Nicotinic Receptor Assembly Requires Multiple Regions throughout the γ Subunit

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Assembly of ionotropic neurotransmitter receptors typified by acetylcholine receptors (AChRs) is thought to be directed by an N-terminal extracellular domain of a subunit. Consistent with this hypothesis, chimeras with the δ subunit N-terminal domain fused to the rest of the γ subunit can substitute for δ , but not γ , subunits during AChR assembly. However, chimeras with the γ subunit N-terminal domain fused to the rest of the δ subunit cannot substitute for γ or δ subunits during assembly. Furthermore, expression of this chimera with the four wild-type subunits prevents the formation of α -bungarotoxin (Bgt) binding

sites. Instead of AChR pentamers, complexes are assembled containing only the chimera and either α or β subunits. Based on the results of additional γ - δ chimeras, there are at least two different regions within the C-terminal half of the chimera required for the dominant-negative effect. Our results indicate that the N-terminal domain of the γ subunit mediates the initial subunit associations, whereas signals in the C-terminal half of the subunit are required for subsequent subunit interactions.

Key words: protein folding; conformational changes; assembly; acetylcholine; α -bungarotoxin; nicotinic receptors

As large oligomeric and polytopic proteins, the folding and assembly of ion channels are complex and must occur with high fidelity because misassembly of even a few channels, each controlling the flow of $\sim 10^7$ ions/sec, can be disastrous for a cell. Moreover, folding and assembly are the rate-limiting steps during channel biogenesis (Green and Millar, 1995) and are likely to play an important role in regulating the number and targeting of functional channels. The muscle-type nicotinic acetylcholine receptor (AChR) was the first and remains among the few ion channels for which the subunit composition and stoichiometry are established definitively. As a member of the family of neurotransmitter-gated ion channels that includes neuronal AChRs, 5HT₃, glycine, and GABA_A and GABA_C receptors, the muscle-type AChR has long served as a model for studies of ion channel assembly.

Muscle-type AChRs are composed of four homologous subunits (α , β , γ , and δ) that assemble into $\alpha_2\beta\gamma\delta$ pentamers (Karlin and Akabas, 1995; Lindstrom, 1995; Changeux and Edelstein, 1998). According to the "heterodimer" model of AChR assembly (Blount et al., 1990; Gu et al., 1991; Saedi et al., 1991; Kreienkamp et al., 1995), α subunits fold into a conformation that can bind α -bungarotoxin (Bgt) before assembling with γ or δ subunits into heterodimers. ACh binding sites form on the heterodimers that associate together with β subunits into $\alpha_2\beta\gamma\delta$ pentamers. Evidence for this model is based on complexes formed in cells expressing less than the full complement of subunits. When cells expressing all four AChR subunits are used (Green and Claudio, 1993; Green and Wanamaker, 1997), it has been found that

AChR subunits rapidly assemble into $\alpha\beta\gamma$ trimers. Afterward, δ subunits are added to form $\alpha\beta\gamma\delta$ tetramers, and then a second α subunit is added to make $\alpha_2\beta\gamma\delta$ pentamers. Another significant difference is that the first Bgt and ACh binding sites appear only after $\alpha\beta\gamma$ trimer assembly, and the second Bgt and ACh binding sites appear after $\alpha_2\beta\gamma\delta$ pentamers form (Green and Wanamaker, 1998).

Regions within the N-terminal extracellular domain of AChR subunits are required for associations between subunits (Yu and Hall, 1991; Sumikawa and Gehle, 1992; Verrall and Hall, 1992; Kreienkamp et al., 1995). The N-terminal regions of glycine (Kuhse et al., 1993) and GABA_C and GABA_A (Hackam et al., 1998) receptors also are involved in receptor assembly. In support of these studies, we find that the chimeric subunit, which contains the N-terminal domain of the AChR δ subunit fused to the rest of the γ subunit, substitutes for δ , but not γ , subunits during AChR assembly. However, the reverse chimera with the N-terminal domain of the γ subunit fused to the rest of the δ subunit does not substitute for γ or δ subunits. Instead, when coexpressed with the four wild-type subunits, the chimera assembles with α or β subunits and blocks the formation of all Bgt binding sites. This dominant-negative effect is specific for the loss of γ subunit regions, not the presence of δ subunit regions, and requires at least two different γ subunit regions in the C-terminal half of the subunit. Our results indicate that regions within an N terminal domain of a subunit mediate the initial rapid subunit associations, whereas regions within the C-terminal half of the γ subunit are required for subsequent subunit interactions.

MATERIALS AND METHODS

cDNA constructs. The γ 215 δ and δ 221 γ chimeras were constructed via standard PCR methods (Yon and Fried, 1989). For γ 215 δ , an oligonucleotide was obtained consisting of the 15 base pairs (bp) of the γ cDNA preceding the region coding for the first transmembrane domain (M1), followed by the first 15 bp of M1 from the δ cDNA. Amplification of γ cDNA using this primer resulted in a product corresponding to the γ sequence up to the start of M1, followed by 15 bp of δ sequence. This product was used in a second PCR step to amplify the δ cDNA, with the short region of δ sequence serving to anneal the sequence to the δ

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template. The $\delta 221\gamma$ chimera was made by the same method, except that both a forward and a reverse chimeric primer corresponding to the chimera junction were used. The $\gamma 215\beta$ chimera was constructed by using a shared *BclI* restriction site located before M1 of the β and γ subunits to join the γ and β cDNAs at the *BclI* site.

All additional chimeras were constructed by an introduction of restriction sites into the subunit cDNA by PCR, using a mutagenic primer with a 1–3 bp mismatch (Ho et al., 1989). The $\gamma 215\alpha$ chimera was produced by introducing the *BclI* site described above into the homologous location in the α subunit to join the γ and α cDNAs at the *BclI* site. To construct $\gamma 215\delta 467\gamma$, we introduced a unique *SylI* restriction site within M4 of δ into the homologous location of the γ subunit to replace the C terminus of $\gamma 215\delta$, starting at amino acid 467, with that of γ . The $\gamma 322\delta$ and $\gamma 215\delta 329\gamma$ chimeras were constructed by introducing the unique *BglII* site of the δ subunit into the corresponding location of the γ subunit. Full-length chimeric subunits were created by cutting with the appropriate restriction enzyme and ligating subunit fragments together at the common sites. Regions of mutant subunits constructed by PCR methods were sequenced to control for errors in the PCR products.

