Assembly of the Mammalian Muscle Acetylcholine Receptor in Transfected COS Cells

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Abstract. We have investigated the mechanisms of assembly and transport to the cell surface of the mouse muscle nicotinic acetylcholine receptor (AChR) in transiently transfected COS cells. In cells transfected with all four subunit cDNAs, AChR was expressed on the surface with properties resembling those seen in mouse muscle cells (Gu, Y., A. F. Franco, Jr., P. D. Gardner, J. B. Lansman, J. R. Forsayeth, and Z. W. Hall. 1990. *Neuron*. 5:147-157). When incomplete combinations of AChR subunits were expressed, surface binding of ^{125}I-α-bungarotoxin was not detected except in the case of $\alpha\beta\gamma$ which expressed <15% of that seen with all four subunits. Immunoprecipitation and sucrose gradient sedimentation experiments showed that in cells expressing pairs of subunits, $\alpha\delta$ and $\alpha\gamma$ heterodimers were formed, but $\alpha\beta$ was not. When three subunits were expressed, $\alpha\delta\beta$ and $\alpha\gamma\beta$ complexes were formed. Variation of the ratios of the four subunit cDNAs used in the transfection mixture showed that surface AChR expression was decreased by high concentrations of δ or γ cDNAs in a mutually competitive manner. High expression of δ or γ subunits also each inhibited formation of a heterodimer with α and the other subunit. These results are consistent with a defined pathway for AChR assembly in which $\alpha\delta$ and $\alpha\gamma$ heterodimers are formed first, followed by association with the β subunit and with each other to form the complete AChR.

TRANSMEMBRANE ion channels comprise several families of proteins with a common structural design in which homologous subunits or protein domains surround a central aqueous pore (Unwin, 1989). The simplest oligomeric channels are homopolymers; others contain as many as four different polypeptide subunits. Although the structure and function of many of these channels is well understood, relatively little is known about how they are assembled. Indeed, the mechanisms of assembly of only a few oligomeric membrane proteins of any type have been extensively investigated (Carlin and Merlie, 1987; Rose and Doms, 1988; Hurtley and Helenius, 1989).

The most completely studied ion channel is the nicotinic actylcholine receptor (AChR)[1] from vertebrate muscle or from *Torpedo* electric organ (McCarthy et al., 1986; Claudio, 1989). The AChR is a pentamer with four different subunits whose stoichiometry is $\alpha_2\beta\gamma\delta$. The subunits have highly homologous sequences and are presumably evolved from a common ancestor that formed a homo-oligomeric channel in which all of the subunits were interchangeable (Raftery et al., 1980; Noda et al., 1983; Numa et al., 1983). Each of the subunits is made as a single polypeptide chain (Anderson and Blobel, 1981), and the four are assembled into the complete oligomer in the endoplasmic reticulum (Smith et al., 1987; Gu et al., 1989*b*). After synthesis of the polypeptide, the α chain undergoes a maturational step before assembly with the other subunits that confers upon it the ability to bind α-bungarotoxin (α-BuTx) (Carlin et al., 1986; Merlie and Lindstrom, 1983; Merlie and Sebbane, 1981). Subsequent binding of the α subunit to the γ and δ subunits result in conformational changes in the α subunit that allow it to bind agonists and antagonists with high affinity (Blount and Merlie, 1989).

To investigate more completely the steps in assembly of the AChR, we have used transiently transfected COS cells in which the number and relative proportions of individual subunits can be easily regulated (Gu et al., 1990). Our results show that assembly of the AChR occurs along a stepwise pathway in which $\alpha\delta$ and $\alpha\gamma$ heterodimers are formed, and then interact with each other and with the β subunit to yield the fully assembled receptor.

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin.

Materials and Methods

cDNAs and Vector

Full-length cDNAs coding for α, β, γ, and δ subunits of mouse muscle nicotinic acetylcholine receptor were obtained from Drs. J. P. Merlie and N. Davidson (α, Isenberg et al., 1986; β, Buonanno et al., 1986; γ, Yu et al., 1986; δ, Lapolla et al., 1984). Each of the cDNAs was subcloned into the SV-40-based expression vector pSM (Brodsky et al., 1990) at the multiple cloning site.

Antibodies

mAb 61 and 124 (Tzartos et al., 1981; Gullick and Lindstrom, 1983), which

recognize the α and β subunit, respectively, were generously given to us by Dr. Jon Lindstrom (The Salk Institute for Biological Studies, San Diego, CA); mAb 14-3-F7, specific for the α subunit, was prepared as described in Dowding and Hall (1987); mAb 88B (Froehner et al., 1983) that recognizes the δ subunit was the generous gift of Dr. Stanley C. Froehner (Dartmouth Medical School, Hanover, NH). Anti-γ 485 antibodies were affinity purified from antisera raised against a synthetic peptide corresponding to the amino acid residues 485–497 of the γ subunit of mouse muscle AChR (Gu and Hall, 1988).

Transfection of COS Cells

Transfection of COS cells was carried out as described previously (Gu et al., 1990), using a modified DEAE-dextran transfection procedure (Seed and Aruffo, 1987). 30–50% confluent dishes of cells were incubated for 4 h at 37°C with the appropriate amount of plasmid cDNA in DME H-16 supplemented with 1% heat-inactivated FBS, 0.4 mg/ml DEAE-dextran, and 0.1 mM chloroquine diphosphate. The amount of plasmid used for each subunit in the transfection was determined empirically to give maximum cell surface AChR expression (see also Fig. 5). The standard mixture for the transfection of a 60-mm dish of cells contained 1.3 μg α, 0.65 μg β, 1.0 μg γ, and 0.26 μg δ subunit plasmids. After the DNA solution was removed, the cells were treated with 10% DMSO in PBS for 2 min at room temperature before being returned to 37°C in growth medium (10% FBS in DME H-16 supplemented with 100 U/ml penicillin and streptomycin). 24 h later, the cells were trypsinized and distributed into the appropriate culture dishes or multiwell clusters and AChR expression determined after an additional 24 h as described.

