Title
Studies on the molecular mechanisms of acetylcholine receptor assembly

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Publication Date
1993

Peer reviewed|Thesis/dissertation

Studies on the Molecular Mechanism of Acetylcholine Receptor Assembly

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Physiology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

For my lovely and handsome son, Christopher H. Gu

For my husband, Yong Gu and parents

Acknowledgement

I would like to take this opportunity to address my acknowledgments to the people who were helpful to me through the years of graduate school. Without them, I could not possibly have finished the degree.

I would like to express my gratitude to my adviser, Dr. Zach W. Hall for his persistent encouragement, advice and support. His help focused not only in science, but also extended to many aspects including my written English. His contributions with his skillful, splendid English to improvement of my language are countless. Over the years of graduate school, Zach left too numerous to enumerate footprints on me of his elegance, kindness, politeness, sharpness, smartness and considerateness.

I owe thanks to my thesis committee members, Dr. Vishwanath R. Lingappa, Dr. Juan I. Korenbrot, for their persistent cooperations and support ever since I started to organize my qualifying examination, and Dr. David J. Julius, for tolerating me to give him a month to finish up all the duties.

I am greatly indebted to Dr. Barry Gumbiner for his advice and support. He patiently taught me a lot when I was rotating in his lab.

I thank the colleagues in the Hall lab for their always critical comments which make me grow fast.

I thank Dr. Yong Gu, my husband for introducing me into the door of AChR assembly and for sharing the receipts of the most techniques I used in the thesis. I also thank him for his understanding and for being patient of having a wife struggling for Ph.D degree over the years.

I thank my parents, Lu-Ze Yu and Jin-Di Xie for their understanding and support. I especially appreciate my mother, Jin-Di Xie for taking over my early motherhood duties, which helped me tremendously.

I thank my lovely son, Christopher H. Gu for his patience. His words "Mommy, you are too slow, I have been ready to go for a long time." have energized me to hurry up all the way through graduate study.

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Xiao-Mei Yu

Abstract

The acetylcholine receptor (AChR), a ligand-gated ion channel, is an oligomeric membrane protein whose composition is $\alpha_2\beta\delta\gamma$ or $\alpha_2\beta\delta\varepsilon$, in the embryonic or adult stage, respectively, of muscle development. The AChR is assembled by a defined pathway: α associates with δ or γ/ε to form heterodimers as the first step of AChR assembly; then the β subunit associates with $\alpha\delta$ or $\alpha\gamma/\alpha\varepsilon$ to form a pentamer. More than about 200 constructs including chimeric, truncated and point-mutated subunits were generated from four different AChR subunits to investigate the sequences mediating either the early or late step of AChR assembly and the sequences mediating interaction with the 43 kD protein.

1. The N- and C-termini of the δ and ε subunits carry all of the subunit-specific information required for assembly of the AChR.

2. For the α subunit, the N-terminal domain appears to mediate the subunit-specific interactions involved in heterodimer and heterotrimer formation. A region of the long cytoplasmic loop and the C-terminal domain seem to participate a late step of assembly.

3. For the β subunit, both the N-terminal domain and the main cytoplasmic loop seem to be important in AChR assembly. The short C-terminal domain of the β subunit is surely dispensable.

4. The C-termini of the α, δ, γ and ε subunits are likely to be related in a late step of assembly as the C-terminus-truncated α, δ and ε subunits can form heterodimers with the α.

5. To explore the molecular mechanism of 43 kD protein-induced AChR clustering, immunocytochemical staining and coexpression of the AChR or AChR incorporating the mutated subunits and 43 kD protein were established. Having studied the role of the each cytoplasmic loop of the AChR subunits by systematic mutagenesis, we found that regions near M4 of the long cytoplasmic loops of both the α and β subunits may be of importance for association with the 43 kD protein. The AChR may provide alternative multiple binding sites to interact with the 43 kD protein directly or indirectly

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Chapter One

General Introduction

Neuromuscular synapse

Neurons communicate with muscles and other neurons mostly by chemical synapses. The synapse consists of a specialized presynaptic membrane, cleft and postsynaptic membrane. In response to an action potential the presynaptic neuron releases neurotransmitters stored in vesicles into the synaptic cleft by a process of exocytosis (Hall et al., 1992). The released transmitters then act on their receptors in the postsynaptic membrane.

The neuron-muscle synapse, called the neuromuscular junction, is a structurally specialized contact that is one of the best-studied and well-understood synapses. The neuromuscular junction consists not only of a neuron and muscle cell, but also a Schwann cell. Each motor neuron branches to innervate hundreds of muscle fibers. At the muscle, the nerve loses its myelin cover and makes a spray of boutons that rest on the muscle fiber surface. The muscle provides the specialized postsynaptic spot which includes the gutter, the junctional folds, which are precisely aligned with the presynaptic active zones, membrane thickenings at the top of the folds which function as the postsynaptic chemoreceptive surface, and clusters of nuclei that lie beneath the postsynaptic membrane (Hall & Sanes, 1993; Sanes et al., 1991; Fontaine et al., 1988). The neurotransmitter mediating the neuromuscular junction transmission is acetylcholine. It acts on a protein in the postsynaptic membrane called the acetylcholine receptor (AChR).

The AChRs are localized on the crests of the folds, not in the troughs, of the postsynaptic membrane. In addition to AChRs, the postsynaptic membrane also contains voltage-sensitive sodium channels (Flucher & Daniels, 1989), β1 integrins (Bozyczko et al., 1989), N-CAM (Covault & Sanes, 1986) and cytoskeletal proteins such as 43 kD protein (Froehner et al., 1981), β-spectrin (Bloch & Morrow, 1989), actin (Hall et al., 1981), vinculin (Bloch & Hall, 1983), dystrophin and dystrophin-related protein (Bewick et al., 1992; Ohlendieck et al., 1991). How these proteins are assembled underneath the nerve at the synaptic site is a major problem in developmental neurobiology. Addressing this may partially depend on understanding AChR clustering.

Structure and function of the AChR

The AChR consists of four different subunits with stoichiometry of $\alpha 2\beta\delta\gamma$ or $\alpha 2\beta\delta\varepsilon$. The receptor binds two molecules of ACh and opens a cation-selective channel. By doing so, it mediates neuromuscular transmission, and plays a significant role in synaptogenesis and even in autoimmune disease (Claudio, 1989).

The large quantity of AChRs existing in Torpedo and an antagonist α-bungarotoxin with an almost irreversible binding constant (10^{-11} M) isolated from elapid snake (Lee, 1972), facilitated studies on the AChR and have made it the best understood member of a superfamily of ligand-gated ion channels which includes the neuronal AChR, serotonin receptor ($5HT_3$), glutamate receptor and the glycine and γ-aminobutyric acid ($GABA_A$) receptors. They

function at synapses by rapidly altering the potential of the postsynaptic membrane in response to their neurotransmitters. The muscle AChR, neuronal AChR, serotonin receptor ($5HT_3$), and glutamate receptor are cation selective ion channels at excitatory synapses, whereas the glycine and γ-aminobutyric acid ($GABA_A$) receptors open chloride channels at inhibitory synapses upon binding the inhibitory neurotransmitters, glycine and GABA. Unlike voltage-gated ion channels, ligand-gated ion channels are not strongly selective for a particular ion. In contrast to gap junction channels or porins which are almost completely nonselective, they are either cation- or anion-selective. Despite their different selectivity, at the level of amino acid sequence, the subunits of the AChR, the neuronal AChR, the serotonin receptor (5-HT3), $GABA_A$ receptor and glycine receptor are all homologous (Unwin, 1993). The subunits of the glutamate-gated channels have less overall homology with these and have been sorted to a separate family.

The structural composition of all the members in both families have been proposed as a pentamer resembling the composition of the AChR. The studies of the molecular mechanism of AChR assembly may thus shed light on the assembly of all family members. In this thesis, I have used the AChR as an example to study assembly and structure-function -relationship of oligomeric membrane proteins.

The structural features of the AChR have been examined using electron images of stained molecules (Ross et al., 1977; Zingsheim et al., 1982b), neutron scattering (Wise et al., 1979; Ross et al., 1977), X-

ray diffraction (Ross et al., 1977), and metal replicas of freeze-fractured AChRs (Zingsheim et al., 1982b; Heuser & Salpeter, 1979). The five subunits of the AChR are arranged around a central pore demonstrated by three-dimensional electron image analysis (Brisson & Unwin, 1985). The AChR, at a resolution of 2.5 nm, is about 12 nm in length with approximately 5.5 nm on the extracellular surface and about 2 nm on the cytoplasmic side. The outside diameter of the molecule is about 8 nm. A central aquous pore extends the whole length of the molecule and has a diameter of about 2.5 nm on the extracellular side but is constricted to about 0.6 nm at or within the lipid bilayer (Brisson and Unwin, 1985; Toyoshima & Unwin, 1990; Unwin, 1993). A recent study demonstrated further the structure of the acetylcholine receptor at 9 Å resolution and described M2, the second transmembrane domain, as the only transmembrane domain forming a α-helix whereas the M1, M3 and M4 appear to be β-sheets in the secondary structure of each subunit (Unwin, 1993).

The AChR interacts with three major pharmacological classes of ligands: agonists, competitive antagonists and non-competitive antagonists. The competitive antagonists are subdivided into two classes: those that are readily reversible (dimethyl-d-tubocurarine, hexamethonium) and those that are slowly reversible (the curaremimetic toxins). Both the agonists and competitive antagonists bind to ACh binding sites on the α subunits of the AChR. Binding of agonists causes channel opening whereas binding of antagonists prevents the AChR from opening. Non-competitive antagonists or inhibitors block the AChR response to ligands in a variety of ways

including binding to the open channel and sterically blocking its closure while preventing the passage of ions, or stabilizing the closed (resting) state, or promoting desensitization (Claudio et al., 1987). Binding sites of non-competitive antagonists have been localized on α (Blanchard & Raftery, 1979), δ (Oswald & Changeux, 1981; Hucho et al., 1986; Giraudat et al., 1986), α and β (Kaldany & Karlin, 1983), β and δ (Muhn & Hucho, 1983) or all four subunits (Lauffer et al., 1979).

The Torpedo AChR, exists a monomer (9s) and dimer (13s) without any differences in functional properties. It has a monomeric molecular weight of 250 kD. In denaturing gels, the α subunit migrates with apparent molecular mass of 40 kD, the β 50 kD, the γ 60 kD and the δ 65 kD (Changeux, 1981; Conti-Tronconi et al., 1982; Barrantes, 1983; Popot & Changeux, 1984; Dolly & Barnard, 1984; Claudio et al., 1987). Partial amino acid sequences provided keys to design degenerate oligonucleotides to screen a Torpedo electric organ cDNA library, resulting in the cloning of all four subunits (Claudio et al., 1987; Sumikawa et al., 1982; Noda et al., 1982; Noda et al., 1983c; Noda et al., 1983b). A novel γ-like subunit, the ε subunit, was retrieved from a fetal bovine library (Mishina et al., 1986). A comparison of a single subunit in different species shows a large degree of sequence conservation. Sequences among the four subunits in a species are also highly homologous. The most highly conserved regions in each subunit are putative transmembrane domains, indicating that they perform a fundamental function shared for all species.

All subunits (α, β, δ, γ and ε) have their own genes, several of which have been mapped to different chromosomes. These include: human α (Noda et al., 1983a); (Numa et al., 1983) and γ (Shibahara et al., 1985); Topedo δ (Hershey et al., 1983); Chicken α (Klarsfeld et al., 1987; Gardner et al., 1987); γ and δ (Nef et al., 1984); rat γ and ε (Witzemann et al., 1987); and mouse γ (Gardner et al., 1987) and δ (Crowder & Merlie, 1986; Evans et al., 1987). Mouse α gene has been mapped on chromosome 17, mouse β on 11, mouse γ and δ on 1 (Heidmann et al., 1986). Their gene structures and intron-exon patterns are very similar, suggesting that they come from the same ancestral gene. The mRNAs transcribed from these genes give rise to polypeptides containing a 18-23 amino acid signal peptide which is cotranslationally cleaved (Mishina, 1986). All subunits are glycoproteins that are cotranslationally glycosylated on the N-terminal domain (Nomoto et al., 1986; Gu & Hall, 1988a). The same overall membrane topology is shared by all the subunits: a long N-terminal domain, followed by four transmembrane domains and a short C-terminal domain. Both the N- and C-terminal domains are located on the extracellular side of the cell. A short loop between M1 and M2 as well as a long loop between M3 and M4 resided on the intracellular side of the membrane (Changeux, 1981; Conti-Tronconi et al., 1982; Mishina et al., 1984; Chavez & Hall, 1991; Chavez & Hall, 1992).

Potential asparagine-linked glycosylation sites in the N-terminal domain of AChR subunits have been identified: the α and β subunits

contain one potential N-linked glycosylation site, the γ contains four and the δ three. Studies, using lectin binding and endoglycosidase F or H digestion, have revealed that the α, β, γ and δ subunits contain one, one, two and three units of asparagine-linked oligosaccharide, respectively (Nomoto et al., 1986; Gu and Hall, 1988a).

Phosphorylation sites in the cytoplasmic domains of the AChR subunits have been identified. PKA, PKC and tyrosine kinase dependent phosphorylation in their cytoplasmic domains have been shown to regulate channel properties (Huganir & Miles, 1989).

Function of individual AChR subunits

α-BTX binds to the α subunit of the AChR. The toxin-binding site has been localized between α 170 and α 200 by using α–BTX to probe either synthetic peptides that contain the primary sequence of various regions of the α subunit (Neumann et al., 1986; Wilson et al., 1988; Gershoni, 1987) or proteolytic fragments from the purified α subunit (Pedersen et al., 1986). A cysteine pair at position αC192-αC193 that is unique to the α subunit forms a disulfide bond (Kao & Karlin, 1986). Newly synthesized α subunits do not have α–BTX-binding activity and undergo a conformational change including disulfide bond formation as well as glycosylation to acquire toxin binding activity (Blount & Merlie, 1988). This entire process takes place in the ER within 15 min (Blount and Merlie, 1988; Blount et al., 1990) after synthesis and confers upon the α subunit an ability to associate with the δ or γ/ε to form a heterodimer. The α subunit alone lacks binding affinity for small ligands such as d-tubocurarine and

carbamylcholine. The association of the α subunit with the γ subunit creates a high-affinity binding site for d-tubocurarine and with the δ subunit a low-affinity binding site (Blount & Merlie, 1989; Pedersen & J.B., 1990). Their binding affinities are the same as those in the complete AChR. These results suggest that the small ligand binding sites are co-formed by the α along with the γ or δ subunits. The αγ and αδ heterodimers provide the molecular basis for non-equivalence of ligand-binding affinity between the two α subunits (Dowding & Hall, 1987; Neubig & Cohen, 1979; Sine & Taylor, 1981).

The function of the other AChR subunits (β, γ/ε and δ) is unknown. The γ or ε subunit expressed in different stages of muscle development has been proposed to be involved in regulating the channel properties (Mishina et al., 1986). The δ subunit has been shown to play a role in determining the conductance property of the AChR (Imoto et al., 1986) and in regulating AChR desensitization as well as small ligand-binding (Unwin et al., 1988).

Developmental changes of the AChR

During the development of the neuromuscular junction, the AChR changes not only in amount and distribution, but also in several of its properties. In the rodent, just before birth, the AChRs at the endplate decrease their metabolic turnover by about 10-fold (Chang & Huang, 1975; Berg & Hall, 1975b); the AChR clusters grow more condensed (Steinbach, 1981) and become resistant to dispersion by collagenase or calcium chelators (Bloch & Steinbach, 1981).

The AChRs at newly formed synapses, as well as those in the extrajunctional membrane have a half-life of 10-20 hours. Several days after clustering, AChRs at the synapse increase their turnover time to about 10 days (Berg and Hall, 1975b; Reiness & Weinberg, 1981; Steinbach, 1981). When adult muscle is denervated, the half life of the AChRs is decreased. Electrophysiological recording experiments showed that the embryonic AChR has a smaller single-channel conductance and a longer opening time than the adult AChR (Schuetze & Role, 1987; Brehm & Henderson, 1988).

The molecular basis for the change of the AChR properties turns out to be the change in subunit composition: synaptic AChRs containing γ subunit (the embryonic form; $\alpha_2\beta\delta\gamma$) are replaced by AChRs containing a homologous ε subunit (the adult form; $\alpha2\beta\delta\varepsilon$) (Mishina et al., 1986; Gu & Hall, 1988b). The biological significance of this transition could be that γ-AChRs are more effective in depolarizing the smaller muscle fibers of embryonic muscles than are ε-AChRs (Jaramillo et al., 1988).

Two models of subunit configuration in the AChR

The two α subunits have been shown not to lie next to each other (Zingsheim et al., 1982a; Karlin et al., 1987; Hamilton et al., 1985) and the δ subunit is not positioned between them. Electron microscopic image of the α subunit in the negative-stained AChR by labeling the α subunit with biotinylated cobra toxin and avidin showed that the avidin molecules were not adjacent (Zingsheim et al., 1982a; Karlin et al., 1987). Using δ–δ cross-linked AChR dimers, a

similar study was performed. The position of avidin molecules relative to the region of two receptor molecules was measured to determine the location of the δ subunits relative to the α subunits. This study demonstrated that the δ subunit is not placed between the two α subunits (Zingsheim et al., 1982a; Karlin et al., 1987). To examine the location of the β subunits, artificially produced β–β cross-linked dimers (disulfide linkage between the two β subunits) were labeled by biotinylated toxin and avidin in the same way as that of the δ–δ AChR dimer. The angles were measured between avidin molecules and the β–β cross-link, indicating that the β was not located between the α subunits (Fairclough et al., 1983). Therefore, these studies indicate that the γ subunit must be located between the α subunits.

In experiments supporting the other model, monoclonal antibodies were used to locate the β subunit between the two α subunits (Tzartos et al., 1981a; Tzartos et al., 1981b). Also, electron image analysis of tubular crystals from Torpedo suggested that the β subunit may lie between the two α subunits (Kubalek et al., 1987). The α subunit was traced with BTX or the monoclonal antibody directed against the extracellular region of the α subunit (Karlin et al., 1987); the β subunit was labeled with the monoclonal antibody directed against its cytoplasmic domain (Merlie & Lindstrom, 1983) and the δ subunit was marked with wheat germ agglutinin which binds specifically to N-acetylglucosamine moieties located exclusively on the δ subunit (Nomoto et al., 1986), and electron micrographs and images were analysed. This result suggested that β subunit was

located between the α subunits. However, if any one of these subunits is tilted in the passages of crossing the membranes, the predication from this study may not be true (Blount and Merlie, 1989).

In the oocyte expression system, the γ subunit appears to be the best subunit to increase toxin binding activity of the α subunit (Kurosaki et al., 1987), and has also been suggested to be located between the two α subunits (Claudio et al., 1987). Another evidence that inhibition of the BTX binding of the αγ and αδ as well as the AChR by small ligands also suggested that the γ subunit is the lone subunit between the two α subunits (Blount and Merlie, 1989). The problem of the configuration of subunits around the channel is still not solved.

Assembly of the AChR

How do the five subunits recognize each other and assemble in a specific order and with the precise positions? Although the subunits are not covalently connected, they are so tightly associated that they cannot be dissociated unless conditions are used that completely denature the polypeptides. Even when individual AChR polypeptides were synthesized in cell-free translation systems, expression of assembled and functional AChRs was not successful (Mendez et al., 1980; Anderson & Blobel, 1981; Chavez et al., 1992). Since reconstitution of purified individual subunits into a functional AChR has not yet been successful and it is impossible to dissociate the

AChR subunits without denaturing the AChR, assembly of the AChR has only been investigated in in vitro expression systems. Three approches have been attempted to determine the function of individual subunits, or partial regions of each subunit in assembly of the AChR: 1. expressing incomplete combinations of fewer than four subunits, the full complement of subunits; 2. expressing hybrid AChRs whose subunits are from different species; 3. constructing chimeric or point-mutated subunits and characterizing them in the presence of the other required normal subunits.

The first successful expression of Torpedo AChRs in Xenopus oocytes was achieved by Sumikawa and his colleagues. They isolated poly $[A]^+$ mRNA from Torpedo electric organ, microinjected it into the oocytes and found that individual subunits were glycosylated and assembled into proper $\alpha 2\beta\delta\gamma$ complexes which were expressed on surface membranes and were able to bind α-BTX (Sumikawa et al., 1981). They also showed that the application of ACh to these AChRs resulted in the opening of a channel whose ionic permeability resembled those of nicotinic AChRs. However, since heterogenous RNA was injected into oocytes, whether the four subunits were sufficient for AChR assembly and function could not be determined.

Numa and his colleagues subsequently solved this puzzle by using cDNA clones and expressing recombinant AChRs in Xenopus oocytes. They found that four subunit cDNAs were sufficient for expressing functional AChRs on the surface (Numa et al., 1983). Furthermore, to determine how subunits were associated, different

combinations of mRNAs of Torpedo AChR subunits were injected into the oocytes and electrical recording as well as α–BTX binding were conducted to characterize expressed toxin-binding complexes (Mishina et al., 1984; Kurosaki et al., 1987). Although toxin-binding was detected, the whole cell current in response to bath-applied ACh showed that the combination of αβγ gave 5 % of normal activity seen in the combination of the four subunits, of αβδ 3%, α alone 0.7%, and of αγδ 0.08% (Mishina et al., 1984). In a later more detailed study, oocyte extracts injected with all combinations of fewer than 4 subunits were used to perform the BuTx binding and results are shown as follows: α 1%; αβ 1%; αγ 26%; αδ 8%; αβγ 69%; αβδ 10%; αγδ 33% (Kurosaki et al., 1987). These data suggested that αγ and αδ formed heterodimers and αβγ and αβδ might form heterotrimers whereas the α-BTX binding of αγδ might be a combination of two heterodimers, αγ and αδ. Moreover, finding that a combination of αβγ generated toxin binding of 36% of normal level on the surface but current response of it is only 10 % of the normal indicates that the δ may play a role in voltage sensitivity of the channel (Kurosaki et al., 1987; White, 1987). Whether these data are useful to determine configuration of subunits around the channel and assembly pathway is still questionable. Subsequent permanent and transient transfectants in fibroblast (Blount et al., 1990), muscle (Blount et al., 1990), Xenopus oocytes (Saedi et al., 1991) and COS cells (Gu et al., 1991b) give a much clear answer to the assembly pathway.

Two lines of primary evidence on the hybrid AChRs suggested that the subunits from different species can cross-assemble into the

functional channel. When the AChRs of mouse δ plus Torpedo αβγ subunits were injected into oocytes, the functional AChR was assembled and showed a 3- to 4-fold greater response to ACh than all-Torpedo AChRs (White et al., 1985). AChR composed of Torpedo αβγ and calf δ had channel open time similar to those of all-calf AChRs (Sakmann et al., 1985), suggesting that δ subunit was responsible for determining the open time of the channel. However, the result of the AChR with reciprocal combination (calf αβγ plus Torpedo δ) has not yet been reported.

Moreover, a region in δ subunit determining ion transport through the AChR was localized by constructing a series of 11 chimeric δ subunits between Torpedo and calf sequence and expressing them with Torpedo αβγ subunits. One δ chimera composed of approximately 500 amino acids of Torpedo and only 29 amino acids of calf produced a calf-like conductance. In constrast, another δ chimera contained only 36 amino acids from Torpedo produced a Torpedo-like conductance. Both sequences responsible for generating the conductance changes are composed of the M2 region plus the bend region between M2 and M3. Examination of these sequences in Torpedo and calf indicated that the M2 domains between both species were very similar. The major difference in the bend region between M2 and M3 was the existence of two positively charged residues in calf compared with one positively charged and one negatively charged residues in Torpedo. This study is consistent with the hypothesis that M2 regions form a channel wall and the M2-M3 bend region is located at the mouth of the channel.

A further study by Imoto (1988) described a large number of point mutations made in each of the four Torpedo AChR subunits. Both outward and inward currents could be affected by primarily changing the three clusters of negatively charged amino acids and glutamine residues: two sites between M1 and M2 and one site between M2 and M3. This study indicates that the three clusters of negatively charged residues form three anionic rings around the external and internal mouths of the channel and determine the AChR channel conductance and ion specificity. In the mammalian muscle AChR, two ends of M2 segment of ε-subunit contains two more net negative charges than the segment in the γ-subunit, one in each of extracellular and intracellular rings. This may account for the higher channel conductance of the ε-AChR than that of γ-AChR. The GABA receptor contains preponderantly positive charged residues at the mouths of the pore, creating its Cl^- permeability.

These studies not only revealed cross-assembly ability of the AChR subunits from different species, but also suggested that determinants for assembly and for channel properties are separately localized. These studies also demonstrated that making chimeric proteins and using in vitro mutagenesis and expression system to study structure-function of AChR subunits are feasible.

Two more recent experiments using hybrid AChR subunits indicate that the N-terminal domains of the subunits influence the efficiency of assembly. One study of expressing hybrid receptors

between rat and mouse was carried out in COS cells (Gu et al., 1991a). When cDNAs for rat α, β and δ subunits plus cDNA for mouse ε subunit were transfected into COS cells, the level of surface AChRs were 10 times higher than the level seen with all rat subunits. Correspondingly, coexpression of mouse α, β and δ subunits with rat ε subunit decreased the surface AChRs by 10-fold. This result suggests that ε subunit from mouse is more efficient than rat ε subunit in supporting expression of surface AChRs. Furthermore, the formation of αε heterodimer from rat is also less efficient than that from mouse. By site-directed mutagenesis, the responsible differences were identified at 2 amino acid positions, 106 and 115 of rat and mouse ε subunits.

A similar experiment of expressing hybrid receptors was performed between human and mouse subunits (Chavez et al., 1992). More than two-fold more hybrid receptors were expressed on COS cells incorporating human α subunit with mouse β, δ and ε subunits than the receptors on COS cells transfected with the wild type mouse AChR subunits. Human α expressed alone in COS cells underwent a conformational change faster and acquired α–BTX binding activity more efficiently than did the mouse α subunit. Site-directed mutagenesis experiments found that the difference of efficiency between human and mouse α subunits resulted from two residues in the N-terminal domain of the α subunits. However, hydrid AChR containing mouse α and human β, δ and ε has not yet been expressed.

Two studies suggest that mutation in the glycosylation site of the N-terminal domain of α subunit can regulate assembly of the AChR. In the mouse muscle-like cell line, BC_3H-1 permanently transfected with mutated α subunits which contain mutations of either the 128 and/or 142 cysteine to either serine or alanine, or with deletion of the entire 14 amino acids in this region, AChR expression on the surface was completely abolished, suggesting that a disulfide bond between cysteines 128 and 142 and oligosaccharide addition at asparagine 141 are required for the normal maturation of α subunit (Blount & Merlie, 1990).

Not-surprisingly, a similar result was reported in Xenopus oocytes as well. Mishina et al., using site-directed mutagenesis, destroyed the one potential asparagine-linked glycosylation site in the α subunit by converting N-141 to an asparatic acid residue. When RNA of this mutated α subunit was mixed with wild-type β, γ and δ RNAs injected into Xenopus oocytes, no α–BTX binding activity was detected in solubilized oocytes and no channel activity was seen when ACh applied to the bathing solution (Mishina et al., 1985). Whether the mutated α subunit was unstable or whether the nonglycosylated α subunit was misfolded is unclear in this case.

Taken together, all the evidence suggests that amino acids in the N-terminal domain of the AChR subunits are importance for AChR assembly.

Based on results in four in vitro expression systems, COS cells (Gu et al., 1990); fibroblast cells, BC_3H-1 muscle cells (Claudio et al., 1987; Claudio et al., 1989; Merlie et al., 1983; Blount et al., 1990); and Xenopus oocytes (Saedi et al., 1991), the AChR assembly pathway has been defined as follows: α subunit associates with δ or γ/ε to form heterodimers as intermediates of assembly; then β subunit interacts with both heterodimers, $\alpha\delta$ and $\alpha\varepsilon/\alpha\gamma$ to accomplish a pentamer (Blount et al., 1990; Gu et al., 1991b; Saedi et al., 1991). A recent report also suggested another pathway of AChR assembly (Green & Claudio, 1993) which does not our expression system.

How these steps take place, how the subunits recognize each other, which domains in the individual subunits contain recognition sites to complete assembly of the AChR specifically and precisely, and where the interaction sites of AChR subunits are for associating with the 43 kD protein in 43 kD-induced AChR clustering are my thesis questions. In this thesis, I will describe more than 200 constructs were made on the four different AChR subunits to investigate the molecular mechanism of AChR assembly. Constructed proteins includes chimeric, truncated and point-mutated subunits. These provide structure-function information at the amino acid sequence level about each subunit.

<u>AChR clustering during neuromuscular junction formation</u>

The other interesting question I asked in my thesis is how the AChR is clustered during neuromuscular junction formation. AChR clustering is one of the earliest events during neuromuscular synapse

formation, occurring within hours after the contact between the growing nerve and the muscle fiber (Bevan & Steinbach, 1977; Steinbach, 1981). Mononucleated myoblasts express little if any AChRs on their surfaces. Soon after fusion of the mononucleated myoblasts to multinucleated myotubes, AChRs appear throughout the membrane (Steinbach, 1981). When the growth cone of an ingrowing motor axon approaches a myotube, AChR clusters form near the contact site within hours (Steinbach, 1981). Then, the amount, distribution, and the metabolic and channel properties of the AChR exhibit changes during further development. The AChR clusters appear and become restricted to the postsynaptic membrane (Bevan and Steinbach, 1977) and the subunit composition of the AChRs changes from $\alpha_2\beta\delta\gamma$ to $\alpha_2\beta\delta\varepsilon$ (Gu and Hall, 1988b). Meanwhile, extrajunctional AChRs diminish; junctional folds appear; the postsynaptic membrane becomes subdivided into receptor-rich crest and receptor-poor troughs; and many other proteins become clustered in the postsynaptic membrane (Bevan and Steinbach, 1977; Hall and Sanes, 1993).

In developing muscle, each postsynaptic site is innervated by several presynaptic terminals. During the second week after birth, all terminals are withdrawn but one neuronal input stays at each postsynaptic site (Redfern, 1970); (Jansen & Fladby, 1990). The synaptic elimination involves activity-dependent, competitive interactions among closely spaced synaptic sites (Goodman & Shatz, 1993; Thompson, 1985; Betz et al., 1980). This retraction is always preceded by the loss of AChR from postsynaptic sites directly

beneath those terminals destined for elimination and the loss may result from diffusion of AChRs away from these sites (Rich & Lichtman, 1989). Thus, studying regulation of AChR clustering is essential not only for understanding neuromuscular junction formation, but also for synapse elimination and remodeling.

Neural control of AChR clustering at the neuromuscular junction

The nerve plays a dominant role in AChR clustering by secreting factors to regulate redistribution of the preexisting AChRs and expression of the newly synthesized AChRs. The factors released by the nerve include agrin (McMahan, 1990), bFGF (Peng et al., 1991), ARIA (Usdin & Fischbach, 1986) and CGRP (Fontaine et al., 1987). Both agrin and bFGF facilitate AChR clustering in the absence of protein synthesis whereas ARIA and CGRP stimulate AChR clustering by increasing the protein level. Some synapse-specific components of basal lamina are involved in maintaining the AChR cluster at the synaptic site (Burden et al., 1979).

Agrin, a basal lamina-associated protein, is synthesized by the motoneurons, released by their nerve terminals, and inserted into the synaptic basal lamina (Magill-Solc & McMahan, 1988; Magill-Solc & McMahan, 1990). It is able to induce AChR clusters in cultured myotubes by redistributing surface membrane AChRs without increasing their number (McMahan, 1990). It also induces accumulations of acetylcholinesterase, lamina, and heparan sulphate proteoglycan (Wallace, 1986; Wallace, 1989). Agrin induces AChR

clustering probably by binding its receptor which somehow generates tyrosine phosphorylation of the AChR (Wallace et al., 1991).

Besides the neurons, muscle, as well as other tissues, also express different forms of agrin (Godfrey, 1991) which differ in their ability to aggregate AChRs. The aggregating activity of agrin is regulated by alternative splicing; its action appears to be affected by proteoglycans or other basal lamina components (Ferns et al., 1992; Ferns & Hall, 1992).

Basic FGF, as well as charged latex beads (Peng & Cheng, 1982), can also induce AChR clustering (Peng et al., 1991). Since FGF- and bead-induced clustering are heparin-inhibitable (Baker et al., 1992), bFGF must bind to heparan sulfate proteoglycans, be sequestered in the basal lamina, then released locally through proteolysis.

CGRP, calcitonin gene-related peptide, is synthesized by motor neurons and transported to motor nerve terminals, where it is stored in dense-core vesicles and released on stimulation (Matteoli et al., 1990; Matteoli et al., 1988). CGRP regulates a cAMP-mediated pathway to increase AChR mRNA and protein level (New & Mudge, 1986; Fontaine et al., 1987; Fontaine et al., 1986).

ARIA, or acetylcholine receptor-inducing activity, is present in brain and spinal cord. How it is synthesized and secreted by motor neurons is still under investigation. Several lines of evidence

demonstrated that ARIA increases levels of AChRs partially by accumulating AChR subunit mRNAs when added to myotube cultures (Usdin and Fischbach, 1986; Falls et al., 1990). ARIA has been cloned and it is a member of the neu ligand family which binds the receptor tyrosine kinase encoded by the neu proto-oncogene (Falls et al., 1993) .

Since CGRP and ARIA are both released by the nerve, AChR subunit synthesis and assembly must occur at substantially higher rates in synaptic sites where the concentration gradients of both would be the highest, than extrasynaptic areas. The evidence indicates that mRNAs encoding the four subunits are highly concentrated near synaptic sites in adult muscle fibers (Merlie & Sanes, 1985; Fontaine et al., 1988; Goldman & Staple, 1989; Brenner et al., 1990). In transgenic mice expressing a reporter gene linked to enhancer and promoter regions of AChR ε and δ subunit genes, the protein encoded by the reporter gene accumulates in or near the nuclei beneath the postsynaptic membrane (Sanes et al., 1991; Simon et al., 1992; Klarsfeld et al., 1991), suggesting that AChR genes are selectively transcribed by synaptic nuclei, and that local synthesis of AChRs contributes to the growth of the synapse as well as to its maintenance.

To enhance transcription in nuclei near synapses, the neural factors apparently alter the nuclei through their receptors and second messenger pathways. Evidence has shown that synaptic nuclei differ from their nonsynaptic counterparts in their

morphology and attachment to the cytoskeleton (Roberts, 1987). Endplate nuclei continue to express AChR ε subunit mRNA selectively many weeks after the nerve has been removed (Brenner et al., 1990). Alternatively, the neural factors induce AChR clustering which may then trigger nuclear changes by causing local changes in the cytoskeleton (Englander & Rubin, 1987) or in calcium concentration (Decker & Dani, 1990).