Transfections. The four *Torpedo* subunit cDNAs in the pRBG4 expression vector (Lee et al., 1991) were transiently transfected into 6 cm cultures of tsA201 cells (Margolske et al., 1993), using a calcium phosphate method (Eertmoed et al., 1998). Unless otherwise indicated, 1 μ g of α , 0.5 μ g of β , 2.5 μ g of γ , and 7.5 μ g of δ subunit cDNAs were used. Cells were maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% fetal bovine serum. Because of the temperature dependence of *Torpedo* AChR expression (Claudio et al., 1987), cells were shifted from 37 to 20°C 24 hr after the transfection. Maximal expression occurred 4 d after the temperature shift.

The $\gamma 215\delta$ chimeric cDNA in pRBG4 was stably transfected into a cell line already expressing the four *Torpedo* subunits (Claudio et al., 1987). Cells were maintained in DMEM supplemented with 10% calf serum, HAT (15 μ g/ml hypoxanthine, 1 μ g/ml aminopterin, and 5 μ g/ml thymidine), and G418 (1 mg/ml) to maintain thymidine kinase and neomycin selection of the stably transfected subunits. To enhance the expression of the four wild-type subunits under the control of SV40 promoters, we also supplemented the culture medium with 20 mM sodium butyrate for 1–5 d before assay. Using calcium phosphate transfection, we cotransfected each 10 cm culture with 15 μ g of the $\gamma 215\delta$ chimeric cDNA and 500 ng per plate of the neomycin resistance gene construct pSV2Neo. Clonal isolates of neomycin-resistant cells were obtained by adding 0.6 mg/ml G418 to the culture medium and were screened for the expression of AChR subunits by ¹²⁵I-Bgt binding.

¹²⁵I-Bgt binding. To assay cell-surface ¹²⁵I-Bgt binding, we washed the cultures with PBS and incubated them at room temperature in PBS containing 4 nM ¹²⁵I-Bgt (140–170 cpm/fmol) for 2.5 hr on a shaker table. Plates were washed again with PBS and solubilized; ¹²⁵I-Bgt counts were determined by γ counting. To assay ¹²⁵I-Bgt binding to solubilized AChRs (surface plus intracellular), we incubated cell lysates with 10 nM ¹²⁵I-Bgt overnight at 4°C, followed by immunoprecipitation with monoclonal antibody (mAb) 35. Nonspecific binding was measured by binding to sham-transfected cells or by adding 10 mM carbamylcholine during binding.

Metabolic labeling and SDS-PAGE analysis. Cultures in 10 cm plates were labeled as described previously (Green and Claudio, 1993; Green and Wanamaker, 1997). Briefly, cultures were pulse-labeled in 2 ml of methionine–cysteine-free DMEM, supplemented with 333 μ Ci of a ³⁵S-methionine ³⁵S-cysteine mixture (NEN EXPRE ³⁵S³⁵S). The labeling was stopped with the addition of DMEM plus 5 mM methionine. To follow the subsequent changes in the labeled subunits, we “chased” the cells by incubation for the indicated times in NB medium at 20°C. The cells were solubilized in (in mM) 150 NaCl, 5 EDTA, 50 Tris, pH 7.4, 2 PMSF, and 2 *N*-ethylmaleimide plus 0.02% NaN₃ and 1.83 mg/ml phosphatidylcholine and 1% Lubrol (LPC). Solubilized AChR subunits and chimeras were immunoprecipitated with rabbit polyclonal antisera, anti- α , anti- δ (Claudio and Raftery, 1977), or anti- β mAb 148, anti- γ mAb 168 (Gullick and Lindstrom, 1983), conformational-dependent mAb 14 (Tzartos and Lindstrom, 1980), or γ and δ subunit-specific mAb 88b (American Type Culture Collection, Rockville, MD).

Antibody–subunit complexes were precipitated with Protein G-Sepharose and electrophoresed on 7.5% SDS polyacrylamide gels, fixed, enhanced for 30 min, dried on a gel dryer, and exposed to film at –70°C with an intensifying screen. Autoradiographs were quantified by scanning densitometry, using a flatbed scanner, and analyzed with the Intelligent Quantifier software from BioImage (Ann Arbor, MI). A

standard, provided by BioImage, with 21 bands ranging from 0.05 to 3.05 optical density units in 0.15 increments was used to calibrate the linearity of the densitometer before using it. To ensure that quantified bands were in the linear range and that the darker signals were not saturated, we took three to five exposures of each autoradiograph; each was scanned to insure that the scanned bands remained in the linear range of the film.

Sucrose gradients. Cell lysates were layered on a 5 ml 5–20% linear sucrose gradient prepared in LPC lysis buffer. Gradients were centrifuged in a Beckman SW 50.1 rotor at 40,000 rpm to $\omega^2 t = 9.0 \times 10^{11}$. Eighteen fractions of 300 μ l each were collected from the top of the gradient. Then the fractions were counted in a gamma counter to determine the amount of ¹²⁵I-Bgt bound to each fraction.