Assays

Surface expression of α-BuTx-binding sites was determined by incubating intact cells for 1.5 h at 37°C with 10 nM ^{125}I-α-BuTx (Amersham Corp., Arlington Heights, IL). Nonspecific binding was measured by the addition of 100-fold excess unlabeled α-BuTx to the incubation mixture. Unbound toxin was then removed by washing the cells with PBS. The amount of bound toxin was determined by solubilizing the cells in 0.1 M NaOH and measuring the radioactivity in a γ counter. Protein concentration was determined with the method of Bradford (1976) with BSA as a standard.

Immunoprecipitation of Toxin-binding Sites with Subunit-specific Antibodies

Immunoprecipitation with subunit-specific antibodies was performed as described previously (Gu and Hall, 1988). Transfected COS cells were solubilized in a buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM sodium tetrathionate, 1 mM *N*-ethylmaleimide, 0.4 mM PMSF, 10 U/ml aprotinin, 20 μg/ml leupeptin (extraction buffer). ^{125}I-α-BuTx was then added to the lysates to a final concentration of 20 nM to label all the toxin-binding sites. For immunoprecipitation with mAb 61 or mAb 124, the cell lysates (80 μl) were incubated with 3 μl of the antibody for 2 h at 4°C. Then 30 μl of Pansorbin (Calbiochem-Behring Corp., San Diego, CA) precoated with appropriate rabbit antibodies (Cappel Labs, Malvern, PA) was added and the incubation continued for two more hours. For precipitation with γ-specific antibodies, uncoated Pansorbin was used. Immunoprecipitation with the δ subunit–specific antibody was performed using mAb 88B coupled to Sepharose. All the precipitates were washed three times with 1 ml of the extraction buffer supplemented with 1 M NaCl before being counted in a γ counter. Nonspecific precipitations were determined using sham-transfected cells.

Sucrose Gradient Sedimentation

To label AChR intermediates in transfected COS cells, the cells were solubilized in extraction buffer (above), incubated on ice with 20 nM ^{125}I-α-BuTx for 2 h, and then applied to a 5–20% sucrose gradient with alkaline phosphatase (6.3 S) and catalase (11.4 S) included as markers. Gradients were centrifuged at 36,000 rpm in SW50.1 rotor for 15-16 h at 4°C and fractionated into 100-μl aliquots. Every second fraction was then immunoprecipitated with anti-δ antibody, mAb 88b, coupled to Sepharose. After incubation for 2 h at 4°C, the beads were washed and associated ^{125}I counted in a γ counter.

Results

When COS cells were transfected with α, β, γ, and δ subunit cDNAs, α-BuTx binding sites were expressed on their surface (Fig. 1 *A*). In previous experiments we have demonstrated that the surface binding sites for α-BuTx in the transfected cells represent fully assembled, functional AChRs with physiological and pharmacological properties resembling those of the embryonic form of the receptor (Gu et al., 1990). Under the conditions used, expression lasts for at least 4 d.

To see if subunits can replace one another in the assembly pathway, we omitted one or more subunit cDNAs from the transfection mixture. In all but one case toxin-binding activity was not detected on the surface of the cells (Fig. 1 *A*).

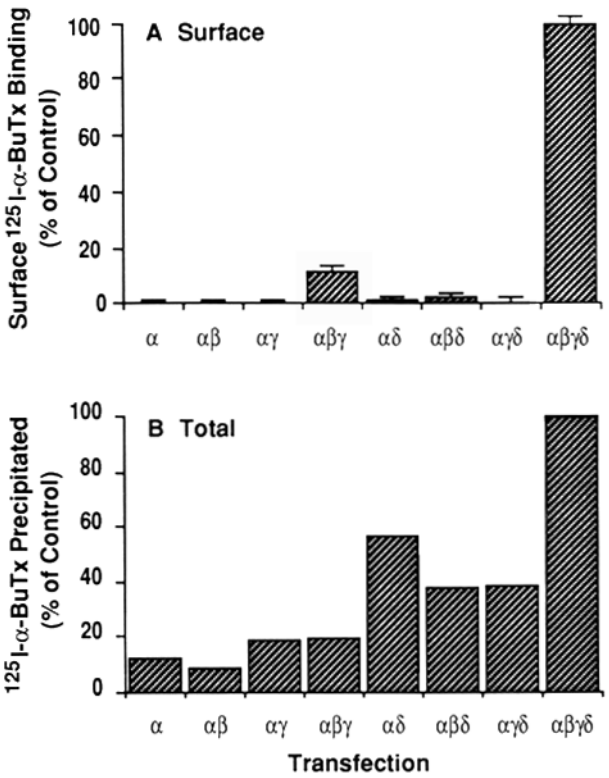

Figure 1. Expression of α-BuTx-binding sites in COS cells transfected with various combinations of mouse AChR subunit cDNAs. (*A*) Surface expression. COS cells in 60-mm dishes were transfected with the indicated combinations of AChR subunit cDNAs and were then trypsinized and redistributed to four wells in 24-well cluster plates. Surface expression of α-BuTx-binding sites were determined 24 h later by incubating intact cells with ^{125}I-α-BuTx. In each case, toxin-binding per milligram total protein was expressed as a percentage of the value obtained in cells transfected with all four subunits. Each value is the mean ± SEM of three determinations. (*B*) Total expression. COS cells in 60-mm dishes transfected as above were lysed in a buffer containing Triton X-100 and the lysate incubated with ^{125}I-α-BuTx to label all the toxin binding sites. The labeled toxin-binding sites were then immunoprecipitated with mAb 61 and in each case were expressed as the percentage of the total sites in cells transfected with all four subunits. Each value is the average of two determinations. Western blot analysis of the whole cell lysate with mAb 14-3-F7 indicated that the total amount of α subunit expressed in the cells was similar in all the transfections (data not shown).