Extrasynaptic AChRs are lost upon formation of a synaptic cluster of AChRs. After adult muscle is denervated, AChRs (mostly the γ-form) reappear in extrasynaptic membrane but are reduced again when the muscle is reinnervated. The appearance of extrasynaptic receptors after denervation results from new synthesis of AChR subunit mRNAs and proteins (Merlie et al., 1984; Brockes & Hall, 1975) rather than from redistribution of synaptic AChRs; the synaptic cluster remains intact for several weeks after the peak of extrasynaptic level of AChRs is achieved. Nerves exert a suppressive effect on the synthesis of extrasynaptic AChRs via a chemical messenger or electrical activity to regulate activity of transcription factors (Goldman et al., 1988; Klarsfeld & Changeux, 1985; Fambrough, 1979; Burden, 1977; Berg & Hall, 1975a).

<u>The role of 43 kD protein in AChR clustering in vivo and in vitro</u>

AChRs are densely packed at the postsynaptic membrane ($10^4/um^2$) (Fertuck & Salpeter, 1974), with fewer in the extrasynaptic membrane ($<10/um^2$) (Salpeter et al., 1988),

suggesting that the AChR is anchored directly or indirectly to the underlying cytoskeleton and to the overlying basal lamina. The 43 kD protein has been proposed to be the anchor (Froehner, 1991). Therefore, the role of the 43 kD protein in the induction of AChR clustering is particularly interesting.

The AChR and 43 kD protein are colocalized in the postsynaptic membrane of electrocytes (Sealock et al., 1984) and neuromuscular synapses (Froehner, 1984; Froehner et al., 1981) and in AChR clusters on cultured myotubes (Bloch & Froehner, 1986; Burden, 1985; Carr et al., 1989; Peng & Froehner, 1985). The AChR and 43 kD protein are present in 1:1 ratio in electrocytes and cultured myotubes (LaRochelle & Froehner, 1987); (LaRochelle et al., 1990). Removal of peripheral membrane proteins such as the 43 kD protein by alkaline extraction increases the mobility of AChR without affecting the functional properties of AChRs (Barrantes, 1980; Cartaud et al., 1981), suggesting that the proteins removed by the alkaline treatment are involved in anchoring AChRs at postsynaptic sites. Freeze fracture immunoelectron microscopy indicates that the 43 kD protein associates with cytoplasmic domains of the AChR. Chemical crosslinking experiment demonstrated that the β subunit of AChR mediates the association between AChR and 43 kD protein (Burden et al., 1983). But a recent study showed that each AChR subunit has an ability to associate with the 43 kD protein (Maimone & Merlie, 1993). All these results indicate that the 43 kD may play a major role in AChR clustering in synaptogenesis by directly or indirectly interacting with the AChR.

The 43 kD protein has been cloned (Carr et al., 1987; Frail et al., 1987; Frail et al., 1988; Froehner, 1989). Molecular studies on 43 kD protein function have gradually advanced understanding of the mechanism of AChR clustering during synaptogenesis. Functional AChRs expressed on the surface of Xenopus oocytes (Froehner et al., 1990), fibroblast cells (Phillips et al., 1991a) and COS cells (Chapter VI; Yu and Hall, unpublished results; Yoshihara & Hall, 1993) are evenly distributed all over the cell surface. In contrast, when both are coexpressed in those systems, the AChR is colocalized with the 43 kD protein and redistributes itself in a cluster. 43 kD protein seems to act directly in the clustering process since 43 kD protein expressed alone in those systems also forms aggregates. These results suggest that the 43 kD protein directly associates with the AChR in regulation of AChR clustering. However, in muscle cells, the coexpression of these two proteins is not sufficient to induce clustering (Gordon et al., 1993), and 43 kD protein does not cluster in a AChR-deficient muscle cell variant (LaRochelle et al., 1989). Investigation of spontaneous AChR clusters in cultured muscle cells suggests that the 43 kD protein is also linked to the actin cytoskeleton through a sequence involving the 58 kD protein and spectrin (Bloch & Pumplin, 1988; Bloch and Morrow, 1989; Bloch et al., 1991). The clusters seen in non-muscle cells are much smaller than those in muscle cells, suggesting that more proteins are involved in AChR clustering in muscle fibers or that the mechanism of 43 kD-induced AChR clustering in non-muscle cells is simply part of a more complex mechanism used in muscle cells. It is possible that the 43 kD

protein-induced AChR clustering in non-muscle cell lines may use endogenous proteins provided by the recipient cells. A number of proteins associated with the postsynaptic membrane have been identified as cytoskeletal proteins and may be involved in 43 kD protein-induced AChR clustering both in vivo and in vitro.

Comparison of the amino acid sequence of the Torpedo (Carr et al., 1987; Frail et al., 1987), mouse (Frail et al., 1988; Froehner, 1989) and Xenopus 43 kD proteins reveals a number of conserved regions that may be important for cluster formation. The amino terminus of the 43 kD protein is highly conserved with the first 19 residues being almost identical. A myristylation occurs on the N-terminal glycine (Carr et al., 1989; Musil et al., 1988) and may anchor the 43 kD protein to the membrane by insertion into the lipid bilayer (Porter & Froehner, 1985). A potential leucine zipper structure, located at the N-terminal domain, may be of importance for dimerization of the 43 kD protein or for interaction with other proteins. A cysteine-rich region at the C-terminus originally identified as having homology with the regulatory region of protein kinase C (Froehner, 1989) may fold into a zinc finger structure and has been proposed to interact with other proteins of the postsynaptic membrane. The residues surrounding serine 406 provide the consensus sequence for both cyclic AMP-dependent (RRSS) and protein kinase C (SMK)-mediated phosphorylation.

In vitro mutagenesis experiment showed that the conversion of Gly to Ala to eliminate N-terminal myristylation reduced the

association of the 43 kD protein with the plasma membrane, but did not disrupt the aggregation of AChRs on the surface. Both N- and C-termini are required for insertion of the 43 kD protein into the plasma membrane, while the central region of the 43 kD protein is involved in AChR aggregation. The mutated 43 kD protein without the central region associates with the membrane normally, but did not induce AChR clustering (Phillips et al., 1991a).

Chapter Two

The Extracellular Domains of the ε Subunit Mediate Subunit Interactions of the Muscle Acetylcholine Receptor During Assembly.

Introduction

Ligand-gated ion channels, a major class of cell-surface proteins, have a pseudosymmetric structure with five highly homologous subunits arranged around a central ion pore (Unwin, 1989). The correct assembly of each channel, whose subunit composition varies with cell type and stage of development, requires specific recognition between the subunits (Betz, 1990; Gu and Hall, 1988a; Mishina, 1986). Assembly of the pentameric form of the acetylcholine receptor from adult muscle proceeds by a stepwise pathway starting with the formation of the heterodimer, αε and αδ. The heterodimers then associate with the β subunit and with each other to form the complete receptor (Blount and Merlie, 1989; Blount and Merlie, 1990; Gu et al., 1991a; Gu et al., 1991b; Saedi et al., 1991). We have now determined which parts of the subunits mediate the interactions during assembly of the adult form of the receptor from mouse muscle by using a chimeric subunit (ε_β) in which the N- and C-terminal extracellular domains are derived from the ε subunit with the remainder from the β subunit. The ε and β subunits were chosen because the ε subunit forms a heterodimer with the α subunit in the pathway for assembly of the receptor, whereas the β subunit does not. The ε_β chimera substitutes for the ε but not the β subunit in the oligomeric receptor, indicating that the α subunit specifically recognizes extracellular domains of the ε subunit.

Results

The ε_β subunit can support surface AChR assembly and expression

To investigate which domains of the ε subunit contain recognition sites mediating interaction and assembly of the AChR, new restriction sites were introduced into both ε and β subunit cDNAs at the 5' end of M1 (SnaBI) and the 3' end of M4 (SpeI) by oligonucleotide-directed mutagenesis (Fig. 1). These sites were chosen to separate the major extracellular domains at the N- and C-termini of each subunit from the intervening transmembrane and intracellular domains. The segments between the new restriction sites were then exchanged between the two subunit cDNAs by conventional methods to generate two complementary chimeric subunit cDNAs: ε_β, whose termini are derived from the ε subunit; and β_ε, with termini from the β subunit (Fig. 1).

COS cells transfected with a combination of cDNAs for α, β, ε and δ subunits expressed the AChR on their surface (Table 1); this receptor has properties that are similar to those of the AChRs at adult endplates (Gu et al., 1990). When either the β, or ε subunit cDNA was omitted from the transfection mixture, no toxin-binding activity was observed on the cell surface, indicating that acetylcholine receptors were not expressed there (Table 1). We then tested the ability of the chimeric cDNAs to restore surface toxin-binding activity to cells transfected with the deficient mixtures. We found that the cDNA encoding ε_β could substitute for the ε, but not the β, subunit cDNA in the transfection assay. Addition of ε_β cDNA to a transfection mixture containing α, β and δ cDNAs increased the toxin-binding activity to

roughly 30% of that obtained with the complete mixture, whereas ε_β added to a mixture containing α, ε and δ cDNAs had no effect (Table 1). The β_ε subunit cDNA failed to produce surface toxin-binding activity in either case. Chimeras in which only the ε N- or C-terminal domain was substituted into the β subunit also failed to yield surface toxin-binding activity. Because chimeric proteins often fold incorrectly (Rose & Doms, 1988; Hurtley & Helenius, 1989), we could not make conclusions about the specificity of subunit interactions for chimeric subunits that did not give rise to surface toxin-binding activity; we did not investigate these further.

The relative effectiveness of ε_β and ε subunits in promoting expression of toxin-binding activity was compared by adding increasing amounts of each to transfection mixtures containing a fixed amount of α, β and δ cDNAs. Complementary DNAs for the ε_β and ε subunits were equally effective in supporting AChR assembly at low cDNA concentrations, but at higher concentrations the amount of surface toxin-binding activity with ε cDNA was about twice that with ε_β cDNA (Fig. 2).

The toxin-binding complex apparently contains all four subunits. Thus, the expression of toxin-binding activity on the surface of COS cells transfected with α, β, δ and ε_β subunits was reduced by over 85% by the omission of either ε_β, β or δ subunit cDNAs. In addition, the surface toxin-binding complex was immunoprecipitated by subunit-specific antibodies to α, β and δ subunits (data not shown). The antibody to the β subunit (monoclonal antibody 124), which recognizes an epitope on the cytoplasmic loop connecting M3 and M4 (Gullick & Lindstrom, 1983), stained both β and ε_β subunits in

immunoblots of total cell extracts (data not shown), and so recognizes both. Consistent with its being a pentamer, sucrose gradient sedimentation showed that the complex has a sedimentation constant of 9.5S, as does the native acetylcholine receptor (Fig. 3, Table 2).

AChRs incorporating the ε_β subunit have similar properties to those of normal AChRs

Many of the other properties of the toxin-binding complex formed by the ε_β subunit are also similar to those of the acetylcholine receptor. The association rate constant of the toxin-binding reaction for the complex and the metabolic half-life of the complex are not significantly different from those of the ε-containing acetylcholine receptor expressed in COS cells (Table 2). Also, the binding of toxin is inhibited by d-tubocurarine; the complex is even more sensitive to d-tubocurarine than is the native receptor (Table 2). But we were unable to detect acetylcholine-sensitive channels in COS cells expressing the ε_β-containing complex.

Taken together, the properties of the toxin-binding complex formed when ε_β is present suggest that the complex has an oligomeric structure analogous to that of the acetylcholine receptor and that it contains the ε_β subunit instead of the ε subunit. The complex is recognized as a complete receptor by the cell, which transports it to the surface, and degrades it with a normal turnover time. In other experiments, we have shown that incompletely or incorrectly assembled subunits are not transported to the surface of COS cells, but are retained intracellularly (Gu et al., 1991b; J. R. Forsayeth and Z. W. Hall, unpublished experiments).

The ε_β subunit, like the ε subunit, associates with the α subunit to form a heterodimer

To examine further the role of the chimeric ε_β subunit in the pathway of assembly, we investigated whether it forms a heterodimer with the α subunit. COS cells were transfected with α subunit cDNA and either ε, β or ε_β subunit cDNA. Extracts of the transfected cells were then incubated with ^{125}I-labeled α-bungarotoxin and immunoprecipitated either with affinity-purified antibodies for the ε subunit, or with the monoclonal antibody 124 that recognizes the long cytoplasmic loop of the β subunit. Immunoprecipitation of toxin-binding activity was taken as evidence of heterodimer formation. Both ε and ε_β subunits, but not β, formed a heterodimer with α subunit (Fig. 4a, b). Thus, substitution of the N- and C-terminal domains of the β subunit with those of the ε subunit enables it to form a specific complex with the α subunit. As the formation of such a complex is the first step in the pathway of the acetylcholine receptor assembly (Blount and Merlie, 1989; Blount et al., 1990; Gu et al., 1991b), the ability of ε_β to associate with α in the absence of other subunits presumably underlies its ability to be assembled into an oligomeric receptor and subsequently transported to the surface.

Discussion

That the terminal extracellular domains of the ε subunit determine the specificity of subunit interactions suggests that the subunits of the acetylcholine receptor recognize each other by direct

interactions among these domains. Indeed, the relative efficiency of rat and mouse ε subunits in promoting association with α subunits in COS cells is determined by two amino acids in the N-terminal extracellular domain (Gu et al., 1991a). In addition, a chimeric subunit similar to ε_β, but with terminal domains from the δ subunit, also forms a heterodimer with the α subunit and supports the expression of the acetylcholine receptor (see chapter III), replacing δ in the assembly pathway. This suggests that recognition by one or both terminal domains may be a general mechanism involved in the assembly of receptors from subunits (Chapter III).

The subunits of several transmembrane olgomeric proteins with simple structures, such as the haemagglutinin protein and neuraminidase of influenza virus (Doyle et al., 1986; Varghese et al., 1983), the insulin receptor (Johnson et al., 1988) and the Na^+, K^+-ATPase (K. J. Renaud, E. M. Inman and D. M. Fambrough, personal communication), interact through their extracellular domains. By constrast, interactions between two subunits of the T-cell receptor complex are mediated by transmembrane domains (Manolios et al., 1990). In the acetylcholine receptor the hydrophobic domains of the subunits are closely associated to form the ion channel (Unwin, 1989). Our experiments clearly show, however, that the extracellular domains carry out a recognition step that is necessary for assembly of this complex oligomeric protein, a step that could occur before association of the hydrophobic domains to form the channel. Further definition of the extracellular domains involved in recognition may lead to understanding of the mechanisms that determine the identity

and relative position of subunits in different members of the ligand-gated receptor family and in other oligomeric membrane proteins.

Experimental Procedures

Antibodies

Monoclonal antibodies, MAb 61, 124 are generous gifts from Dr. Jon Lindstrom. Anti-ε 360 was generously given by Dr. Yong Gu. The antibodies recognize the long cytoplasmic loops of the α, β and ε subunits, respectively.

cDNAs

Full-length cDNA clones coding for the α, β, γ and δ subunits of mouse muscle nicotinic AChR were gifts from Drs. J. P. Merlie and N. Davidson: α, (Isenberg et al., 1986); β, (Buonanno et al., 1986); γ, (Yu et al., 1986) and δ, (LaPolla et al., 1984). The full-length cDNA for the mouse ε subunit was isolated as described (Gu et al., 1990). Each of the cDNAs were subcloned into the SV40 based expression vector pSM (Brodsky et al., 1990) at the multiple cloning sites. A M13 origin in this vector was used to generate uracil-containing single-stranded DNAs for site-directed mutagenesis (Geisselsoder et al., 1987; Kunkel, 1985).

Site-directed mutagenesis

Site-directed mutagenesis was conducted according to previously described methods (Geisselsoder et al., 1987; Kunkel, 1985), using synthetic oligodeoxyribonucleotides prepared with an automatic DNA synthesizer. Uracil-containing single-stranded DNAs, prepared by

infecting plasmid-transformed E.coli CJ236 (dut-ung-F') cultures with M13 helper phages, were used as the templates. Each of constructed cDNAs was analyzed by restriction enzymes. In some case, plasmids were confirmed by DNA sequencing.

Construction procedures: SnaBI and SpeI restriction sites were introduced into homologous locations in pSMβ and pSMε by oligonucleotide-directed mutagenesis. The responsible oligonucleotides overlapped the wild-type sequence for 10 bases on either side of the last base(s) changed. In pSMβ, the nucleotide sequence TACCTG (encoding the β residues Tyr 225 and Leu 226) was mutagenized to TACGTA (Tyr 225 and Val 226) to introduce the SnaBI restriction site. The changes in the pSMβ nucleotide sequence required for the introduction of the SpeI site did not alter the primary β sequence of Thr 461, Leu 462 and Val 463 (original, ACCCTAGTC; mutant, ACACTAGTC). In pSMε, the SnaBI site was introduced into the nucleotide sequence coding for the ε residues Tyr 222, Val 223 by changing TACGTC to TACGTA without primary sequence alterations, whereas the SpeI site was created by changing the nucleotide sequence TCTACTCTC (corresponding to ε residues Ser 450, Thr 451, Leu 452) into TCACTAGTC (ε residues Ser 450, Leu 451, Val 452). When either the pSMβ or pSMε construct containing the SnaBI and SpeI sites was cotransfected into COS cells with the other acetylcholine receptor subunit cDNAs, wild-type levels of ^{125}I-labeled BTX binding to the surface was detected (data not shown). After the mutated pSMβ and the pSMε cDNAs were digested with SnaBI and SpeI, the resulting 0.7-kilobase (kb) fragments, encoding the region between the start of M1 and the end of M4, were purified

and ligated into the purified vectors from the other construct, containing the N- and C-termini, to generate the desired chimeric subunits.

Transfection

Transfection in COS cells was a modified DEAE-dextran transfection procedure (Seed & Aruffo, 1987), performed as previously described (Gu et al., 1990). Cells at 30-50% confluence in a 60-mm dish were incubated for 3-5 hrs at 37 °C with a transfection mixture consisting of an appropriate amount of plasmid cDNAs added in DME-H21 supplemented with 1.0 % heat-inactivated fetal bovine serum, 0.10 mM chloroquine diphosphate and 0.4 mg/ml DEAE-Dextran. The amount of plasmid used for each subunit in the transfection mixture was determined empirically to produce maximal cell surface AChR expression. For analysis of cell surface AChR expression, 3 ml of the transfection mixture was added to a 60 mm dish containing cDNAs of α: 1.32 ug, β: 0.66 ug, γ or ε: 1.0 ug and δ: 0.26 ug. For immunoprecipitation of heterodimer, 5 ml (3 ml) of the transfection mixture was added to a 100 mm (60 mm) dish containing 2.0 ug (1.0 ug) of each cDNA of the α and the indicated subunits. After 3-4 hrs of incubation, the transfection solution was removed, the cells were treated with 10% DMSO-PBS for 2 min at room temperature and replaced with 5 ml (60 mm dish) or 10 ml (100 mm dish) of the growth medium (10% fetal bovine serum in DME H21 supplemented with 100 u/ml penicillin and streptomycin). After twenty-four hours at 37 °C, the cells were trypsinized and distributed into 3 wells of 24 well plate (surface AChR assay) or into

60 mm dishes (heterodimer immunoprecipitation assay). The surface AChR assay and heterodimer immunoprecipitation analysis were carried out after an additional 24 hours.

Surface AChR Assay

Surface AChR expression was determined by incubating intact transfected cells for 90 min at 37 °C with 10 nM ^{125}I-α-BTX (Amersham Corp., Arlington Heights, IL). Nonspecific binding was measured by addition of more than 100X excess of cold α-BTX to the 10 nM ^{125}I-α-BTX medium, or by sham transfections. Unbound α-BTX was removed by washing the cells with PBS. The amount of bound toxin was measured by solubilizing the cells in 0.1 M NaOH and counting the radioactivity in a gamma counter.

Immunoprecipitation of heterodimers

Immunoprecipitation with subunit-specific antibodies was performed as previously described (Gu and Hall, 1988a). The COS cells transfected with the α and β or ε or ε_β subunits were lysed in a solubilizing buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1.0 % Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM Na tetrathionate, 1 mM N-ethylmaleimide, 0.4 mM PMSF, 10 U/ml aprotinin, 20 ug/ml leupeptin. Eighty ul of the cell lysate samples were incubated for 2 hr at 4 °C with anti-ε 360 or MAb 124 in the presence of 10 nM of ^{125}I-α-BTX. Samples of the all reaction mixtures were then added with 50 ul slurry of rabbit anti-rat IgG conjugated Sepharose 4B and incubated with rocking for 2 more hours at 4 °C. All the precipitates were washed three times with washing buffer containing 50 mM

Tris-HCl pH 7.4, 1 M NaCl and 1% Triton X-100, before being counted in a gamma counter. Control immunoprecipitations were carried out using sham transfected COS cells.

Sucrose gradient sedimentation

Surface AChRs were labeled with 10 nM ^{125}I-α-BTX in growth medium for 90 min at 37 °C. The cells were scraped off the plate with a rubber policeman, pelleted and solubilized in the extraction buffer. The cell lysates, mixed with the gradient markers: bovine alkaline phosphatase (6.3S) and catalase (11.4S), were applied to 5-20 % sucrose gradients. Gradients were centrifuged at 36 K rpm in a Beckman SW50.1 rotor for 15-16 hr at 4 °C. and fractionated in 100 µl aliquots. Fractions were counted in a gamma counter.

dTC inhibition

The α-BTX association rate constant (K_t) was determined by a previously described method (Gu et al., 1985) according to the second-order rate equation:

$$K_t t = \frac{1}{T_o - R_o} \ln \frac{R_o T}{T_o R}$$

R_o: the initial concentration of receptor, expressed in α-BTX-binding sites.

R and T: the concentrations of AChR and ^{125}I-α-BTX at time t.

R and T were determined by subtracting the amount of toxin-receptor complex present at time t from the initial concentrations, R_o and T_o, respectively.

COS cells (1.89×10^6/dish) were plated into a 10 cm dish. Four dishes of COS cells were transfected with 8 ml of transfection mixture containing cDNAs for α (3.5 ug), β (1.8 ug), δ (0.68 ug) and ε or ε_β (2.4 ug). The transfected cells were trypsinized, pooled and plated into 30 wells of 24 well-plates 24 hours later. After another 24 hours, the cells were preincubated with 200 ul of growth medium with different indicated concentrations of dTC for 30 min, and replaced with the same medium containing 5 nM ^{125}I-α-toxin and incubated for 25 min at room temperature. The cells were then washed three times with PBS and dissolved in 0.5 ml of 0.1N NaOH and counted. At each concentration of dTC, K_t was calculated and ploted to determine IC_{50}.

α-BTX association rate

The α-BTX association rate of the ε-AChR or ε_β-AChR was determined by the equation listed above. The transfected cells were incubated with α-BTX for 25 min without preincubation of dTC.

Turnover rate

COS cells expressing ε-AChRs or ε_β-AChRs were labeled with ^{125}I-α-BTX by surface assay. The cells were washed three times with growth medium. Then 2 ml of growth medium in each dish was added, collected after 2 hours of incubation at 37°C. The cells were replaced with 2 ml of flesh growth medium every 2 hours through

Fig. 2-1. a. A general model of the transmembrane topology of acetylcholine receptor subunits proposed on the basis of primary sequence hydrophobicity. b. Structure of chimeric ε_β and β_ε subunits.

The structures of chimeric ε_β and β_ε subunits are diagrammatically shown with the hydrophobic segments M1-M4 aligned, and with the length difference between them (8 amino acids) ignored. Open boxes, mouse β subunit amino acid sequences; filled boxes, mouse ε subunit amino acid sequences. The locations of the introduced SnaBI and SpeI sites are indicated. The chimeric ε_β subunit is made up of ε residues -20-222, β residues 225-461 and ε residues 452-473, whereas the chimeric β_ε subunit is made up of β residues -23-224, ε residues 223-451 and β residues 462-478.

Figure 2-1

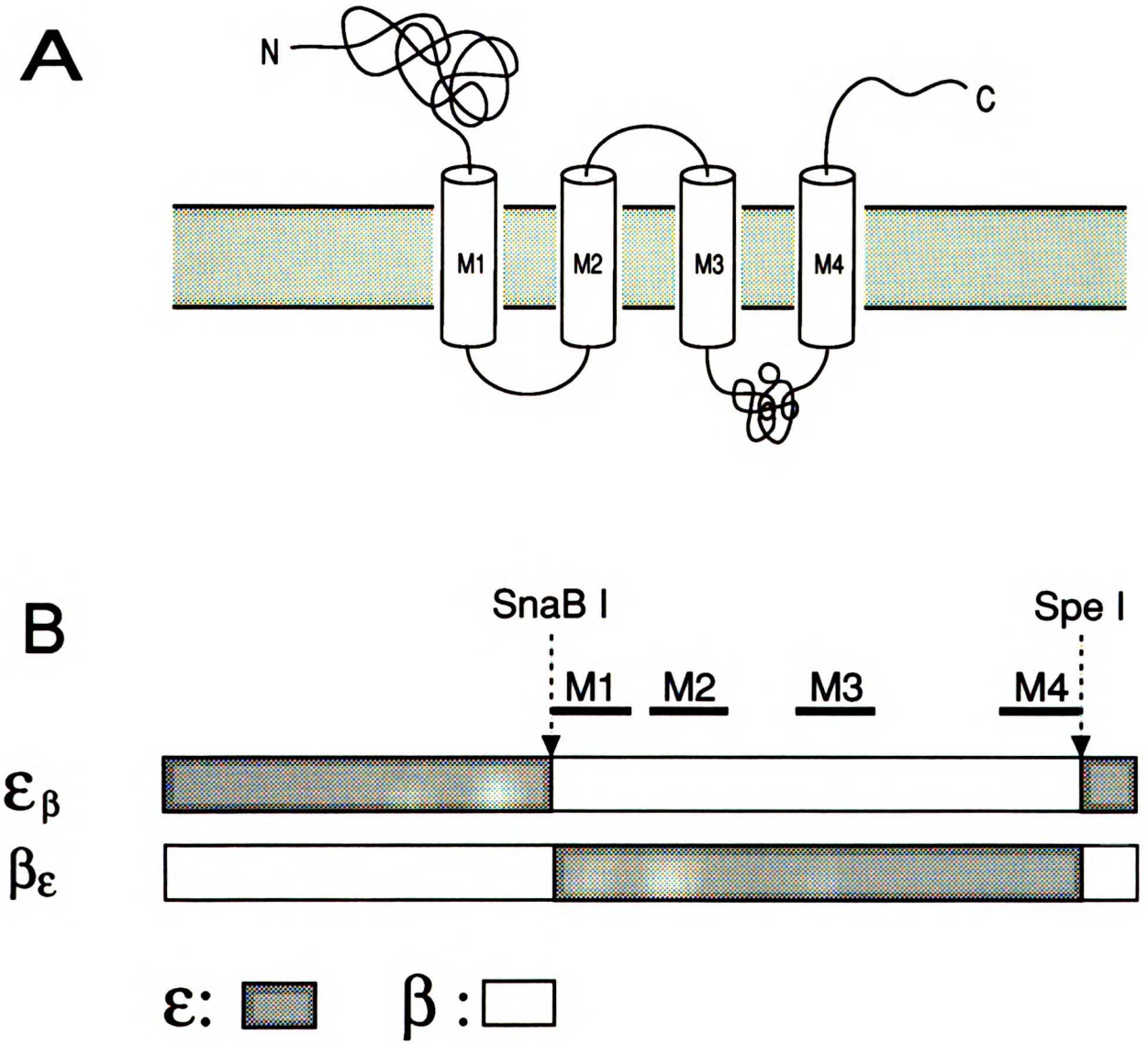


Table 2-I legend:

COS cells in 60 mm dishes with 50% confluency were transfected with various combinations of mouse AChR subunit cDNAs as shown. The transfected cells were replated into 4 wells in a 24-well plate 24 hr after the transfection and were incubated with ^{125}I-BTX 24 hr later. ^{125}I-BTX binding to the intact cells was determined as previously described (Gu et al., 1990). Each value is the mean ± SEM of three determinations.

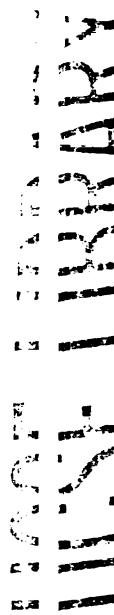


Table 2-I: ^{125}I-BTX Binding on the Surface of COS Cells Transfected with Different Combinations of AChR Subunit cDNA

Subunits expressed	Surface AChR (fmol/well)
$\alpha\beta\varepsilon\delta$	46.7 ± 13.3
$\alpha\beta\delta$	1.0 ± 0.6
$\alpha\beta(\varepsilon_\beta)\delta$	13.6 ± 0.6
$\alpha\beta(\beta_\varepsilon)\delta$	0.3 ± 0.4
$\alpha\varepsilon\delta$	0.8 ± 1.1
$\alpha(\varepsilon_\beta)\varepsilon\delta$	0.6 ± 0.1
$\alpha(\beta_\varepsilon)\varepsilon\delta$	0.2 ± 0.8

Fig. 2-2. AChR surface expression as a function of the amount of ε (square) or ε_β (diamond) cDNA.

COS cells in 60-mm dishes were transfected with fixed amounts of α (1.32 ug), β (0.66 ug), and δ (0.26 ug) cDNAs plus the indicated amount of ε or ε_β subunit cDNA. The cells at each concentration were then trypsinized and replated into 3 wells of a 24-well cluster, and the surface ^{125}I-α-BTX binding determined 24 hours later. Each point represents the mean ± SEM of 3 determinations.

Figure 2-2

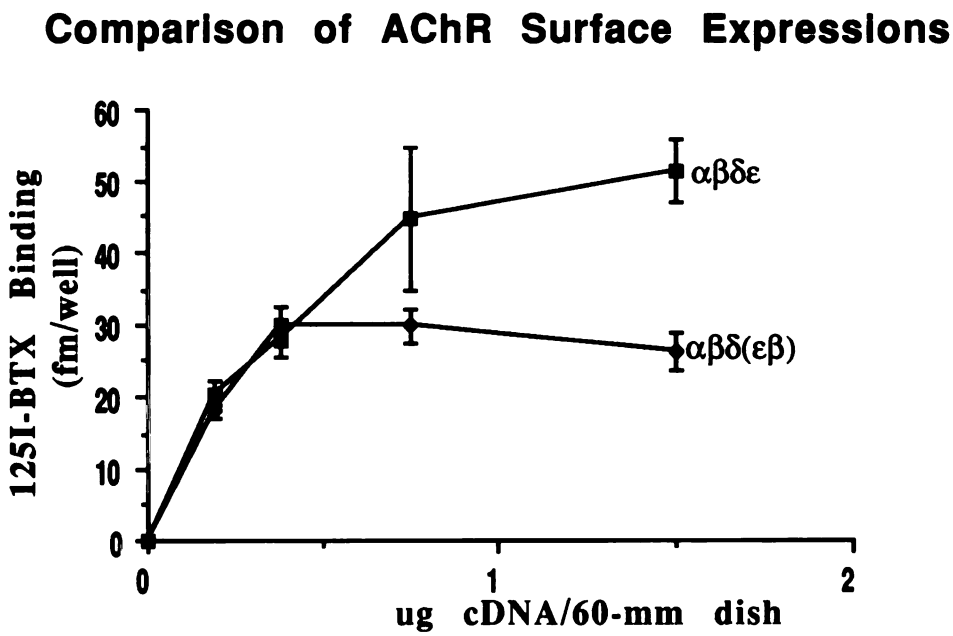

Comparison of AChR Surface Expressions
125I-BTX Binding (fm/well)
60
50
40
30
20
10
0
0
1
2
ug cDNA/60-mm dish
αβδε
αβδ(εβ)

Fig. 2-3. Sedimentation coefficient of AChRs containing ε_β subunit examined by sucrose gradient.

COS cells transfected with cDNAs for α, β, δ and ε or ε_β subunits were labelled with ^{125}I-α-BTX in 0.5% saponin buffer. The cell pellets were lysated and loaded to the sucrose gradient column which then spun for 15 hours. Each column was fractionated into 100 ul of fractions which were counted in a gamma counter.

Figure 2-3

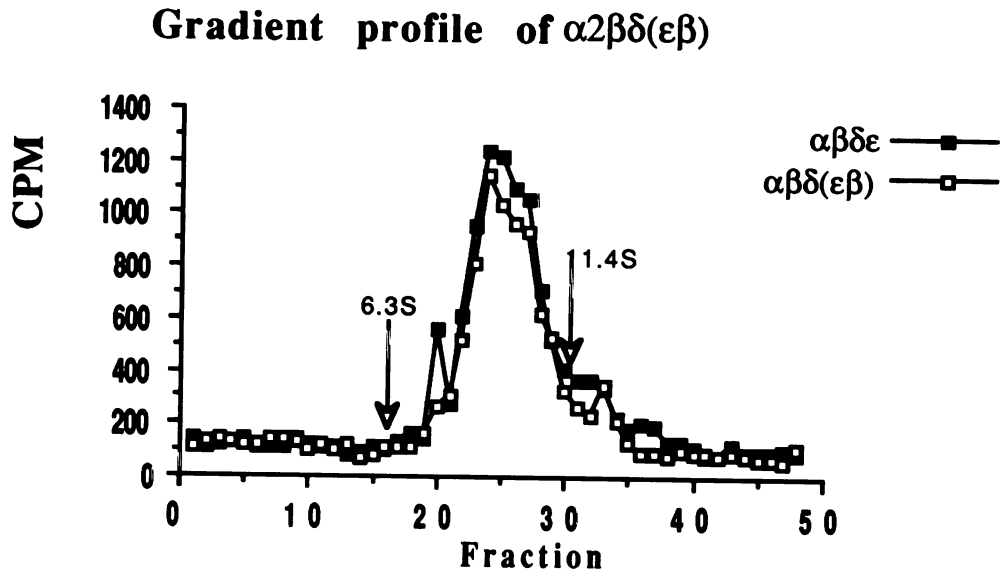

Gradient profile of α2βδ(εβ)
CPM
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Fraction
6.3S
11.4S
αβδε
αβδ(εβ)

Table 2-II. Properties of the ε-AChR and the ε_β-AChR expressed on the surface of COS cells

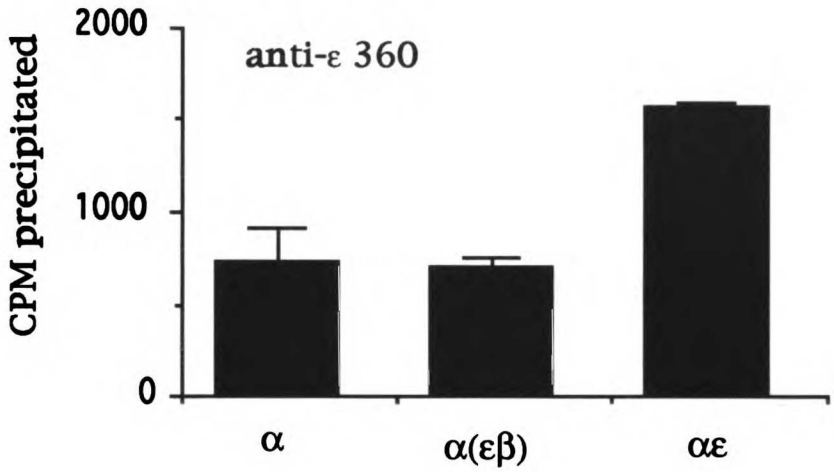
	ε-AChR	ε_β-AChR
Sedimentation coefficient (S)[a]	9.5	9.5
Association rate constant for α-BTX (10^4 M^{-1} S^{-1})[b]	2.3 ± 0.1	2.6 ± 0.2
IC_{50} for d-tubocurarine (M)[b]	2.5 x 10^{-7}	3.0 x 10^{10}
Metabolic half life of AChR (hr)[c]	2 3	1 9

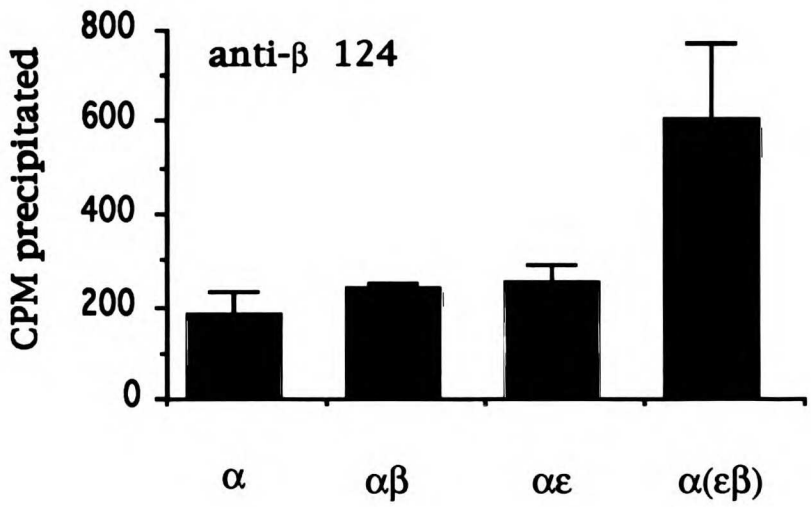
Values were determined in each case as previously described methods (see Material and Methods): a) a single determination; b) the mean ± SEM of six determinations from two separate experiments; and c) the average of two determinations.