RESULTS

The δ subunit N-terminal extracellular domain is sufficient to direct δ subunit assembly

Of the four AChR subunits, γ and δ subunits are the most similar. Nonetheless, γ subunits rapidly associate with α and β subunits, while δ subunits remain unassembled (Green and Claudio, 1993). To determine regions on the subunits responsible for these differences, we constructed subunit chimeras in which homologous regions of the γ and δ subunits were swapped. In the chimera $\delta 221\gamma$, regions of the δ subunit were replaced by regions of the γ subunit starting at amino acid 221, which is at the junction between the extracellular N-terminal domain and the first transmembrane region, M1 (Fig. 1A). As expected, the molecular weight of the chimera appears to be between that of the γ and δ subunits (Fig. 1A). Cotransfection of the $\delta 221\gamma$ chimera with wild-type α , β , and γ subunits yielded expression of Bgt binding receptors on the cell surface at levels similar to those seen with all four wild-type subunits (Fig. 1B). Consistent with previous studies (Kurosaki et al., 1987; Sine and Claudio, 1991), surface Bgt binding receptors were observed with the transfection of the wild-type subunit cDNAs without either the γ or δ subunit cDNAs (Fig. 1B). However, the levels of surface Bgt receptors lacking γ or δ subunits were very low, ~10% or less of control, reflecting very inefficient assembly without all four subunits. The addition of $\delta 221\gamma$ subunits to wild-type α , β , and δ subunits resulted in little to no increase in the number of Bgt binding sites above what was observed with the expression of α , β , and δ subunits alone (Fig. 1B).

Further experiments tested whether AChRs containing the $\delta 221\gamma$ chimera were pentamers with two ACh binding sites. The size of surface receptors was examined by using sucrose gradients (Fig. 1C). A single peak, at 9 S, was indistinguishable from the location where AChRs composed of the four wild-type subunits migrated. This finding indicates that $\delta 221\gamma$ -containing AChRs are pentamers with a stoichiometry of $\alpha_2\beta\gamma(\delta 221\gamma)$, with $\delta 221\gamma$ replacing the δ subunit in the *Torpedo*, wild-type $\alpha_2\beta\gamma\delta$ complexes. To test whether the $\delta 221\gamma$ -containing AChRs have two distinguishable ACh binding sites like wild-type AChRs, we used mAb 247G, which recognizes and blocks Bgt binding to one ACh site, but not the other (Mihovilovic and Richman, 1987; Green and Wanamaker, 1998). When cells expressing $\delta 221\gamma$ -containing AChRs were incubated with increasing amounts of mAb 247G, ~50% of the Bgt binding sites were blocked, confirming the presence of two different ACh binding sites. Similar results were obtained previously by using a mouse $\delta 221\gamma$ chimera (Sine, 1993) and, together with our results, indicate that the $\delta 221\gamma$ chimera can substitute for the δ subunit, but not the γ subunit, during AChR assembly.

Figure 1. The δ subunit N-terminal extracellular domain directs δ subunit assembly. *A*, The $\delta 221\gamma$ chimera. The N-terminal half of the δ subunit (filled bar) up to amino acid 221 was fused to the C-terminal half of the γ subunit (open bar) to create the $\delta 221\gamma$ chimera. The δ subunit domain is entirely extracellular. The γ subunit domain is composed of three membrane-spanning regions, M1, M2, M3 (broadened areas), followed by a cytoplasmic domain, a fourth membrane-spanning region, M4 (broadened area), and the extracellular C terminus. Cells were transiently transfected with 2.5 μg of γ , δ , or $\delta 221\gamma$ cDNA per 6 cm plate, metabolically labeled, and immunoprecipitated with mAb 88b. SDS-PAGE analysis of the $\delta 221\gamma$ chimera relative to the γ and δ subunits is displayed. *B*, The $\delta 221\gamma$ chimera can substitute for the δ subunit during AChR assembly. The indicated amounts of $\delta 221\gamma$ chimera cDNA were cotransfected with set amounts of the α , β , and γ (filled squares) or α , β , and δ (open squares) subunit cDNAs, and cell-surface ^{125}I -Bgt binding was assayed. The addition of the chimera to α , β , and δ subunit cDNAs did not increase cell-surface ^{125}I -Bgt binding. When cotransfected with α , β , and γ subunits, ^{125}I -Bgt binding increased to the level obtained with the transfection of all four wild-type subunit cDNAs. Data are expressed as the percentage of the binding observed for cells expressing the four wild-type subunits (20 fmol of Bgt binding sites for this experiment). Filled squares represent the mean of two plates from a single experiment. Similar results were obtained in three other experiments. Open squares are a mean of four plates from two experiments. Error bars $\pm$ SEM are smaller than the symbols. *C*, Sedimentation of $\delta 221\gamma$ AChRs. Surface ^{125}I -Bgt binding was performed on cells expressing α , β , γ , and $\delta 221\gamma$ subunits (filled symbols; $\alpha\beta\gamma + \delta 221\gamma$); the ^{125}I -Bgt-bound $\delta 221\gamma$ AChRs were size-fractionated on 5–20% linear sucrose gradients. The results are plotted as the fraction of the maximum value to facilitate comparison with cells expressing wild-type AChRs (open symbols; $\alpha\beta\gamma\delta$). The fraction number runs from the top to the bottom of the gradient, and the arrows mark the peak fractions of the standards: alkaline phosphatase (5.4 S), catalase (11 S), and $\alpha_2\beta\gamma\delta$ AChRs (9 S). *D*, $\delta 221\gamma$ AChRs have two ligand binding sites. Intact cells expressing the $\delta 221\gamma$ chimera and the α , β , and γ subunits were incubated with mAb 247G for 3 hr before cell-surface ^{125}I -Bgt binding was assayed. Data are expressed as a percentage of ^{125}I -Bgt binding to cells not incubated with mAb 247G (94 fmol of Bgt binding sites). For receptors containing the $\delta 221\gamma$ chimera, ~50% of the binding sites remain with saturating amounts of mAb 247G. Values represent the mean $\pm$ SEM of four plates from two experiments. Because mAb 247G blocks Bgt binding to the α_γ , but not the α_δ , ligand binding site on AChRs, the results indicate that both sites are present on $\delta 221\gamma$ AChRs.