The sole exception was cells transfected with a combination of α, β, and γ cDNAs, in which a small, but significant level of activity (10–15%) was detected. Because pairwise combinations of $\alpha\beta$ and $\alpha\gamma$ cDNAs did not yield surface toxin-binding activity, the activity seen when all three are expressed presumably results from an oligomer containing each of the three subunits. This activity was not further investigated.

Association of the α Subunit with Other Subunits

We then investigated whether intracellular associations between subunits occurred when only two or three subunits were expressed together. In this way, we hoped to detect intermediates in the assembly process that were accumulated in the absence of complete assembly. We first measured the binding of ^{125}I-α-BuTx in lysates of cells transfected with various combinations of subunit cDNAs. Previous experiments have shown that the glycosylated primary translation product of the α subunit does not bind α-BuTx, but that it acquires the ability to do so before assembly with other subunits (Carlin and Merlie, 1987; Blount and Merlie, 1988). In agreement with these results, we found toxin-binding activity in extracts of COS cells transfected only with α subunit cDNA, as measured by the ability of an antibody to the α subunit (mAb 61) to precipitate ^{125}I-α-BuTx from transfected cell extracts (Fig. 1 *B*).

The amount of α subunit converted to the toxin-binding form was increased significantly when α was coexpressed with δ subunit. A smaller increase was seen when α and γ were coexpressed; coexpression with β subunit did not increase the amount of α converted to the toxin-binding form. Immunoblotting of the whole cell extract showed that the total amount of α subunit expressed was unchanged by coexpression of α with the other subunits (data not shown). Thus coexpression with γ or δ subunits increased the proportion of α accumulated in the toxin-binding form. Addition of β cDNA to cells expressing either the $\alpha\delta$ pair or the $\alpha\gamma$ pair resulted in no further increase (Fig. 1 *B*). The largest effect on toxin binding was seen when all four subunits were coexpressed. Under these conditions, toxin binding was increased ~10-fold over that seen with the α subunit alone. Even under these conditions, however, conversion was relatively inefficient. By comparing the amount of metabolically labeled α subunit precipitated by mAb 61 with that precipitated by Sepharose-bound α-BuTx, we estimate that COS cells transfected with all four subunits convert only about 5% of the total α subunit to the toxin-binding form (data not shown).

The ability of γ and δ subunits to increase the proportion of α subunit accumulated in the toxin-binding form implies that they associate with α in the absence of other subunits. We tested this directly by examining the ability of antibodies to other subunits to immunoprecipitate toxin-binding activity in transfected cell extracts. When α and δ were coexpressed, toxin-binding activity was immunoprecipitated by mAb 88, which recognizes the δ subunit, indicating that the two subunits are physically associated (Fig. 2 *A*). The amount of immunoprecipitated toxin-binding activity was not increased by the addition of β or of γ subunit alone, but was increased approximately twofold when all four subunits were expressed (Fig. 2 *A*). The twofold increase presumably