Fig. 2-4. Association of α subunit with either chimeric ε_β or β_ε subunit determined by immunoprecipitation with either ε subunit-specific or β subunit-specific antibody.

COS cells transfected with subunit cDNAs as indicated were lysed in a 10-cm dish and the cell lysates incubated with ^{125}I-labelled α-BTX to label toxin-binding sites. Toxin-binding activity was then immunoprecipitated with anti-ε 360 antibodies, which are specific for ε subunit, or with monoclonal antibody 124, which is specific for the large cytoplasmic loop of the β subunit.

Figure 2-4





Chapter Three

The N- and C-terminal Domains Specify the Identity of the δ Subunit in AChR Assembly.

Abstract

The acetylcholine receptor (AChR) assembly undergoes a defined pathway: α subunit associates with the δ or ε/γ subunit to form a heterodimer; and then β associates with two heterodimers to complete AChR assembly. The previous work about chimeric ε_β subunit has demonstrated that the lumenal domains of the ε subunit contain sufficient information to finish the αε heterodimer formation and the AChR pentamer assembly. The dominant negative approach has showed that the N-terminal fragments of the α, δ and γ subunit contain recognition signal to compete normal heterodimer and AChR formation. However, as none of the partial proteins can complete AChR assembly in the presence of the other normal subunits, we continue to search for the smallest fragment of the δ subunit which can sufficiently replace its parental subunit to finish AChR assembly. Using the approach of making chimeric subunit, three chimeric subunits derived from the δ subunit (δ_β, δ_γ and δ_ε) were constructed in such a way that they all contain the N- and C-terminal domains from the δ subunit and the intervening region from the β, γ and ε subunits, respectively. Because the β subunit does not associate with the α subunit to form a heterodimer, the δ_β subunit becomes the best representative. By characterizing the chimeric δ subunits, we found that only the N- and C-terminal domains of the δ subunit are required for both heterodimer formation and AChR assembly, consistant with the previous work of the ε_β subunit. We also found that the IC_{50}s of the heterodimers containing either ε_β or δ_β subunit are similar to those of their parental heterodimers and the IC_{50}s of the AChRs containing either or both chimeric subunits are similar to

that of the normal AChR, consistant with the previous observation that allosteric interactions play little role in the binding of antagonist to two nonequivalent sites.

Introduction

Experiments in Chapter II showed that a chimeric subunit (ε_β) containing the extracellular domains of the ε subunit and the rest from the β subunit was able to replace the wild type ε subunit so that an intact AChR-like oligomer was expressed on the cell surface. The receptor containing the chimeric ε_β subunit was a pentamer (sedimentation coefficient of 9.5S), that exhibited an association rate constant for α-BTX and a metabolic half-life similar to those of the wild type AChR. Moreover, the chimeric ε_β subunit formed a stable heterodimer with the α subunit. These experiments suggest that the extracellular domains of the ε subunit possess the structural domains specifically responsible for its association with the α subunit and for subsequent steps of AChR assembly (Yu & Hall, 1991). Dominant negative inhibition experiments illustrated that N-terminal fragments of the α, γ and δ subunits that terminate just distal to the M1 domain inhibit both surface AChR expression and heterodimer formation, suggesting that the N-terminal domains of AChR subunits contain recognition signals for the initial steps of AChR assembly (Verrall & Hall, 1992). However, none of the partial proteins can replace their wild type counterparts to produce intact AChR. To determine which regions of the δ subunit are required for heterodimer and AChR formation, we have constructed a chimeric subunit (δ_β) for the δ subunit in which the regions M1 through M4, inclusive, are replaced by the corresponding regions from the β subunit. We find that this chimera, like ε_β, can substitute for the corresponding wild type subunit to produce both a receptor-like

complex and a heterodimer with the α subunit. We also find that both the chimeric ε_β and δ_β subunits are able simultaneously to assemble into the AChR, suggesting that the fragments from M1 to M4 of both the δ and ε subunit are not required for specific subunit recognition during AChR assembly. More importantly, the formation of a heterodimer between αM1 and δ_β provides clear evidence that heterodimer formation, the first step of assembly, is mediated by the N-terminal domain of the α subunit and the extracellular domains of the δ subunit.

Results

Chimeric δ_β, δ_ε and δ_γ subunits can substitute for δ subunit in the AChR assembly pathway.

To search for the δ sequences that will confer δ identity during assembly, several chimeric subunits, δ_β, δ_γ, δ_ε and β_δ, were constructed. The chimeric δ_β subunit, the most important of these, contains the N- and C-terminal domains of the δ subunit and the remainder (M1-M4, inclusive) from the β subunit. The chimeric δ_γ and δ_ε subunits have a similar structural scheme except that the M1-M4 part is from the γ or ε subunits, respectively. The chimeric β_δ subunit was made in the opposite way by incorporating the N- and C-terminal domains of the β subunit and the remainder from δ (Fig. 1, not shown). When cDNAs for the α, β and ε subunits were transfected into COS cells without δ cDNA, no α-BTX binding activity was detected on the cell surface (Table I), consistent with the previous results showing that all four subunits of the AChR are all

required to generate the surface AChR (Gu et al., 1991b; Yu and Hall, 1991). Addition of δ subunit cDNA to this basic transfection mixture, however, resulted in the surface AChR (60.9 fm/well). This receptor has similar properties to those of AChRs at adult endplates (Gu et al., 1990). When cDNA for either the chimeric δ_{β}, or δ_{ε}, or δ_{γ}, or β_{δ} subunit was then substituted for δ subunit cDNA, surface AChRs were expressed in all cases but that of β_{δ} (Table I, not shown). Thus the chimeric δ_{β}, δ_{ε} and δ_{γ} subunits can substitute for the δ subunit in AChR assembly, but the β_{δ} can not. In contrast, when cDNA for either the δ_{β} or β_{δ} subunit was added to a transfection mixture containing α, ε and δ cDNAs but lacking β cDNA, toxin binding was not detected in either case (Table I). Thus only the N- and C-terminal domains of the δ subunit contain subunit-specific information that is necessary for AChR assembly.

To investigate the properties of the surface toxin-binding complex assembled with the chimeric δ_{β} subunit, we examined its sedimentation coefficient in a sucrose gradient as described in chapter II. The sedimentation constant of the receptor complexes (9.5S) was identical to that seen for the normal AChR, (Table II). When γ, instead of the ε subunit cDNA was used in the transfection mixture, the sedimentation coefficient of the complex was also 9.5S (not shown). The receptor complexes formed with the chimeric subunit, δ_{β}, thus have a sedimentation coefficient that is consistent with a pentameric structure.

The chimeric δ_β subunit stably associates with the α subunit to form a heterodimer

Immunoprecipitation and sucrose gradient sedimentation were used to determine whether δ_β, like δ, forms a heterodimer with the α subunit. COS cells were transfected with α cDNA alone, with α plus δ cDNAs, or with α plus δ_β cDNAs. The lysates were then incubated with ^{125}I-α-BTX and ability of the δ subunit specific antibody MAb88B (Fig. 2A) and the β subunit specific antibody MAb124 (Fig. 2B) to immunoprecipitate toxin from each lysate was tested. In extracts of cells coexpressing α and δ_β subunits, MAb124 (which recognizes the cytoplasmic loop of β) but not MAb88B (which recognizes the cytoplasmic loop of δ) precipitated toxin-binding activity. Thus δ_β is associated with α. Sedimentation analysis of cell extracts showed a toxin-binding complex of 6.3S, consistent with formation of a heterodimer (Fig. 3).

An N-terminal fragment of the α subunit, αM1, has been previously shown to specifically associate with the δ to form a heterodimer which can be immunoprecipitated by MAb88B and can be visualized as a peak of 6S in the sucrose gradient (Verrall and Hall, 1992). To explore whether the δ_β subunit can associate with αM1, first, the lysates of the COS cells transfected with cDNAs for the α plus δ_β, α plus δ, or αM1 plus δ subunits were precipitated with MAb88B (Fig. 4A); second, the extracts from the COS cells transfected with cDNAs for the α plus β, αM1 plus δ_β or αM1 plus β subunits were subjected to immunoprecipitation with MAb124 (Fig. 4B). These experiments showed that MAb88B was able to precipitate

toxin-binding activity when either α plus δ or αM1 plus δ were expressed, consistent with heterodimer formation, and MAb124 was also able to immunoprecipitate toxin-binding acitvity when αM1 and δ_β or α and δ_β were expressed, indicating that in this case as well, a heterodimer was formed. These results give strong evidence that the N-terminal domain of the α subunit and the extracellular domains of the δ subunit are sufficient for heterodimer formation, the first step in AChR assembly.

Receptor assembly can occur with both δ_β and ε_β

We then tested whether the heterodimers, $\alpha\varepsilon_\beta$ and $\alpha\delta_\beta$, can associate along with the β subunit to form a complete receptor complex. When either of the chimeric subunit cDNA was omitted, toxin binding on the surface was not detected, but when α, β, ε_β and δ_β subunits were coexpressed in COS cells, significant toxin-binding activity was expressed on the cell surface (Table II). As the toxin-binding activity is provided by the α subunit, and the β subunit is required for surface toxin-binding activity (data not shown), the complex must contain all four subunits. As with the other complexes, its sedimentation constant (9.8S) is consistent with the formation of a pentamer. Thus the M1-M4 segment of neither the δ or ε subunit is required for AChR assembly.

The relative assembly efficiencies of the 4 different forms of the AChRs containing the wild type or either or both chimeric subunits are compared in Table II. The surface expression levels of the AChRs containing either or both chimeric subunits are only 20% of that of

the normal AChR. The heterodimers, $\alpha\delta_\beta$, $\alpha\varepsilon_\beta$, were also formed at 20-30% of the normal efficiency of the $\alpha\delta$ or $\alpha\varepsilon$ (data not shown). It is interesting to note that acumulative or combinatorial defect of the chimeric δ_β and ε_β subunits in AChR assembly was not observed.

dTC can inhibit the toxin binding of the heterodimer as well as the AChRs containing either δ_β, ε_β or both subunits

Association of the α and δ subunits, $\alpha\delta$, creates a low affinity site to dTC, and of the α and γ subunits, $\alpha\gamma$, generates a high affinity site (Blount & Merlie, 1989). The similar two nonequivalent sites also exist in AChR (Blount & Merlie, 1989; Dowding & Hall, 1987; Pedersen & Cohen, 1990). The binding sites were localized on the boundary of the two subunits (Pedersen & Cohen, 1990).

We then investigated whether or not the $\alpha\varepsilon_\beta$ and $\alpha\delta_\beta$ heterodimers as well as the AChRs including the AChR containing either or both ε_β and δ_β can still provide nonequivalent binding to agonist and antagonist. COS cells transiently transfected with indicated cDNAs were permeabilized and the effects of increasing concentrations of dTC on toxin-binding were determined (Fig. 5). For $\alpha\varepsilon_\beta$, an IC_{50} for dTC of 5×10^{-7} M was obtained, similar to the value of 1×10^{-7} M found for the $\alpha\varepsilon$ heterodimer. For $\alpha\delta_\beta$, the IC_{50} was 5×10^{-6} M, the same as the value found for $\alpha\delta$ (Fig. 5). The values for the normal heterodimers reported here are very similar to these reported previously (Blount and Merlie, 1989; Gu et al., 1990).

As the values of dTC inhibition of toxin-binding for heterodimers containing the chimeric subunits are very similar to those formed for the normal subunits, the values found for receptor complexes on the surface are quite resembling. The complete curves of dTC inhibition seen with the normal AChR and AChR containing either ε_β or δ_β is shown in Fig. 6. For the normal AChR and the AChRs with the δ_β, ε_β or both subunits inhibition occurs over a broad range, as seen previously (Blount and Merlie, 1989; Gu et al., 1990; Sine and Taylor, 1981) and can be described by a curve for binding to two sites.

Discussion

This work accomplishes two things. We find that the N- and C-termini of the δ subunit are sufficient to replace the δ subunit to complete AChR assembly. We provide strong evidence to draw a conclusion that the heterodimer formation is mediated by association through the N-terminal domains of the two subunits.

AChR assembly

The results reported in this chapter extend the results reported in Chapter II for ε subunit to the δ subunit. In both cases, chimeric subunits containing only the N- and C-terminal domains of the subunit, with the intervening portion from another subunit, can replace the intact subunit in the AChR assembly pathway. Both types of chimeric subunits form heterodimers with the α subunit, and in both cases, heterodimer formation is associated with the formation of a small ligand binding site, as originally reported by

Blount and Merlie. Moreover, both chimeric subunits can substitute for the appropriate parental subunit in forming a complete receptor-like complex. These complexes apparently contain all four subunits and have a sedimentation coefficient consistent with a pentameric structure. These results thus establish that the extracellular N- and C-terminal domains of the ε and δ subunits contain all the subunit-specific information necessary for heterodimer and AChR assembly. This conclusion is likely to be also true for the γ subunit as well, although we were unable to obtain direct information on this point.

The relative unimportance of the segment M1-M4, inclusive, is also emphasized by two further observations. First, we have found that an AChR complex can be formed in which both ε_β and δ_β are substituted for their parental subunits. In this case, three of the five subunits in the complex have an M1-M4 segment derived from the β subunit, and two have such a segment derived from the α subunit. As shown in Chapter IV, attempts to obtain a complex in which all of the M1-M4 segments were derived from the β subunit were unsuccessful. Second, we found that a heterodimer could be formed between αM1 and δ_β. Experiments by Verrall and Hall had shown that an αM1δ complex could be formed. Our results indicate that specific formation of the αδ heterodimer requires an M1-M4 segment from neither the α nor the δ subunit.

Our experiments raise the question of whether both N- and C-terminal regions of the δ and ε subunits are required for AChR assembly. Experiments by Verrall and Hall (1992) showed that

heterodimer formation requires only the N-terminal domain. Experiments reported in Chapter V suggest that the C-terminal domain functions in a later step of assembly.

dTC inhibition.

One aspect of our studies is the effect of the chimeric subunits on dTC binding in the heterodimers and the intact AChR complex. As dTC binding of the heterodimers formed by ε_β and δ_β appears to have the characteristic affinities shown by the heterodimers formed with their parental subunits, in both cases, the intact complexes formed by the chimeric subunits show similar behavior from that seen with the normal AChR. This result suggests that the mutation in the δ_β subunit does not influence the toxin binding site and is consistant with the result reported previously that allosteric interactions play little if any role in the binding of antagonist to these two nonequivalent sites (Blount and Merlie, 1989).

Experimental Procedures

Antibodies

Monoclonal antibodies, MAb 61, 124 are generous gifts from Dr. Jon Lindstrom. MAb88B is given by Dr. Stanley C. Froehner. The antibodies recognize the long cytoplasmic loops of the α, β and δ subunits, respectively.

cDNAs

Full-length cDNA clones coding for the α, β, γ and δ subunits of mouse muscle nicotinic AChR were gifts from Drs. J. P. Merlie and N. Davidson: α, (Isenberg et al., 1986); β, (Buonanno et al., 1986); γ, (Yu et al., 1986) and δ, (LaPolla et al., 1984). The full-length cDNA for the mouse ε subunit was isolated as described (Gu et al., 1990). Each of the cDNAs were subcloned into the SV40 based expression vector pSM (Brodsky et al., 1990) at the multiple cloning site. An M13 origin in this vector was used to generate uracil-containing single-stranded DNAs for site-directed mutagenesis (Geisselsoder et al., 1987; Kunkel, 1985).

Site-Directed Mutagenesis

Site-directed mutagenesis was conducted according to previously described methods (Geisselsoder et al., 1987; Kunkel, 1985), using synthetic oligodeoxyribonucleotides prepared with an automatic DNA synthesizer. Uracil-containing single-stranded DNAs, prepared by infecting plasmid-transformed E.coli CJ_{236} (dut-ung-F') cultures with

M13 helper phages, were used as the templates. Each of constructed cDNAs was analyzed by restriction enzymes. In some case, plasmids were confirmed by DNA sequencing.

Construction procedures: Two novel restriction enzyme sites, SnaBI and SpeI, were introduced into pSMβ, pSMγ, pSMδ and pSMε plasmids, SnaBI at the beginning of M1 and SpeI at the ending of M4 of each subunit. The plasmids with the introduced sites were then digested with the two restriction enzymes. The plasmids for the chimeric subunits, β_δ, δ_β, δ_γ and δ_ε were constructed by purifying the corresponding fragments, and ligating them to the pSMδ vectors that had been digested with the same restriction enzymes. The plasmids were analyzed and mapped by restriction enzymes.

Transfection

Transfection in COS cells was a modified DEAE-dextran transfection procedure (Seed and Aruffo, 1987), performed as previously described (Gu et al., 1990). Cells grown to 30-50% confluence in a 60-mm dish were incubated for 3-5 hrs at 37 °C with the following transfection mixture: an appropriate amount of plasmid cDNAs added in DME-H21 supplemented with 1.0 % heat-inactivated fetal bovine serum, 0.10 mM chloroquine diphosphate and 0.4 mg/ml DEAE-Dextran. The amount of plasmid used for each subunit in the transfection mixture was determined empirically to produce maximal cell surface AChR expression. For analysis of cell surface AChR expression, 3 ml of the transfection mixture was added to a 60 mm dish containing cDNAs of α: 1.32 ug, β: 0.66 ug, γ or ε: 1.0 ug and δ: 0.26 ug. For immunoprecipitation of heterodimers, 5 ml (3 ml) of the

transfection mixture was added to a 100 mm (60 mm) dish containing 2.0 ug (1.0 ug) of each cDNA of the α and δ or δ_β subunits. After 3-4 hr of incubation, the transfection solution was removed, the cells were treated with 10% DMSO-PBS for 2 min at room temperature and replaced with 5 ml (60 mm dish) or 10 ml (100 mm dish) of the growth medium (10% fetal bovine serum in DME H21 supplemented with 100 u/ml penicillin and streptomycin). After twenty-four hours at 37 °C, the cells were trypsinized and distributed into 3 wells of 24 well plates (surface AChR assay) or into 60 mm dishes (heterodimer immunoprecipitation assay). The surface AChR assay and heterodimer immunoprecipitation analysis were carried out after an additional 24 hours.

Surface AChR Assay

Surface AChR expression was determined by incubating intact transfected cells for 90 min at 37 °C with 10 nM ^{125}I-α-BTX (Amersham Corp., Arlington Heights, IL). Nonspecific binding was measured by addition of more than 100X excess of cold α-BTX to the 10 nM ^{125}I-α-BTX medium, or by sham transfections. Unbound α-BTX was removed by washing the cells with PBS. The amount of bound toxin was measured by solubilizing the cells in 0.1 M NaOH and counting the radioactivity in a gamma counter.

Immunoprecipitation of heterodimers

Immunoprecipitation with subunit-specific antibodies was performed as previously described (Gu and Hall, 1988). The COS cells transfected with the α and β, α and δ_β, αM1 and β, αM1 or δ_β

subunits were lysed in a solubilizing buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1.0% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM Na tetrathionate, 1 mM N-ethylmaleimide, 0.4 mM PMSF, 10 U/ml aprotinin, 20 ug/ml leupeptin. ^{125}I-α-BTX was then added to the lysates to a final concentration of 10 nM to label all the toxin binding sites. Eighty ul of the cell lysate samples were incubated with MAb124 in the presence of 10 nM of ^{125}I-α-BTX for 2 hr at 4 ^{o}C. Samples of all reaction mixtures were then added with 50 ul slurry of rabbit anti-rat IgG conjugated sepharose 4B and incubated with rocking for 2 more hours at 4 ^{o}C. Immunoprecipitations of the α and δ or αM1 and δ heterodimers were performed by incubating the lysate samples with MAb88B-coupled sepharose in the presence of 10 nM ^{125}I-α-BTX for 2 hr at 4 ^{o}C. All the precipitates were washed three times with washing buffer containing 50 mM Tris-HCl pH 7.4, 1 M NaCl and 1% Triton X-100, before being counted in a gamma counter. Control immunoprecipitations were carried out using sham transfected COS cells.

Sucrose Gradient

To label assembled intermediates which are localized intracellularly, the transfected COS cells in 60 mm dishes were incubated for 1-2 hours on ice with 0.5% saponin buffer containing 10 nM ^{125}I-α-BTX, 10 mM HEPES pH 7.4, 0.1% BSA and 0.5% saponin, then washed 3 times in the same buffer without ^{125}I-α-BTX. Surface AChR was labeled with 10 nM ^{125}I-α-BTX in the growth medium for 90 min at 37 ^{o}C. The cells were scraped off the plate with a rubber policeman, pelleted and solubilized in the

extraction buffer. The cell lysates, mixed with the gradient markers: bovine alkaline phosphatase (6.3S) and catalase (11.4S), were applied to 5-20% sucrose gradients. The gradients were centrifuged at 36 K rpm in a Beckman SW50.1 rotor for 15-16 hr at 4 ^{o}C and fractionated in 100 ul aliquot. Fractions were counted in a gamma counter.

dTC inhibition

COS cells expressed with either the receptors and the heterodimers in 100 mm dishes were trypsinized after the transfections, and split to wells in 24 well plates. Duplicate wells were performed for each concentration of dTC. Twenty hour later, the cells were preincubated for 1 hour with 200 ul of the 0.5% saponin buffer containing the indicated final concentration of dTC, were then added with 10 ul of 100 nM ^{125}I-α-BTX to make a final concentration of ^{125}I-α-BTX about 5 nM, and incubated for another 30 min. Then the cells were washed three times with the saponin buffer, dissolved in 0.5 ml of 0.1 N NaOH and counted. The cells expressing the surface AChRs were preincubated for 1 hour with 200 ul of the growth medium containing the indicated final concentration of dTC, were then added with 10 ul of 100 nM ^{125}I-α-BTX to make a final concentration of ^{125}I-α-BTX about 5 nM, and incubated for another 30 min. The cells were then washed three times with PBS, dissolved in 0.5 ml of 0.1 N NaOH and counted. Nonspecific binding was defined by addition of 1 uM cold α-BTX. Specific binding was defined by total binding minus nonspecific binding. Total binding was defined by addition of only ^{125}I-α-BTX.

Figure 3-1. a. A general model of the transmembrane topology of acetylcholine receptor subunits proposed on the basis of primary sequence hydrophobicity. b. Structure of chimeric δ_β, δ_γ and β_δ subunits.

The structures of chimeric δ_β, δ_γ and β_ε subunits are diagrammatically shown with the hydrophobic segments M1-M4 aligned. Open boxes, mouse β subunit amino acid sequences; filled boxes, mouse δ subunit amino acid sequences. The locations of the introduced SnaBI and SpeI sites are indicated.

Figure 3-1

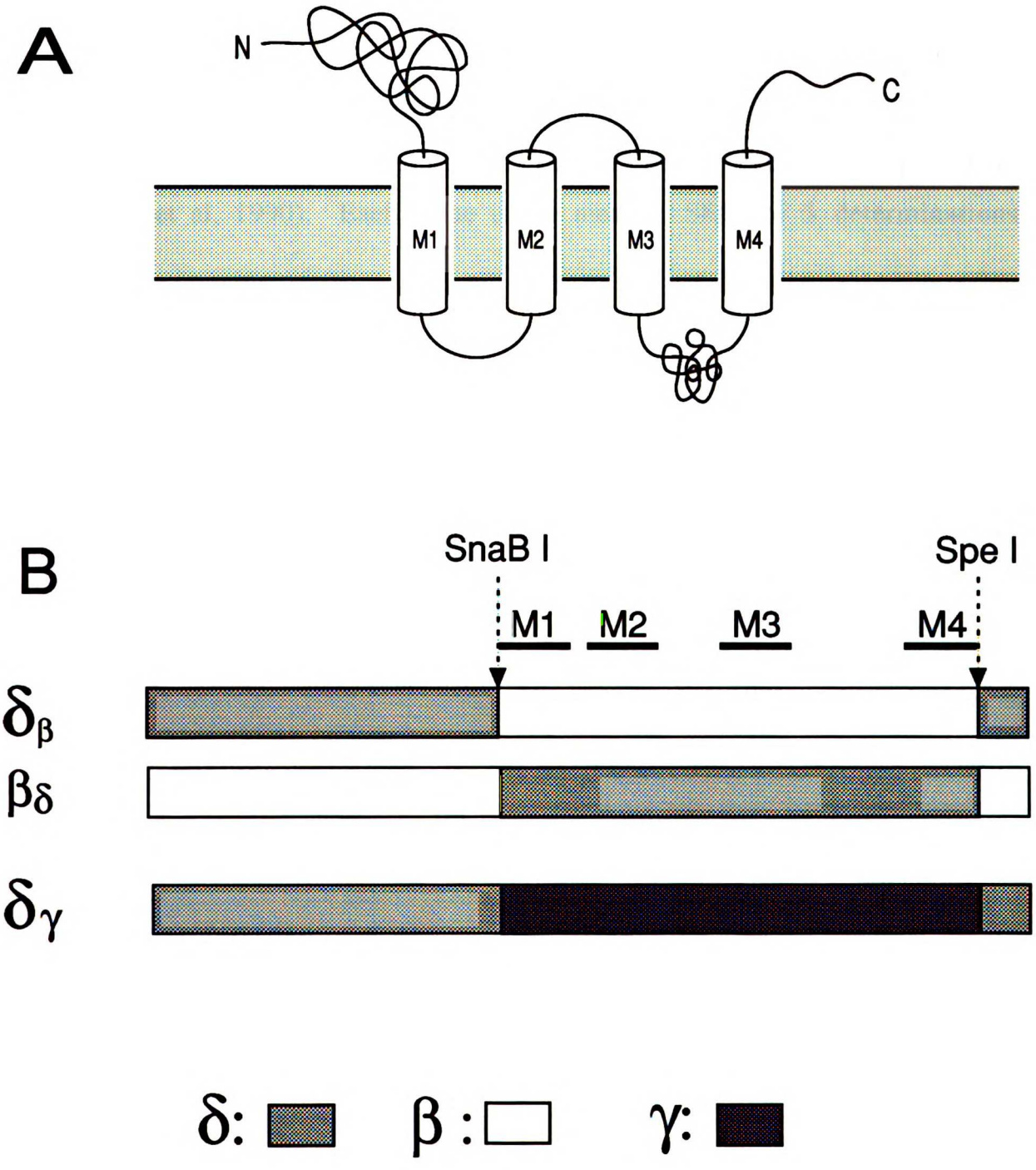


Table 3-I legend:

COS cells in 60 mm dishes with 50% confluency were transfected with various combinations of mouse AChR subunit cDNAs as shown. The transfected cells were replaced to 3 wells in 24-well plates after 24 hours, and were incubated with ^{125}I-BTX 24 hour later. ^{125}I-BTX binding to the intact cells was determined as previously described (Gu et al, 1990). Each value is the mean ± SEM of 3 determinations.

Table 3-I: ^{125}I-BTX binding on the surface of COS cells transfected with different combinations of the AChR subunit cDNAs

Subunits expressed	Surface AChR (fm/well)
$\alpha\beta\varepsilon$	0
$\alpha\beta\varepsilon(\delta_\beta)$	12.4 ± 0.4
$\alpha\beta\varepsilon(\beta_\delta)$	0.4 ± 0.1
$\alpha\beta\varepsilon\delta$	60.9 ± 1.6
$\alpha\beta\varepsilon(\delta_\gamma)$	41.6 ± 1.1
$\alpha\varepsilon\delta$	0
$\alpha\varepsilon\delta(\delta_\beta)$	0.4 ± 0.1
$\alpha\varepsilon\delta(\beta_\delta)$	3.6 ±1.0

Figure 3-2. The heterodimer $\alpha\delta_\beta$ was precipitated by MAb88B (A) and MAb124 (B).

A. COS cells were transfected with the cDNAs for α, α plus δ and α plus δ_β subunits, extracted 48 hr later and subjected to immunoprecipitation with MAb88B. B. COS cells were transfected with the cDNAs for α alone or α plus δ_β subunits, extracted 48 hr later and immunoprecipitated with MAb124.

Figure 3-2A.

$\alpha\delta_\beta$ heterodimer was not precipitated by MAb88B

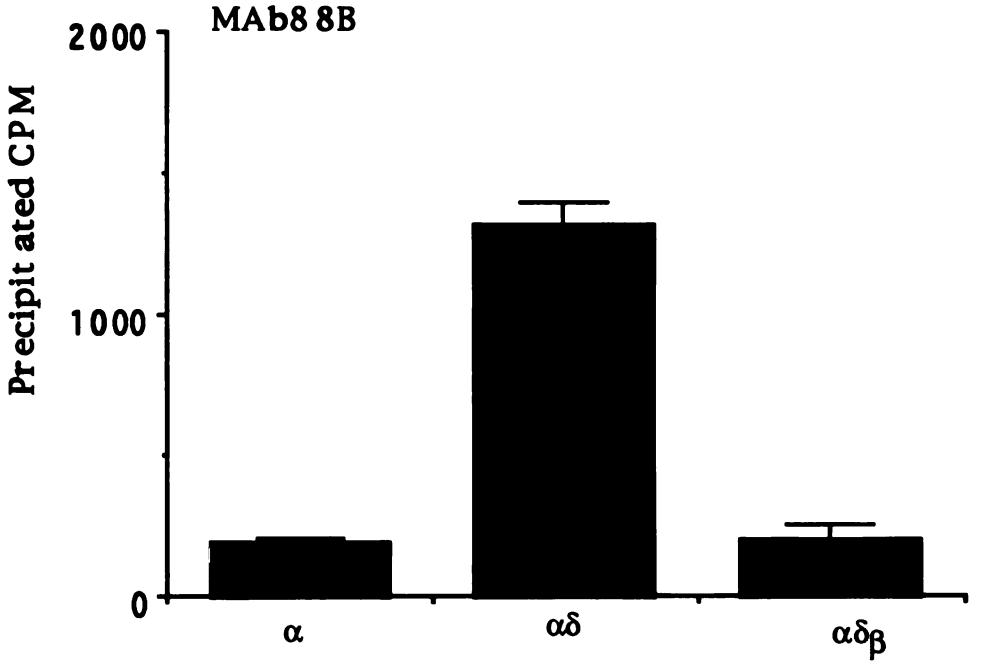


Figure 3-2B.

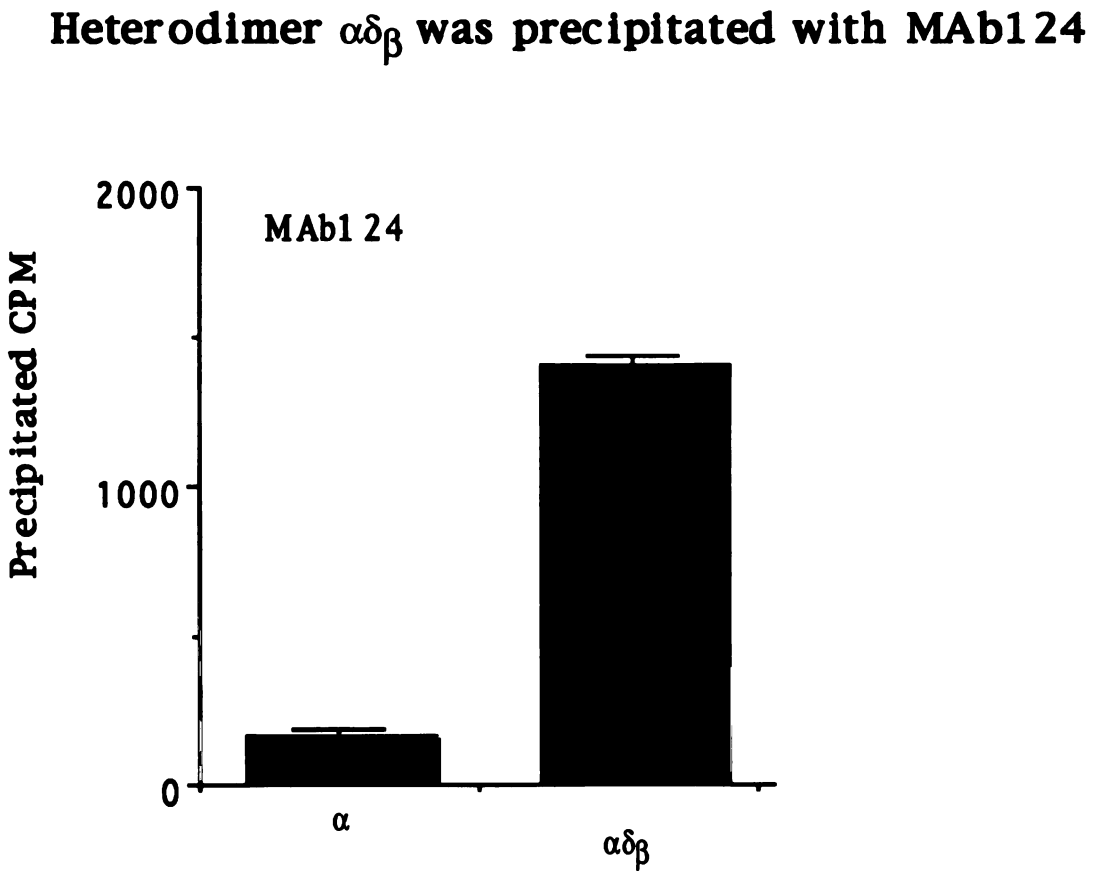

Heterodimer αδβ was precipitated with MAb124
Precipitated CPM
2000
1000
0
MAb1 24
α
αδβ

Figure 3-3. The sedimentation coefficients of the heterodimers, $\alpha\delta$ and $\alpha\delta_\beta$ were determined in sucrose gradients as 6.3S.