The C-terminal half of the γ subunit is required for Bgt binding site formation

The chimera $\gamma 215\delta$ was constructed by replacing the δ subunit N-terminal domain with that of the γ subunit (Fig. 2*A*). Like the $\delta 221\gamma$ chimera, $\gamma 215\delta$ migrates as expected between the γ and δ subunits on SDS-PAGE gels (Fig. 2*A*). Unlike the $\delta 221\gamma$ chimera,

the $\gamma 215\delta$ chimera does not substitute for the γ subunit during AChR assembly because the addition of $\gamma 215\delta$ to α , β , and δ subunits did not increase AChR expression (data not shown). However, the $\gamma 215\delta$ chimera is not inert during assembly, as evidenced by a complete loss of surface AChRs when $\gamma 215\delta$ chimera cDNAs are transfected with the four subunit cDNAs

Figure 2. The C-terminal half of the γ subunit is required for Bgt site formation. **A**, The $\gamma 215\delta$ chimera. The N-terminal extracellular domain of the γ subunit (open bar) up to amino acid 215 was fused to the C-terminal half of the δ subunit (filled bar) to create the $\gamma 215\delta$ chimera. The γ subunit domain is entirely extracellular, whereas the δ subunit domain extends from the first membrane-spanning region, M1 (broadened area), to the C terminus. SDS-PAGE analysis of the $\gamma 215\delta$ chimera was performed as in Figure 1A. **B**, The $\gamma 215\delta$ chimera inhibits AChR surface expression and Bgt binding site formation. Cultures were transfected with the four wild-type subunit and the indicated amount of $\gamma 215\delta$ cDNA. ^{125}I -Bgt binding to both intact cells (triangles) and cell lysates (circles) was assayed. Also assayed was the effect of additional δ subunit expression on surface ^{125}I -Bgt binding. The indicated amounts of δ cDNA were added to the 7.5 μg that yielded maximum AChR expression (squares). Values are expressed as a percentage of the ^{125}I -Bgt binding observed in the absence of chimera or additional δ subunit expression. Each point represents the mean of 10 6 cm plates from five experiments (Surface) and six plates from three experiments (Total). Error bars $\pm$ SEM are smaller than the symbols for all $\gamma 215\delta$ values. **C**, The $\gamma 215\alpha$ and $\gamma 215\beta$ chimeras. The N-terminal extracellular domain of the γ subunit (open bar) up to amino acid 215 was fused to the C-terminal half of the α (striped bar) or β (shaded bar) subunit to create the $\gamma 215\alpha$ and $\gamma 215\beta$ chimeras, respectively. SDS-PAGE analysis of the $\gamma 215\alpha$ and $\gamma 215\beta$ chimeras was performed as in Figure 1A except that α and $\gamma 215\alpha$ were immunoprecipitated with polyclonal anti- α Ab, and β and $\gamma 215\beta$ were immunoprecipitated with polyclonal anti- β Ab. **D**, $\gamma 215\alpha$ and $\gamma 215\beta$ chimeras inhibit AChR expression. Cells were transfected with all four subunit cDNAs plus the indicated amount of each chimera cDNA and assayed for surface ^{125}I -Bgt binding as in **B**. Values are expressed as a percentage of the ^{125}I -Bgt binding observed in the absence of chimera (43 and 45 fmol for the experiments with $\gamma 215\alpha$ and $\gamma 215\beta$, respectively). Each point represents the mean of six cultures from three experiments ($\gamma 215\alpha$) or the mean of seven cultures from three experiments ($\gamma 215\beta$). For comparison, cell-surface ^{125}I -Bgt binding to cells transiently transfected with all four subunit cDNAs plus $\gamma 215\delta$ is repeated from **B**. **E**, The $\gamma 215\beta$ chimera cannot substitute for the γ subunit. The indicated amounts of $\gamma 215\beta$ chimera were cotransfected with a set amount of the α , β , and δ subunit cDNAs (open diamonds), and cell-surface ^{125}I -Bgt binding to the cells was assayed. Also displayed are results from cells transfected with varying amounts of γ subunit cotransfected with a set amount of the α , β , and δ subunit cDNAs (open squares). Data are expressed as the percentage of the maximum binding for cells expressing the four wild-type subunits, which occurred at 5 μg of γ subunit cDNA, yielding 146 fmol of Bgt sites. Each point represents the mean $\pm$ SEM determined from four cultures from two separate experiments. Note that the $\gamma 215\beta$ chimera error bars are smaller than the symbols.

(Fig. 2*B*). In addition to blocking the expression of surface AChRs, $\gamma 215\delta$ chimeras blocked the formation of all intracellular Bgt binding sites (Fig. 2*B*). The loss of surface Bgt binding sites because of the $\gamma 215\delta$ chimera thus is caused by a failure of the sites to form and not by a block of their transport to the cell surface. The dominant-negative effect of the $\gamma 215\delta$ chimera differs from the inhibition of expression observed when a single AChR subunit is overexpressed relative to the other three subunits (Gu et al., 1991). Overexpression of the δ subunit, which most affected AChR expression, did not alter significantly the number of intracellular Bgt binding sites (Gu et al., 1991), unlike the effect of adding the $\gamma 215\delta$ chimera (Fig. 2*B*). Additionally, overexpression of the δ subunit caused, at most, a 40–50% decrease in expression (Gu et al., 1991). In our experiments, transfection of an additional 10 μ g of the δ subunit cDNA caused a <20% decrease in AChR expression, whereas transfection of the same amount of the $\gamma 215\delta$ chimera cDNA decreased expression by >90% (Fig. 2*B*).

Two more chimeras were constructed to test whether the dominant-negative effect of the $\gamma 215\delta$ chimera is caused by the loss of γ subunit regions or by the addition of δ subunit regions. The chimeras $\gamma 215\alpha$ and $\gamma 215\beta$ contained the N-terminal domain of the γ subunit and the C-terminal half contributed by either the α or β subunit (Fig. 2*C*). The translated product of both chimera cDNAs appeared to be full-length, because $\gamma 215\alpha$ migrates on gels between the γ and α subunits and $\gamma 215\beta$ migrates between the γ and β subunits (Fig. 2*C*). When cotransfected with all four wild-type subunits, both the $\gamma 215\alpha$ and $\gamma 215\beta$ chimeras blocked the formation of surface AChRs (Fig. 2*D*). The dominant-negative effect of the $\gamma 215\beta$ chimera was less pronounced than that of the $\gamma 215\delta$ or $\gamma 215\alpha$ chimeras. This difference was not caused by the assembly of any $\gamma 215\beta$ -containing AChRs, because the transfection of $\gamma 215\beta$ chimeras with α , β , and δ subunits did not increase the surface expression of AChRs (Fig. 2*E*). In summary, a dominant-negative effect occurs regardless of whether the replaced regions are from the α , β , or δ subunits. Regions from these subunits, therefore, do not contribute, and the dominant-negative effect results from a loss of signals within the C-terminal half of the γ subunit.

