>
<th>aδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface binding*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific</td>
<td>605 ± 83</td>
<td>406 ± 30</td>
</tr>
<tr>
<td>Nonspecific</td>
<td>549 ± 54</td>
<td>459 ± 35</td>
</tr>
<tr>
<td>Extracted + precipitated†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface binding</td>
<td>95</td>
<td>17</td>
</tr>
<tr>
<td>Total binding</td>
<td>37,450</td>
<td>10,142</td>
</tr>
</tbody>
</table>

* 35mm dishes of aγ and aδ cell lines were incubated with 5 nM 125I-BTX for 3 h with (Nonspecific) or without (Specific) 500 nM unlabeled BTX. The cells were washed, harvested, and counted in a gamma counter (Beckman Instruments Inc.). The values shown are mean cpm of three values ± SD.
† Two plates each of the aγ and aδ specific surface binding described above were solubilized with PBS, 1% Triton X-100. The extracts were immunoprecipitated with the α specific mAb210 in the presence (Specific) or absence (Nonspecific) of 5 nM 125I-BTX. Total binding time was 3 h. The immunoprecipitates were washed as described in Materials and Methods and counted in a gamma counter (Beckman Instruments Inc.). Nonspecific controls of only second antibody and S. aureus have been subtracted in each case.

Assembled a subunit, we were able previously to selectively block BTX binding sites in unassembled a, thereby enriching for BTX binding sites composed of aγ and aδ complexes (Blount and Merlie, 1989). To measure the sedimentation coefficient of aγ and aδ complexes without interference of unassembled a subunit, we have used a similar technique. After permeabilization with saponin, a, aγ, and aδ-producing cells were treated with 20 nM unlabeled BTX and either 100 μM carbamylcholine (carb selected) or 10 μM d-tubocurarine (curare selected) for 1 h. Under these conditions, BTX should bind to most of the unassembled aγ subunit. Since binding is very slowly reversed (Blount and Merlie, 1988), this block with unlabeled BTX is essentially irreversible. On the other hand, because carbamylcholine and d-tubocurarine bind with high affinity to aγ and aδ complexes but not unassembled aγ, most of the heterooligomeric complexes should be selectively protected by carbamylcholine or d-tubocurarine from BTX blockade. The cells were rinsed free of the reversibly bound carbamylcholine or d-tubocurarine and unbound BTX, and the remaining toxin binding sites, enriched for aγ and aδ complexes, were labeled with [125I]BTX. Table I shows the percentage of total [125I]BTX binding sites remaining in the a, aγ, and aδ cell lines when unassembled aγ, is preblocked in this manner. We previously reported that the aγ complex has a higher relative affinity for d-tubocurarine and the aδ complex has a higher relative affinity for carbamylcholine (Blount and Merlie, 1989); therefore, it is not unexpected that more BTX binding sites would be protected in the curare-treated aδ cells and the carb-treated aδ cells. As expected, neither carb nor curare selection protected the a subunit from the BTX blockade (Table I). Cells treated in this manner were extracted in 1% Triton X-100 in PBS and the extracts were analyzed by sucrose gradient velocity sedimentation. In three independent experiments, the selectively labeled [125I]BTX complexes in extracts from aδ cells consistently contained a peak at 6.3S without the shoulder at 5S (Fig. 2 B, compare filled vs. open squares). More dramatically, peaks of [125I] at 6.3 and 8.5S were revealed in extracts from aγ cells; these peaks were observed only as shoulders in extracts from untreated cells (Fig. 2 C, filled vs. open circles) probably because of the large excess of 5S aγ. When extracts from selectively labeled aγ cells were incubated at 4°C for increasing times up to 30 h before velocity sedimentation, the 6.3 and 8.5S peaks decreased relative to the 5S peak (not shown). This decomposition, which could be due either to proteolysis or the instability of the aγ complex in extraction buffer, may account for the significant amount of 5S [125I] observed. We have presented only the curare selection data for aγ, and only the carb selection data for aδ in Fig. 2 because of the higher signal-to-noise ratio (see Table I); however, both carb and curare selection decreased the relative size of the 5S peak in both the aγ and aδ cell lines.