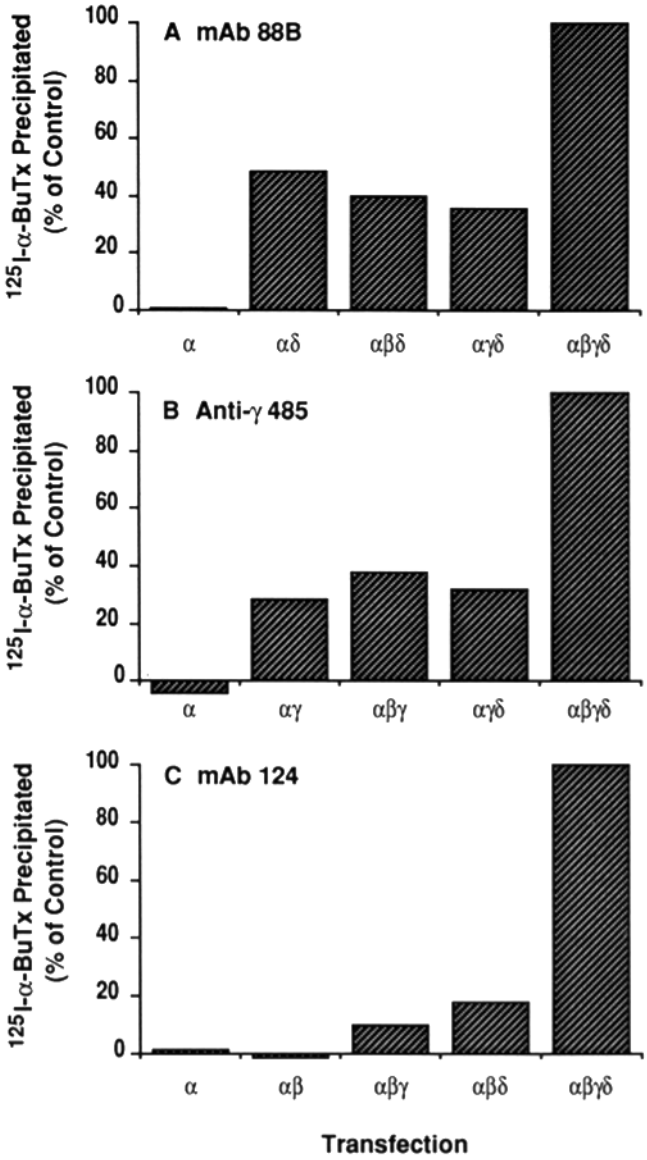

Figure 2. Association of α subunit with other subunits in transfected COS cells. COS cells transfected with the indicated combinations of mouse AChR subunit cDNAs were lysed in extraction buffer. The lysate were incubated with ^{125}I-α-BuTx to label all the toxin-binding sites and were then immunoprecipitated with mAb 88B (*A*), anti-γ 485 (*B*), or mAb 124 (*C*). These antibodies are specific for δ, γ and β subunit, respectively. The results for each antibody were expressed as percentages of sites precipitated from cells transfected with all four subunits. Each value is the average of two determinations. Western blotting of the whole cell lysate with mAb 14-3-F7 showed that the total amount of α subunit expressed in these cells was similar for different combinations of transfections (data not shown).

reflects assembly of the complete receptor which contains two α subunits for each δ subunit. These results suggest that α and δ subunits, when expressed alone, form a heterodimer.

When γ subunit was coexpressed with α, and antibodies specific for the γ subunit were used to immunoprecipitate toxin-binding activity, results that were qualitatively similar to those seen with the δ subunit were observed (Fig. 2 *B*). Thus immunoprecipitation of toxin-binding activity was ob-

served when only α and γ subunits were expressed; addition of β or δ did not significantly increase the immunoprecipitated activity; and both added together caused a substantial increase. One difference between the results observed with anti-γ and anti-δ antibodies is that formation of the $\alpha\delta$ heterodimer appeared to be more efficient than formation of the $\alpha\gamma$ heterodimer when expressed as a percentage of the toxin-binding activity seen when all four subunits are present. An important point is that the results obtained with both antibodies (Fig. 2, *A* and *B*) showed that coexpression of α, γ, and δ subunits resulted in no increase over that seen when either γ or δ was coexpressed with α alone. Thus, $\alpha\delta$ and $\alpha\gamma$ heterodimers appear not to associate with each other in the absence of the β subunit.

The same experiment with β subunit cDNA and mAb 124, specific for the β subunit, gave a different pattern of results (Fig. 2 *C*). In this case, no toxin-binding activity was precipitated when α and β were co-expressed, unless either γ or δ was also present. These results suggest that β does not associate with α, but can form a complex with either $\alpha\delta$ or $\alpha\gamma$ heterodimers. The surface expression of toxin-binding activity when α, β and γ subunits are expressed together is consistent with this idea (Fig. 1 *A*). In either case of β expression with a heterodimer pair, the amount of toxin-binding activity immunoprecipitated by the anti-β antibody was <20% of that seen when all four subunits were expressed, indicating that association of β with either heterodimer alone is relatively weak.

Sucrose Gradient Sedimentation of $\alpha\delta$ and $\alpha\delta\beta$ Complexes

We further examined the properties of the complexes formed in COS cells transfected with combinations of α, δ, and β subunits by sucrose gradient sedimentation. In control experiments, lysates of COS cells transfected with all four subunits were incubated with ^{125}I-α-BuTx, sedimented in a 5–20% sucrose gradient, and the fractions immunoprecipitated with mAb 88B-Sepharose. A major peak at 9.7 S (Fig. 3) migrated at the same position as fully assembled AChR on the cell surface (data not shown) and a minor peak migrated at about 6 S. When the same experiment was performed with cells transfected with only α and δ subunits, a single peak of toxin-binding activity was obtained at 6 S. The peak contains both α subunit (toxin binding) and δ subunit (immunoreactive with mAb 88B), and its size is consistent with its being a heterodimer. In cells transfected with α, δ and β subunits, a 6 S peak was again observed (Fig. 3). Also present was additional, higher molecular weight material which migrated as a broad shoulder between 6 and 9 S. Attempts to detect $\alpha\gamma$ oligomers after sucrose gradient sedimentation were unsuccessful, perhaps because this complex is less stable (see Discussion).

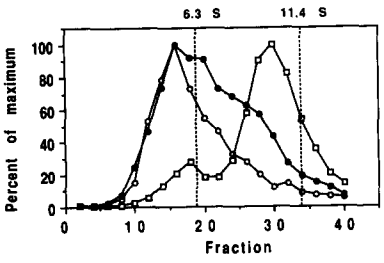

Figure 3. Sucrose velocity sedimentation of $\alpha\delta$, $\alpha\beta\delta$ complexes and complete AChR. COS cells, transfected with α, β, γ, and δ (*open squares*), α and δ only (*open circles*), or α, β and δ (*solid circles*) were extracted with Triton, labeled with ^{125}I-α-BuTx, and separated on a 5–20% sucrose gradient. Even-numbered gradient fractions (100 μl) were immunoprecipitated with mAb 88B coupled to Sepharose. Maximum cpm in each gradient in the representative experiment shown was 2,473 cpm for $\alpha\beta\gamma\delta$, 2,061 cpm for $\alpha\delta$, and 545 cpm for $\alpha\beta\delta$.