The effect of the $\gamma 215\delta$ chimera on AChR assembly

Because Bgt binding sites form during AChR assembly (Merlie and Lindstrom, 1983; Green and Claudio, 1993; Green and Wanamaker, 1998), the block of Bgt binding site formation by the $\gamma 215\delta$ chimera appears to be altering AChR assembly. To determine more precisely how the $\gamma 215\delta$ chimera affects AChR assembly, we stably transfected the $\gamma 215\delta$ chimera into a AChR-expressing cell line we have used previously to characterize AChR assembly ($\alpha\beta\gamma\delta$ cells; Claudio et al., 1987). Eighteen cell lines were isolated after stable transfection of the $\gamma 215\delta$ cDNA into $\alpha\beta\gamma\delta$ cells. All isolated cell lines displayed a significant reduction in surface AChR expression as assayed by Bgt binding. A single cell line was chosen for characterization in which Bgt binding was not totally eliminated but was greatly reduced. As displayed in Figure 3*A*, cell-surface Bgt binding to these cells was reduced sevenfold as compared with the parent cell line. There was also an approximately twofold decrease in the levels of the wild-type subunits relative to the $\alpha\beta\gamma\delta$ cells as determined by metabolically labeling the subunits (Fig. 3*B*). The decrease in wild-type subunit levels, apparently caused by the added $\gamma 215\delta$ chimera synthesis, may be contributing to the loss of Bgt binding, but it is unlikely to be the major factor. The reduction in the

subunit levels is much less than the decrease in Bgt binding. Moreover, we have shown in Figure 2*B* that the $\gamma 215\delta$ chimera causes a specific inhibition of AChR expression that is not duplicated by the overexpression of other AChR subunits.

Subunit assembly was assayed by metabolically labeling the subunits for 30 min and following changes in the labeled subunits for the indicated times (Fig. 3*C–E*). When labeled subunits from the $\alpha\beta\gamma\delta$ cells were immunoprecipitated with α subunit-specific polyclonal antibodies, β and γ subunits coprecipitated with α subunits consistent with the assembly of $\alpha\beta\gamma$ trimers during the label period (Fig. 3*C*, left). Initially, the α subunit signal was larger than that of the other subunits because both assembled and unassembled α subunits were precipitated by the anti- α antibodies. Excess unassembled α subunits degraded with a half-life of 8–10 hr at this temperature (Fig. 3*F*) (Green and Claudio, 1993; Green and Wanamaker, 1997), whereas the assembled α subunits were stable. Labeled subunits also were immunoprecipitated with mAb 14 (Fig. 3*C*, right), which recognizes a conformation-dependent epitope formed as δ subunits are added to $\alpha\beta\gamma$ trimers, and only precipitates complexed subunits well after the assembly of $\alpha\beta\gamma$ (Green and Claudio, 1993). Subunit assembly was changed significantly by the addition of $\gamma 215\delta$ chimeras to the parent cell line (Fig. 3*D*). When the labeled subunits were immunoprecipitated with the α subunit-specific antibodies, only $\gamma 215\delta$ chimeras significantly coprecipitated (Fig. 3*B,D*). Also, β subunit-specific mAb coprecipitated predominantly $\gamma 215\delta$ chimeras, and the γ/δ subunit-specific mAb coprecipitated α and β subunits with $\gamma 215\delta$ chimeras (Fig. 3*B,E*). Thus, instead of AChR assembly, $\gamma 215\delta$ chimeras associated with α or β subunits, and complexes with all three subunits were not observed.

The $\gamma 215\delta$ -containing complexes were not stabilized like normal assembly intermediates and were degraded at the same rate as unassembled wild-type subunits (Fig. 3*F*). Furthermore, subunit folding events that normally occur on partially assembled complexes during AChR assembly were not observed for the $\gamma 215\delta$ -containing subunit complexes. None of the $\gamma 215\delta$ -containing complexes was precipitated by mAb 14 (Fig. 3*D*, right) and thus lacked the mAb 14 epitope. In the $\gamma 215\delta$ -expressing cells, a small number of subunit complexes were precipitated by mAb 14 (see last lane in Fig. 3*D*). Importantly, these complexes contained only the four wild-type subunits and appear to be the small number of AChRs that do assemble and are transported to the cell surface (Fig. 3*A*). Because the addition of $\gamma 215\delta$ chimeras also blocks the formation of Bgt binding sites (Figs. 2*B*, 3*A*), neither the mAb 14 epitope nor the Bgt binding site forms on $\gamma 215\delta$ -containing complexes. All together, our data indicate that associations between either α or β subunits and $\gamma 215\delta$ chimeras prevent the assembly of $\alpha\beta\gamma$ trimers, the first stable assembly intermediate. The mAb 14 epitope and Bgt binding sites fail to form on these α - $\gamma 215\delta$ and β - $\gamma 215\delta$ complexes and, as misfolded partially assembled complexes, they are degraded at a rate similar to unassembled subunits.