We used immunoprecipitation to confirm the subunit composition of the peaks observed from the aδ-producing cells. As expected, both the 5 and the 6.3S peaks contained a subunit-associated 125I, and the 6.3S peak contained a subunit-associated 125I (see legend to Fig. 2). In addition, the peaks of 125I between 5 and 9.5S are observed only in the aγ and aδ cell lines and are enhanced by carb or curare selection. Therefore, we conclude that the peak of 125I at 6.3S observed in the aγ and aδ cell lines, and the 8.5S peak observed only in the aγ cell line are discrete heterooligomer complexes that appear to be representative of normal assembly intermediates.

αγ and aδ Complexes Are Intracellular

To determine whether AChR assembly intermediates could be transported to the cell surface in transfected fibroblasts we measured the ability of [125I]BTX to bind to the cell surface. 5 nM of [125I]BTX was added to the media of aγ and aδ cells in the absence or presence of 500 nM unlabeled BTX. The cells were incubated for 3 h, rinsed, harvested, and counted in a gamma counter (Beckman Instruments Inc.). As seen in Table II, no specific BTX binding was observed on the cell surface. When Triton X-100 extracts of these cells were assayed for internal binding sites by incubation with 5 nM [125I]BTX and immunoprecipitation with the a subunit-specific antibody, mAb210, a large population of BTX binding sites not present on the surface was revealed (Table II). Quantitation of these data indicate that <0.5% of the total BTX binding sites are expressed on the cell surface in the aγ and aδ cell lines. Under similar conditions, ~65% of BTX binding sites in BC3H-1 cells were found to be on the cell surface (Boulter and Patrick, 1977).

Covalent Modification of the δ Subunits in BC3H-1 and aδ Cells

The availability of the δ-specific monoclonal antibody, mAb88B, allowed for the study of the normal maturation of the δ subunit expressed in the AChR-producing BC3H-1 cell line, and for the comparison of the maturation of the BC3H-1 δ with that expressed in aδ and aδ-producing fibroblasts. As seen in Fig. 3, when surface or total AChR from [35S]methionine-labeled BC3H-1 cells were precipitated and analyzed on SDS-PAGE, four specific bands were revealed. The identity of the aγ, aγ, and δ subunits were confirmed by denaturing the immunoprecipitated AChR in SDS and reimmunoprecipitating the dissociated subunits with subunit-specific monoclonal antibodies (Fig. 3, Reprecipitated); the identity of the γ subunit could not be confirmed because γ subunit-specific antibodies capable of immunoprecipitation of the mouse γ
Figure 3. Migration of BC3H-1 AChR subunits in SDS-PAGE. BC3H-1 cells were labeled with [35S]methionine and also incubated with 10 nM [125I]BTX in the media for 6 h. The cells were then rinsed, harvested, and extracted. Antitoxin was used to precipitate surface receptor bound to [125I]BTX. Toxin antitoxin (BTX) and the δ subunit specific mAb88B were used to immunoprecipitate total AChR. The NS lanes for BTX were incubated with 100-fold excess BTX (500 nM) but the normal amount of antitoxin so that virtually all antibodies would bind free BTX. The NS lane for mAb88B was incubated in the absence of this primary antibody. Washed immunoprecipitates of total AChR precipitated with toxin antitoxin were eluted with 2% SDS, diluted 10-fold in 1% Triton X-100 in PBS, and reimmunoprecipitated (Reprecipitated) with the δ-specific mAb61, the β-specific mAb148, the γ-specific mAb88B, or in the absence of primary antibody (NS). The α, β, γ, and δ subunits are labeled.

The δ subunit migrated as a heterogeneous band between 60 and 66 kD on SDS-PAGE. The heterogeneity was especially pronounced in the combined pools of surface and intracellular AChR. One interpretation of these data is that posttranslational modification(s) leads to electrophoretic heterogeneity of the δ subunit.

One obvious posttranslational modification that may lead to electrophoretic heterogeneity of proteins is the addition of complex oligosaccharides. Carbohydrate analysis has demonstrated that the Torpedo californica AChR α and δ, but not α and β subunits, contained complex N-linked oligosaccharides (Nomoto et al., 1986). To determine if complex carbohydrates altered the migration of the AChR subunits in SDS-PAGE, we treated BC3H-1 cells with swainsonine, a drug that inhibits mannosidase II and prevents subsequent addition of complex carbohydrates (Tulsiani et al., 1982). BC3H-1 cells were incubated in the presence or absence of swainsonine, pulse labeled for 5 h with [35S]methionine and the labeled AChR subunits were analyzed (Fig. 4). Swainsonine treatment revealed a species of the δ subunit (δm) that migrated faster in SDS-PAGE than the normally processed, mature form of the δ subunit (δa). A slight mobility shift and decrease of heterogeneity in SDS-PAGE was also observed for the γ subunit, consistent with this subunit also containing complex oligosaccharides. No difference was observed in the migration of the α and β subunits, consistent with the finding that these subunits contain only high mannose oligosaccharide chains (Merlie et al., 1982; Merlie, J. P., and M. M. Smith, unpublished results). These experiments implied that δm and the similarly incompletely processed γ subunit could assemble into a pentameric complex and be expressed on the cell surface.