COS cells were transfected with the cDNAs for α plus δ or α plus δ_β subunits, labeled with ^{125}I-α-BTX, extracted and loaded to 5-20% linear sucrose gradients. Each gradient was fractionated and the fractions were counted. According to the two gradient markers, alkaline phosphatase (6.3S) and catalase (11.4S), the AChR peaks are determined as 6.3S. Points are plotted as percent CPM of the greatest fraction.

Figure 3-3.

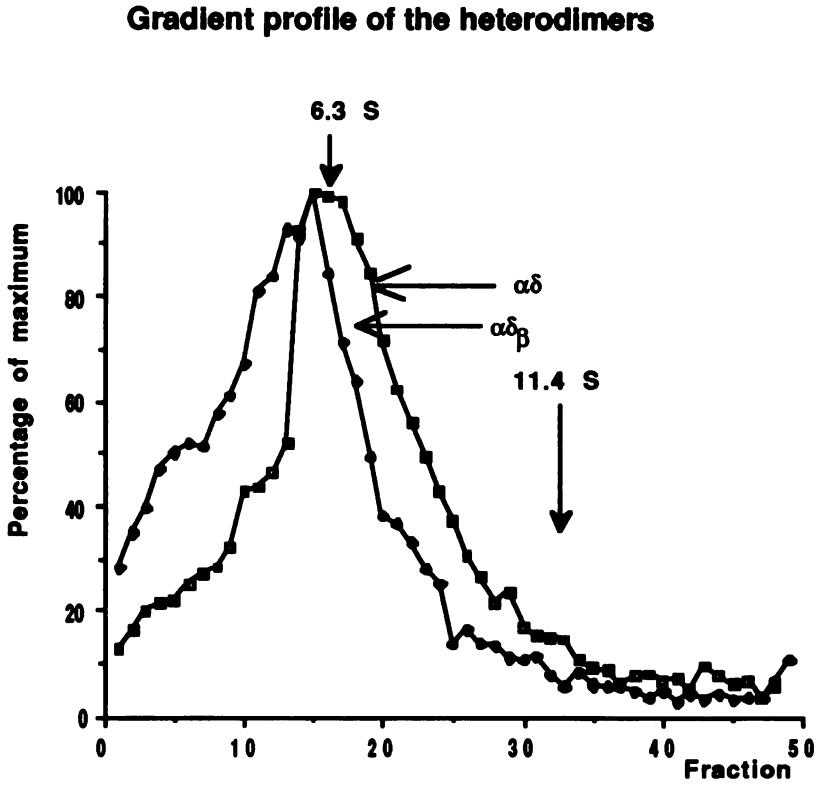

Gradient profile of the heterodimers
6.3 S
11.4 S
αδ
αδβ
Percentage of maximum
100
80
60
40
20
0
0
10
20
30
40
50
Fraction

Figure 3-4. Heterodimer formation is mediated by the association through the N-terminal domain of the α subunit and the extracellular domains of the δ subunit. The heterodimers, αδ, αM1δ, not $\alpha\delta_\beta$, were able to immunoprecipitated with MAb88B (A) while the heterodimers, $(\alpha M1)(\delta_\beta)$ were immunoprecipitated with MAb124 (B).

COS cells were transfected with the cDNAs for either the α plus δ_β, α plus δ, αM1 plus δ subunits (A); or the α plus β, αM1 plus δ_β, or αM1 plus β subunit (B). The transfected COS cells were then extracted and subjected to immunoprecipitation with either MAb88B (A) or with MAb124 (B). Each value represents the mean ± SD of three determinants.

Figure 3-4A.

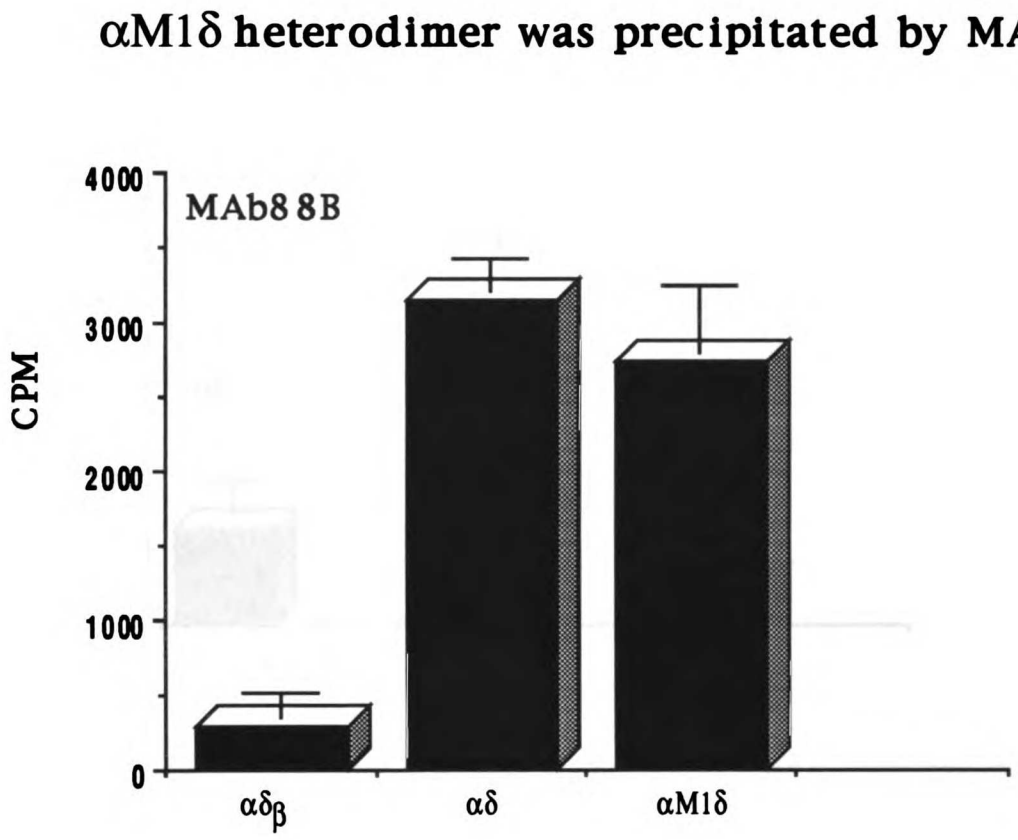

αM1δ heterodimer was precipitated by MAb88B
MAb88B
CPM
4000
3000
2000
1000
0
αδβ
αδ
αM1δ

Figure 3-4B.

αM1 associates with δ_β to form a heterodimer

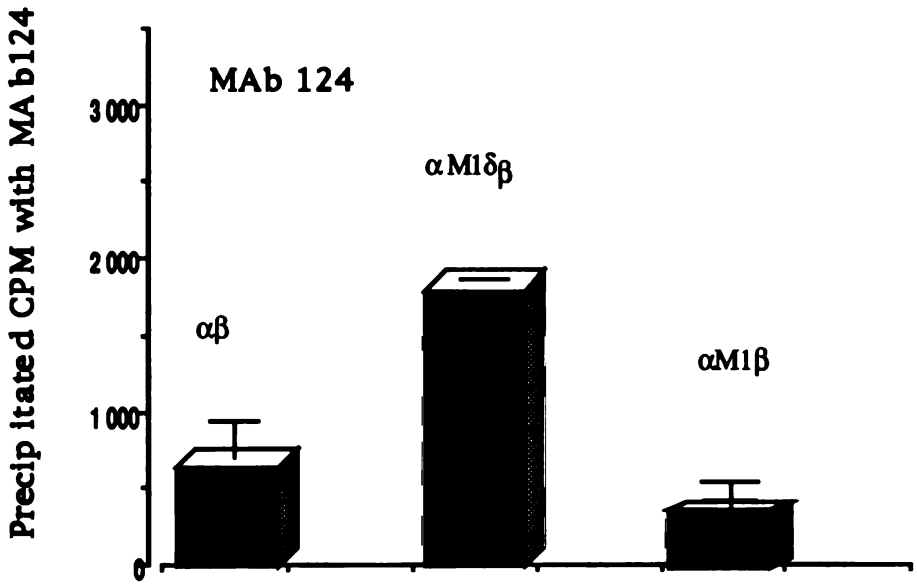


Table 3-II. The relative efficiencies of assembly for four different forms of the AChRs were compared. The AChRs incorporating either δ_β or ε_β or both subunits exhibit a similar efficiency, about 20% of that of the normal AChR. The sedimentation coefficients of the AChRs containing either δ_β, ε_β or both subunit determined in sucrose gradients show that the complexes are pentamers.

COS cells were transfected with the cDNAs for the subunits as indicated, and labeled with ^{125}I-α-BTX 48 hr later. The redioactivity was counted in a gamma counter. Each value represents the mean ± SD of triplicate determinants.

COS cells were transfected with the cDNAs for the α, β, δ and ε subunits, α, β, δ_β and ε subunits, α, β, δ and ε_β subunits or the α, β, δ_β and ε_β subunits. Then the cells were labeled with ^{125}I-α-BTX 48 hr later, extracted and loaded to the gradients. Each gradient was fractionated and the fractions were counted. According to the two gradient markers, alkaline phosphatase (6.3S) and catalase (11.4S), the AChR peaks are determined as 9.5S.

Table 3-II. Comparison of the relative assembly efficiencies of AChRs formed with chimeric δ and ε subunits

Transfection mixture	Surface AChR expression (% of control)	Sedimentation coefficient (S)
$\alpha\beta\varepsilon_\beta$	0.0	
$\alpha\beta\delta_\beta$	0.0	
$\alpha\beta\delta_\beta\varepsilon$	15.1 ± 3.8	9.5
$\alpha\beta\delta\varepsilon_\beta$	23.8 ± 0.3	9.5
$\alpha\beta\delta_\beta\varepsilon_\beta$	12.7 ± 2.5	9.8
$\alpha\beta\delta\varepsilon$	100 ± 9.9	9.5

Figure 3-5. The heterodimer $\alpha\delta_\beta$ has the same affinity to dTC as $\alpha\delta$ while $\alpha\varepsilon_\beta$ does as $\alpha\varepsilon$.

COS cells were transfected with cDNAs for either $\alpha\delta$, $\alpha\delta_\beta$ or $\alpha\varepsilon$ or $\alpha\varepsilon_\beta$ subunits, then permeablized, preincubated with different concentrations of dTC, coincubated with ^{125}I-α-BTX, washed three times and counted. Each value in the top panel represents the average of four determinants in two experiments.

Figure 3-5.

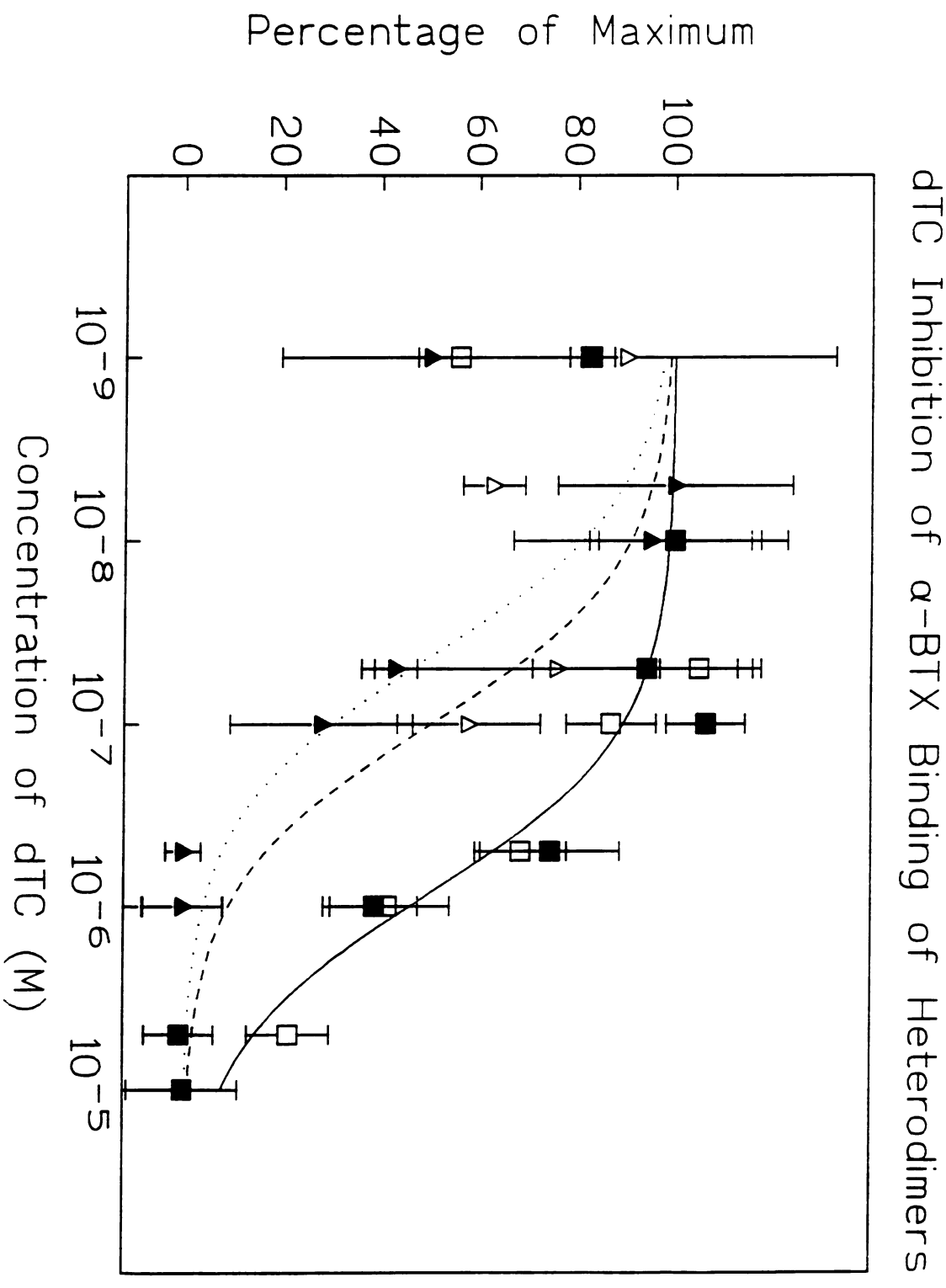
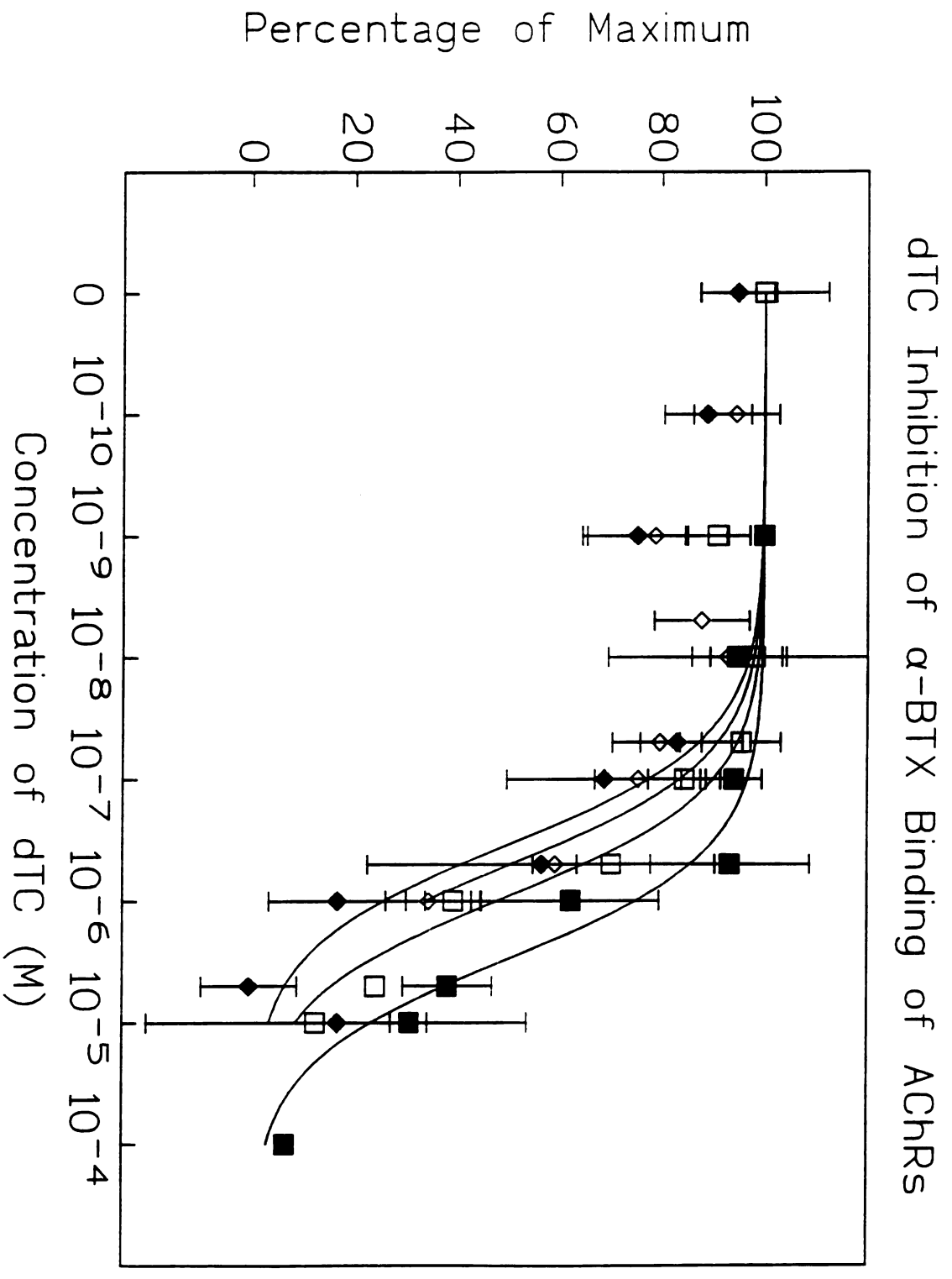

Percentage of Maximum
0
20
40
60
80
100
dTC Inhibition of α-BTX Binding of Heterodimers
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
Concentration of dTC (M)

Figure 3-6. The toxin binding activity of the AChRs incorporating the normal or δ_β or ε_β subunit or both can be inhibited by dTC. The AChRs incorporated the δ_β subunit shift the curve to the right and the AChRs incorporated the ε_β subunit shift the curve to the left, compared to the normal AChR curve.

COS cells were transfected with the cDNAs for the $\alpha\beta\delta\varepsilon$ or $\alpha\beta\delta_\beta\varepsilon$ or $\alpha\beta\delta\varepsilon_\beta$ or $\alpha\beta\delta_\beta\varepsilon_\beta$ subunits as indicated, 48 hr later were precipitated with different concentrations of dTC as indicated, coincubated with ^{125}I-α-BTX, and washed three times and counted. The total binding was determined by incubating the transfected cells with ^{125}I-α-BTX after preincubation of different concentrations of dTC. The non-specific binding was determined by incubating the transfected cells in the presence of cold α-BTX. The specific binding was determined by subtraction of the nonspecific binding from the total binding. Each value is the mean ± SD of six determinations.

Figure 3-6


dTC Inhibition of α-BTX Binding of AChRs
Percentage of Maximum
0
20
40
60
80
100
Concentration of dTC (M)
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴

Chapter Four

Portions of the Main Cytoplasmic Domains of the α and β Subunits are Required for Assembly of the Nicotinic Acetylcholine Receptor

Abstract

The acetylcholine receptor (AChR), a ligand gated ion channel, is an oligomeric membrane protein whose subunit composition is $\alpha 2\beta\delta\varepsilon$ or $\alpha 2\beta\delta\gamma$. The AChR is assembled by a defined pathway in which the first step is the association of the α subunit with the δ or ε subunit to form a heterodimer. Each of the two heterodimers, $\alpha\delta$ and $\alpha\varepsilon$, then interacts with the β subunit to form the fully assembled receptor. Chimeric and truncated proteins of the α subunit were constructed by using in vitro mutagenesis. A chimeric $\alpha\beta$ subunit which contains the N- and C-terminal domains of the α subunit and the remainder from the β subunit does not support AChR assembly when coexpressed with the other required subunits in COS cells. Another chimeric α–β1 subunit which contains only the cytoplasmic loop between M3 and M4 from the β subunit also does not support AChR assembly. Because this chimera can bind α-BTX and can associate with the δ to form a heterodimer, its failure to support AChR assembly must result from its inability to undergo the final steps of AChR assembly. Systematic mutational analysis of the cytoplasmic loop of the α subunit by sequentially mutating or deleting 7-15 amino acid segments in the loop between M3 and M4 demonstrated that a sequence of about 17 amino acids near M4 is required for completing AChR assembly. α subunits with a mutation or deletion in this sequence bind the toxin, associate with δ subunits to form heterodimers, but are incompetent in the last step of the assembly. We conclude that a sequence of about 17 amino acids near M4 in the cytoplasmic loop of the α subunit plays a role in the final step of AChR assembly. Similar information was found in the β subunit loop.

Systematic deletion and mutations of 7-15 amino acid fragments made from the beginning to the end of the loop reveal two important regions, a 28 amino acid sequence at the beginning (β310-338) and a 25 amino acid region near the end (β408-418; β 430-445) of the loop. The finding that the corresponding regions in the loops of both the α and β subunits are required, combined with the results that neither loops of the δ or ε subunit are required for AChR assembly, raised the possibility that the loops of the α and β subunit must interact.

Introduction

The nicotinic acetylcholine receptor (AChR) of vertebrate muscles is one of a family of oligomeric ligand-gated ion channels whose subunits have a common structure (Betz, 1990). Each has a large N-terminal domain followed by three transmembrane domains, a long cytoplasmic loop, and a fourth transmembrane domain, ending in a short extracellular region (Noda et al., 1982; Claudio, 1989; Devillers-Thiery et al., 1983; Chavez and Hall, 1991; Chavez and Hall, 1992). Most of the receptors in the family are formed by several different subunits that assemble around a central aqueous pore through which ions flow. The assembly of the subunits in the proper stoichiometry and in the proper order around the ring clearly requires highly specific interactions between them.

We have recently investigated the mechanism of assembly of the mouse muscle AChR, a pentameric ion channel with four different subunits (Hall, 1992). In embryonic muscles, the stoichiometry of the subunits is $\alpha_2\beta\gamma\delta$ (Karlin,1989); during postnatal development, ε subunit replaces γ (Takai et al., 1985; Mishina et al., 1986; Gu and Hall, 1988a). Each of the subunits is synthesized in the endoplasmic reticulum (ER) and becomes assembled there into a hetero-oligomeric receptor (Anderson and Blobel, 1981; Smith et al., 1987; Gu et al., 1989). Before assembly, the α subunit undergoes a folding reaction involving disulfide bond formation that enables it to bind α-bungarotoxin (α-BTX) (Merlie and Lindstrom, 1983; Blount and Merlie, 1990). Assembly of the AChR then occurs by a defined

pathway in which the folded α subunit first forms heterodimers with γ (or ε) and δ subunits; the heterodimers then assemble with the β subunit and with each other to form the complete receptor (Blount and Merlie, 1989; Blount and Merlie, 1990; Gu et al., 1991b; Saedi et al., 1991).

We have shown previously, using chimeric subunits and dominant negative experiments, that heterodimer formation occurs principally through interactions between the N-terminal, lumenal domains of the α, δ and γ (ε) subunits (Yu and Hall, 1991; Verrall and Hall, 1992). In initial experiments, we used chimeric subunits in which the N- and C-termini of the subunits were derived from one subunit and the intervening portion or mid-region, consisting of the four transmembrane domains (M1-M4) and the cytoplasmic domains, was derived from another subunit. We have reported previously (Chapter II) that when the mid-region of the β subunit was substituted for that of the ε subunit, the resulting chimeric subunit (termed ε_β) was able to replace the ε subunit in the assembly reaction. Thus, COS cells transfected with cDNAs for the α, β, ε_β and δ subunits expressed α-BTX-binding activity on its surface at 25-30% of AChR level seen with the normal ε subunit cDNA (Yu and Hall, 1991). Similar results were obtained when the mid-region of the β subunit was substituted for that of the δ subunit: the resulting chimeric subunit δ_β was able to substitute for the δ subunit in all steps of AChR assembly (Chapter III). Even when both chimeric subunits, δ_β and ε_β, replaced δ and ε subunits, respectively, toxin-binding activity was observed at approximately 25-30% of the level

seen when all four normal subunits were expressed. The cytoplasmic domains of the ε and δ subunits are thus not required for assembly of the AChR.

In contrast to assembly of the heterodimers, little is known about the interactions that mediate the later assembly steps and about how β subunit comes to the complex. We investigate here the role of the cytoplasmic domains of the subunits in AChR assembly. We find that a region in the long cytoplasmic loop of the α and β subunits, but not the other subunits is required for one of the final steps of AChR assembly.

Results

The cytoplasmic loop of α subunit is required for AChR assembly

When a chimeric subunit for the α subunit was constructed in a way similar to that described for the ε_β and δ_β subunits, i.e the N- and C-terminal domains were derived from the α subunit and the intervening region from the β subunit, and was coexpressed into COS cells along with β, γ and δ subunits, toxin-binding activity was not detected on the surface of the transfected COS cells (Fig. 1). Thus the transmembrane and cytoplasmic domains of the α subunit seem important for assembly. A similar result was observed with a chimeric subunit cDNA (α–β1) in which only the cytoplasmic loop between M3 and M4 of the α subunit was replaced by the corresponding loop of the β subunit (Fig. 1). The failure of the chimeric subunit to support AChR assembly could be explained in two ways. One possibility is that alteration of the cytoplasmic loop of

the α subunit might prevent it from folding correctly. To test this possibility, we examined the ability of the α–β1 chimeric subunit to bind α-BTX and to associate with the δ subunit. The α and α–β1 subunits were each expressed in COS cells, both alone and with the δ subunit. Because toxin-binding activity is generally not detected on the surface of COS cells when incomplete combinations of subunits are expressed (Gu et al., 1991b; Yu and Hall, 1991), we measured internal toxin-binding activity (presumably in the ER) after permeabilization of the cells with saponin (Blount et al., 1990). After transfection with either α or α–β1 cDNAs, toxin-binding activity was detected (Fig. 2), indicating that the α–β1 subunit, like α, is capable of folding correctly. The toxin-binding activity of both α and α–β1 was increased significantly by co-expression with the δ subunit (Fig. 2). The increased toxin-binding activity for the α subunit seen under these conditions has previously been shown to occur because association with δ subunit decreases its rate of degradation (Blount et al., 1990; Chavez and Hall, 1991). The increase seen for the α–β1 (Fig. 2) thus suggests that it associates with δ subunit. The α–β1 subunit, like α, also associates with δ_β and ε_β (results not shown).

We tested directly for association between α–β1 and δ subunits by immunoprecipitation (Fig. 3). When either α or α–β1 subunits were coexpressed with the δ subunit, toxin-binding activity was immunoprecipitated with a monoclonal antibody (MAb88B, Froehner et al., 1983) that specifically recognizes the δ subunit. Taken together, the toxin-binding and immunoprecipitation experiments show that the α–β1 chimeric subunit can fold correctly to acquire

toxin-binding activity, and can associate with the δ subunit. Its inability to support AChR assembly must therefore arise from its lack of competence at later stages of the assembly pathway.

α subunit without a cytoplasmic loop can form a heterotrimer with the δ subunit

To test further the role of the cytoplasmic loop in the later steps of AChR assembly, we examined a fragment of the α subunit, αM1, that contains only the N-terminal domain and the first transmembrane domain. Our laboratory has previously shown that αM1 expressed in COS cells can fold to acquire toxin-binding activity and can associate with the δ subunit (Verrall and Hall, 1992; Chavez and Hall, 1992). To see whether αM1 can participate in heterotrimer formation, we transfected COS cells with cDNAs for αM1, β and δ subunits. Extracts of the transfected cells were then incubated with ^{125}I-α-BTX and immunoprecipitated with a monoclonal antibody specific for the β subunit (MAb124). The ability of the antibody to immunoprecipitate toxin-binding activity indicates that a heterotrimer was formed (Gu et al., 1991b; Saedi et al., 1991), as toxin-binding activity was not precipitated when the δ subunit was not expressed (Fig. 4). The extent of heterotrimer formation seen when αM1 was expressed was comparable to that seen when the intact α subunit was expressed. Although αM1 can form a heterotrimer with β and δ subunits, AChR is not expressed on the surface (not shown). This experiment suggests that the cytoplasmic loop of the α subunit is not needed for the heterodimer and heterotrimer formation, but is required for assembly of a pentamer.

A region near M4 in the long, cytoplasmic loop of the α subunit carries subunit-specific information for assembly

To define which parts of the long, cytoplasmic loop of the α subunit are required for AChR assembly, systematic deletions or substitutions were made throughout the loop (Fig. 5), and the ability of the mutated subunits to support assembly of the AChR in transfected COS cells was examined. Because the entire β loop was inactive when substituted into the α loop, all smaller substitutions were made using the corresponding β loop sequences. Examination of the deletion mutants showed that surface AChR expression was sensitive to removal of only two regions, a 10 amino acid region near the N-terminus of the loop (amino acids 312-322), and a longer region of about 40 amino acids at the C-terminus that included the amphipathic helix. In both cases, the possibility of a defect in transport of a fully assembly receptor was ruled out by the failure to find assembled AChR in cell extracts. When the corresponding sequence from the β sequence was substituted for the α sequence at the regions of amino acid 312-322 and 399-409, surface expression was observed, showing that these regions were not responsible for the failure of α–β1 to assemble. Likewise, substitution for amino acids α367-382 with the β sequence also resulted in surface expression. In contrast, substitution of the next nine amino acids (α382-391) with the β sequence did not result in AChR expression. Substitution of the region (α391-399) also abolished AChR assembly. Thus the defect in assembly appears to be within a 17 amino acid

region (α382-399) at the C-terminus of the loop, just before the fourth transmembrane region.

To determine which steps of assembly were affected by the deletions at the C-terminal end of the loop, the ability of the mutated subunits to fold correctly (Fig. 6), to associate with the δ subunit (Fig. 7, 8) and to form heterotrimer with the β and δ subunits (Fig. 9) was examined as described above. In all cases, the subunits behaved as did the intact α subunits. The mutated α subunits acquired toxin-binding activity, as determined by saponin assay (Fig. 6, not shown). The toxin-binding activity of the mutated α subunits was increased by the association with the δ subunit (Fig. 7, not shown). Heterodimers of the mutated α subunits with the δ subunit were successfully precipitated by the δ subunit specific antibody (Fig. 8). Furthermore, the mutated α subunits were able to associate with the β and δ subunit to form heterotrimers (Fig. 9, not shown). Thus the region near M4 must be important for a late step in AChR assembly.

The cytoplasmic domain of β subunit is required for AChR assembly

A chimeric β_α subunit was constructed in the opposite way as described for δ_β, ε_β and α_β, with the N- and C-terminal domains derived from the β and the mid-region from the α subunit. When the cDNA of this chimeric subunit was transfected into COS cells along with cDNAs of the α, δ and ε subunits, surface AChR was not detected, suggesting that the cytoplasmic and transmembrane domains are important. Similar results were found for chimeric β_γ, β_ε, and β_δ which have the same structure schemes as the chimeric β_α except

that the M1-M4 fragments were from γ, ε and δ, respectively (data not shown). We then constructed a chimeric β–α1 subunit with a smaller region, the long cytoplasmic loop, replaced by the corresponding α sequence. The chimeric β–α1 subunit, made in the same way as described for α–β1, contains all of the β subunit except the long loop from the α subunit. When this chimeric subunit was coexpressed in COS cells with the α, δ and ε subunit, no surface AChR was detected, clearly indicating that the cytoplasmic loop is necessary for completing AChR assembly(data not shown).

Systematic deletions and mutations of 7–15 amino acid fragments were made from the beginning to the end of the loop as described previously for the α subunit. We found two regions that were important, a 28 amino acid sequence at the beginning (β310-338) and a 25 amino acid region near the end (β408-418; β430-445) of the loop. The β subunit with deletion or mutation of those regions did not support AChR assembly when coexpressed with the α, δ and ε subunits (Fig. 10). This result suggests that only these regions, not the whole loop, are involved in assembly of AChR.

Discussion

Chapters II and III have shown: a) that the lumenal domains of the ε and δ subunits are sufficient to mediate heterodimer formation, the first step in AChR assembly; and b) that the transmembrane and cytoplasmic domains of the ε and δ subunits carry no subunit-specific information that is necessary for AChR assembly. The results in this Chapter show that specific sequences in the cytoplasmic loops of the α and β subunits are required for complete AChR assembly and that the sequences are important for a step in assembly that is subsequent to heterodimer formation.

The conclusion that subunit-specific sequences in the loop are required for AChR assembly arises from the study of chimeric subunits in which either the entire regions between M1 and M4, the cytoplasmic loops between M3 and M4, or short sequences within the loop, were exchanged between α and β subunits. In constrast to the ε and δ subunits, in which the M1 through M4 regions could be replaced without affecting AChR assembly, chimeric subunits in which this region from α and β were exchanged were inactive in assembly. Mutagenesis experiments then narrowed the required sequences for each subunit. For the α subunit, a specific sequence of 17 amino acids near the C-terminal end of the loop appears to carry subunit-specific information. For the β subunit, three specific sequences were found: a 10 and a 15 amino acid sequence near the C-terminal end that are separated by 12 non-sensitive amino acids,

and a 28 amino acid sequence near the M3 segment at the beginning of the loop.

One difficulty in interpreting the failure of chimeric or mutated subunits to support assembly is in distinguishing the absence of specific sequences that are required for assembly from the failure of the subunits to fold properly. In the case of the α subunit, whose folding confers toxin-binding upon it (Blount & Merlie, 1988), folding can be measured independently of assembly. In the case of each of the chimeric or mutated α subunits that failed to suppport AChR assembly, we showed not only that the subunit acquired toxin-binding and therefore was folded correctly, but that it could associate with the δ subunit to form a heterodimer. Thus the failure of these subunits to be assembled into the intact AChR can not be ascribed to a nonspecific, global alternation in subunit structure.

Unfortunately, there is no comparable measure for folding of the β subunit. Because the sequences involved, however, are at least partially homologous to those in the α subunit that are required for assembly, it seems reasonable to suppose that they also participate in specific subunit interactions.

In the case of the α subunit we were also able to test whether the cytoplasmic regions were required for formation of the αδβ heterotrimer. We found that each α subunit with a short sequence substituted from the β subunit that failed to support assembly was competent for heterotrimer formation with the δ and β subunits.