Multiple assembly signals within the C-terminal half of the γ subunit

Additional chimeras were constructed to identify discrete regions within the C-terminal domain important for assembly. It was reported previously that a chimera composed of the ϵ subunit N-terminal and β subunit C-terminal domains substituted during assembly for the ϵ subunit, which is the mammalian adult isoform of the γ subunit (Yu and Hall, 1991). The ϵ/β chimera differed from the $\gamma 215\beta$ chimera in that its short extracellular C terminus

Figure 3. The effect of the $\gamma215\delta$ chimera on AChR assembly. *A*, 125 I-Bgt binding to cells stably expressing $\gamma215\delta$ in addition to the four AChR subunits ($\alpha\beta\gamma\delta + \gamma215\delta$ cells). The $\gamma215\delta$ chimera cDNA was stably transfected into a cell line expressing the four AChR subunits ($\alpha\beta\gamma\delta$ cells). Surface 125 I-Bgt binding to $\alpha\beta\gamma\delta$ cells was 77 fmol per plate, whereas binding to the $\alpha\beta\gamma\delta + \gamma215\delta$ cells was reduced to 11 fmol (mean $\pm$ SEM of seven plates from three experiments). *B*, Subunit synthesis and coprecipitation. The $\alpha\beta\gamma\delta$ and $\alpha\beta\gamma\delta + \gamma215\delta$ cells were labeled for 30 min, solubilized to preserve subunit associations, and immunoprecipitated with α -specific polyclonal antibodies (lanes 1 and 2), β -specific polyclonal antibodies (lanes 3 and 4), or γ - and δ -specific mAb 88b (lanes 5 and 6), which also recognizes the $\gamma215\delta$ chimera. The precipitated subunits from one-half of a 10 cm plate were loaded onto each lane. *C*, Assembly of AChR subunits in $\alpha\beta\gamma\delta$ cells. The $\alpha\beta\gamma\delta$ cells were labeled metabolically as in *B* and then followed for the indicated times at 20°C before being immunoprecipitated with either an α -specific polyclonal antibody (left) or the conformation-dependent mAb 14 (right). The precipitated subunits from a 10 cm plate were loaded onto each lane. The band labeled α' just above the δ band previously was shown to be different from the δ subunit (Green and Claudio, 1993; Green and Wanamaker, 1997). The band that migrates between the α and β subunit bands is actin. *D*, The α subunit associations with the $\gamma215\delta$ chimera inhibit its folding and assembly. The $\alpha\beta\gamma\delta + \gamma215\delta$ cells were labeled metabolically and followed for the indicated times; the subunits were immunoprecipitated as in *C*. Only the chimera coprecipitates with the α subunit (left) and prevents formation of the mAb 14 epitope (right). Qualitatively similar results were obtained two times with the $\alpha\beta\gamma\delta + \gamma215\delta$ cell line shown and two more times with a second $\alpha\beta\gamma\delta + \gamma215\delta$ cell line. *E*, $\gamma215\delta$ chimeras associate with α or β subunits. The $\alpha\beta\gamma\delta + \gamma215\delta$ cells were labeled metabolically and followed as in *C*. Subunits were immunoprecipitated with β -specific mAb 148 (left) or γ - and δ -specific mAb 88b (right), which also recognizes the $\gamma215\delta$ chimera. Qualitatively similar results were obtained two times with the $\alpha\beta\gamma\delta + \gamma215\delta$ cell line shown and two more times with a second $\alpha\beta\gamma\delta + \gamma215\delta$ cell line. *F*, The rate of subunit degradation in $\alpha\beta\gamma\delta + \gamma215\delta$ cells. The time course of subunit degradation is shown quantitatively from the scanned values of the experiment shown in *C* (left) for the α subunits and in *D* (left) for $\gamma215\delta$ (filled circles) and α subunits (filled triangles). The data are plotted on a semilog scale. The decay of the α subunit signal from the $\alpha\beta\gamma\delta$ cells (filled squares) is biphasic. The fast component corresponds to degrading unassembled α subunits. To estimate the unassembled α rate of decay, we subtracted the 48 hr value from the other values and plotted it as open squares. The half-life was estimated to be 8.1 hr, based on a least-squares fit of an exponential function to the three data sets.

Figure 4. Multiple assembly signals within the C-terminal half of the γ subunit. **A**, The $\gamma 215\delta 467\gamma$ chimera. The C terminus of the $\gamma 215\delta$ chimera starting at amino acid 467 was replaced by the corresponding region from the γ subunit (open bar) to create the $\gamma 215\delta 467\gamma$ chimera. The C-terminal γ subunit domain extends from the end of the fourth membrane-spanning region, M4 (broadened area), to the C terminus. Immunoprecipitation and SDS-PAGE analysis of the $\gamma 215\delta 467\gamma$ chimera were performed as in Figure 1A. **B**, The $\gamma 215\delta 467\gamma$ chimera blocks the formation of Bgt binding sites when cotransfected with all four wild-type subunits. Cells were transiently transfected with $\alpha\beta\gamma\delta$ and the indicated amount of chimera cDNA as in Figure 2B and were assayed for cell-surface ^{125}I -Bgt binding. Values are a mean $\pm$ SEM of four plates from two experiments, expressed as a percentage of the amount of ^{125}I -Bgt bound in the absence of the chimera, which was 66 fmol. Results with the $\gamma 215\delta$ chimera from Figure 2B are shown also. **C**, The $\gamma 215\delta 329\gamma$ and $\gamma 322\delta$ chimeras. The $\gamma 215\delta$ chimera starting at amino acid 329 was replaced by the corresponding region from the γ subunit (open bar) to create the $\gamma 215\delta 329\gamma$ chimera. This part of the γ subunit extends from just after the third membrane-spanning region (broadened area) to the C terminus. The γ subunit starting at amino acid 322 was replaced by the corresponding region from the δ subunit (filled bar) to create the $\gamma 322\delta$ chimera. Immunoprecipitation and SDS-PAGE analysis of the chimeras were performed as in Figure 1A. **D**, The $\gamma 215\delta 329\gamma$ and $\gamma 322\delta$ chimeras block the formation of Bgt binding sites when cotransfected with all four wild-type subunits. Cells were transiently transfected with $\alpha\beta\gamma\delta$ and the indicated amount of chimera cDNA as in Figure 4B and were assayed for cell-surface ^{125}I -Bgt binding. Values are a mean $\pm$ SEM of seven plates from three experiments, expressed as a percentage of the amount of ^{125}I -Bgt bound in the absence of the chimera, which was 57 fmol. Results with the $\gamma 215\delta$ chimera from Figure 2B are shown also.

was from the ϵ subunit, not the β subunit. To test whether the C terminus contributes to the dominant-negative effect of $\gamma 215\delta$, we replaced the C terminus of $\gamma 215\delta$ by that from the γ subunit to construct $\gamma 215\delta 467\gamma$ (Fig. 4A). Transfection of the $\gamma 215\delta 467\gamma$ chimera along with the four wild-type subunits completely blocked AChR expression (Fig. 4B), and the addition of the γ subunit C terminus does not reverse the dominant-negative effect.