Pulse-chase labeling experiments were performed to determine if the kinetics of appearance of the two different δ subunit species were consistent with conversion of δa to δm. When BC3H-1 cells were labeled for 15 min with [35S]methionine, harvested immediately, and analyzed by immunoprecipitation, newly synthesized, unassembled, α, β, and δ subunits were observed (Fig. 4). In contrast, when cells were chased for 4 h with conditioned media supplemented with 1 mM unlabeled methionine after labeling but before immunoprecipitation, all four subunits were coimmunoprecipitated indicating assembly. As shown in Fig. 5, the assembled form of the δ subunit migrated on SDS-PAGE in the same position as the δm subunit, and the newly synthesized δ subunit migrated identical to δm observed in swainsonine-treated cells. We conclude that the δa subunit normally acquires the slower mobility of δm by addition of complex oligosaccharides; however, as demonstrated in Fig. 4, this modification is not required for assembly or surface expression of the AChR.
Transfected fibroblasts allowed us to address the question of whether expression of the δ subunit alone, or the assembly of δ with α subunit, was sufficient for complex oligosaccharide modification. As shown in Fig. 5, the δ subunit produced by either the αδ cell line or a cell line producing only δ had a motility in SDS-PAGE identical to that of δα. Therefore, we conclude that the expression of δ and the assembly of δ with α subunit in QT-6 fibroblasts is not sufficient for the complex oligosaccharide modification of the δ subunit; the subsequent assembly of β and/or γ is a likely requirement for this modification.

**Lectin Binding to AChR and Complexes of αγ and αδ Subunits**

To better define the complex carbohydrate content of the αγ and αδ complexes and the AChR, we tested their ability to bind to lectins. Curare and carb treatment described above was used to selectively enhance 125I-BTX labeling of αγ and αδ complexes (see Table I). Surface AChR was labeled by addition of 125I-BTX into the media of BC3H-1 cells. Extracts from each of these cell lines were then incubated with lectins conjugated to agarose beads. After binding, the beads were washed, precipitated, and counted in a gamma counter. Fig. 6 shows the percentage of bound 125I radioactivity precipitated by lectin gels. Con A, a lectin that has a specificity for mannose and glucose (Goldstein and Hayes, 1978), bound essentially all 125I-labeled complexes in the αγ, αδ and BC3H-1 cell lines. In contrast, wheat germ agglutinin, a lectin with specificity for galactosylated glycopeptides containing N-acetylgalactosamine linked to the β-linked mannose residue of the core (Cummins and Kornfeld, 1982), bound 43% of the surface BC3H-1 AChR-associated 125I-BTX, but <3% of the 125I-BTX associated with αγ or αδ complexes. To determine if 43% binding of AChR to PHA E was indicative of heterogeneity, or, alternatively, due to low affinity of PHA E for AChR, supernatant from a similar PHA E binding experiment (now containing the remaining 125I-labeled AChR) was rebound to PHA E. The lectin bound 51% of the remaining AChR, suggesting that essentially all of the BC3H-1 AChRs could bind PHA E, and that the low efficiency of binding was due to low affinity. These results demonstrate that PHA E specifically binds to mature AChR from BC3H-1 cells but not αγ or αδ complexes, confirming that the mature AChR possesses complex oligosaccharides that are absent in αγ and αδ assembly intermediates.

**Discussion**

The normal expression of a multimeric transmembrane receptor ultimately requires the specific association of mature subunits into a functional complex that is transported to the cell surface. However, the mechanism involved in specific subunit associations and transport are poorly understood. For example, several possibilities exist for the relationship between subunit maturation and assembly; subunit maturation may require assembly, or, at the other extreme, subunits may be able to achieve a mature conformation only
after assembly. Also, interactions between subunits may lead to the formation of large, incorrect homo- or heterooligomers that must either be degraded or disassembled before correct assembly (Anderson and Blobel, 1983); alternatively, subunit associations may be very specific leading to the formation of only correct dimers, trimers, and correctly assembled receptors. Finally, to prevent surface expression and the potential uncoupling of ligand binding from biological response, incompletely and incorrectly assembled subunits may be retained in either the ER or the Golgi apparatus. Because of our current knowledge about the structure of the AChR, this molecule is an ideal subject for studying these issues.