Further confirmation of the dispensibility of the cytoplasmic loop for this interaction is the finding that αM1, lacking the long cytoplasmic loop, also formed an αδβ heterotrimer. We have shown in Chapter 3 that αM1 can form a heterodimer with the chimeric subunit δ_{β}. As noted there, the specific interpretation of this result depends upon the particular model for the order of the subunits which is being considered.

Since α subunits in which the cytoplasmic loop is missing or mutated can form αδ heterodimer and αδβ heterotrimer, the sensitive sequence may be required for the final step of assembly, in which the heterotrimers and/or heterodimers associate to form the intact receptor. Alternatively, they could be involved in αγβ heterotrimer formation. We attempted to measure whether the mutated α subunits could undergo heterotrimer formation with the γ or ε subunits, but because of the low level of this species (Gu et al, 1991), we were unable to do so. Thus the loop sequences could be required for either the last step of the assembly pathway or for one of the heterotrimer steps. The second possibility is particularly plausible since according to either scheme of subunit order, the α and β subunits are adjacent in the αγβ heterotrimer.

The finding of a sensitive sequence in the β subunit at a comparable position, near transmembrane region M4, and another near transmembrane region M3, raises the possibility that the cytoplasmic regions in the α and β subunits interact. The previous finding that neither of the other two subunits (δ or ε) contain

subunit-specific information in this region (Chapter III) is consistent with this interpretation. As the cytoplasmic loop between M1 and M2 is exceedingly short, the interactions between AChR subunits that form the channel vestibule on the cytoplasmic side of the membrane are likely to involve the cytoplasmic loops between M3 and M4.

Experimental Procedures

cDNAs

Full-length cDNA clones coding for the α, β, γ and δ subunits of mouse muscle nicotinic AChR were gifts from Drs. J. P. Merlie and N. Davidson: α, (Isenberg et al., 1986); β, (Buonanno et al., 1986); γ, (Yu et al., 1986) and δ, (LaPolla et al., 1984). The full-length cDNA for the mouse ε subunit was isolated as described (Gu et al., 1990). Each of the cDNAs were subcloned into the SV40 based expression vector pSM (Brodsky et al., 1990) at the multiple cloning site. An M13 origin in this vector was used to generate uracil-containing single-stranded DNAs for site-directed mutagenesis (Geisselsoder et al., 1987); (Kunkel, 1985).

Site-Directed Mutagenesis

Site-directed mutagenesis was conducted according to previously described methods (Geisselsoder et al., 1987); (Kunkel, 1985), using synthetic oligodeoxyribonucleotides prepared with an automatic DNA synthesizer. Uracil-containing single-stranded DNAs, prepared by infecting plasmid-transformed E.coli CJ236 (dut-ung-F') cultures with M13 helper phages, were used as the templates. Each of constructed cDNAs was analyzed by restriction enzymes.

Construction procedures: Two novel restriction enzyme sites, SnaBI and SpeI, were introduced into pSMα, pSMβ, pSMγ, pSMδ and pSMε, SnaBI at the beginning of M1 and SpeI at the ending of M4 of

each subunit. The pSMα, pSMβ, pSMγ, pSMδ and pSMε with the introduced sites were then digested with the two restriction enzymes. The chimeric plasmids for chimeric α_β, β_α, β_γ, β_ε, β_δ subunits were constructed by purifying fragments, and ligating them to the corresponding vectors that had been digested with the same restriction enzymes. They are also analyzed and mapped by restriction enzymes.

Another two new restriction enzyme sites, Afl II and Mlu I, were introduced into pSMα and pSMβ. Afl II was inserted to right after M3, and Mlu I to right before M4. The chimeric α–β1 and β–α1 were constructed by digesting the resulting pSMα and pSMβ with the Afl II and Mlu I, the fragments were purified and inserted to the corresponding vectors which have been cut with the same restriction enzymes. The chimeric α–β1 and β–α1 subunits without Afl II and Mlu I sites were constructed by purifying the uracile-containing single-stranded pSM α–β1 and pSM β–α1 as the templates to which oligonucleotides annealed which return the mutated amino acids to the original sequences to remove Afl II and Mlu I sites.

Where appropriate, @ is used to indicate a deleted segment; bases that are altered to produce amino acid changes or to introduce a restriction site are underlined.

Mutations in the cytoplasmic loop of the β subunit:

β(Δ310–323). The 13 amino acids, HHRSPHTHQMPFW, of the β subunit loop were deleted by the oligonucleotide CTT AGT GTT GT<u>A</u>GT<u>A</u>CTC

AAC CTG @ GTC CGC CAG ATC TTC. Sca I site was created without amino acid change.

β(Δ323–338). The 15 amino acids of the β loop, VRQIFIHKLPPYLGL, were deleted with the oligonucleotide CAA ATG CCC TTT TGG @AAG AGG CCC AAA CCC. Bgl II site in the position of amino acid sequence, QIF, was deleted.

β(*405–420). Next 15 amino acids GLPQELREVISSISY were mutated to LKHPEVKSAIEGVKY, corresponding to the sequence in the amphipathic helix of the loop of the α subunit, by oligonucleotide CCA ACC CGG GCT GTA CTT AAG CAT CCG GAG GTA AAA TCG GCC ATT GAG GGA GTC AAA TAC ATG GCC CGA CAG. BspE I site underlined was created.

β(Δ408–428). 20 amino acids in the putative amphipathic helix of the β loop, QELREVISSISYMARQLQEQ, were deleted by the oligonucleotide CCA ACC CGG GCT GTA GGC CTG CCT @ GAG GAC CAC GAC GCA CTG. Stu I site was created without any amino acid change.

β(Δ430–445). 15 amino acids, HDALKEDWQFVAMVV, were deleted by oligonucleotide CAG GAA CAG GAG GAC @ GAC CGT CTT TTT CTG. BstX I site in the position of amino acid sequence, AMVVD, was deleted. The constructions of the remaining mutated β subunits have been described in Chapter VI.

Mutations in the cytoplasmic loop of the α subunit:

α(Δ312–322). The 10 amino acids VRKVFIDTIP were deleted by the oligonucleotide ACC CAC ATC ATG CCC GAA TTC @ AAC ATC ATG TTT TTC. EcoR I site was introduced.

α(Δ369–390). The 21 amino acids PEVKSAIEGVKYIAETMKSDQ covering the putative amphipathic helix of the loop in the α subunit were deleted by the oligonucleotide TCT CCG CTG ATC AAG CTT @ GAG TCC AAT AAC GCC GCT. Hind III site was introduced.

α(*382–391). The 9 amino acids IAETMKSDQ in the amphipathic helix were mutated to MARQLQEQE, corresponding to the sequence of the β subunit, by the oligonucleotide GAG GGC GTG AAG TAC ATGGCA AGG CAG CTG CAG GAA CAA GAG GAG TCC AAT AAC GCC. Pvu II site was introduced.

α(Δ391–399). The 8 amino acids ESNNAAEE were deleted by the oligonucleotide ATG AAG TCA GAC CAG @CTG AAG TAT GTT GCC ATG. Pvu II site was introduced.

α(*391–399). The 8 amino acids ESNNAAEE were substituted to the corresponding sequence EDHDALKED of the β subunit with the oligonucleotide AAG TCA GAC CAG GAG GAC CAT GAT ATC CTT AAG GAG GAT TGG AAG TAT CTT GCC. EcoR V site was created.

α(Δ399–409). The 10 amino acids WKYVAMVMDH were deleted by the oligonucleotide AAC GCC GCT GAG GAA @TTC CTC CTC GGA GTC TTT.

EcoR I site was introduced by changing I to F, the amino acid right after the last amino acid of the 10 amino acids.

α(*399–409). The 10 amino acids WKYVAMVMDH were mutated to the corresponding sequence WQFVAMVVDR of the β subunit with oligonucleotide GCT GAG GAA TGG CAG TTT GTT GCC ATG GTG GTC GAC CGC ATC CTC CTC. Sal I site was created.
The constructions of the remaining mutated α subunits have been described in Chapter VI.

Transfection

Transfection in COS cells was performed as previously described (Gu et al., 1990) and it is a modified DEAE-dextran transfection procedure (Seed and Aruffo, 1987). Cells with 30-50% confluence in a 60-mm dish were incubated for 3-5 hrs at 37 °C with a transfection mixture: an appropriate amount of plasmid cDNAs added in DME-H21 supplemented with 1.0 % heat-inactivated fetal bovine serum, 0.10 mM chloroquine diphosphate and 0.4 mg/ml DEAE-Dextran. The amount of plasmid used for each subunit in the transfection mixture was determined empirically to produce maximal cell surface AChR expression. For analysis of cell surface AChR expression, 3 ml of the transfection mixture was added to a 60 mm dish containing cDNAs of α: 1.32 ug, β: 0.66 ug, γ or ε: 1.0 ug and δ: 0.26 ug. For immunoprecipitation of heterodimer, 5 ml (3 ml) of the transfection mixture was added to a 100 mm (60 mm) dish containing 2.0 ug (1.0 ug) of each cDNA of the α and δ or δ_β subunits. For intracellular ^{125}I-α-BTX binding assay, 3 ml of the transfection

mixture was added to a 60 mm dish containing indicated plasmid cDNAs. After 3-4 hrs of incubation, the transfection solution was removed, the cells were treated with 10% DMSO-PBS for 2 min at room temperature and replaced with 5 ml (60 mm dish) or 10 ml (100 mm dish) of the growth medium (10% fetal bovine serum in DME H21 supplemented with 100 u/ml penicillin and streptomycin). Twenty-four hours later in 37 °C, the cells were trypsinized and distributed into 3 wells of 24 well plates (surface AChR assay and intracellular ^{125}I-α-BTX assay) or 60 mm dishes (heterodimer immunoprecipitation assay). The surface AChR assay and heterodimer immunoprecipitation analysis were carried out after an additional 24 hours.

Surface AChR Assay

Surface AChR expression was determined by incubating intact transfected cells for 90 min at 37 °C with 10 nM ^{125}I-α-BTX (Amersham Corp., Arlington Heights, IL). Nonspecific binding was measured by sham transfections. Unbound α-BTX was removed by washing the cells with PBS. The amount of bound toxin was measured by solubilizing the cells in 0.1 M NaOH and counting the radioactivity in a gamma counter.

Intracellular ^{125}I-α-BTX binding assay

The intracellular ^{125}I-α-BTX binding was measured by incubating the transfected cells for 90 min on ice with 0.5% saponin buffer containing 10 nM ^{125}I-α-BTX. The cells were then washed for three times with the 0.5% saponin buffer and solubilized with

0.1N NaOH and counted. Nonspecific binding was measured by sham transfection.

Immunoprecipitation of heterodimers

Immunoprecipitation with subunit-specific antibodies was performed as previously described (Gu and Hall, 1988a). The COS cells transfected with the α alone, or α and δ, or α–β1 and δ subunits were lysed in a solubilizing buffer containing 50 mM Tris-HCl, pH7.4, 50 mM NaCl, 1.0 % Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM Na tetrathionate, 1 mM N-ethylmaleimide, 0.4 mM PMSF, 10 U/ml aprotinin, 20 ug/ml leupeptin. The cell lysate samples (80 ul) were incubated with MAb88B coupled sepharose in the presence of 10 nM ^{125}I-α-BTX for 2 hr at 4 ^{o}C. All the precipitates were washed three times with washing buffer containing 50 mM Tris-HCl, pH 7.4, 1 M NaCl and 1% Triton X-100, before being counted in a gamma counter. Control immunoprecipitations were carried out using sham transfected COS cells.

Immunoprecipitation of heterotrimers in the transfected cells was carried out by β subunit specific antibody, MAb124. Immunoprecipitation procedure is almost the same as the previous one except COS cells were transfected with an equal amount of three plasmids, α, β and δ.

Figure 4-1. A schematic diagram of the structures of chimeric α subunits and their surface and intracellular expression when cotransfected with cDNAs for the β, δ and ε subunits into COS cells.

Figure 4-1.

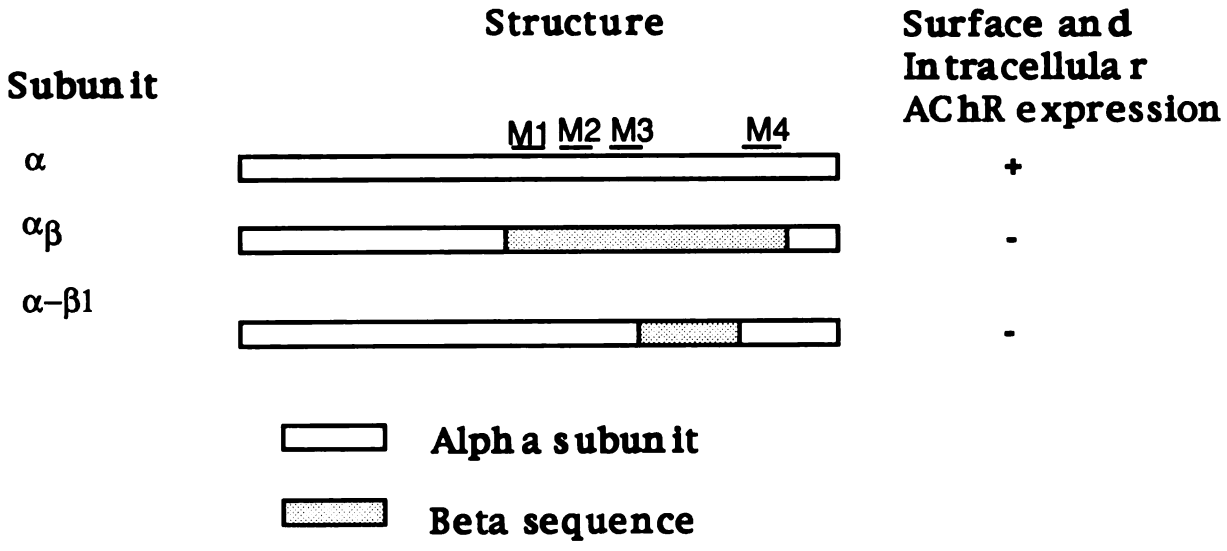

Structure
Surface and Intracellular AChR expression
Subunit
M1 M2 M3 M4
α
+
αβ
-
α–β1
-
Alpha subunit
Beta sequence

Figure 4-2. Heterodimer formation of α–βl with δ subunit, as determined in a toxin-binding assay.

COS cells were transfected with cDNAs as indicated. Twenty-four hours later the cells were split. Triplicate samples of the cells were incubated with ^{125}I-labeled α-BTX, washed and counted in a gamma counter.

Figure 4-2.

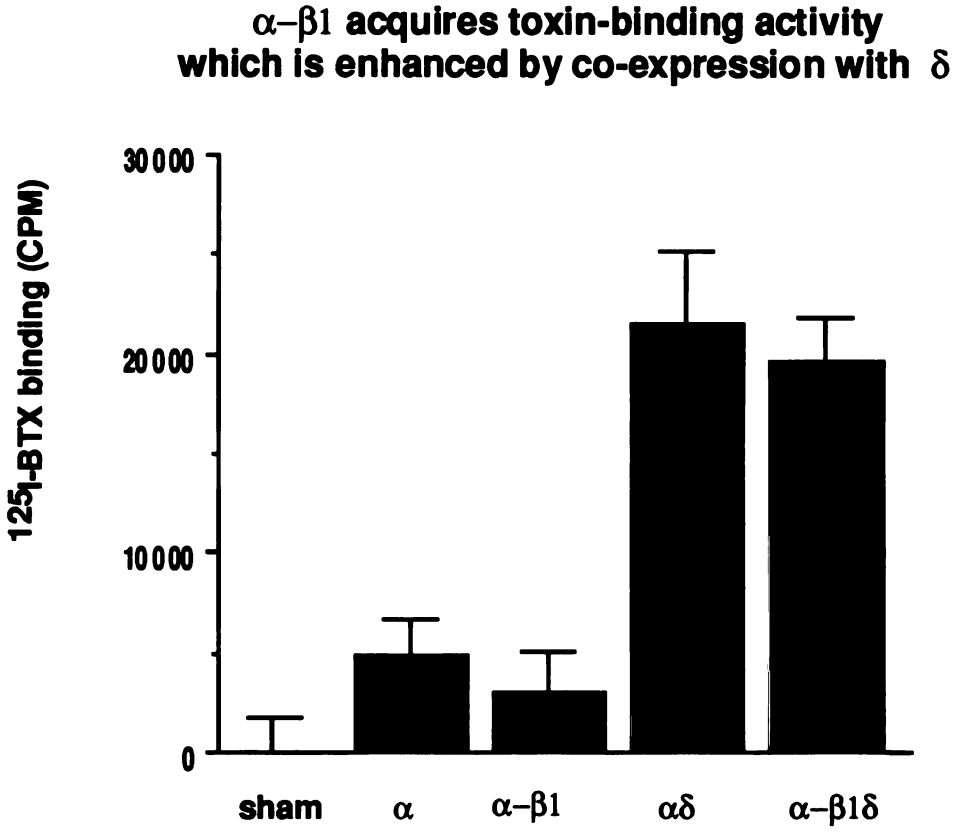

α–β1 acquires toxin-binding activity
which is enhanced by co-expression with δ
125I-BTX binding (CPM)
30000
20000
10000
0
sham
α
α–β1
αδ
α–β1δ

Figure 4-3. Association of α–βl with δ demonstrated by immunoprecipitation.

COS cells were transfected with cDNAs as indicated. Fourty-eight hours later cells were pelleted and resuspended in extraction buffer. Triplicate samples of the extracts were incubated with ^{125}I-labeled α-BTX and MAb88-Sepharose at 4 ^{o}C for 2 hours, pelleted, washed and counted in a gamma counter.

Figure 4-3.

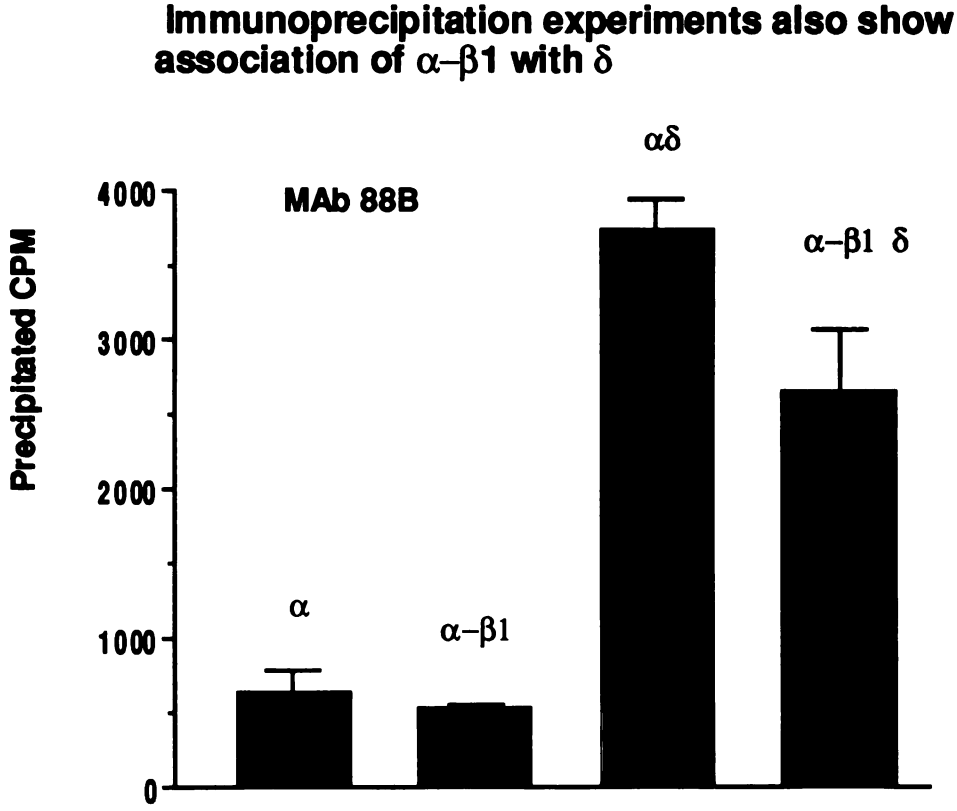

Immunoprecipitation experiments also show association of α–β1 with δ
αδ
MAb 88B
α–β1 δ
Precipitated CPM
4000
3000
2000
1000
0
α
α–β1

Figure 4-4. Heterotrimer formation of αM1, δ and β subunits determined by coimmunoprecipitation.

COS cells were transfected with cDNAs as indicated. Fourty-eight hours later cells were pelleted and resuspended in extraction buffer. Triplicate samples of the extracts were incubated with ^{125}I-labeled α-BTX and MAb124 at 4 ^{o}C for 2 hours, then incubated with anti-rat IgG-linked sepharose, pelleted, washed and counted in a gamma counter.

Figure 4-4.

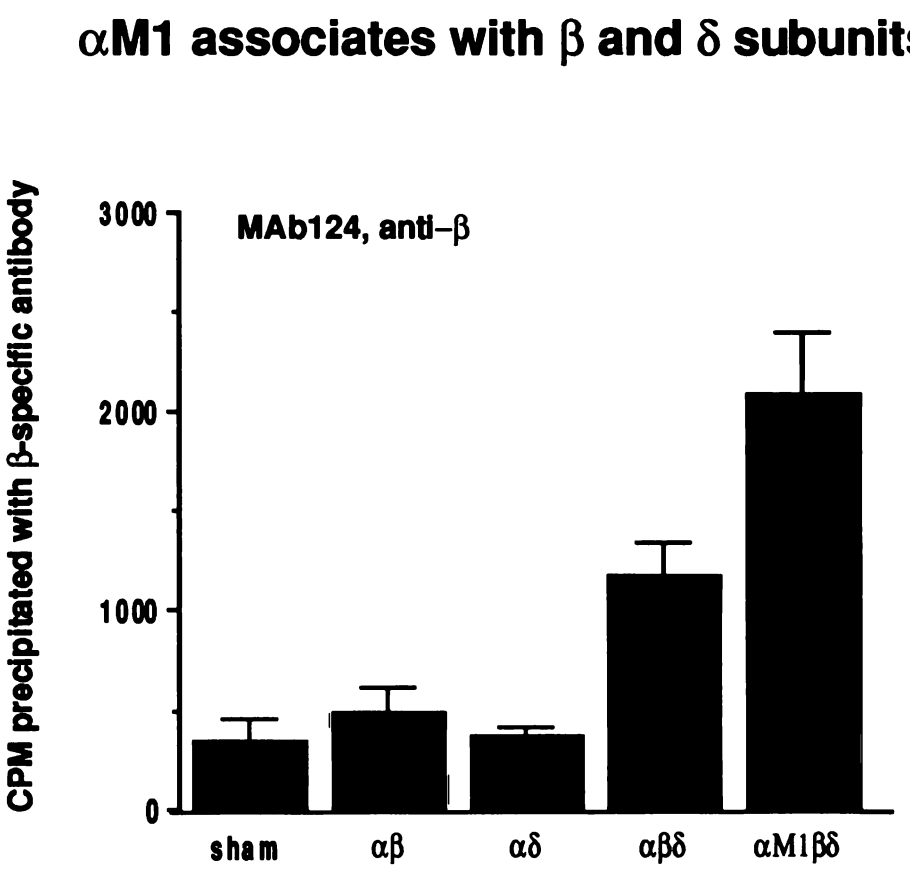

αM1 associates with β and δ subunits
CPM precipitated with β-specific antibody
3000
2000
1000
0
MAb124, anti-β
sham
αβ
αδ
αβδ
αM1βδ

Figure 4-5. A schematic diagram of the structures of mutated α subunits and their surface expression when each was coexpressed with the β, δ and ε subunits into COS cells. The surface expressions were determined in surface assay.

Figure 4-5

Summary of mutations in the long cytoplasmic loop of the α subunit and their effect on AChR assembly

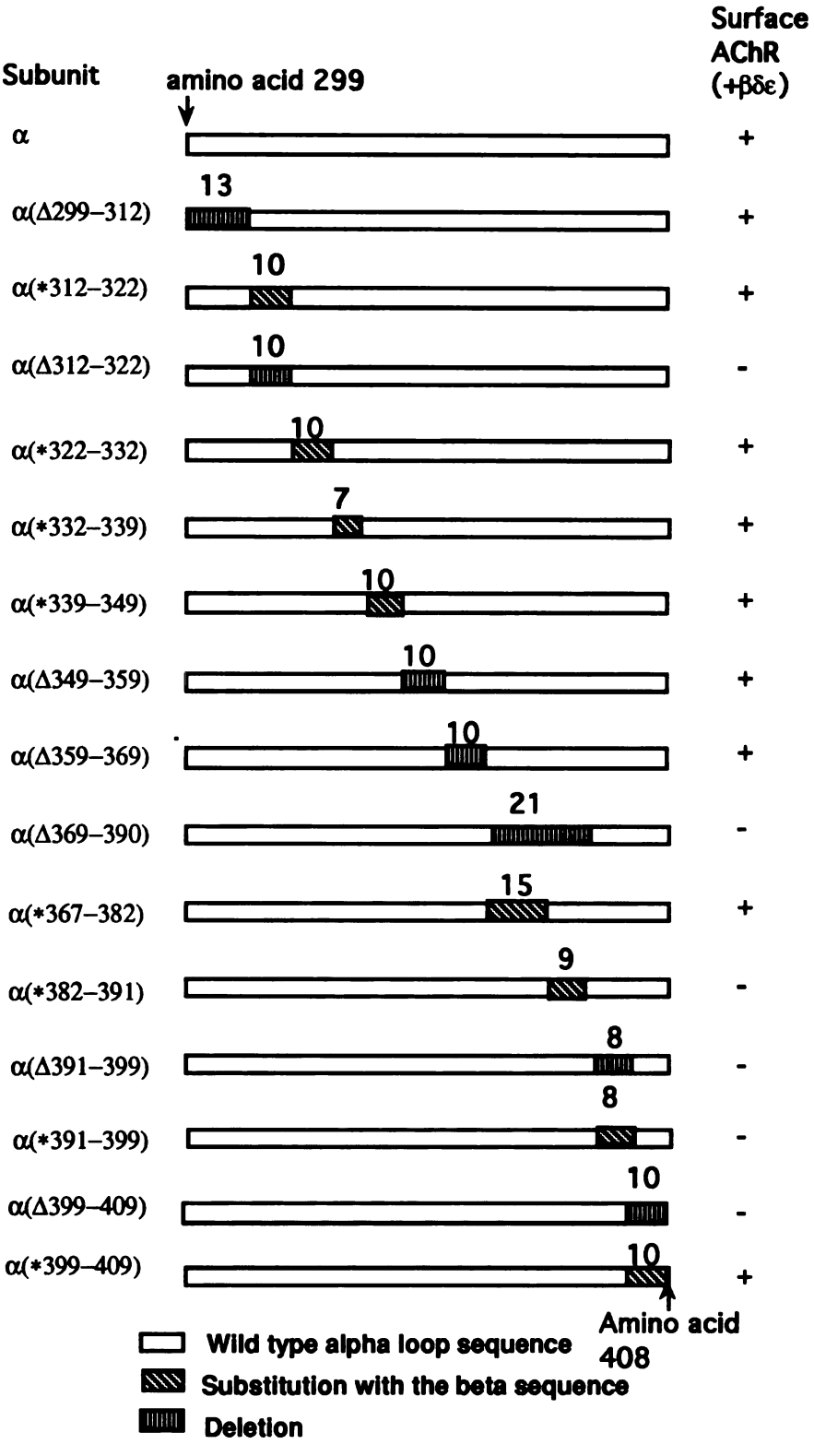


Figure 4-6. Toxin-binding activity of the mutated α subunits which fail to support AChR assembly.

The experimental procedure was the same as described in Figure 4-2.

Figure 4-6.

α mutants fold correctly to acquire toxin-binding activity

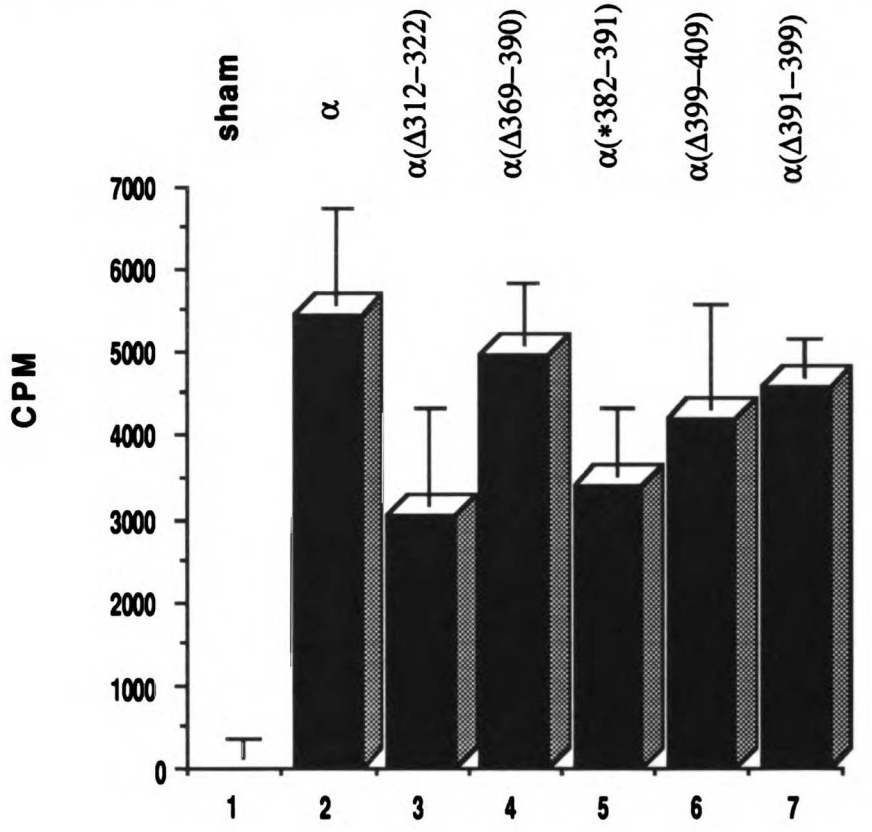


Figure 4-7. Association of the mutated α subunits which fail to support AChR assembly with δ subunits to form heterodimers as determined by saponin assay.

Experiments were carried out as described in Figure 4-2.

Figure 4-7.

Mutant α subunits associate with δ subunits as measured by increased toxin-binding

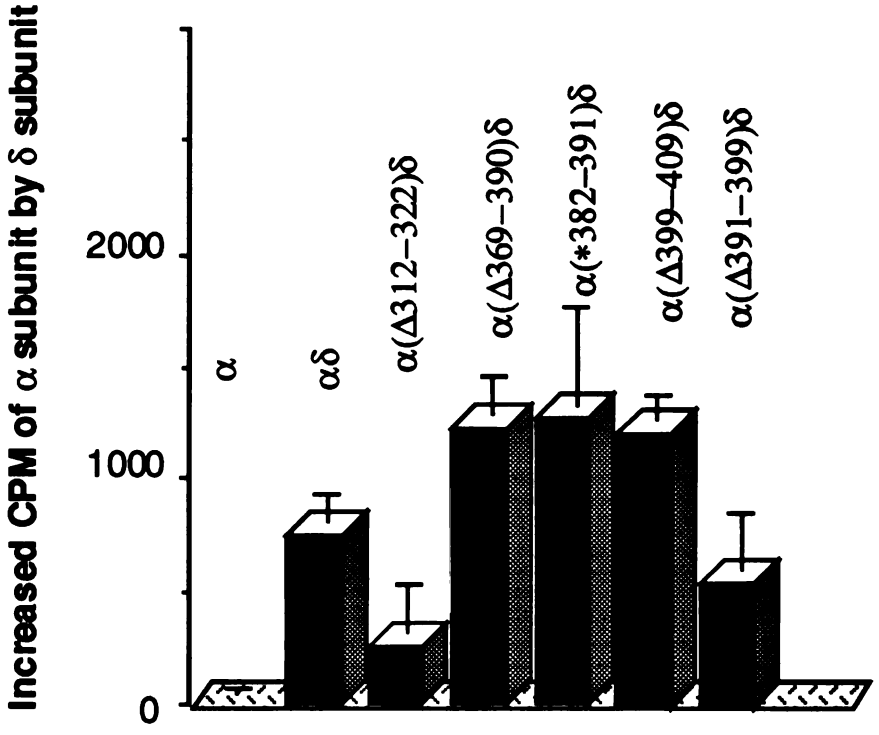


Figure 4-8. Heterodimer formation by all five mutated α subunits with δ subunits as determined by immunoprecipitation.

Experiments were performed as described in Figure 4-3.

Figure 4-8.

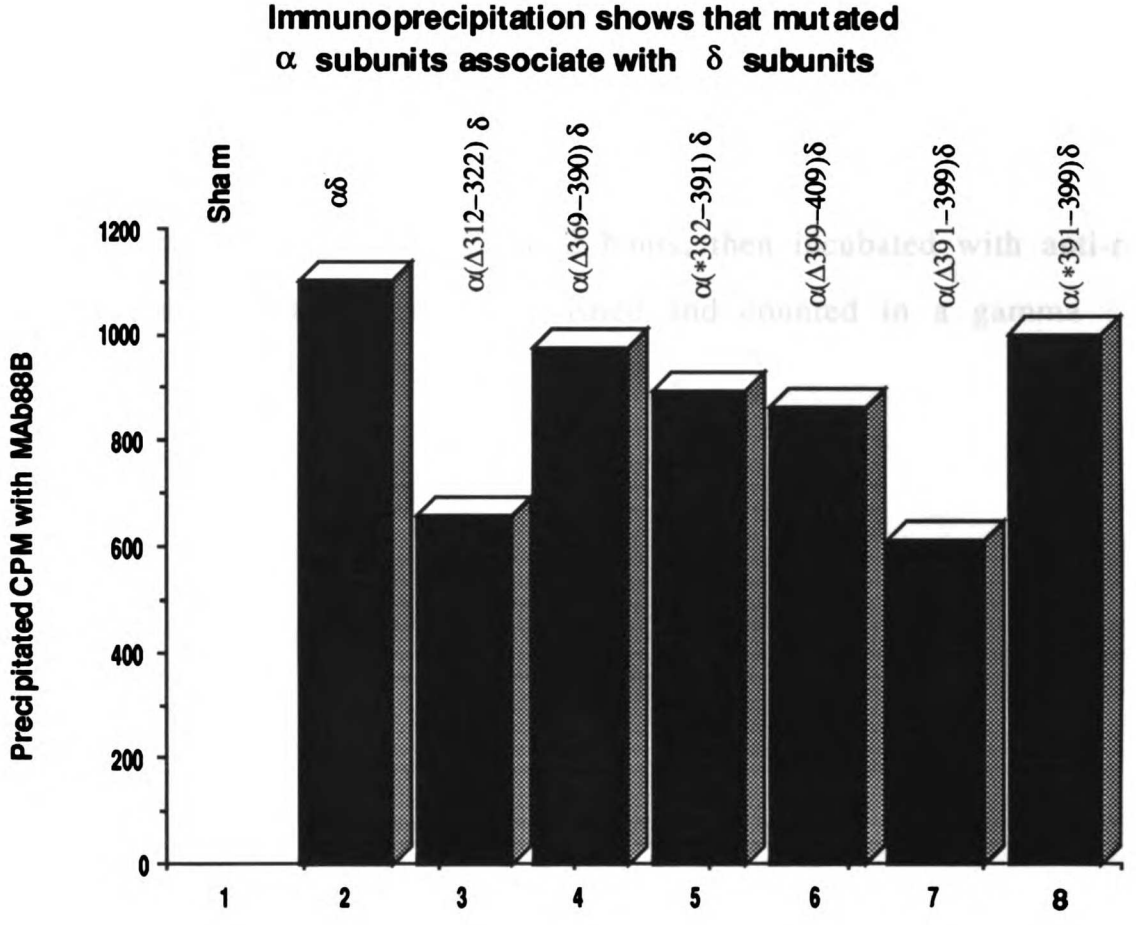

Immunoprecipitation shows that mutated
α subunits associate with δ subunits
Precipitated CPM with MAb88B
1200
1000
800
600
400
200
0
Sham
αδ
α(Δ312–322) δ
α(Δ369–390) δ
α(*382–391) δ
α(Δ399–409)δ
α(Δ391–399)δ
α(*391–399)δ
1
2
3
4
5
6
7
8

Figure 4-9. Heterotrimer formation of the mutated α subunits with β and δ subunits determined by immunoprecipitation

COS cells were transfected with cDNAs as indicated. Fourty-eight hours later cells were pelleted and resuspended in extraction buffer. Triplicate samples of the extracts were incubated with ^{125}I-labeled α-BTX and MAb124 at 4 ^{o}C for 2 hours, then incubated with anti-rat IgG-linked Sepharose, pelleted, washed and counted in a gamma counter.