It is unclear why our results differ from those with the ϵ/β chimera.

Two more chimeras were constructed and characterized. The γ subunit region that contains the first three transmembrane domains was replaced by the homologous region of the δ subunit to produce the $\gamma 215\delta 329\gamma$ chimera, and the γ subunit region just after the third transmembrane domain to the C terminus was

replaced by the homologous δ subunit region to construct the $\gamma 322\delta$ chimera (Fig. 4). Surprisingly, expression of either the $\gamma 215\delta 329\gamma$ or $\gamma 322\delta$ chimera along with the four wild-type subunits again completely blocked expression of cell-surface AChRs (Fig. 4D). On the basis of these results, we conclude that multiple regions within the γ subunit C-terminal half are required to overcome the dominant-negative effect observed when this domain is replaced. Minimally, two separate regions are required: one that contains the first three transmembrane domains and a second that extends from the cytoplasmic loop just after the third transmembrane domain to the C terminus.

DISCUSSION

A large number of studies have identified regions and specific residues within the AChR subunit N-terminal domain that mediate subunit associations during assembly (Yu and Hall, 1991, 1994a; Sumikawa and Gehle, 1992; Verrall and Hall, 1992; Sumikawa and Nishizaki, 1994; Kreienkamp et al., 1995). These studies did not assay the process of receptor assembly directly. Instead, assembly was assayed indirectly, either by monitoring cell-surface expression or by studying associations between subunits and/or subunit fragments in isolation of the other subunits. Thus, it remains to be determined when N-terminal regions participate during assembly and whether other subunit regions also could be involved in assembly (see also Yu and Hall, 1994b).

We found that the membrane-spanning and the cytoplasmic domains of the γ subunit are essential for complete AChR assembly. When these regions of the γ subunit are replaced by the homologous regions of the α , β , or δ subunits, AChRs no longer fully assemble, as shown by a complete block of AChR surface expression and Bgt binding site formation. Our approach differed from previous work in that methods were used that directly assayed full AChR assembly and allowed us to determine which steps in assembly were altered. We found that, by altering the C-terminal half of the γ subunit, assembly was blocked at a step that prevented the appearance of $\alpha\beta\gamma$ trimers. Instead of assembling into trimers, $\gamma 215\delta$ chimeras associated with α and β subunits, but not with γ and δ subunits. These results indicate that the initial subunit associations required for the assembly of $\alpha\beta\gamma$ trimers, i.e., the recognition of α and β by the γ subunit, are preserved when $\gamma 215\delta$ chimeras replace γ subunits. Thus, the N-terminal domain of the γ subunit in the $\gamma 215\delta$ chimera appears to mediate the initial subunit associations. Importantly, $\gamma 215\delta$ chimeras only interact with α or β subunits, not both together. This finding further indicates that $\gamma 215\delta$ chimeras block AChR assembly at a step in which heterodimers specifically recognize a third subunit for assembly into $\alpha\beta\gamma$ trimers.

The results from this paper, together with data from other studies, indicate that the first steps of AChR assembly occur as shown in Figure 5. We have shown previously that α , β , and γ subunits rapidly associate into $\alpha\beta\gamma$ trimers during or just after subunit synthesis, and interactions between N-terminal domains may be in part cotranslational (Green and Claudio, 1993). The data from this study further indicate that $\alpha\gamma$ and $\beta\gamma$ heterodimers precede the rapid assembly of $\alpha\beta\gamma$ trimers, which suggests that assembly is initiated when the N-terminal domains of two subunits interact. The space between ribosomes on the endoplasmic reticulum (ER) membrane is ~ 500 Å, too large a distance to allow associations between two partially synthesized subunits (Hurtley and Helenius, 1989) but, as shown in Figure 5, would allow the N-terminal domain of a subunit undergoing synthesis to interact with a subunit no longer associated with the translocation

machinery. A similar process also may initiate K^+ channel assembly in which a region of the subunit N-terminal domain is required for rapid, perhaps cotranslational, interactions between subunits (Deal et al., 1994). According to the “heterodimer” model of AChR assembly (Blount et al., 1990; Gu et al., 1991; Saedi et al., 1991), α and the other subunits remain unassembled for a considerable time, during which the subunits fold and the Bgt binding site is created on the α subunits. This feature of the assembly model is difficult to reconcile with our results, in particular our finding that γ subunit chimeras prevent the formation of Bgt binding sites when added to wild-type subunits.

Interactions between subunits show a lack of subunit specificity, because homomeric associations occur when each of the four AChR subunits is expressed alone (Paulson et al., 1991) or when any heteromeric pair of subunits is expressed (Green and Claudio, 1993). These subunit associations also appear to form rapidly (Green and Claudio, 1993) and involve the N-terminal domain of the subunit (Verrall and Hall, 1992). The lack of subunit specificity, together with the rapidity by which associations occur, suggests that the function of the initial subunit associations is to protect critical subunit domains from exposure to the membrane or the aqueous environment and to prevent the misfolding of these domains. As shown in Figure 5, *A* and *B*, a lack of subunit specificity also can help to explain why AChRs assemble inefficiently such that only 20–30% of the synthesized subunits assemble into AChRs and the rest of the subunits are degraded (Merlie and Lindstrom, 1983; Ross et al., 1991). As modeled in Figure 5*A*, productive subunit associations occur with the assembly of $\alpha\beta$, $\alpha\gamma$, or $\beta\gamma$ heterodimers. If each potential dimer assembles with equal probability, only 30% of the initial interactions would form productive subunit pairs. Dimers assembled from other subunit pairs, i.e., $\alpha\alpha$, $\beta\beta$, $\gamma\gamma$, $\delta\delta$, $\alpha\delta$, $\beta\delta$, or $\gamma\delta$, would not continue into $\alpha\beta\gamma$ trimers. Although these dimers can form, they are not detected when all four subunits are present (Green and Claudio, 1993; Green and Wanamaker, 1998), indicating that the associations are relatively weak and short-lived. The transient nature of these subunit interactions could allow some of the subunits to reassociate with other subunits, perhaps to form a productive pairing. However, many of the subunits that formed unproductive pairs would be expected to misfold and be rapidly degraded (Fig. 5*B*).