The discovery that the subunit of the AChR in B3H-1 acquired high affinity BTX binding and assembled with other subunits in a time-dependent manner (Merlie and Lindstrom, 1983) implicated subunit maturation as a rate-limiting step in AChR assembly. Subsequently, by expressing only the subunit in stably transfected fibroblasts, we confirmed that the time-dependent maturation of the primary translation product of the subunit (Oto) to otrx could occur independent of other subunits (Blount and Merlie, 1988). Studies described here demonstrate that the kinetics of assembly of and in fibroblasts are similar to the AChR assembly previously described in BC3H-1. As previously suggested (Merlie and Lindstrom, 1983), slow association of subunits may reflect a requirement for folding of newly synthesized subunits before assembly; alternatively, association may be limited by low subunit concentrations in the ER.

Figure 5. Migration of the fibroblast subunit in SDS-PAGE relative to the precursor and mature subunit in BC3H-1. BC3H-1 cells were labeled with [35S]methionine for 15 min. These cells were then either immediately harvested (P) or chased for 4 h in conditioned media supplemented with 1 mM unlabeled methionine (C) before harvesting. The harvested cells were extracted and immunoprecipitated using toxin antitoxin (BTX), the -specific mAb148, and the -specific mAb88B. Cells labeled with [35S]methionine for 4 h from the cell line and from a cell line producing only the subunit were immunoprecipitated with mAbg8B. The immunoprecipitates from BC3H-1 and transfected cell lines were loaded in adjacent lanes on a gel for SDS-PAGE; however, because of differences in band intensities different fluorographic exposures are shown. The band labeled actin is a common nonspecific band that was observed in controls. The , , , subunits are labeled.

Figure 6. Lectin binding to AChR, , and complexes. Surface AChRs in BC3H-1 cells were labeled by incubating in 5 nM [125I]-BTX for 4 h. Cells from - and -producing fibroblast cell lines were treated with a 10 mM phosphate buffer with 2 mg/ml BSA and 0.5% saponin for 10 min. As in Table I and Fig. 2, the cells were incubated in the same buffer with 20 nM BTX and 100 µM carbamylcholine for 1 h; the cells were incubated in the same buffer with 20 nM BTX and 10 µM d-tubocurarine for 1 h. These cells, now enriched for and complexes, were washed and incubated with 20 nM [125I]-BTX. The surface labeled BC3H-1 cells (AChR) and the and cells were then solubilized in Tris (pH = 8.0) buffer with 1% Triton X-100 and bound to 40 µl of Con A, PHA E, or wheat germ agglutinin. All lectins were covalently linked to agarose beads. The agarose beads were precipitated by centrifugation and washed twice in PBS, 1% Triton X-100 before counting in a gamma counter (Beckman Instruments Inc.). The values are presented as percent control where the control is [125I] cpm immunoprecipitated from the extract with the -specific mAb210 (10,600, 32,500, and 17,400 cpm for , , and AChR, respectively). [125I]-BTX was diluted in extraction buffer and precipitated with the lectin gels in an identical fashion (B/X); these values are presented as percent control where the control is total input counts (36,400 cpm).
membrane and an intrinsic rate constant for the process. Analysis of the association rates of $\alpha$ and $\gamma$ and other subunit pairs await the availability of an anti-mouse-$\gamma$-specific antibody that is efficient at immunoprecipitation and the production of fibroblasts expressing other subunit combinations. However, the observation that the rate of maturation of the $\alpha$ subunit, association of $\alpha$ with $\delta$ subunit in fibroblasts, and assembly of the AChR in BC3H-1 cells, are all similar suggests the possibility that $\alpha$ subunit maturation and/or $\alpha/\delta$ association may be rate limiting in normal AChR assembly.