Figure 4-9.

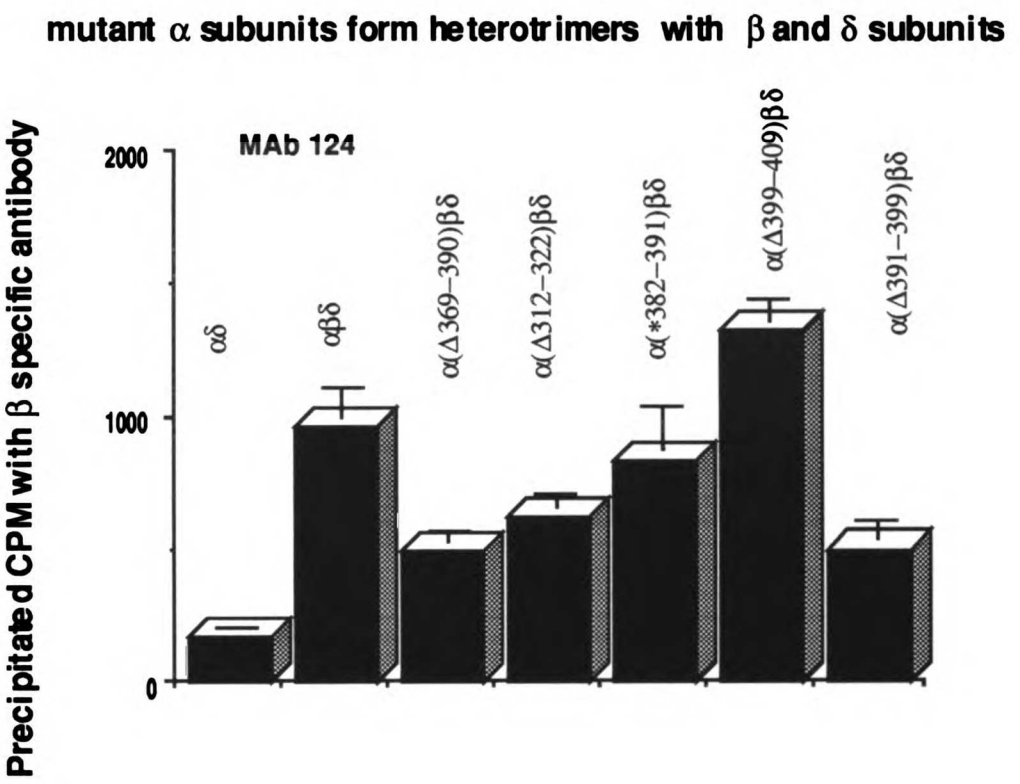
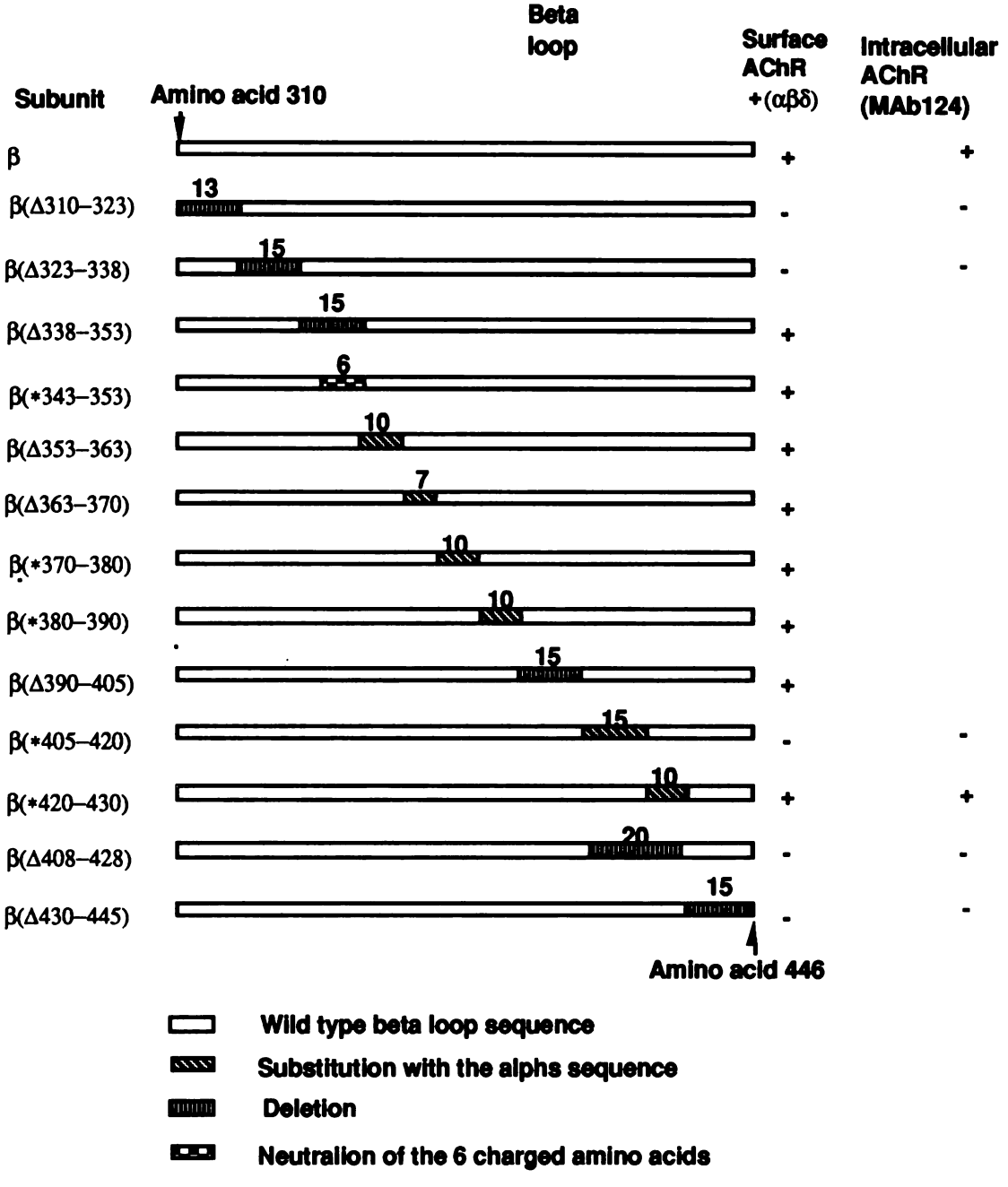

mutant α subunits form heterotrimers with β and δ subunits
Precipitated CPM with β specific antibody
MAb 124
2000
1000
0
αδ
αβδ
α(Δ369–390)βδ
α(Δ312–322)βδ
α(*382–391)βδ
α(Δ399–409)βδ
α(Δ391–399)βδ

Figure 4-10. A schematic diagram of the structures of mutated β subunits and their surface expression when each was coexpressed with the α, δ and ε subunits into COS cells. The surface expressions were determined in surface assay.

Figure 4-10.


Beta loop
Surface AChR +(αβδ)
Intracellular AChR (MAb124)
Subunit
Amino acid 310
β
β(Δ310–323)
β(Δ323–338)
β(Δ338–353)
β(*343–353)
β(Δ353–363)
β(Δ363–370)
β(*370–380)
β(*380–390)
β(Δ390–405)
β(*405–420)
β(*420–430)
β(Δ408–428)
β(Δ430–445)
13
15
15
6
10
7
10
10
15
15
10
20
15
Amino acid 446
Wild type beta loop sequence
Substitution with the alphs sequence
Deletion
Neutralion of the 6 charged amino acids

Chapter Five

The C-termini of the α and δ Subunits Participate in a Late Step of ACh Receptor Assembly

Introduction

AChR assembly requires that each of the four subunits recognize each other so that they can associate together correctly. As described in Chapter II, the extracellular domains (the N-terminus before M1 and the C-terminus after M4) are sufficient for the ε subunit to recognize the α subunit to form a heterodimer and then to support assembly of the intact receptor in the presence of other required subunits. A similar structure-function relationship was demonstrated for the δ subunit in Chapter III. The dominant negative approach demonstrated remarkably that the N-terminal fragments of the α, γ and δ subunits can inhibit surface AChR expression and heterodimer formation (Verrall and Hall, 1992). Therefore, the N-terminal regions of the AChR subunits contain the recognition information for the first step of assembly. However, the N-terminal domains of the AChR subunits alone do not support AChR assembly and expression on the surface. The remaining portions of each subunit of the AChR must therefore play a role in AChR assembly. Experiments described in Chapter IV established that the main intracellular domains (loops) of the α and β subunits play a role in a late step of assembly. Although they are short, the C-termini of the subunits also seem to be important structurally and functionally. According to the amino acid sequence, these domains in the δ, γ and ε subunits, but not the α subunit, are proline-rich domains. A number of known transcriptional factors in their C-terminal domains contain similar proline-rich domains that have been proposed to be involved in protein-protein interactions (Santoro et al., 1988; Paonessa et al.,

1988; Gil et al., 1988). Here, we report that the C-terminal domains of the α and δ subunits appear to play a role in the last part of AChR assembly, and that the C-terminus of the δ subunit can replace that of the α subunit to accomplish AChR assembly.

Results

The C-terminus of the α subunit is required for AChR assembly

To study what role the C-terminal domain of the α subunit plays in assembly, we specifically removed it by truncating the α subunit right after M4 (αT). When cDNA for the truncated αT was alone transfected into COS cells, extracts of the transfected cells were immunobloted with MAb61 which specifically recognizes the loop of the α subunit. Two bands of the truncated α subunit were found, one glycosylated and one not (not shown) just as with the native α subunit. COS cells, transfected with cDNA encoding either for αT, or α, were permeabilized with saponin and labeled with ^{125}I-α-BTX as described previously (Chapter III). The intracellular ^{125}I-α-BTX binding showed that the αT had about 30-50% of the ^{125}I-α-BTX binding activity of the normal α subunit (Fig. 1). The toxin binding activity of the αT subunit suggested that it is folded correctly. To explore whether or not αT associates with the δ or ε subunit to form a heterodimer, COS cells were transfected with the cDNAs for the α plus δ, αT plus δ, α plus ε, and αT plus ε subunits. The intracellular ^{125}I-α-BTX binding with the transfected COS cells demonstrated that the toxin binding activity of the αT subunit was slightly, but significantly, elevated by its coexpression with either δ or ε subunits (Fig. 2), suggesting association between the αT and δ or ε subunits.

The stably formed heterodimer αTδ was further precipitated by MAb88B which specifically recognizes the intracellular domain of the δ subunit (Fig. 3). However, αT was not able to support AChR assembly in the presence of the other required subunits, β, δ and ε subunits (Fig. 4). All these results suggested that the truncated αT is competent for αδ heterodimer and for αδβ trimer formation, but is not competent for the final step of assembly, presumably due to the missing the C-terminal domain. Therefore, the C-terminal domain of the α subunit must be involved in a late step of assembly.

The C-terminus of the δ subunit is required for assembly

To explore the function of the C-terminal domain of the δ subunit, it was deleted and the truncated δ subunit (δM4) characterized in the following ways. cDNA for the truncated δ subunit was transfected alone into COS cells, and the cells were labeled metabolically. A cell extract was then immunoprecipitated with MAb88B and subjected to SDS-PAGE and immunobloted. A band that was a little smaller than the δ subunit was detected, showing that the protein was expressed in COS cells. When cDNA for the truncated δ subunit was cotransfected with the cDNAs for the α, β and ε subunits into COS cells, no surface AChRs were detected (data not shown). When cDNA for the truncated δ subunit was transfected into COS cells along with the cDNA of the α subunit, immunoprecipitation of a heterodimer was not successful. The C-terminus of the δ subunit was further deleted at 3 different positions: removing last 8 amino acids, SEQDKRFI (δ/SED); removing last 17 amino acids, PFPGDPFSYSEQDKRFI

(δ/PFP) and removing last 24 amino acids, YNQPPLQPFPGDPFSYSEQDKRFI (δ/YNQ) (Fig. 5). When cDNA for each of the truncated δ/SEQ, δ/PFP and δ/YNQ subunits was transfected into COS cells, the proteins were expressed and then immunobloted after precipitation with MAb88B and SDS-PAGE (Fig. 6). The sizes of their bands became gradually smaller as the deletion got bigger. Their behavior in the gel was almost the same as with the normal δ subunit with respect to heterogeneity, indicating that they had appropriate post-translational modifications. When cDNA for the δ/SEQ was cotransfected with cDNAs for the α, β and ε subunits, the surface ^{125}I-α-BTX binding was found to be about 66% of that for the wild type AChR, suggesting that the AChR was able to be assembled with the truncated δ subunit and that the last 8 amino acids are not required for assembly. However, when cDNA for the δ/PFP or for δ/YNQ replaced the δ cDNA in the presence of the cDNAs for the α, β and ε subunits, no surface toxin binding was seen, indicating that neither of these truncated subunits was able to direct AChR expression (Table I). In contrast, mutating glycine to methionine in the C-terminus of the δ subunit (δG471M) decreased, but did not abolish, AChR assembly. Since glycine makes a turn in the primary structure of protein, G may determine the position of the C-terminal domain of the δ, so that it can recognize its partner. Mutating alanine to leucine and tryptophan to valine, in combination with mutating isoleucine to valine (δI227V, δA465L and δW466V) did not affect AChR assembly (Table I). Taken together, the data indicated that sequence of 9 amino acids next to the terminal

fragment of 8 amino acids in the δ subunit are critical for AChR assembly.

We further evaluated the function of the C-terminus of the δ subunit at the heterodimer level. When the cDNA for the δ/SEQ subunit was cotransfected with the α subunit alone, it generated 36% of the amount of heterodimer seen with the wild-type δ after immunoprecipitation with MAb88B. The δ/PFP exhibited a corresponding value of 18%, whereas the δ/YNP only showed only a negligible amount of heterodimer (Table I). It is very interesting that the δ/PFP was able to produce 18% of the normal amount of heterodimer with the α subunit, but could not support assembly of the whole AChR. These results indicated that the C-tail of the δ subunit is clearly involved in a late step of assembly, and is not needed for the first step of assembly, consistent with the results from dominant negative experiments (Verrall and Hall,1992).

Heterodimer formation of the α with the C-terminus-truncated δ subunits was further studied in sucrose gradients. When COS cells were transfected with cDNA for the α subunit along with that for each of the truncated δ subunits, and the transfected cells were labeled with ^{125}I-α-BTX, extracted with the solubilizing buffer and the extracts loaded onto the sucrose gradients. The δ/SEQ was found to be associated with the α subunit and formed a heterodimer peak of 6.3 S whereas the δ/PFP and δ/YNQ were not (Table I). The discrepancy in detection of the heterodimer, αδ/PFP, by the methods of immunoprecipitation and sucrose gradient sedimentation may

result from the long course of centrifugation (15.5 hr) which may cause dissociation of the heterodimer. These results also suggest that the C-tail of the δ subunit is important and that the 9 amino acids, PFPGDPRPY, may be involved in assembly.

The importance of the C-terminal domain of the δ subunit is confirmed by several chimeric δ proteins

The function of the C-tail of the δ subunit in assembly was also investigated by making chimeric subunits. The 9 amino acids, PFPGDPRPY, were also identified as crucial ones in this approach.

Using in vitro mutagenesis, the C-terminus of the δ subunit was substituted by the corresponding tail of either the α, β, γ or ε subunit (structure seen in Fig. 7), named as δ_α^s, δ_β^s, δ_γ^s, and δ_ε^s, respectively. All were expressed in COS cells and immunobloted after precipitation with MAb88B and SDS-PAGE (Fig. 6, not shown). When cDNA for each of them was cotransfected with cDNAs for the α, β and ε subunits, only the δ_γ^s produced significant surface AChR (Table II). When each of them were coexpressed with the α subunit into COS cells and the resulting heterodimers were immunoprecipitated with MAb88B, only the δ_γ^s and δ_α^s were able to form a heterodimer with the α subunit (Table II). The sucrose gradient experiments also confirmed that only the δ_γ^s subunit generated a heterodimer peak (6.3 S) (Table II), consistent with the results of immunoprecipitation. Thus the C-tail of the γ subunit can substitute for the δ C-tail in assembly. We then compared the amino acid sequences between the C-termini of the δ and γ subunits (Fig. 8) and found that the fragment

of 9 amino acids next to the last fragment of 8 amino acids in the C-termini of both subunits are almost identical. This presumably explains the functional replacement of the C-terminus of the δ subunit by that of the γ subunit.

All these experiments with chimeric δ subunits confirmed the results from the truncated δ subunits that the C-tail of the δ subunit is essential for whole receptor assembly. The fragment of 9 amino acids PFPGDPRPY, which is almost identical to the corresponding fragment in the γ C-tail, plays a role in late assembly. It coincidently contains high proline content which has been suggested to mediate protein-protein interaction in the transcription machinery.

The C-terminus of the δ subunit can replace the C-terminus of the α subunit to rescue the C-terminus-truncated α subunit to be assembled into the AChR

To investigate further the role of the C-terminus in assembly, we transfered the C-terminus of the δ subunit to the corresponding position of the β subunit to make a chimeric β subunit containing the C-terminus from the δ subunit ($\beta\delta^s$, Fig. 9). It is known that the α and β subunits do not associate with each other to form a heterodimer (Gu et al., 1991; Chapter II, III, IV). If the α subunit could associate with the $\beta\delta^s$ subunit, this data would suggest that the C-terminus of the δ subunit alone would contain recognition information to associate with α subunit. When cDNA for the $\beta\delta^s$ was cotransfected with cDNA for the α subunit, no heterodimer was detected by immunoprecipitation with MAb124 (data not shown).

This failure is not the result of misfolding of the $\beta\delta^s$, because it was readily seen as a band at the position of the β subunit in an immunoblot (Fig. 6) and because the $\beta\delta^s$ can function as the β subunit to support AChR assembly (data not shown) in the presence of α, δ and ε subunits. This suggested that the C-terminus alone of the δ subunit was not capable of associating with the α subunit, and that the $\beta\delta^s$ chimera could not replace the δ subunit in the steps of assembly.

To study whether the C-terminus of the δ subunit alone acts in a late step of assembly, we then connected the C-terminus of the δ subunit to the M4 of the α subunit to replace the C-terminus of the α subunit ($\alpha\delta^s$)(Fig. 9) because it is known that the N-terminal domain of the α subunit is sufficient for the first step of assembly (Chapter II, III, IV, Verrall and Hall, 1992). When cDNA for this chimeric $\alpha\delta^s$ subunit was cotransfected with cDNAs for the β, δ and ε subunit, the assembled surface AChR was successfully expressed (Fig. 10). In contrast, when the C-terminus of the β subunit was substituted for that of the α subunit (Fig. 9), this chimeric α subunit with the β C-tail was not able to support AChR assembly (Fig. 10). This result is not surprising as the C-terminus of the β subunit is not required for AChR assembly (Yu and Hall, unpublished results). This suggests that the C-terminus of the δ subunit, but not of the β, can replace the C-terminus of the α subunit, rescue the function of the truncated α subunit and must therefore be involeved in the late step of assembly.

Comparison of the C-terminal domains of the α and δ subunit indicates that they are not homologous except for two identical amino acids, G and Q (Fig. 11). The mutation of glycine to methionine in the C-terminus of the δ subunit did not abolish AChR assembly (Table I), suggesting that G is not involved in the recognition site. Although the C-termini of the α and δ subunit may both interact with the β subunit, they do so in different ways and in different places. The mechanism of the salvage may be that the C-terminal domain of the δ subunit rescues the interaction between the C-terminus of the α with the β subunit by establishing new interaction between the C-terminus of δ subunit with the β subunit or whatever it should be.

Discussion

This chapter clearly concluded that the C-termini of both α and δ subunits are of importance in late assembly and the function of the α C-terminus can be restored by the C-terminal domain of the δ subunit. The 9 amino acids have been identified from the C-terminal domain of δ subunit to be involved in the late step of assembly.

The C-terminal part of α subunit is involved in the late part of assembly

From the C-terminus-truncated α subunit, we clearly see that the C-terminus plays a role in assembly. It is structurally not dispensable. Removal of it does not cause protein misfolding because the truncated subunit has toxin binding activity, can associate with either δ or ε subunit, and can form a heterotrimer with β and δ

subunits. Its inability to direct AChR assembly only suggests that the C-terminal portion contains necessary assembly structure.

Consistently, another truncated α subunit, αM1, which is only composed of the N-terminal half part of the subunit, perfectly performs the first step, but not late step of assembly (Chapter IV).

In chapter four, we also provided a lot of evidence to demonstrate that the portion of the intracellular domain (loop) of the α subunit is involved in late step of assembly. Combining this conclusion with the results of the C-terminal domain of the α subunit, I would imagine that a fragment starting from the portion of the loop to the end of the C-terminus may play a very important role in assembly. All these make sense in a way that mutating either the portion of the loop near M4, or the M4, or the C-terminus shatters the integrity of the fragment and prevents the fragment from exerting recognition interaction, which leads to the failure of late assembly.

Having analysed the sequence of this fragment of the α subunit, we found that the C-terminal sequence from the M4 to the end actually forms an amphipathic α-helix with leucine residue in every seventh amino acid position and contains 4 leucine residues in the proper position. This sequence is coincidently consistent with a structural motif, the leucine zipper. DNA-binding proteins, such as the jun, fos, myc gene products, the yeast transcriptional activator GCN4, and C/EBP enhancer-binding protein all contain a region with 4

or 5 leucine residues spaced exactly seven amino acid residues apart. The leucine zipper consists of two separate α-helices, one from each monomer, that constitute the dimerization. In the AChR, the two α subunits probably provide two C-terminal α-helices to form a leucine zipper which could mediate the last step of AChR assembly. However, surprisingly, mutating each of four leucines to isoleucines did not influence AChR assembly (Yu and Hall, observation). Only way to confirm experimentally that the C-terminal portion of the α subunit forms a leucine zipper motif is to replace it with a typical known leucine zipper sequence to examine whether the α subunit with the known leucine zipper motif supports AChR assembly or not (Personal communication with Dr. Struhl).

Substitution of the C-terminus of the δ subunt, not of β subunit, for that of the α subunit suggests either that the leucine zipper motif, or whatever it is, was not disrupted by this change (rarely possible because the β C-terminus did not rescue the function of the α C-terminus), or that the C-terminal domain of the δ subunit by establishing its own interaction with the β subunit or with another region functionally replaces the α C-terminus, or that both simultaneously take place.

Because of massive mutational analysis, it is evident that several structural domains separately localize in different places of the primary amino acid sequence, each independently exerting its own function. The N-terminal part of the subunit sufficiently performs the first step of assembly. The C-terminal portion of the subunit

which undergoes conformational change after the first step of assembly, is required for the late step of assembly.

The C-terminal domain of the δ subunit is required for the late step of AChR assembly

In both aspects of research of making chimeric and truncated proteins, the C-terminus of δ subunit is evaluated to participate late assembly.

The fragment of 9 amino acids, PFPGDPRPY, has been identified as the critical one to be involved in recognition. When deletion extents to the fragment of 9 amino acids, the truncated δ subunit does not support AChR assembly any more. When the C-terminus of γ subunit replaces that of the δ subunit, the chimeric δ_{γ}^{S} subunit successfully directs AChR assembly because both termini share the fragment which is almost identical. None of the other termini have this ability as they do not have this segment either. From these results, we conclude that the fragment is very likely involved in recognition.

It happens to contain a high content of prolines which have been proposed to mediate protein-protein interaction. Transcription factors in CTF/NF-1 family all have the proline-rich region on their C-terminal domain (Santoro et al., 1988; Paonessa et al., 1988; Gil et al., 1988). Steroid receptors for progesterone and estrogen also contain proline-rich sequences in regions of the molecule that participate in their transcriptional activity (Kumar et al., 1987, Gronemeyer et al., 1987). Some proteins involved in the

developmental control of gene expression contain a region of 50-100 amino acids with high proline content (Laughon & Scott, 1984; Rosenberg et al., 1986).

Therefore, it is very likely that the C-teminus of the δ subunit mediating late assembly acts through this proline-rich region.

Experimental procedures are the same as described in Chapter III and IV except for construction of the constructs.

Construction of the mutated subunits:

The chimeric α_{δ}^{s}, α_{β}^{s}, β_{δ}^{s}, δ_{β}^{s}, δ_{α}^{s}, δ_{γ}^{s}, and δ_{ε}^{s} subunits were constructed by digesting pSMα, pSMβ, pSMγ, pSMδ and pSMε with introduced SpeI and endogenous ScaI located in the pSM vector, purifying the bigger fragments which contain the main body of each subunit and the smaller fragments which contain the C-terminal domain of each subunit, and ligating the corresponding bigger and smaller fragments. All the constructs were confirmed by restriction enzyme mapping.

The C-terminus-truncated α subunit, αT, was generated by oligo-directed mutagenesis and putting a stop codon to the first amino acid after M4 in oligonucleotide. The oligonucleotide is CTG GCT GTG TAA GCT TGT CGG CTC AT. HindIII site was created.

δΔSEQDKRFI (δΔSEQ) was generated by oligo-directed mutagenesis and putting a stop codon at amino acid S position in an oligonucleotide. The oligonucleotide is C TTC TCC TAT TAA GCT TAG GAC AAG CGC. HindIII site was created.

δΔPFPGDPFSYSEQDKRFI (δΔPFP) was constructed by oligo-directed mutagenesis and putting a stop codon at amino acid P position in oligonucleotide. The oligonucleotide is C CCA CTC CAG TAG TAC TCT GGG GAC CC. ScaI site was created.

δΔYNQPPLQPFPGDPFSYSEQDKRFI (δΔYNQ) was constructed by oligo-directed mutagenesis and putting a stop codon at amino acid Y position in oligonucleotide. The oligonucleotide is AG GGT GTC TAA AAG CTT CCC CCA CTC C. HindIII was created.

δ G471M was made by oligonucleotide, ATC TTC CTG CAA ATG GTC TAC AAC C, directed mutagenesis. The endogenous Pst I site was killed.

Figure 5-1

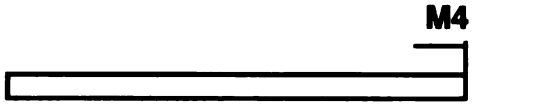
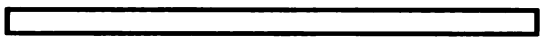
Structure of truncated αM4	Name of the subunits	Toxin binding of the subunit (% of control)
	αM4	32.6±13.2
	α	100±14.2

Figure 5-2. αM4 acquires the toxin binding activity which is increased by coexpression with either the δ or ε subunit.

COS cells were transfected with the cDNAs for either α, αM4, or α plus δ, or αM4 plus δ, or α plus ε, or αM4 plus ε, as indicated, 48 hr later permeabilized with saponin and labeled with ^{125}I-α-BTX. The transfected COS cells were washed three times with the same saponin buffer, dissolved in NaOH and counted in a gamma counter. In each case, triplicates were determined and each value represents a mean ± SD of the three determinants.

Figure 5-2

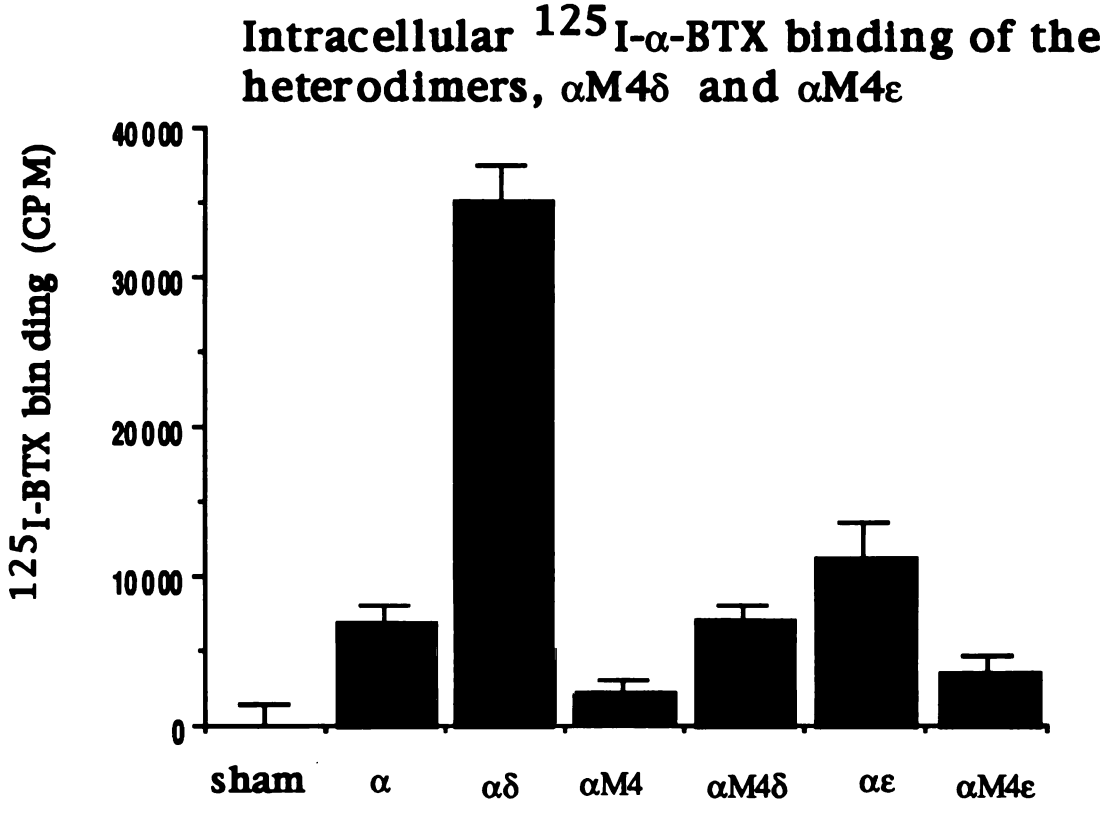

Intracellular 125I-α-BTX binding of the heterodimers, αM4δ and αM4ε
125I-BTX bin ding (CPM)
40000
30000
20000
10000
0
sham
α
αδ
αM4
αM4δ
αε
αM4ε

Figure 5-3. αM4 forms a heterodimer with the δ subunit.

COS cells were transfected with the cDNA for either α, αM4, α plus δ, or αM4 plus δ. 48 hr later, the transfected cells were extracted with the solubilizing buffer and incubated with MAb88B-sepharose plus ^{125}I-α-BTX for 2 hr. The sepharose beads were then washed twice with the washing buffer and counted in a gamma counter. Each value represents a mean ± SD of three determinations.

Figure 5-3

Heterodimer formation between αM4 and δ subunits as shown by immunoprecipitation

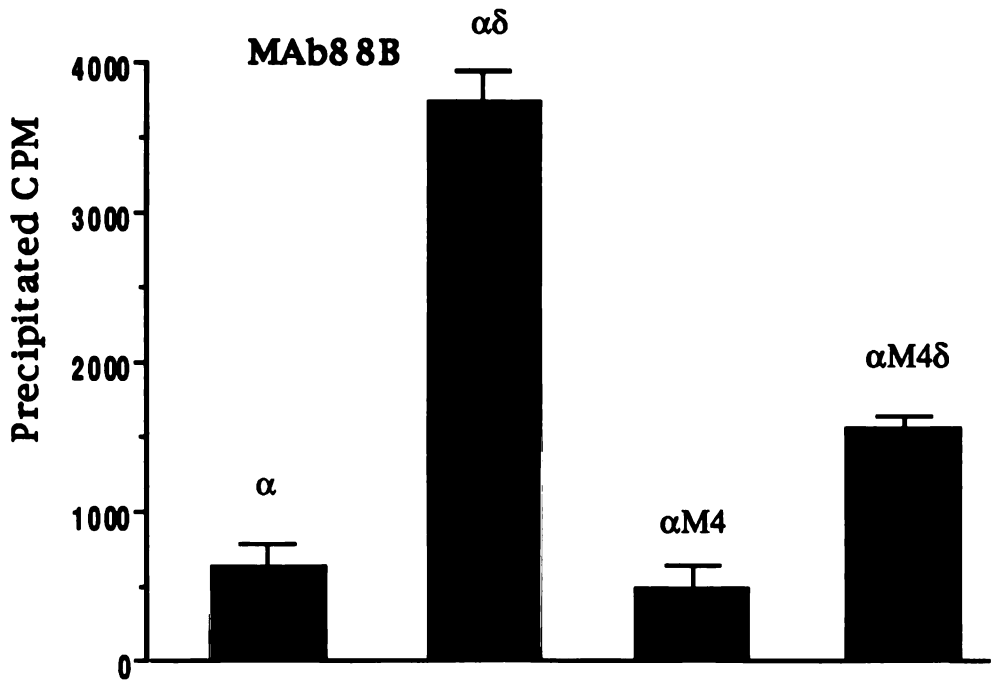


Figure 5-4. αM4 does not support AChR assembly when it is coexpressed with the β, δ and ε subunit.

COS cells were transfected with the cDNAs for either βδε, αM4βδε and αβδε as indicated. 24 hr later, the cells were split to wells of 24 well plates. The cells were then labeled with ^{125}I-α-BTX, washed three times with PBS and counted. Each value represents a mean ± SD of three determinations.