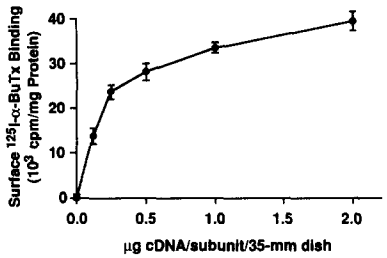

Figure 4. Surface AChR expression as a function of the total amount of AChR subunit cDNA. COS cells in 60-mm dishes were transfected with the indicated amount of cDNA for each subunit and were distributed to four wells in 24-well plates. Surface toxin-binding sites per milligram protein was determined as described in the legend to Fig. 1. Each value represents the mean ± SEM of three determinations.

Competition between γ and δ Subunits in AChR Assembly

We next examined the level of surface AChR expression in COS cells transfected with all four subunit cDNAs in various ratios. To determine the dependence of AChR expression on total cDNA level, we first held the ratio of the four subunit cDNAs at 1:1:1:1 ratio and varied the total amount of cDNA. Under these conditions, as the total amount of cDNA was increased surface expression initially increased linearly and then reached a plateau (Fig. 4).

To determine the optimal ratio of subunit cDNAs, COS cells were then transfected with varying amounts of a single subunit cDNA, along with fixed amounts of the other three

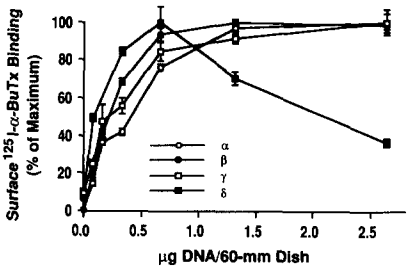

Figure 5. Surface AChR expression as a function of the ratio between different subunit cDNAs. COS cells were transfected with the indicated amounts of α (*open circles*), β (*solid circles*), γ (*open squares*), or δ (*solid squares*) subunit cDNAs along with a fixed amount of the other three subunit cDNAs as described in the legend to Fig. 1. The cells were then trypsinized and redistributed to 24-well multiwell plates. Surface AChR expression per milligram protein was determined 24 h later. The data were expressed as percentages of the maximal expression for each subunit cDNA. Each value is the mean ± SEM of three determinations.

cDNAs. When β, γ, and δ cDNAs were held constant and the cDNA for the α subunit was increased, the amount of surface AChR expression initially increased, then reached a plateau, presumably because one of the other subunits became limiting (Fig. 5, *open circles*). A similar dependence of surface AChR expression on cDNA concentration was seen for β and γ subunits. Maximal expression was seen at DNA levels of approximately 1.3, 0.7, and 1 μg per 60 mm dish for α, β, and γ subunits, respectively. The slightly higher level of α subunit cDNA required may reflect the presence of two subunits of α in the fully assembled AChR.

When the cDNAs for α, β, and γ subunits were held constant and the δ cDNA was varied, a different result was obtained (Fig. 5). Surface AChR expression initially increased with increasing amounts of cDNA, as with the other subunit cDNAs; expression then reached a maximum at 0.3–0.6 μg δ cDNA per dish and declined at higher amounts.

The inhibition of surface expression of the AChR at higher levels of δ cDNA was not due to diminished formation of $\alpha\delta$ complexes. When toxin-binding activity was immunoprecipitated with mAb 88B from the lysates of cells that had been transfected with a constant amount of α, β, and γ cDNAs and increasing amounts of δ cDNA, the number of toxin-binding sites in association with the δ subunit increased to a plateau with no significant decrease at higher amounts of δ cDNA (Fig. 6 *A*).

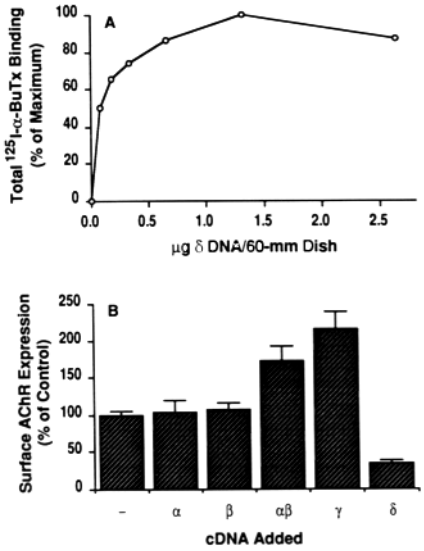

Figure 6. (*A*) Increased δ cDNA concentration does not decrease the expression of total toxin-binding sites in transfected COS cells. COS cells transfected with varied amounts of cDNA for the δ subunit, along with fixed amounts of α, β, and γ subunit cDNAs, were lysed and the lysates incubated with ^{125}I-α-BuTx. The labeled toxin-binding sites were then immunoprecipitated with mAb 88B, specific for the δ subunit. The data per milligram total protein were expressed as percentages of the maximal expression. Each value is the average of two determinations. (*B*) Reversal of the effect of increased δ cDNA concentration by increased γ cDNA. COS cells transfected with an inhibitory amount of δ cDNA, along with the normal amount of α, β, γ cDNAs with or without the further addition of twice the normal amount of α, β, γ, or δ cDNAs were assayed for surface toxin-binding sites. The data per milligram total protein was expressed as the percentage of expression in the absence of the additional subunit cDNAs. Each value is the mean ± SEM of three determinations. in this experiment, the increased amount of δ cDNA used decreased the surface AChR expression by ~50% compared to the optimal amount of δ subunit cDNA (not shown).

One explanation for the reduced surface AChR expression with higher levels of δ cDNA is competition of δ with γ subunit in the formation of $\alpha\delta$ and $\alpha\gamma$ heterodimers. Since γ and δ compete for the same pool of α, high expression of either γ or δ should result in a reciprocal reduction in the formation of the opposite ($\alpha\delta$ or $\alpha\gamma$) pair.