The necessity for assembly of homologous subunits, and the potential substitution of alternate subunits in the formation of ligand-gated ion channels draw attention to the issue of specificity (or lack of specificity) in subunit assembly. Specifically, are incorrect homo- and heterooligomeric subunit complexes formed during normal assembly? When individual AChR subunits were translated in a cell-free system, the formation of large complexes with sedimentation coefficients of 9–13S led to the hypothesis that large homooligomers were formed in the course of normal assembly (Anderson and Blobel, 1983). However, studies of assembly in tissue culture systems have not supported this hypothesis. Only two forms of high affinity BTX binding sites have been observed in the muscle cell line, BC3H-1. The 9.5S AChR is found both intracellularly and at the cell surface; another, strictly intracellular, 5S $\alpha$ subunit species has also been observed (Merlie and Lindstrom, 1983; Carlin et al., 1986). Pulse-chase labeling and immunoprecipitation demonstrated that the 5S $\alpha$ subunit was the first species detected and had the kinetic properties of a precursor to the fully assembled 9.5S AChR (Merlie and Lindstrom, 1983). Finally, transfected fibroblasts produce a 5S $\alpha$ subunit in the absence of other subunits (Blount and Merlie, 1988). Thus, all data are consistent with the 5S BTX binding site being the unassembled, probably monomeric, $\alpha$ subunit. Assuming that the 5S $\alpha$ subunit was monomeric, Merlie and Linstrom (1983) previously predicted that dimers would be ~6.5S, a value close to the 6.3S BTX binding sites observed in both the $\alpha\gamma$ and $\alpha\delta$ fibroblast cell lines. The additional complex observed at 8.5S in $\alpha\gamma$ producing fibroblasts suggests that $\alpha$ and $\gamma$ subunits have more than one binding site for each other, leading to the formation of hetero-trimers or tetramers. More than one site of contact with the $\alpha$ subunit is a property expected only of the lone subunit between the two closest $\alpha$ subunits in the pentameric complex. The finding that $\alpha$ and $\gamma$ can form heterotrimers or tetramers is consistent with the proposal that the $\gamma$ subunit is the lone subunit between the two $\alpha$ subunits in the assembled AChR (Karlin et al., 1983; but see Kubalek et al., 1987). Thus, the coexpression of the $\alpha$ and $\gamma$ or the $\alpha$ and $\delta$ subunits in fibroblasts leads to the formation of only discrete complexes, probably dimers and trimers or tetramers, that appear to be representative of normal assembly intermediates of the AChR. The finding that $\alpha$ and $\beta$ do not efficiently form heteromer complexes (Blount and Merlie, 1989) suggests that $\beta$ may be incorporated as the final step of assembly.

The surface expression of incompletely assembled receptors could have detrimental results by uncoupling ligand binding from normal biological response. Here we have demonstrated that little if any of the unassembled $\alpha$ (Blount and Merlie, 1988), or complexes of $\alpha\gamma$ or $\alpha\delta$ AChR subunits are expressed on the surface of transfected fibroblasts. These results are in agreement with observations in BC3H-1 cells suggesting the absence of complexes <9.5S on the cell surface (Carlin et al., 1986; Blount and Merlie, 1988). However, our results are in contrast with previous results suggesting that a small but significant amount of partially assembled and unassembled Torpedo $\alpha$ subunit is transported to the surface of injected oocytes (Kuroskai et al., 1987; Sumikawa and Miledi, 1989). This apparent contradiction is likely to be due to the difference in the species from which the subunit clones were derived or the expression system used.