Figure 5-4

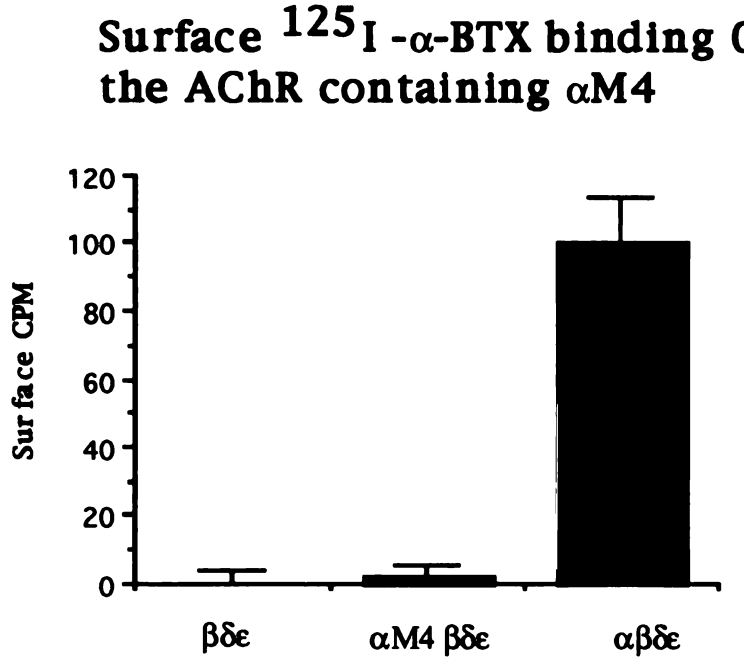

Surface ^{125}I -α-BTX binding Of the AChR containing αM4
Surface CPM
120
100
80
60
40
20
0
βδε
αM4 βδε
αβδε

Figure 5-5. A schematic diagram shows the structures of the truncated δ subunits. Three different oligonucleotides designed contain the stop codon TGA after the last amino acid indicated in the diagram. In vitro mutagenesis was used to construct δΔSEQ, δΔPFP and δΔYNQ.

Figure 5-5.

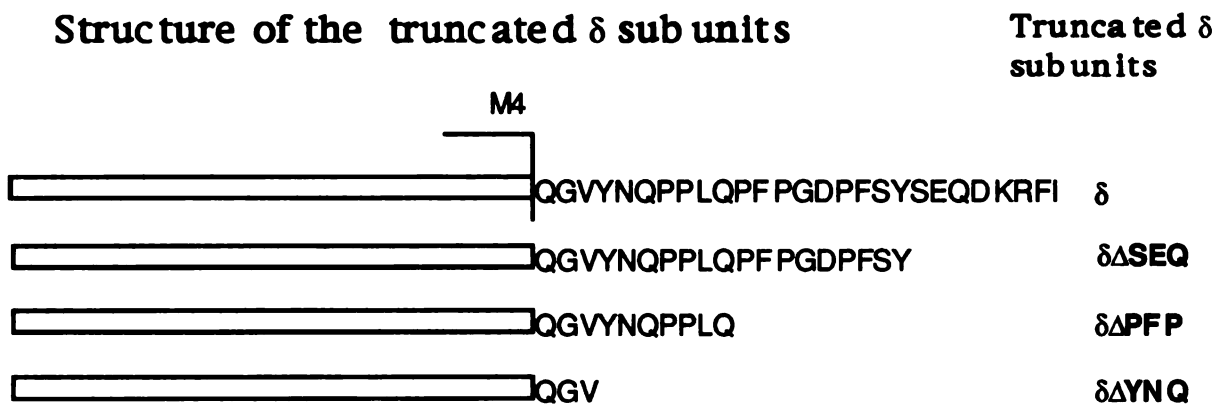

Structure of the truncated δ sub units
Truncated δ sub units
M4
QGVYNQPPLQPF PGDPFSYSEQD KRFI
δ
QGVYNQPPLQPF PGDPFSY
δΔSEQ
QGVYNQPPLQ
δΔPFP
QGV
δΔYNQ

Figure 5-6. The truncated δ subunits, chimeric δ subunits (A) and a chimeric β_{δ}^{s} (B) are successfully expressed in COS cells determined in immunoblot.

COS cells were transfected with the cDNA for each subunit as indicated. The extracts from the transfected COS were subjected to immunoprecipitation with δ specific antibody, MAb88B-linked sepharose. The sepharose beads were extracted with SDS-PAGE loading buffer which was then loaded to the 9% separating gel. After drying the PAGE, X-ray film was exposed and developed.

Figure 5-6.

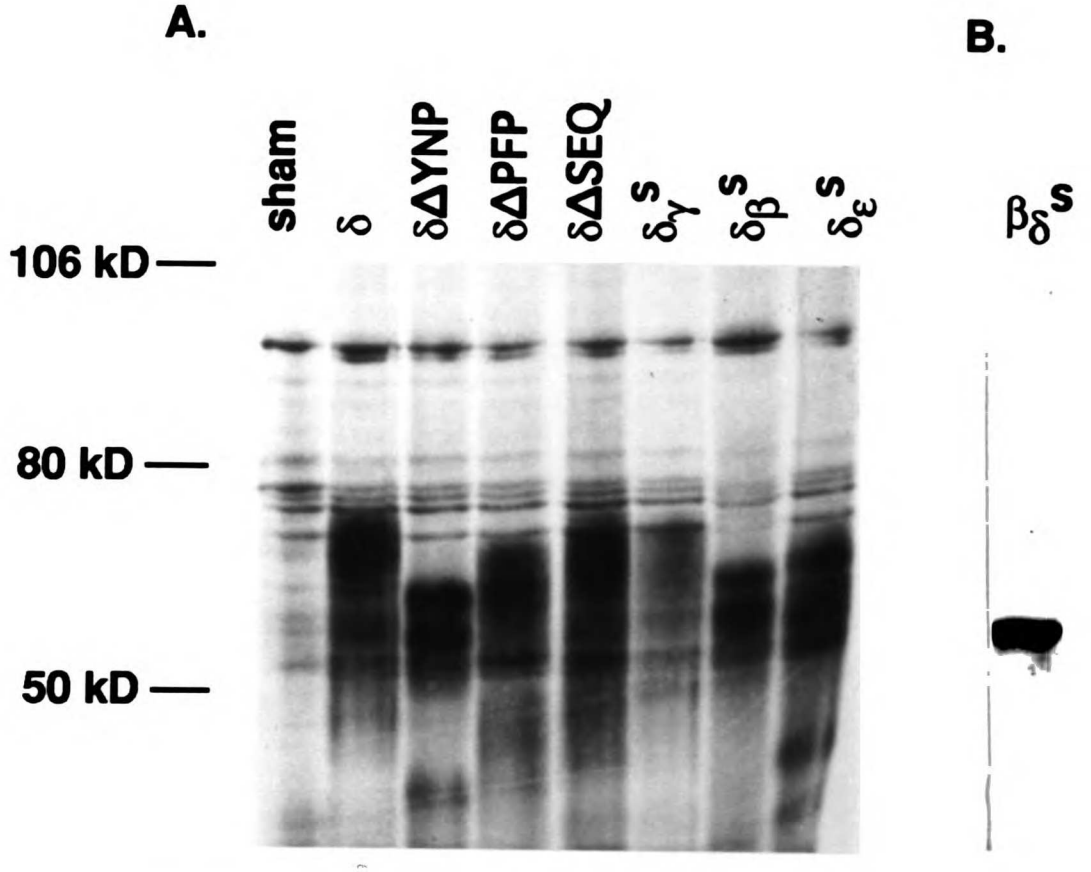

A.
B.
sham
δ
δΔYNP
δΔPFP
δΔSEQ
δ_γ^s
δ_β^s
δ_ε^s
β_δ^s
106 kD
80 kD
50 kD

Table 5-I legend:

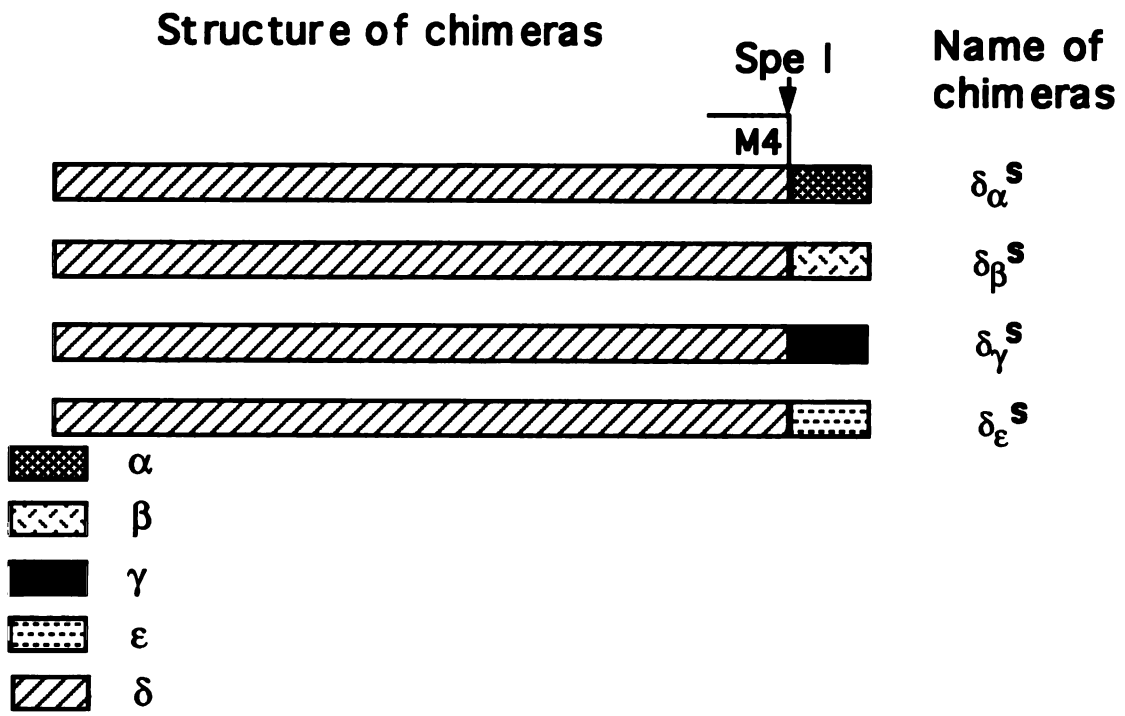
Surface AChR expression of each of the mutated δ subunits with the other required subunits, α, β and ε, heterodimer formation of each mutated δ subunit with the α subunit, and sedimentation coefficient of the heterodimer containing each of the mutated δ subunits were examined. For surface AChR expression, each of the mutated δ , as well as the normal δ as a control, was coexpressed with the other required subunits, α, β, and ε into COS cells. The surface AChRs were measured by surface assay. Triplicates of each sample were determined. For heterodimer formation, each of the mutated δ subunits, as well as the normal δ subunit, was coexpressed with the α subunit, the resulting heterodimers were precipitated with MAb88B. Duplicates of each sample was performed. The values were normalized to either the normal AChR or heterodimer as percentage of the normal controls. For sedimentation coefficient of the heterodimer, COS cells were transfected with cDNAs for the α and each of the mutated δ subunits and labeled with ^{125}I-α-BTX. The extracts of the transfected COS cells were loaded to gradients. The gradients were fractionated and the fractions were counted. According to the sedimentation coefficients of the known protein, alkaline phosphatase (6.3S), and catalase (11.4S), the sedimentation coefficeints of the heterodimers were determined.

Table 5-I: Assembly function of the C-terminus-truncated δ subunits

Transfected subunits	Surface AChR expression (% of Control)	Heterodimer formation with the α subunit	Sedimentation coefficient of heterodimer(S)
α	--	0	5.0
αβε	0		
+δ	100 ± 0.5	100	6.3
δI227V A465L W466V	55.8 ± 5.2		
δG471M	59.4 ±4.2	106	6.3
δΔSEQP...	66.3 ± 3.8	36	6.3
δΔPFP...	0.7 ±1.0	18	5.0
δΔYNP...	-1.3 ± 1.8	1.0	5.0

Figure 5-7. A schematic diagram shows the structures of the chimeric δ subunits with the different C-terminal tails from the α subunit ($\delta_\alpha{}^s$), from the β ($\delta_\beta{}^s$), from the γ ($\delta_\gamma{}^s$) and from the ε ($\delta_\varepsilon{}^s$).

Figure 5-7.


Structure of chimeras
Spe I
M4
Name of chimeras
δ_α^s
δ_β^s
δ_γ^s
δ_ε^s
α
β
γ
ε
δ

Tabel 5-II legend:

Surface AChR expression of each of the chimeric δ subunit with the other required subunits, α, β and ε, heterodimer formation of each chimeric δ subunit with the α subunit, and sedimentation coefficient of the heterodimer containing each of the chimeric δ subunits were examined. For surface AChR expression, each of the chimeric δ , as well as the normal δ as a control, was coexpressed with the other required subunits, α, β, and ε into COS cells. The surface AChRs were measured by surface assay. Triplicates of each sample were determined. For heterodimer formation, each of the mutated δ subunits, as well as the normal δ subunit, was coexpressed with the α subunit, the resulting heterodimers were precipitated with MAb88B. Duplicates of each sample was performed. The values were normalized to either the normal AChR or heterodimer as percentage of the normal controls. For sedimentation coefficient of the heterodimer, COS cells were transfected with cDNAs for the α and each of the chimeric δ subunits and labeled with ^{125}I-α-BTX. The extracts of the transfected COS cells were loaded to gradients. The gradients were fractionated and the fractions were counted. According to the sedimentation coefficients of the known protein, alkaline phosphatase (6.3S), and catalase (11.4S), the sedimentation coefficeints of the heterodimers were determined.

Table 5-II: Assembly ability of the C-terminus-substituted δ subunits

Transfected subunits	Surface AChR expression (% of control)	Heterodimer formation with the α subunit (MAb88B)	Sedimentation coefficient of heterodimer(S)
α	-	0.0	5.0
αβε	0.0		
+δ	100 ± 7.7	100	6.3
$\delta_{\alpha}{}^{s}$	5.2 ± 1.4	20.0	
$\delta_{\gamma}{}^{s}$	30.5 ± 2.5	58.9	6.3

Figure 5-8. The sequences of the C-terminal domains of γ and δ subunits are aligned and identical or highly conserved amino acids are shaded.

Figure 5-8.

Alignment of the C-terminal domains of the gamma and delta subunits

Figure 5-9. A schematic diagram exhibits the structure of the chimeric β subunit with the δ C-terminus (β_{δ}^{s}) and the structures of the chimeric α subunits with the δ C-terminus (α_{δ}^{s}) or with the β C-terminus (α_{β}^{s}).

Figure 5-9.

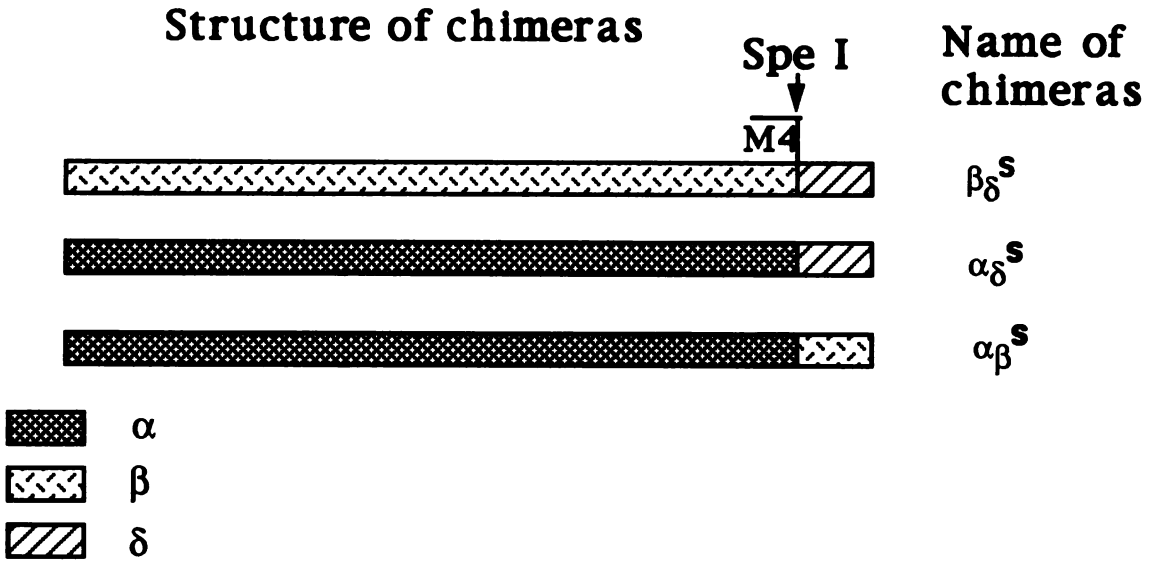

Structure of chimeras
Spe I
M4
Name of chimeras
β_δ^s
α_δ^s
α_β^s
α
β
δ

Figure 5-10. The chimeric $\alpha_{\delta}{}^{s}$ subunit supports AChR assembly whereas the chimeric $\alpha_{\beta}{}^{s}$ subunit does not.

COS cells were transfected with cDNAs for the subunits as indicated, 24 hr later split to wells of 24 well plates, and labeled with ^{125}I-α-BTX in 24 hr. The labeled cells were then washed twice and counted. Triplicates of each sample were determined. Each value represents a mean ± SD of the triplicate determinants.

Figure 5-10.

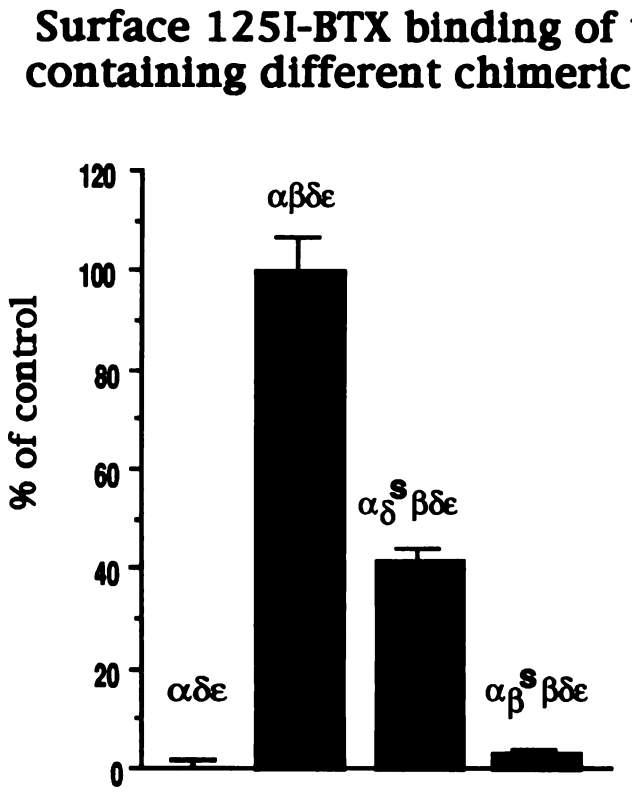

Surface 125I-BTX binding of the AChRs containing different chimeric subunits
% of control
120
100
80
60
40
20
0
αβδε
αδε
αδ^sβδε
αβ^sβδε

Figure 5-11. The sequences of the C-termini of the α and δ subunits were aligned with the identical or conserved amino acids shaded.

Figure 5-11.

Alignment of the C-terminal domains of the alpha and delta subunits

Chapter Six

The Role of The Cytoplasmic Domains of Individual Subunits of The Acetylcholine Receptor in 43 kD Protein-Induced Clustering in COS cells

Summary

The 43 kD protein, a cytoplasmic peripheral membrane protein, is closely associated with the acetylcholine receptor (AChR) at the neuromuscular junction where it is thought to anchor the receptor in the postsynaptic membrane. We have used the 43 kD protein-induced clustering of AChRs that occurs when both proteins are transiently expressed in COS cells to investigate which parts of the AChR might interact with the 43 kD protein. By constructing chimeric subunits, we showed that the cytoplasmic domains of neither the γ nor δ subunits are required for 43 kD protein-induced clustering. Systematic mutational analysis of the long cytoplasmic loops of the α and β subunits showed that most of the loops can be altered without affecting the ability of the AChR to be clustered; in each case, however, one or more sequences could not be tested, because mutation in these regions prevented AChR assembly. Our results suggest either that these regions are involved in clustering or that the 43 kD protein can interact with multiple, alternative sites on the cytoplasmic surface of the AChR. Our experiments also show that the postulated sites of tyrosine phosphorylation in the β subunit and of serine phosphorylation in the α subunit can be mutated without affecting 43 kD protein-induced AChR clustering.

Introduction

The appearance of clusters of acetylcholine receptors (AChRs) in muscle cells near sites of nerve contact is the earliest detectable sign of postsynaptic differentiation during development of the neuromuscular junction (Hall and Sanes, 1993). A cytoplasmic peripheral membrane protein, the 43 kD protein, is closely associated with the AChR at adult and developing synapses (Froehner et al., 1981; Noakes et al., 1993), and has been proposed to anchor it to the cytoskeleton in the postsynaptic membrane (Froehner, 1991). The 43 kD protein is present in approximately 1:1 stoichiometry with the AChR (LaRochelle and Froehner, 1987), and is exactly co-distributed with it, both in the postsynaptic membrane in vivo (Sealock et al., 1984; Flucher and Daniels, 1989) and in spontaneous and nerve-induced AChR clusters in cultured muscle cells (Burden, 1985; Bloch & Froehner, 1987; Gordon et al., 1993). Removal of the 43 kD protein and other peripheral membrane proteins from the membrane by alkaline treatment increases the lateral mobility of the AChR within the cluster (Barrantes et al., 1980; Rousselet et al., 1982), suggesting that one or more of the extracted proteins, such as 43 kD protein, may attach the AChR to the cytoskeleton.

Recent experiments suggest that the 43 kD protein not only stabilizes the AChR in clusters, but also induces AChR clusters to form. When AChRs are expressed in non-muscle cells after transfection or after mRNA injection, they are diffusely distributed on the cell surface; when expressed with the 43 kD protein, however,

the AChRs are found in clusters that are coincident with aggregates of the 43 kD protein (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992). The 43 kD protein forms clusters when expressed alone; AChRs thus presumably become clustered via their association with the 43 kD protein.

Although firm evidence is lacking, several observations suggest that the AChR interacts directly with the 43 kD protein. A protein that appears to be in direct contact with the AChR in reconstituted images of the postsynaptic electroplax membrane (Toyoshima and Unwin, 1988; Mitra et al., 1989) is likely to be the 43 kD protein, as it is the only protein in purified Torpedo membranes that is present in equimolar amounts with the AChR (LaRochelle and Froehner, 1987; LaRochelle & Froehner, 1986). Also, the 43 kD protein can be cross-linked to the β subunit of the AChR by treatment of synaptic membranes with a bifunctional cross linking agent (Burden et al., 1983). The β subunit is of particular interest because agrin, a neurally-derived factor that causes AChRs to cluster (McMahan, 1990; Reist et al., 1992), stimulates phosphorylation of a tyrosine residue in this subunit (Wallace et al., 1991).

Each of the subunits of the AChR has a stereotyped structure with a long N-terminal, extracellular domain, four transmembrane domains (M1-M4) and an extracellular C-terminal domain (Karlin, 1980); Changeux, 1981; Claudio, 1989). Either of the two cytoplasmic domains that link the transmembrane segments M1 and M2, or M3 and M4, respectively, provide potential sites for interaction with the

43 kD protein. To identify the specific subunits and the sites on these subunits that interact with the 43 kD protein, we have expressed AChRs containing chimeric or mutated subunits in COS cells along with the 43 kD protein. Our results demonstrate that the cytoplasmic domains of the ε and δ subunits and most of the intracellular loops of the α and β subunits are not required for 43 kD-induced AChR clustering. The 43 kD protein may thus react with specific sequences on the α and β subunits that we were unable to test or react with multiple, alternative sites on one or more subunits.

Materials and Methods

Vectors

Full-length cDNAs coding for the α, β, γ and δ subunits of the AChR were obtained from Dr. 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). The cDNA clone for the mouse ε subunit was isolated as described (Gu et al., 1990). The cDNA coding for CD8 was a generous gift from Dr. D. Littman. Each of the cDNAs was subcloned into the multiple cloning site of pSM, an SV40-based expression vector (Brodsky et al., 1990) that also contains an M13 origin for oligonucleotide-directed mutagenesis.

cDNA for the mouse 43 kD protein was a generous gift of Dr. S. Froehner (Froehner, 1989). The full length coding sequence minus the 5' untranslated sequences was subcloned into the SV40-based expression vector, pcDLSRα296, under the control of a SRα promotor (Takebe et al., 1988). The pSM vector with the 43 kD cDNA insert was cut with EcoRI, and the 43 kD cDNA dropout (1.5 kb) was purified and cloned into the EcoRI-linearized pcDLSRα296 vector.

Mutagenesis

Oligonucleotide-directed mutagenesis was performed according to protocols described previously (Geisselsoder et al., 1987; Kunkel, 1985a). Synthetic oligodeoxyribonucleotides for each mutated segment were designed with 10-15 flanking nucleotides on either side of the mutation site and were prepared with an automatic DNA synthesizer. The detailed changes for each mutation are given below.

Where appropriate, @ is used to indicate a deleted segment; bases that are altered to produce amino acid changes or to introduce a restriction site are underlined.

Mutations in the cytoplasmic loop of the alpha subunit:

α(Δ299–312): The first 13 amino acids, HHRSPSTHIMPEW, of the long cytoplasmic loop were deleted using the oligonucleotide ATC GTC ATC AAC ACA @ GTG CGC AAG GTT TTT ATC GAC. The base substitution created an FspI site.

α(*312–322): The 10 amino acids, VRKVFIDTIP, were mutated to VRQIFIHKLP, corresponding to the amino acid sequence in the loop of the β subunit, using the oligonucleotide CCC GAG TGG GTG CGG CAG ATT TTT ATC CAC AAG CTT CCA AAC ATC ATG TTT. A HindIII site was created by the change.

α(*322–332): The 10 amino acids, NIMFFSTMKR, were mutated to SLSPRSGWGR, corresponding to the amino acid sequence in the loop of the β subunit, using the oligonucleotide ATC GAC ACT ATC CCA TCC CTC TCG CCT CGC TCC GGA TGG GGA AGA CCA TCC AGA GAT AAA. A BspEI site was created by the change.

α(*332–339): The 7 amino acids, PSRDKQE, were mutated to GTDEYFI, corresponding to the amino acid sequence in the loop of the β subunit, using the oligonucleotide TCC ACA ATG AAA AGA GGT ACC GAT GAA TAT TTT ATT AAA AGG ATT TTT ACA. A KpnI site was introduced by the change.

α(*339–349): The 10 amino acids, KRIFTQDIDI, were mutated to RPPPSDFLFP, corresponding to the sequence of the β subunit, using

the oligonucleotide AGA GAT AAA CAA GAG AGG CCT CCT CCT TCA GAT TTC CTA TTT CCA. A StuI site was introduced by the change.

α(Δ349–359): The10 amino acids, SDISGKPGPP, were deleted using the oligonucleotide GAA GAC ATA GAT ATC @ CCT ATG GGC TTT CAC. A EcoRV site was introduced without any amino acid change.

α(Δ359–369): The 10 amino acids, PMGFHSPLIK, was deleted using the oligonucleotide TCT GGG AAG CCG GGC CCT CCA @ CAC CCT GAG GTG AAA AGC. An Apa I site was introduced without any amino acid change.

α(*367–382): The 15 amino acids, IKHPEVKSAIEGVKY, in the amphipathic helix were also mutated to GLPQELREVISSISY, corresponding to the sequence of the β subunit, using the oligonucleotide TTT CAC TCT CCG CTG GGC CTG CCC CAG GAG CTG AGA GAG GTC ATC TCA TCG ATC AGC TAC ATT GCA GAG ACC ATG. A Cla I site was introduced by the change.

Mutations in the cytoplasmic loop of the beta subunit:

β(Δ338–353): The 15 amino acids, KRPKPERDQLPEPHH, in the β loop were deleted using the oligonucleotide CCT CCA TAC CTA GGC CTG @ TCT CTT TCT CCA. A StuI site was created without amino acid change.

β(*343–353): The 6 charged amino acids were neutralized to hydrophobic amino acids over a window of 10 amino acids to mutate ERDQLPEPHH to VLVQLPVPLL using the oligonucleotide CCC AAA CCC GTA CTA GTC CAA CTC CCT GTA CCA CTT CTC TCT CTT TCT. A SpeI site was introduced by the change.

β(*353–363): The 10 amino acids, SLSPRSGWGR, were mutated to NIMFFSTMKR, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide CCT GAA CCA CAT CAC AAT ATT ATG TTT TTT AGT ACC ATG AAA AGA GGA ACT GAT GAA TAT. A SspI site was introduced.

β(*363–370): The 7 amino acids, GTDEYFI, including the tyrosine phosphorylation site were mutated to PSRDKQE, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide AGT GGC TGG GGC AGG CCT TCT CGT GAT AAA CAA GAA CGG AGG CCT CCA AGT. A StuI site was created.

β(*370–380): The 10 amino acids, RKPPSDFLFP, were mutated to KRIFTQDIDI, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide GAT GAA TAT TTC ATC AAG AGG ATT TTT ACT CAA GAT ATC GAC ATT AAA CTT AAC AGG TTT. A EcoRV site was created.

β(*380–390): The 10 amino acids, KLNRFQPESS, were mutated to SDISGKPGPP, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide GAT TTT CTT TTC CCT TCA GAT ATC TCG GGT AAG CCT GGA CCA CCT GCC CCG GAC CTG CAG. A EcoRV site was created.

β(Δ390–405): The 15 amino acids, APDLRRFIDGPTRAV, were deleted using the oligonucleotide CAG CCT GAA TCA TCT @ GGT CTG CCT CAG GAG. The endogenous SmaI in the position of amino acid sequence, TRA, was also deleted.

β(*420–430): The 10 amino acids, MARQLQEQED, were mutated to IAETMKSDQE, corresponding to the sequence in the loop of the α subunit, using the oligonucleotide TCC TCA ATC AGC TAC ATT GCC

GAA ACG ATG AAG TCT GAT CAG GAA CAC GAC GCA CTG AAG. A BclI site was created.

β(*405–420): The 15 amino acids in the amphipathic helix of the loop of the β subunit, GLPQELREVISSISY, were also mutated to QASPAIQACVDACNL, corresponding to the sequence of the amphipathic helix of the γ subunit, using the oligonucleotide CCA ACC CGG GCT GTA CAG GCC TCT CCG GCG ATT CAA GCG TGC GTT GAC GCA TGC AAC CTC ATG GCC CGA CAG CTT. A StuI site was created.

Cell culture and transient transfection

COS cells were maintained in growth medium (DMEM H21 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin). COS cells were transiently transfected as described previously (Gu et al., 1990), using a modified DEAE-dextran transfection procedure (Seed and Aruffo, 1987). About 18 hours before transfection, COS cells were trypsinized and $2X10^5$ cells were plated into one well in a 12 well plate. The transfection medium was DMEM H21 supplemented with 2 mM L-glutamine mixed with 1% heat inactivated FBS, 0.15 mM chloroquine diphosphate, 0.6 mg/ml DEAE-dextran and routine amounts of plasmid cDNAs for AChR subunits and the 43 kD protein. The routine cDNA concentrations for the AChR subunits and the 43 kD protein were empirically determined for maximum response (α: 0.66 ug/ml; β: 0.33 ug/ml; δ: 0.13 ug/ml; ε: 0.5 ug/ml and 43 kD: 0.2 ug/ml). COS cells were incubated in the transfection medium for about 4 hours in a 37 ^{0}C incubator with 8 % CO_2 and 100% humidity. In all transfections, the transfection mixtures were removed after about 4

hours, and the cells were treated in 10% dimethyl sulfoxide in PBS for 2 min at room temperature, and washed once with PBS before being returned to 37 ^{0}C in 2 ml of growth medium. Twenty-four hours later, the transfected cells were trypsinized and distributed into two wells in a 24 well plate containing round glass coverslips (12 mm in diameter). The staining procedures were carried out the next day. Transfections for coexpression of the α subunit alone with the 43 kD protein, or for the β subunit alone with the 43 kD protein, or for the CD8 protein with the 43 kD protein were performed in the same way as above except that the cDNA concentrations for the α, β subunits and for CD8 were 1 ug/ml.

For the assay of surface AChRs, transfections were carried out in almost the same way as above except that the size of the dishes and the amounts of cells and plasmids were different. $7X10^5$ cells were plated into one 60 mm dish. 3 ml of transfection mixtures was added to each dish containing the routine amounts of cDNAs for the AChR subunits (α: 1.32ug; β: 0.66ug; δ: 0.26ug; ε: 1.0ug). The transfected cells in one 60 mm dish were split into 3 wells of a 24 well plate for triplicate determinations. Assay for surface AChRs was performed 24 hours later.

Immunofluorescence and histochemistry

Immunocytochemical staining for the surface AChRs was carried out on COS cells grown on 12 mm round coverslips and transfected with cDNAs of the AChR subunits with or without cDNA for the 43 kD protein. The transfected COS cells were fixed in 2% paraformaldehyde

in Ca^{++}, Mg^{++} free PBS for 20 min, then were rinsed with PBS, blocked with blocking bufffer (10%FBS/4%BSA/PBS) for 1 hr. to inhibit nonspecific binding of the antibodies, incubated for 2 hr. with a monoclonal antibody, MAb210, that recognizes a site on the N-terminus of the α subunit (Ratnam et al., 1986) at a concentration of 215 nM diluted in the blocking buffer. The COS cells were washed twice with PBS and once with the blocking buffer for 30 min, then incubated for 1 hour with the secondary antibody, FITC-conjugated goat anti-rat IgG, at a 1:200 dilution in the blocking buffer. After washing three times with PBS, the coverslips were mounted on glass slides with p-phenylenediamine in glycerol (Platt & Michael, 1983). The slides were viewed with a Leitz Orthoplan II microscope under epifluorescence illumination equipped with a Vario-Orthomat camera system. Staining of COS cells expressing CD8 with or without the 43 kD protein was carried out in a similar manner, except that the first antibody was OKT8 (Hoffman et al., 1980) against the CD8 protein and the second antibody was FITC-conjugated sheep anti-mouse.

The staining procedure for the 43 kD protein was a modification of the procedure of LaRochelle et al. (1989). Transfected COS cells were fixed in 2% paraformaldehyde, rinsed once with PBS, permeabilized by incubation with 1% Triton/PBS for 10 min, blocked with the blocking buffer for 1 hour, and incubated for 2 hours with a mixture of three monoclonal antibodies against the 43 kD protein (1234A, 19nM; 1201C, 5nM; 1579A, 11nM; Peng and Froehner, 1985). The cells were then washed twice with PBS and once with the blocking buffer for 30 min, and incubated for 1 hour with the second

antibody, FITC-conjugated sheep anti-mouse IgG, at a 1:200 dilution in the blocking buffer. The coverslips were washed three times with PBS, mounted with p-phenylenediamine in glycerol, and visualized in fluorescence microscopy.