We tested the idea of competition between γ and δ by attempting to reverse the inhibition of surface AChR expression seen at high concentrations of δ cDNA with additional γ subunit cDNA (Fig. 6 *B*). At a concentration of δ cDNA that gave about 50% inhibition of surface AChR expression, a twofold increase above the normal amount in γ subunit cDNA increased AChR expression almost twofold. Addition of neither α nor β subunit cDNA alone was effective, and addition of more δ cDNA decreased expression further. Although neither alone was effective, α and β cDNAs added together also increased AChR expression.

One observation not accounted for by the competition hypothesis is the failure of high concentrations of γ subunit cDNA to inhibit AChR expression. If γ and δ are competitive, we reasoned that inhibition by γ might become evident at lower concentrations of δ. This prediction proved to be correct. When the amount of δ cDNA in the transfection mixture was reduced from 0.26 to 0.13 μg, inhibition by high concentrations of γ subunit cDNA became evident (Fig. 7). Taken together, these experiments show that γ and δ subunits compete with each other when either is present in excess, and suggest that these two subunits play similar roles during assembly of the AChR.

We then tested directly whether high expression of γ or δ subunits could interfere with the ability of the other subunit to form a heterodimer with α. When COS cells were transfected with fixed amounts of α and γ subunit cDNAs and increasing amounts of δ subunit cDNA, a progressive inhibition of $\alpha\gamma$ heterodimer formation was observed (Fig. 8 *A*). The concentration dependence of this inhibition coincided almost exactly with the curve for inhibition of surface AChR expression by high concentrations of δ subunit cDNA. In a similar way, high concentrations of γ subunit inhibited the formation of the $\alpha\delta$ heterodimer (Fig. 8 *B*). Again, the concentration

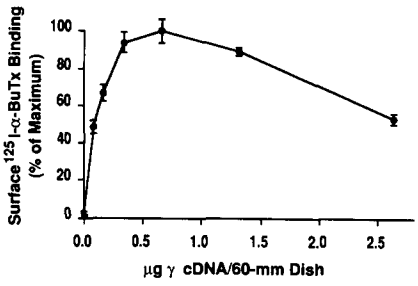

Figure 7. Inhibition of surface AChR expression by increased γ cDNA. COS cells were transfected with the varied amount of γ cDNA as indicated along with the normal amounts of α, β subunit cDNAs and half the normal amount of δ cDNA were assayed for surface ^{125}I-α-BuTx binding. The data were normalized to total protein and were expressed as percentages of maximal expression. Each value is the mean ± SEM of three determinations.

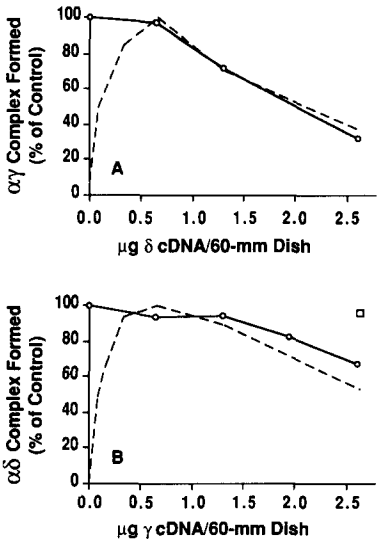

Figure 8. Inhibition of heterodimer formation by γ or δ subunits. (*A*) Inhibition of αγ dimer formation by δ subunit. COS cells in 60-mm dishes were transfected with α and γ subunit cDNAs together with the indicated amounts of δ cDNA. Cell lysates were then incubated with ^{125}I-α-BuTx and immunoprecipitated with anti-γ 485 antibodies (*open circles*). The data is expressed as percentages of precipitation in the absence of δ cDNA. Each point is the average of two determinations except the point at δ cDNA concentration of 0.65 μg, which was a single point determination. The dashed line represents surface AChR expression under similar conditions when β cDNA was also included in the transfection and was reproduced from Fig. 5. (*B*) Inhibition of αδ dimer formation by γ subunit cDNA. COS cells in 60-mm dishes were transfected with the normal amount of α (1.3 μg) and a reduced amount of δ (0.13 μg) subunit cDNAs together with the indicated amounts of γ cDNA. Cell lysates were then incubated with ^{125}I-α-BuTx and immunoprecipitated with mAb 88B coupled to Sepharose (*open circles*). The effect of β cDNA at the indicated concentration was also shown for comparison (*open square*). The data is expressed as percentages of precipitation in the absence of γ or β cDNA. Each point is the average of two determinations. The dashed line represents surface AChR expression under similar conditions when β cDNA (0.65 μg) was also included in the transfection and was reproduced from Fig. 7.

range for this inhibition was similar to that seen for inhibition of surface AChR expression by high concentrations of γ cDNA.

Discussion

We have used a transient transfection system in a mammalian cell line to investigate the mechanisms of assembly and transport to the surface of the nicotinic muscle AChR. Previous investigations of these processes have used muscle cell lines (reviewed in Carlin and Merlie, 1987), permanently transfected cell lines (Blount and Merlie, 1988, 1989; Blount et al., 1990) and expression in *Xenopus* oocytes (Kurosaki et al., 1987; Sumikawa and Miledi, 1989). The COS cell system that we have used offers the advantage of a mammalian cell in which the number and relative proportions of each of the subunits of the AChR can be easily varied. By using subunit-specific antibodies in immunoprecipitation experiments, we have investigated the interactions of subunits under conditions in which the intact AChR is not formed.