Complex carbohydrates have proven to be reliable markers of glycoprotein transport through the Golgi apparatus. Therefore our studies on the oligosaccharide processing of the $\alpha\gamma$ and $\alpha\delta$ complexes formed in transfected fibroblasts versus the normal AChR help to determine if sub-receptor complexes are retained in the Golgi apparatus or in a pre-Golgi compartment. Torpedo californica AChR $\alpha$ and $\beta$ subunits each have only one high mannose asparagine linked oligosaccharide. In contrast, the $\gamma$ and $\delta$ subunits have 2 and 3 oligosaccharide units respectively, and contain complex-type carbohydrates (Nomoto et al., 1986). Although a comparable chemical analysis is lacking, several lines of evidence, including Endo H sensitivity of the single glycosylation on both the $\alpha$ and the $\beta$ subunits (Merlie et al., 1982; Merlie, J. P., and M. M. Smith, unpublished results) and the swainsonine study presented here in Fig. 4, suggest that the mouse AChR subunits are similar to Torpedo in their glycosylation patterns. The data presented here indicate that at least two forms of the $\delta$ subunit can be distinguished by their electrophoretic mobility in SDS-PAGE. The faster migrating $\delta$ subunit, $\delta_m$, has the kinetic properties of a precursor to the slower migrating species, $\delta_s$. In addition, swainsonine, a drug that inhibits mannosidase II and subsequent complex oligosaccharide addition, inhibits the maturation of $\delta_s$ to $\delta_m$. However, assembly and surface expression of AChR can occur independent of the maturation of $\delta_s$ into $\delta_m$ (Fig. 4). These data are in agreement with a study demonstrating that functional receptors are produced in swainsonine-treated BC3H-1 cells (Covarrubias et al., 1989). The $\delta$ subunit expressed in fibroblasts migrates as the $\delta_s$ form, and we have observed no difference in migration of the $\delta$ subunit in these transfected fibroblasts upon assembly with the $\alpha$ subunit (Blount and Merlie, 1989; also see $\delta$ coimmunoprecipitated with $\alpha$ in Fig. 1). Furthermore, we have tested the $\alpha\gamma$ and $\alpha\delta$ complexes for complex oligosaccharides by determining their lectin binding properties. Our data indicate that neither the $\alpha\gamma$ nor the $\alpha\delta$ complexes bind to PHA E, a lectin that binds mature AChR from BC3H-1 cells. Thus, our data are consistent with the absence of complex oligosaccharides on the $\alpha\gamma$ and $\alpha\delta$ complexes formed in fibroblasts. Although we cannot rule out the possibilities that abnormally high expression of $\gamma$ or $\delta$ subunit may decrease the efficiency of subunit maturation, or that $\alpha\gamma$ and $\alpha\delta$ complexes are transported to the Golgi complex but not recognized by enzymes responsible for oligosaccharide processing, we believe the failure of the oligosaccharide maturation of the $\delta$ (and probably the $\gamma$) subunit is most likely due to the retention of partially assembled subunit complexes in a pre-Golgi compartment. This latter interpretation is consistent with the demonstration that maturation and assembly of AChR $\alpha$ subunit occurs in the ER of BC3H-1 cells (Smith et al., 1987). Thus, the data suggest that the $\alpha\gamma$ and $\alpha\delta$ complexes expressed in fibroblasts...
are formed in the ER, and that transport through the Golgi to the plasma membrane requires formation of a pentameric complex.

Many studies have suggested that unassembled or partially assembled multi-subunit transmembrane glycoproteins are retained in an intracellular compartment, often determined to be the ER (for review see Carlin and Merlie, 1986; Hurtley and Helenius, 1989). One of the best studied oligomeric membrane proteins is the T cell receptor (TCR). Several lines of evidence suggest that many of the unassembled subunits of the TCR are retained in the ER and undergo a rapid degradation that is pharmacologically distinct from normal lysosomal degradation (Lippincott-Schwartz et al., 1988). Recently, a transmembrane sequence of 23 amino acids within the α chain of the TCR was reported to be necessary and sufficient for rapid degradation of proteins in the ER (Bonifacino et al., 1990). Rapid degradation of unassembled AChR subunits has been previously demonstrated (Merlie and Lindstrom, 1983). In addition, with a one amino acid insertion, the second putative transmembrane region (M2) of all the subunits of the AChR shares a 20% identity and 50% homology with a 20 amino acid stretch of the sequence reported as the targeting sequence for rapid, pre-Golgi degradation of the α chain of the TCR. None of the other three transmembrane domains in the subunits of the AChR share this homology. Several lines of evidence suggest that the M2 domain lines the channel of the AChR (see Dani, 1989 for a review). Thus, it is tempting to speculate that unassembled AChR subunits are degraded before transport to the Golgi complex by the same pathway as the α chain of the TCR. Upon assembly, the polypeptide sequence responsible for targeting the unassembled protein for this rapid degradation would be buried in the molecule; for the AChR, burial might mean that the M2 sequence lines the inside of the receptor, thus forming the channel.

In conclusion, we have used a eukaryotic expression system to study the assembly of the AChR. We found that the assembly intermediates formed in fibroblasts are the discrete heterooligomers expected of partially assembled AChRs. The evidence presented here on the maturation and glycosylation state of the γ and δ subunits suggest indirectly that these assembly intermediates are formed in and confined to a pre-Golgi compartment. In the accompanying paper (Blount and Merlie, 1990), in an attempt to determine some of the regulatory mechanisms leading to the expression of only correctly processed and assembled AChR, we exploit the association of α and δ subunits in fibroblasts as an assay for testing the ability of mutated α subunits to assemble into stable complexes.

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