Because there appears to be little subunit specificity in the initial subunit associations among AChR subunits, we would expect the $\alpha\beta$, $\alpha\gamma$, and $\beta\gamma$ heterodimer associations to be no tighter than any other combination. What then stabilizes the productive $\alpha\beta$, $\alpha\gamma$, and $\beta\gamma$ heterodimers? In previous studies we showed that the addition of δ subunits to $\alpha\beta\gamma$ trimers is preceded by the folding of the trimer subunits, as evidenced by the appearance of the Bgt binding site and mAb 14 epitope. Similarly, the addition of the second α subunits to $\alpha\beta\gamma\delta$ tetramers is preceded by the folding of the tetramer subunits (Green and Claudio, 1993; Green and Wanamaker, 1997, 1998). We propose that a similar set of events occurs after a productive heterodimer forms and is required for $\alpha\beta\gamma$ trimer assembly (Fig. 5*A*). The correct subunit combination in the dimer allows the subunits to fold into a conformation that strengthens the association and also allows the appropriate third subunit to bind tightly to the dimer (Fig. 5*A*).

The $\alpha(\gamma 215\delta)$ and $\beta(\gamma 215\delta)$ heterodimers form a tightly associated complex but fail to recognize the third subunit needed for assembly into $\alpha\beta\gamma$ trimers (Fig. 5*C*). This finding indicates that the γ subunit C-terminal regions are involved in the subunit folding that creates a new subunit recognition site and are not

A Productive Associations

B Unproductive Associations

C The $\gamma_{215\delta}$ Chimera

Figure 5. The early steps in AChR assembly are diagrammed, consistent with the results of this paper. Regions within the N-terminal domain of the subunit mediate rapid, perhaps cotranslational, and promiscuous associations. Productive subunit associations, as shown in *A*, result from a subset of the subunit pairings: the $\alpha\beta$, $\alpha\gamma$, or $\beta\gamma$ heterodimers. These heterodimers undergo further folding, which strengthens the association, allows for the assembly of $\alpha\beta\gamma$ trimers, and thereby the subsequent steps in AChR assembly. Unproductive subunit associations, shown in *B*, result from the other subunit pairings: $\alpha\alpha$, $\beta\beta$, $\gamma\gamma$, $\delta\delta$, $\alpha\delta$, $\beta\delta$, or $\gamma\delta$ heterodimers. These heterodimers fail to undergo further folding and are free to associate with other subunits after dissociation or are degraded. The α and β subunit associations with the $\gamma_{215\delta}$ chimera, shown in *C*, result in tightly associated complexes that fail to assemble further. These results suggest that the C-terminal half of the γ subunit is required for subsequent folding and assembly.

necessarily part of the site where the third subunit associates with the heterodimer. Expression of the $\gamma_{215\delta}$ chimera has a dominant-negative effect, therefore, because association with α or β subunits allows for strengthening of the association, but the

dimer fails to fold into a conformation that will allow assembly of the next subunit. It is possible that the chimera can associate properly with α or β subunits but cannot undergo subsequent conformational changes because the C-terminal half of the chi-

mera is misfolded. Although the difference between starting in a misfolded state versus a failure to fold correctly is subtle, several of our findings suggest that the $\gamma 215\delta$ chimera is not misfolded. The chimera is not recognized by the cells as misfolded because it is degraded at the same rate as unassembled wild-type subunits (see Fig. 3F). Furthermore, similar effects on surface expression were observed when different regions of the C-terminal half of the γ subunits were substituted by the corresponding δ subunit region (see Fig. 4). More generally, it is possible that all of the nonproductive dimers as well as the $\gamma 215\delta$ -containing dimers are unable to fold correctly and contribute to the assembly of $\alpha\beta\gamma$ trimers because they lack the appropriate C-terminal regions. This conclusion about the role of the γ subunit C-terminal regions is supported by the dominant-negative effect of all of the additional γ - δ subunit chimeras described in Figure 4. We found that replacing any part of the C-terminal half of the δ subunit on the $\gamma 215\delta$ chimera by the appropriate γ regions failed to prevent the dominant-negative effect. This failure to rescue cell-surface expression by replacing piece-by-piece all of the δ regions by γ regions argues that there is no general C-terminal motif that is part of the association site. Instead, it is consistent with the idea that the site where the third subunit associates is created by rearrangement of the heterodimer subunits, and the ability to undergo the rearrangement requires the C-terminal regions of the γ subunit.

The picture that emerges from this work is that associations between AChR subunits are at first weak and nondiscriminating. If the purpose of the initial subunit associations is, indeed, to protect critical subunit domains and prevent misfolding, then a relatively weak and promiscuous type of association fits with an apparent chaperone role of the subunit associations. Other proteins, such as immunoglobulin-binding protein (BiP) and calnexin, that are endogenous ER chaperones also transiently interact with newly synthesized AChR subunits (Blount and Merlie, 1991; Paulson et al., 1991; Forsayeth et al., 1992; Gelman et al., 1995; Keller et al., 1996). Only the correct associations between AChR subunits subsequently are strengthened and stabilized as the subunits continue to fold and mature during assembly of the AChR. Because these assembly events occur in the plane of ER membrane (Smith et al., 1987), cells must be solubilized to purify assembling subunits. Previously, we showed that the ability to purify intact assembly intermediates is dependent on the detergent used and the presence of phospholipids during the solubilization (Green and Claudio, 1993). These milder solubilization conditions allow us to observe intact $\alpha\beta\gamma$ trimers and $\alpha\beta\gamma\delta$ tetramers, but we have never observed intact heterodimer precursors. It is likely, therefore, that even the mildest of solubilization protocols disperses the heterodimers because of the weakness of the interaction and that subunit associations strengthen as assembly continues from dimer to trimer to tetramer to the final pentamer, which can be dispersed only by SDS.

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