The Acquisition of Toxin Binding by the α Subunit

After peptide chain synthesis and glycosylation, the α subunit undergoes a folding reaction that confers upon it the ability to bind α-BuTx. In muscle cells, ~30% of the α subunit is converted to the toxin-binding form and assembled into the intact AChR. The remainder is degraded (Merlie and Lindstrom, 1983; Gu et al., 1989*a*). Conversion occurs before assembly with other subunits (Merlie et al., 1982; Merlie and Lindstrom, 1983), and does not require their presence, as it occurs in *Xenopus* oocytes or in permanently transfected fibroblasts that express only the α subunit (Kurosaki et al., 1987; Blount and Merlie, 1988). In agreement with these results, we found that a small proportion of the α subunit expressed in the absence of other subunits in COS cells acquired toxin-binding activity. The reason for the low rate of conversion to the toxin-binding form in COS cells is not known. Perhaps accessory proteins are needed for this conversion (Rothman, 1989) that in COS cells exist in lower amounts or bind poorly to the mouse α subunit.

Although other subunits are not required for conversion of the α subunit to the toxin-binding form, the proportion of α in the toxin-binding form can be increased by coexpression with γ or δ subunits. The largest increase, however, is seen when all four subunits are expressed together (Fig. 1 *B*). Blount et al. (1990) have observed in transfected fibroblasts that the association with the δ subunit decreases the degradation rate of the α subunit. The toxin-binding form of the α subunit may be similarly stabilized by association with other subunits in our experiments. Alternatively, heterodimer formation may promote the formation of the toxin-binding form of α.

Although we have no direct evidence, it seems likely that the other subunits also undergo conformation changes before assembly, and that this process is likewise inefficient in COS cells. Only a small proportion of the β, γ, or δ subunits become associated with the toxin-binding form of α, even under conditions in which α is in large excess (Gu, Y., and Z. W. Hall, unpublished experiments).

Association of the α Subunit with Other Subunits

When AChR subunits are expressed in COS cells, they associate nonspecifically with each other and with other proteins as determined by coprecipitation experiments (Gu, Y., J. R. Forsayeth, and Z. W. Hall, unpublished experiments). These complexes, which also appear to be formed in an in vitro translation system (Chavez, R., and Z. H. Hall, unpublished experiments), may resemble those seen in other systems when proteins are misfolded, or when incomplete combinations of protein subunits are expressed (Rose and Doms, 1988; Hurtley and Helenius, 1989). Specific binding was seen only when association with the toxin-binding form of the α subunit was measured (Fig. 2).

Our experiments show that coexpression of α with either γ or δ subunit results in the formation of a complex that is detectable by immunoprecipitation of toxin-binding activity with either γ or δ antibody, respectively. Sucrose gradient sedimentation demonstrates a major peak of toxin-binding activity at ~6 S, consistent with the formation of a heterodimer. Cotransfection of α and β cDNAs, in contrast, did not result in association between β and the toxin-binding form of the α subunit. The failure of the β subunit to associate is

evidence for the specificity of the association of γ and δ subunits with the toxin-binding form of α.

The β subunit does associate with $\alpha\delta$ and $\alpha\gamma$ heterodimers, however, to form what are probably heterotrimers (Figs. 2 and 3). The uncertainty in the order of the subunits in the intact oligomer (Fig. 9) makes speculation about the exact structure of these oligomers difficult. According to either model, however, β must associate with the $\alpha\gamma$ dimer through the α subunit. The capacity of α to interact with β is thus not an intrinsic property of the subunit, but is induced by its association with γ. This induction may be related to the changes in the ligand-binding site of α subunit that is induced by its association with γ (Blount and Merlie, 1989). A similar induction presumably underlies the interaction of β with the $\alpha\delta$ heterodimer, but in this case the uncertainty in subunit order makes it difficult to know whether β interacts with the heterodimer through α or through δ.

When α, γ, and δ are expressed in the absence of β, the two heterodimers apparently do not associate. Thus neither antibodies to γ or δ subunits immunoprecipitated more toxin-binding sites when all three subunits were present together than when either γ or δ, respectively, were coexpressed without α. Since the two heterodimers occupy adjacent positions in the intact oligomer, the subunit-subunit interactions that join them must also be induced, in this case by the β subunit.

The results on the formation of the heterodimers and heterotrimers that we have obtained in COS cells which transiently express subunits of the AChR are generally similar to those obtained in quail fibroblasts permanently transfected with subunits of the mouse muscle AChR (Blount and Merlie, 1989; Blount et al., 1990), and in *Xenopus* oocytes injected with *Torpedo* AChR subunit mRNA (Kurosaki et al., 1987; Sumikawa and Miledi, 1989; Saedi et al., 1991). In each case, γ and δ, but not β, form heterodimers with the AChR, and β can add to the heterodimers to form a heterotrimer. The specific associations observed must reflect characteristics of the subunits themselves and are likely to represent steps in a pathway of AChR assembly that is common to all of these cells.

Competition between γ and δ Subunits

A novel aspect of our observations is the apparent competition between γ and δ subunits during AChR assembly. The observed inhibition of AChR assembly at high ratios of δ to γ subunit is not due to nonspecific saturation of the expression system, since the inhibition can be relieved by further addition of γ subunit cDNA. The inhibition of assembly by an excess of either γ or δ subunits is apparently due to inhibition of heterodimer formation by the other subunits, which occurs over the same concentration range as inhibition of surface expression of the AChR. These experiments support the role of heterodimer intermediates in assembly of the AChR. The molecular mechanism of the inhibition is unclear. One possible hypothesis is that the γ and δ subunits compete with each other for the α subunit; the inability of the α subunit cDNA to overcome the inhibition by δ cDNA suggests that this is not the case. The two subunits could also compete for a protein that facilitates folding. Both competition data and the immunoprecipitation experiments indicate that the $\alpha\delta$ heterodimer forms more efficiently or is more stable than the $\alpha\gamma$ heterodimer. The balance in γ and δ subunit expression required for efficient AChR assembly underlines the importance of coordinate regulation of these subunits in cells that express the receptor.

Transport to the Surface

In COS cells in which all four subunits are expressed, approximately half of the toxin-binding activity associated with the assembled AChR is on the surface (Gu et al., 1990). When one or more subunits is absent, little or no toxin-binding activity is found on the surface, in all cases but one. In many cases, partial assembly occurs, but the partial oligomers are not transported to the surface. These results are similar to those found with the T cell receptor, in which omission of one subunit blocks the transport of other subunits to the cell surface (Bonifacino et al., 1989). The partially assembled AChR is presumably located in the endoplasmic reticulum, since AChR assembly occurs there (Smith et al., 1987; Gu et al., 1989*b*). In the one case in which detectable toxin-binding activity is expressed on the surface ($\alpha\beta\gamma$), sucrose gradient sedimentation indicated that the toxin-binding activity is associated with a heterogeneous mixture with a predominant peak at ~5 S (Forsayeth, J. R., Y. Gu, and Z. W. Hall, unpublished experiments). Whether this represents transport of an incompletely assembled AChR, or whether an unstable pentameric species is transported to the surface and then dissociates is not known.

Several laboratories have described the surface expression in *Xenopus* oocytes of AChRs made with incomplete combination of subunits (Mishina et al., 1984; White et al., 1985; Kurosaki et al., 1987; Kullberg et al., 1990). The discrepancy between these results and those reported here may reflect the greater sensitivity of physiological methods, or may result from a higher stringency of regulation of surface expression in COS cells.

AChR Assembly

To produce a correctly assembled AChR in which each subunit is present in the proper stoichiometry and position clearly requires specific recognition between subunits. In principle, this could be accomplished by having a specific recognition site built into the primary or secondary structure of each subunit. In the AChR, however, this is not a viable solution because the α subunit, which is represented twice, has two sets of neighbors. The subunit between the two α subunits (either β or γ, depending on the model; Fig. 9), must be recognized as the correct partner by each α, but on opposite sides. If this information were in the original α subunit, then there is the possibility of a repeating heterooligomer, i.e., $\alpha\beta\alpha\beta$ . . . In the assembly pathway of the AChR, this problem is evidently solved by having two types of interactions between subunits: unconditional interactions that do not depend on the presence of other subunits; and conditional interactions that do. For example, the interaction of α and δ, or of α and γ, subunits, are unconditional. The interaction of β subunit with α, however, is a conditional reaction. Thus, α does not form a specific complex with β except in the presence of either γ or δ subunit. Whether the interactions between γ and δ, or between β and either γ or δ are conditional or nonconditional is less certain because specific associations between these subunits cannot be deter-

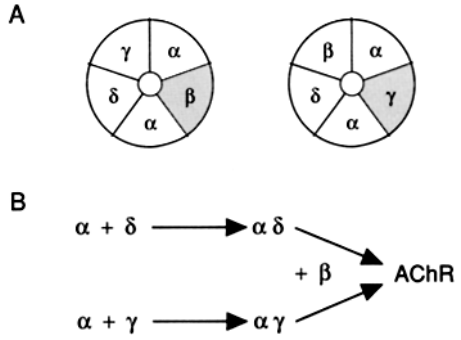

Figure 9. (*A*) Two schemes for the arrangement of subunits in the AChR. The two arrangements differ in the assignment of either the β or γ subunit to a position between the two α subunits. (*B*) Postulated pathway of AChR assembly. The γ and δ subunits compete for available α subunit. The two heterodimers bind β subunit. Some combination of heterodimers and opposing heterotrimers interact to produce complete AChR.

mined. As discussed above, most of each of the subunits in COS cells is probably incorrectly folded and, in that form, nonspecifically associated with other subunits and other proteins. Only in the case of interactions with α, in which the correctly folded form can be recognized by its ability to bind a-BuTx, can we detect specific associations. Also, because of uncertainty about the order of the subunits in the oligomeric AChR, we cannot specify the precise interactions in the heterotrimers (i.e., in $\alpha\delta\beta$, the β subunit could be bound through either the α or the δ subunits). Other experiments in our laboratory suggest that the extracellular NH_2-terminal domain may be important in determining the specificity and efficiency of the interactions between subunits (Gu et al., 1991; Yu and Hall, 1991; Verrall, S., and Z. W. Hall, manuscript in preparation).

Because some subunit-subunit interactions are conditional, the AChR must follow a defined pathway of assembly, in which at least some of the steps of assembly are ordered. Our experiments, and those of others (Blount and Merlie, 1989; Blount et al., 1990) suggest that the first step in AChR assembly is the formation of the two heterodimers, $\alpha\delta$ and $\alpha\gamma$. The β subunit is then added to one or both heterodimers. This scheme is consistent with observations on heterodimer assembly seen in all systems that have been studied, and on the mutual antagonism between γ and δ subunits that we have observed in COS cells. Several possibilities exist for the final step of assembly. Either one or the other heterotrimer could associate with the complementary heterodimer, or the two heterotrimers could associate together with the expulsion of one β subunit (Fig. 9 *B*). On the basis of the data now available, we are unable to choose between these alternatives.

Because other ligand-gated ion channels are oligomeric and have subunits whose overall structure and sequence are homologous to those of the nicotinic muscle AChR, the general principles of assembly of these proteins are likely to be similar to those seen here. Studies on the assembly of the muscle AChR may thus be a guide for further investigations of other receptors.

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