Double-labeling experiments were performed with a protocol modified from Ralston and Hall, (1989). COS cells cotransfected with cDNAs of the AChR and the 43 kD protein were incubated with RH-αBTX at a 1:200 dilution in growth medium at 37°C for 1 hour, rinsed with PBS, fixed in 2% paraformaldehyde in PBS for 20 min, rinsed once with PBS, then permeabilized with 1% Triton in PBS for 10 min, washed with PBS once, and incubated with the blocking buffer for 1 hour. COS cells were then incubated for 2 hours with a mixture of the first antibody, a polyclonal rabbit anti-αBTX at 1:100 dilution and three monoclonal mouse antibodies against 43 kD protein at the same concentrations as above. COS cells were washed twice with PBS and once with the blocking buffer, incubated for one hour with a mixture of the second antibodies at 1:200 dilutions, FITC-conjugated sheep anti-mouse IgG and RH-conjugated goat anti-rabbit IgG. The coverslips were washed three times with PBS, mounted and viewed as described above. The same COS cells expressing both RH-AChR and FITC-43 kD protein signals were photographed.

Staining protocols for COS cells expressing either the α or β subunit with the 43 kD protein were as follows: COS cells transfected with cDNAs for either the α or β subunit and the 43 kD protein were fixed for 20 min with 2% paraformaldehyde, rinsed once with PBS,

then permeabilized for 10 min with 1% Triton-PBS, rinsed once with PBS, and blocked for 1 hour with the blocking buffer. COS cells in one well were then incubated for 2 hours with the first antibody, either MAb210 for the α subunit or MAb124 for the β subunit, respectively, then washed twice with PBS and once with the blocking buffer for 30 min. The COS cells were then incubated for 1 hour with the second antibody, FITC-conjugated goat anti-rat IgG, washed three times with PBS, mounted and visualized under fluorescence microscopy. COS cells in a neighboring were stained for the 43 kD protein using the 43 kD protein staining procedure described above.

All the staining procedures were carried out at room temperature, unless otherwise stated.

AChR surface expression

Surface expression of α-bungarotoxin (αBTX) binding sites was measured by incubating intact transfected COS cells with 10 nM [^{125}I]-αBTX (Amersham, Arlington Heights, IL) for 1.5 hour at 37^{o}C. Sham transfections were used to determine nonspecific binding. Unbound toxin was then removed by washing the cells twice with PBS. The cells were then solubilized with 0.1M NaOH and the radioactivity was measured in a gamma counter.

Results

Coexpression of the 43 kD protein with the AChR in COS cells induces AChR clustering

When cDNAs for the four subunits of the adult mouse AChR ($\alpha\beta\delta\varepsilon$) are transfected into COS cells, AChRs appear on the surface that resemble those seen at adult mouse endplates (Gu et al., 1990). The distribution of the AChRs on the surface of the transfected COS cells was examined by staining them either with rhodamine-conjugated α-BTX (results not shown), or with a monoclonal antibody to an epitope on the extracellular N-terminal domain of the α subunit (Fig. 1A), followed by FITC-conjugated second antibody. Only about 20-30% of the cells expressed the AChR at high enough levels to be detected. In virtually all of these cells, however, the receptors were diffusely distributed on the cell surface (Fig. 1A; Table I). When a cDNA for the 43 kD protein was included in the transfection mixture, a different pattern of staining was observed in which the AChRs appeared in small clusters on the surface of the COS cells (Fig. 1B). This pattern of staining was seen in approximately half the number of the cells that showed strong staining for the AChR (Table I). When the 43 kD protein was expressed in COS cells in the absence of the AChR, and the cells were permeabilized and stained using antibodies to the 43 kD protein, a similar pattern of staining was seen (Fig. 1C). The 43 kD protein appeared to be associated with the surface membrane in small aggregates or clusters that resemble those seen with antibodies to the AChR when both proteins were expressed. 43 kD protein aggregates were also seen in the cytoplasm and diffuse

staining was observed in the nucleus. When ε subunit cDNA was replaced by γ subunit cDNA in the transfection mixture so that the embryonic form of the AChR was expressed (Gu et al., 1990), similar results were obtained (not shown). To test whether AChRs were specifically clustered, we transfected COS cells with a cDNA for CD8, an integral membrane protein expressed on the surface of cytotoxic lymphocytes (Littman, 1987), with and without the cDNA for the 43 kD protein. Whether expressed with (Fig. 1D) or without (not shown) the 43 kD protein, CD8 was always seen to be diffusely distributed on the cell surface (Table I).

43 kD protein-induced AChR clustering was also investigated in double labeling experiments. cDNAs for the γ or ε subunits and for the α, β and δ subunits were cotransfected with or without the 43 kD protein cDNA into COS cells. The AChRs were labeled with RH-α-BTX, followed by rabbit anti-BTX and RH-conjugated goat anti-rabbit IgG; the 43 kD protein was labeled with a mixture of mouse monoclonal antibodies against the 43 kD protein, followed by FITC-conjugated sheep anti-mouse IgG. In the case of both the ε-AChR (Fig. 2A, B) and the γ-AChR (not shown), the receptor and the 43 kD protein were colocalized on the surface. Our results in COS cells, which are consistent with those found by others in non-muscle cells (Froehner et al., 1990; Phillips et al., 1991; Brennan et al., 1992), thus suggest that the 43 kD protein alone forms aggregates at the surface membrane and that the AChR is induced to aggregate by its association with the 43 kD protein. The results with CD8 suggest that

the AChR clustering is a specific effect that does not occur with other membrane proteins.

AChR clustering does not require the cytoplasmic loops of either the γ or δ subunits.

If the 43 kD protein induces AChR clustering by direct interaction with one or more of the AChR subunits, the interaction is likely to be mediated by their cytoplasmic loops. To test whether individual subunits interact with the 43 kD protein, we constructed chimeric subunits in which the N- and C-terminal domains were derived from one subunit and the intervening sequence from another subunit (Yu and Hall, 1991). In many cases, the resulting chimeric subunits did not support AChR assembly when expressed in COS cells and no surface AChR was obtained. When a cDNA of the chimeric subunit, ε_β, containing the N- and C-terminal domains of the ε subunit and the transmembrane and cytoplasmic domains of the β subunit was cotransfected into COS cells with the cDNAs of the α, β, and δ subunits, surface AChRs were detected (Yu and Hall, 1991). These AChRs were diffusely distributed on the cell surface (Fig. 3A); when coexpressed with the 43 kD protein, however, clusters of the AChR were readily observed (Fig. 3B). The clusters occured in approximately the same proportion of AChR-expressing cells as seen with the native AChR (Table I). Similar results were obtained when the corresponding chimera for the δ subunit, δ_β, was expressed with α, β and ε subunits. As with the ε_β receptor, receptors containing the δ_β subunit were diffusely distributed on the surface, but became clustered when coexpressed with the 43 kD protein (Fig. 3C, 3D).

Moreover, even when both ε_β and δ_β subunits replaced their normal counterparts, the AChR expressed on the surface could be induced to form clusters by coexpression with the 43 kD protein (Fig. 3E, 3F). In the case of each chimera, the toxin binding activity expressed on the cell surface was sensitive to d-tubocurarine, and sedimented in a sucrose gradient at a position identical to that of the authentic AChR (Yu and Hall, 1991; Yu and Hall, in preparation). We also observed colocalization of the receptors containing chimeric subunits and of the 43 kD proteins in double labeling experiments (not shown). Since 43 kD protein-induced clustering of the assembled receptor required the cytoplasmic regions of neither the ε nor δ subunits, and occurred in the absence of both, we conclude that interaction of the 43 kD protein with these regions is not necessary for clustering to occur.

To investigate whether the cytoplasmic domains of α and β subunits are required for clustering, we also constructed chimeras in which either the entire region between the extracellular N- and C-terminal domains, or simply the long cytoplasmic loop of each of the subunits was replaced by the corresponding region from other subunits. Cotransfection of these chimeric cDNAs with the other required subunit cDNAs did not result in surface expression of AChRs.

<u>Expression of neither the α nor the β long cytoplasmic loop exerts a dominant negative effect on 43 kD protein-induced clustering of the AChR</u>

If the long cytoplasmic loop of either the α or the β subunit interacted directly with the 43 kD protein, then expression of an altered subunit that contained the loop but was not incorporated into the AChR might exert a dominant negative effect on clustering by competing with the intact AChR for complex formation with the 43 kD protein. Increasing the concentration of either the α or the β subunit cDNA in the transfection mixture by up to 5-fold, however, gave no significant change in either the amount of surface AChR (not shown) or in its ability to be clustered by the 43 kD protein (Fig. 4A, B). In each case, immunoblotting experiments established that the amount of subunit expressed was increased in proportion to the increase in cDNA (data not shown).

We then tested whether expression of α and β subunits in which the N-terminal domains were deleted would exert a dominant negative effect on 43 kD protein-induced AChR clustering. Such deletions should prevent incorporation of the mutated subunits into the AChR (Yu and Hall, 1991; Verrall and Hall, 1992), but leave the cytoplasmic portions of the subunits intact. Although analysis by immunoblotting showed that both truncated proteins were expressed at levels that were comparable to those of the corresponding intact subunit, neither decreased the frequency of AChR clustering (data not shown).

Finally, we sought evidence for intracellular association between the 43 kD protein and the α or the β subunit by expressing them in COS cells in the absence of other subunits and examining

their distribution by immunofluorescence. The α (Fig. 5A) and β subunits (Fig. 5B), identified by staining with specific monoclonal antibodies, were diffusely distributed throughout the cell, in a pattern characteristic of ER staining (Gu et al., 1989). No aggregates were seen. In contrast, the 43 kD protein, stained with a combination of specific monoclonal antibodies, appeared to be concentrated in aggregates on the cell surface and in the cytoplasm (Fig. 5C, 5D). The clearly different patterns of distribution give no evidence for intracellular association between the α or β subunits and the 43 kD protein, and suggest that these subunits do not associate with the 43 kD protein before their incorporation into the AChR.

Mutational analysis of the long cytoplasmic loops of the α and β subunits identifies small regions that may be required for clustering.

If a unique segment of either the α or β subunits was required for AChR clustering, then its removal or replacement by a corresponding segment from the other subunit might be expected to block AChR clustering. We thus used conventional in vitro mutagenesis to make a systematic analysis of the long cytoplasmic loops of both the α and β subunits by deleting successive segments of the loops or by replacing the segments with corresponding sequences from the other subunit (Fig. 6, 7). Because the loops are poorly conserved, the changes made by the replacements were in each case significant (see Materials and Methods). For each deletion or replacement, the construct was tested with the other three subunits, with and without the 43 kD protein, in the COS cell expression system. Surface

expression of the AChR was monitored by ^{125}I-α-BTX binding and the distribution of the AChR containing the mutated α or β subunit by staining with MAb 210. The results are summarized in Fig. 6 and 7. In all cases in which expression of the AChR on the surface was obtained, clustering induced by the 43 kD protein was seen. For the α subunit, substitution or deletion in 7-15 amino acid segments through a length of 83 amino acids, extending from α299 to α382, did not affect the ability of the AChR to be clustered. Altered α subunits with deletions and substitutions within a region of 26 amino acids (α382-408) at the C-terminal end of the loop, however, did not become assembled into AChR expressed on the surface. The possible participation of this segment in clustering could thus not be eliminated.

For the β subunit, deletions or substitutions over a sequence extending from β338 to β430 did not prevent AChRs containing these subunits from being clustered. Deletion of the segment from β405-420 disrupted AChR assembly, as did substitution of the corresponding α sequence. When the corresponding γ sequence for this segment was substituted into the β subunit, however, surface AChR expression did occur, and the resulting AChR was clustered when coexpressed with the 43 kD protein. Mutation of specific non-conserved amino acids within this sequence did not affect 43 kD-induced AChR clustering (Fig. 7). Two regions at each end of the loop, β310-338 and β430-448, could not be mutated or deleted without disrupting AChR assembly. Thus either of these segments could participate in AChR clustering.

One of the substitutions that we made is of particular interest in view of the observation that agrin, a neurally-derived, extracellular matrix protein that induces clustering in muscle cells stimulates the phosphorylation of the β subunit of the AChR in chick (Wallace et al., 1991). When the seven amino acids, GTDEYFI, containing the site of tyrosine phosphorylation on the β cytoplasmic loop were mutated to those of the α subunit, PSRDKQE (containing no tyrosine), AChR clustering induced by the 43 kD protein was not affected (Fig. 7; see subunit β(*363-370)). Thus tyrosine phosphorylation of the β subunit is not required for the coclustering of AChRs with the 43 kD protein that is seen in COS cells. Moreover, when 7 amino acids, PSRDKQE, including a serine in the loop of the α subunit which is phosphorylated by protein kinase C (Huganir, 1989), were mutated to GTDEYFI, no effects on clustering of the AChR was seen (Fig. 6; see subunit α(*332-339)). Thus phosphorylation at these two sites is not required for 43 kD protein-induced AChR clustering.

Discussion

Our results show that in COS cells, as in other non-muscle cells, AChRs expressed alone are diffusely distributed on the cell surface, but become clustered when co-expressed with the 43 kD protein. Because the 43 kD protein forms aggregates when expressed alone, and because the AChR clusters are co-extensive with the 43 kD protein aggregates when the two are expressed together, AChR clustering is likely to arise from direct interaction with the 43 kD protein. This interaction is probably specific, as CD8, another

membrane protein, does not become co-clustered with the AChR when the two are expressed together.

Previous experiments have used in vitro mutagenesis to define the domains of the 43 kD protein that are required for clustering of the AChR (Phillips et al, 1991). We have attempted to carry out similar experiments for the subunits of the AChR. Because the 43 kD protein is a peripheral membrane protein on the cytoplasmic side of the membrane (Froehner, 1991), we have focused largely on the cytoplasmic domains of the AChR subunits, particularly those connecting M3 and M4.

Our experimental approach is based on the hypothesis that the 43 kD protein interacts with the AChR through a specific region of a particular subunit, so that removal or alteration of this region would abolish the ability of the AChR to be clustered by coexpression with the 43 kD protein. We first made receptors lacking the cytoplasmic domains of the ε and δ subunits and found that AChRs containing these mutated subunits could still be clustered by the 43 kD protein. Thus neither the cytoplasmic nor transmembrane domains of the ε or δ subunits are required for clustering. Similar results have been obtained in experiments in which the AChR and 43 kD protein were expressed in Xenopus oocytes (P. Scotland and S. Froehner, Personal communication).

Because cytoplasmic regions of the ε and δ subunits were not required, we then examined the α and β subunits, focusing

particularly on their long cytoplasmic loops. Expression of chimeric subunits in which the loops were exchanged between α and β subunits did not support AChR assembly, so that we were unable to rule out interactions of the 43 kD protein with either. Because of the potential importance of the cytoplasmic loops, we then made a systematic series of deletions or substitutions in them. In each case, deletions or substitutions throughout most of their lengths did not alter the ability of the AChR to be clustered in the presence of the 43 kD protein. For the α subunit, deletions from α299 to α382 had no effect on clustering; for the beta subunit, deletions or substitutions from β338 to β430 were also without effect. For each subunit, however, we were unable to make conclusions about a sequence of 16-26 amino acids that occurs near the junction of the cytoplasmic loop and M4. Removal or alteration of these sequences prevented AChR assembly. In other experiments, we have suggested that this region is required for steps in the assembly pathway subsequent to heterodimer formation (Yu and Hall, in preparation). Also in the case of the β subunit, deletions and substitutions in a short region near the N-terminus of the loop (amino acids 310-338) failed to support surface expression.

Our experiments thus are unable to identify a specific sequence that is required for 43 kD-induced AChR clustering. These results can be interpreted in two ways. First, one or more of the sequences that we were unable to test could mediate the interactions required for clustering. Second, there may not be a specific sequence that is required for clustering. In this case, the 43 kD or other proteins

could interact with multiple or alternate sites on the receptor subunits, so that removal of a single sequence would not abolish clustering. The interactions leading to clustering might thus not depend on any single sequence. The proposed location of the 43 kD protein beneath the AChR (Toyoshima and Unwin, 1988; Mitra et al., 1989) would be consistent with multiple sites of interaction.

One clear result of our experiments is to show that phosphorylation of the tyrosine residue on the cytoplasmic loop of the β subunit is not required for 43 kD protein-induced clustering. This tyrosine, which can be removed without affecting clustering, is postulated to be the site at which the β subunit is phosphorylated in response to the neurally-released clustering factor, agrin (Wallace et al, 1991). We also show that a serine residue (α333) in the cytoplasmic loop of the α subunit of the AChR can be removed without affecting clustering.

In our experiments, we made several unsuccessful attempts to detect interactions between individual subunits and the 43 kD protein. Thus overexpression of neither α nor β subunits detectably decreased AChR clustering as might be expected if the isolated subunits competed with intact AChRs for the 43 kD protein. Also no relation was found between the intracellular distribution of the 43 kD protein and the β subunit. These observations suggest that these subunits may not interact with the 43 kD protein before their incorporation into the AChR.

An unanswered question is the relation between the clustering of AChRs induced by the 43 kD protein in nonmuscle cells and the AChR clustering seen in muscle cells. Although the 43 kD protein is found in aggregates when expressed alone in nonmuscle cells, its distribution in muscle cells appears to be regulated in a different way. Thus the 43 kD protein is not aggregated or clustered in variants of the C_2 muscle cell line that do not form AChR clusters, either because they lack AChRs (LaRochelle et al., 1989), or because they make a defective extracellular matrix (Gordon et al., 1993). Moreover, muscle cells in which the 43 kD protein is overexpressed, show a decrease in the number of large clusters that are normally seen and an increased number of small clusters similar to those seen in COS cells (Yoshihara and Hall, 1993). These experiments raise the possibility that the 43 kD-induced clustering seen in non-muscle cells may occur by a mechanism that is different from that seen in muscle cells. Alternatively, the mechanism of 43 kD protein-induced clustering seen in non-muscle cells may resemble that seen in muscle cells, but may only be part of the more complex system that regulates AChR clustering in muscle cells. In either case, expression in non-muscle cells provides a simplified system in which interactions between the AChR and the 43 kD protein can be mapped in experiments such as those presented here.

Figure 6-1. Clustering of the AChR and the 43kD protein in transfected COS cells.

COS cells were transfected with cDNAs for the α, β, δ and ε subunits minus (A), or plus (B) 43kD protein cDNA. AChRs on the surface (A and B) were stained with MAb 210 against the α subunit, followed by FITC-conjugated goat anti-rat IgG and visualized with fluorescence microscopy. (C): COS cells were transfected with the 43 kD protein cDNA alone, permeabilized and then stained with a mixture of three monoclonal antibodies against the 43kD protein, followed by FITC-conjugated sheep anti-mouse IgG and visualized with fluorescence microscopy. (D): COS cells were transfected with cDNAs for the 43 kD protein and CD8 peptide, permeabilized and then stained with the monoclonal antibody against CD8 protein, OKT8, followed by FITC-conjugated sheep anti-mouse IgG and visualized with fluorescence microscopy. Further details are given in Materials and Methods. Bar=10μM

Figure 6-1. Colocalization of the surface AChR and the 43kD protein.

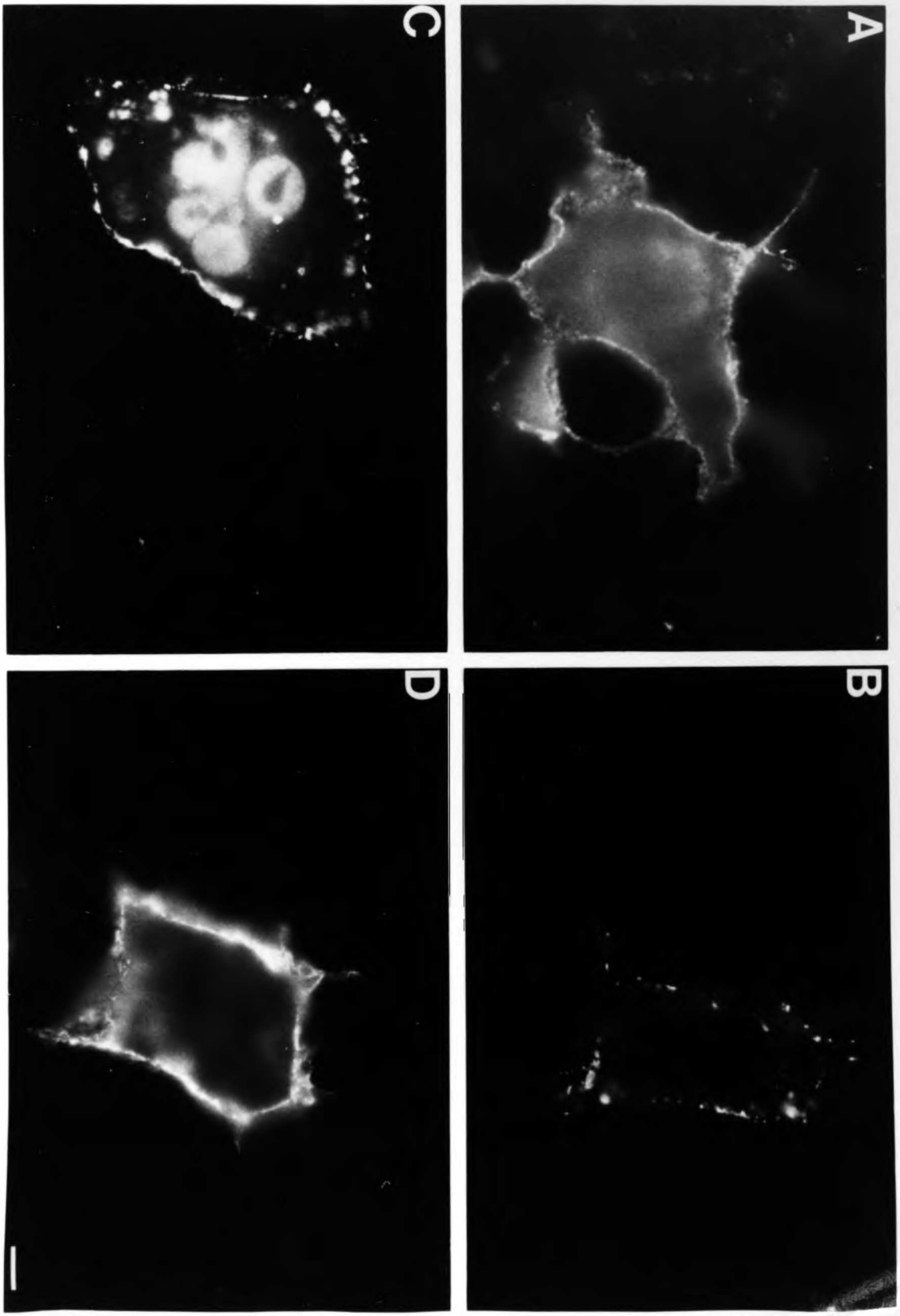


Figure 6-2. Colocalization of the surface AChR and the 43kD protein.

COS cells were transfected with cDNA for the 43 kD protein and with cDNAs for the α, β, δ and ε subunits. Surface AChR was labeled by RH-α–BTX, followed by rabbit anti-α–BTX, and RH-conjugated goat anti-rabbit IgG. The 43 kD protein was stained with a mixture of the three monoclonal antibodies against the 43 kD protein, followed by FITC-conjugated sheep anti-mouse IgG. The same cells expressing both the AChR and the 43kD protein were photographed in the flurescence microscope. (A): RH optics; (B): FITC optics. Bar=10μM

Figure 6-2.

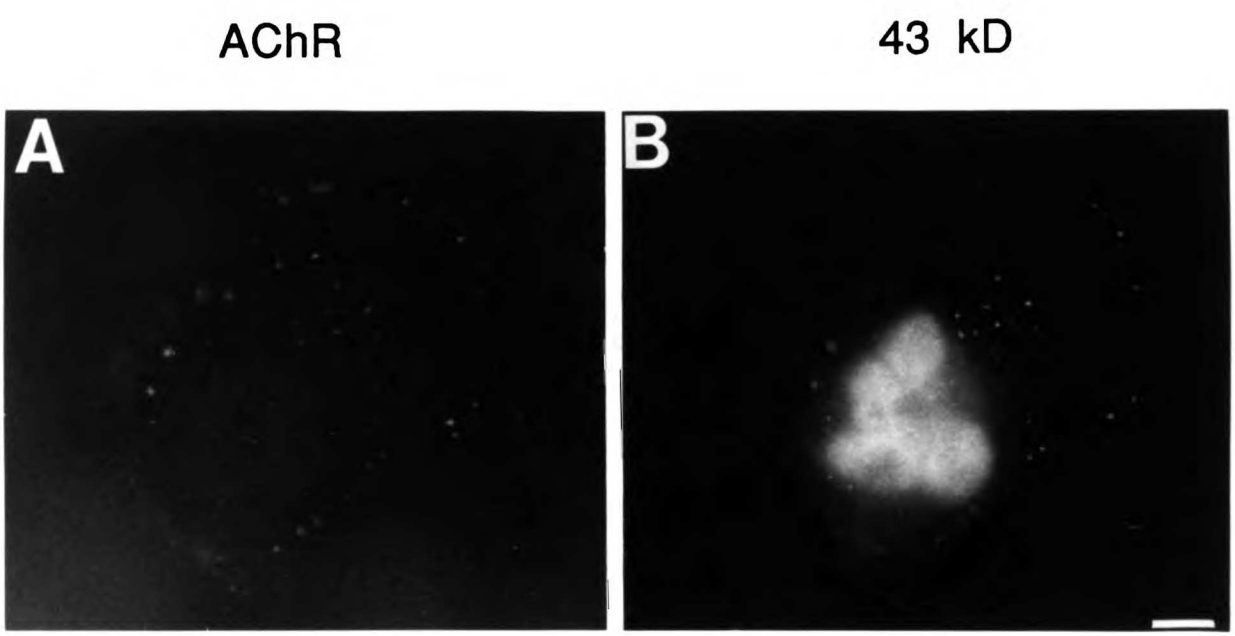
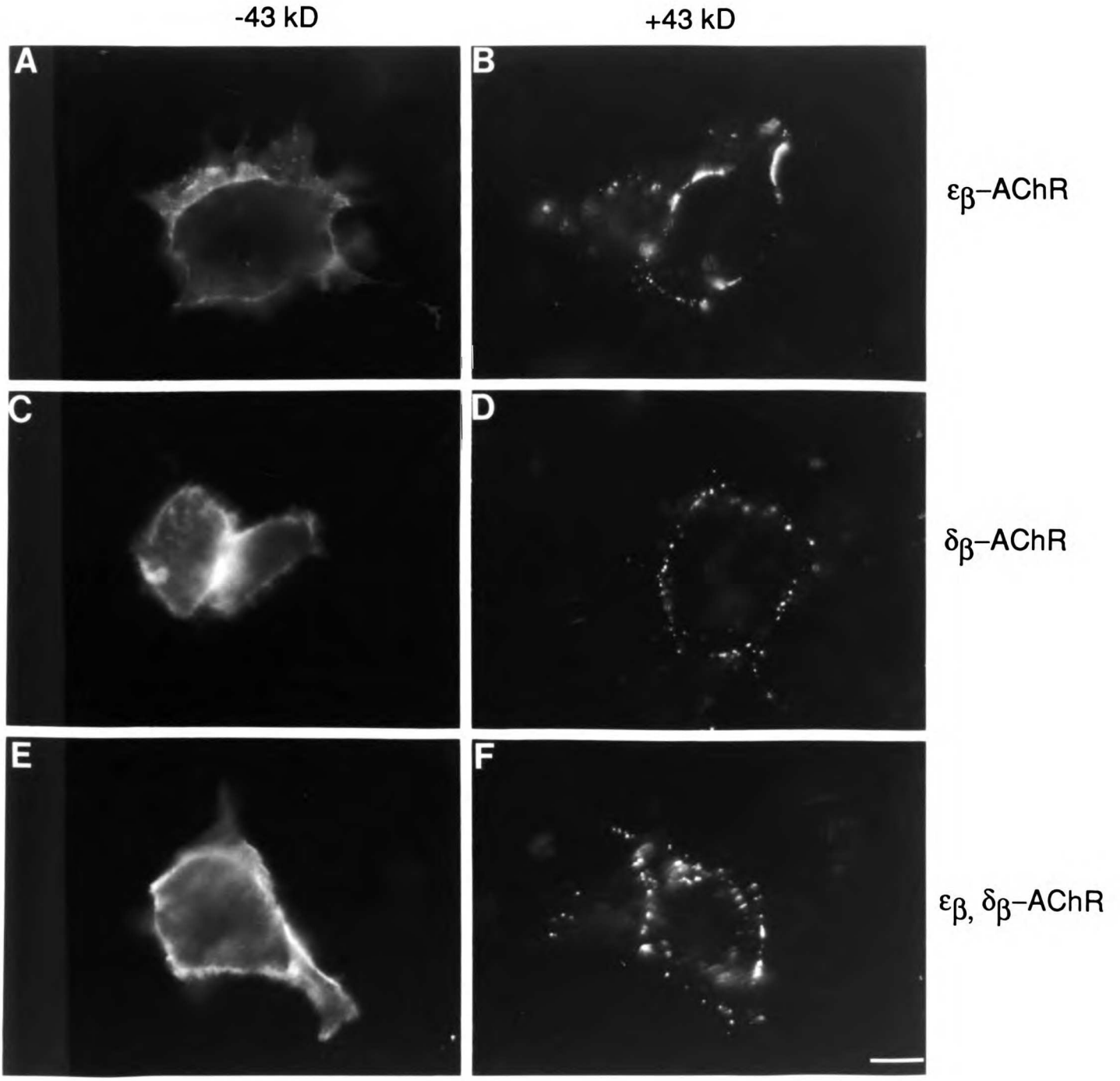


Figure 6-3. 43 kD protein-induced clustering of AChRs containing chimeric subunits in the absence (A, C and E) and presence (B, D and F) of the 43 kD proteins.

COS cells were cotransfected with cDNAs for AChRs containing either chimeric (ε_β) (A, B), or (δ_β) (C, D) or both subunits (E, F) and the distribution of the AChRs was examined by immunocytochemistry. In all cases, the surface AChRs were stained with MAb 210, followed by FITC-conjugated goat anti-rat IgG, and visualized under fluorescence microscopy. More information is given in Materials and Methods. Bar=16μM

Figure 6-3.



Legend of Table 6-I:

cDNAs of the AChR subunits or CD8 protein shown in the table were transfected with or without the cDNA of the 43kD protein into COS cells. AChRs on the surface were stained with mAb210, followed by FITC-conjugated goat anti-rat IgG and visualized with fluorescence microscopy. CD8 protein on the surface was stained with OKT8 antibody against CD8 protein, followed by FITC-conjugated sheep anti-mouse IgG and visualized with fluorescence microscopy. A certain amount of AChR or CD8 protein positive cells were counted, fraction of which containing clusters were also counted.

Table 6-I: 43 kD protein-induced clustering of AChRs with chimeric subunits and of CD8

Membrane protein	43 kD protein-	Cells with AChR clusters / AChR-positive cells
AChR	+	48 /107
	-	3 /106
$\delta\beta$–AChR	+	55 /105
	-	4 /103
$\varepsilon\beta$–AChR	+	57 /102
	-	2 /105
$\delta\beta$, $\varepsilon\beta$–AChR	+	64 /100
	-	3 /104
CD8	+	12 /200
	-	0 /200

Figure 6-4. 43 kD-induced clustering of the surface AChRs was not inhibited by excess expression of the α or β subunit. COS cells were transfected with standard concentrations of the cDNAs for the α, β, δ and ε subunits and for the 43 kD protein plus a 2-fold excess of the α subunit cDNA (A) or with a 3-fold excess of the β subunit cDNA (B). The surface AChRs were stained with MAb 210, followed by FITC-conjugated goat anti-rat IgG and photographed with fluorescence microscopy. Bar=10μM

Figure 6-4.

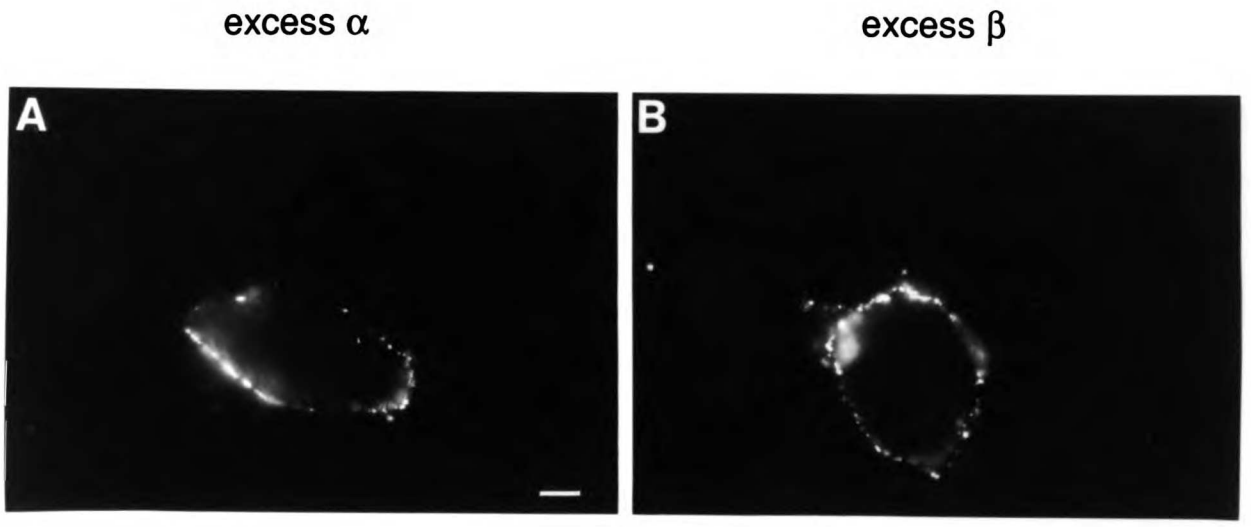


Figure 6-5. Different distributions of the α or β subunit and the 43 kD protein were observed when both were coexpressed in COS cells.

A. Distribution of the α subunit. The α subunit was coexpressed in COS cells with the 43 kD protein, and the cells stained with MAb210 against the N-terminus of the α subunit, followed by FITC-conjugated goat anti-rat IgG, and visualized in fluorescence microscopy.

B. Distribution of the β subunit. The β subunit was coexpressed into COS cells with 43 kD protein, and the cells stained with MAb124 against the loop of the β subunit, followed by FITC-conjugated goat anti-rat IgG, and visualized in fluorescence microscopy.

C. Distribution of the 43 kD protein. The 43 kD protein was coexpressed into COS cells with the α subunit, and the cells stained with the monoclonal antibodies to the 43 kD protein (see Materials and Methods), followed by FITC-conjugated sheep anti-mouse IgG and photographed in fluorescence microscopy.

D. Distribution of the 43 kD protein. The 43 kD protein was coexpressed into COS cells with the β subunit, stained with the monoclonal antibodies, followed by FITC-conjugated sheep anti-mouse IgG and photographed in fluorescence microscopy. Bar=10μM

Figure 6-5

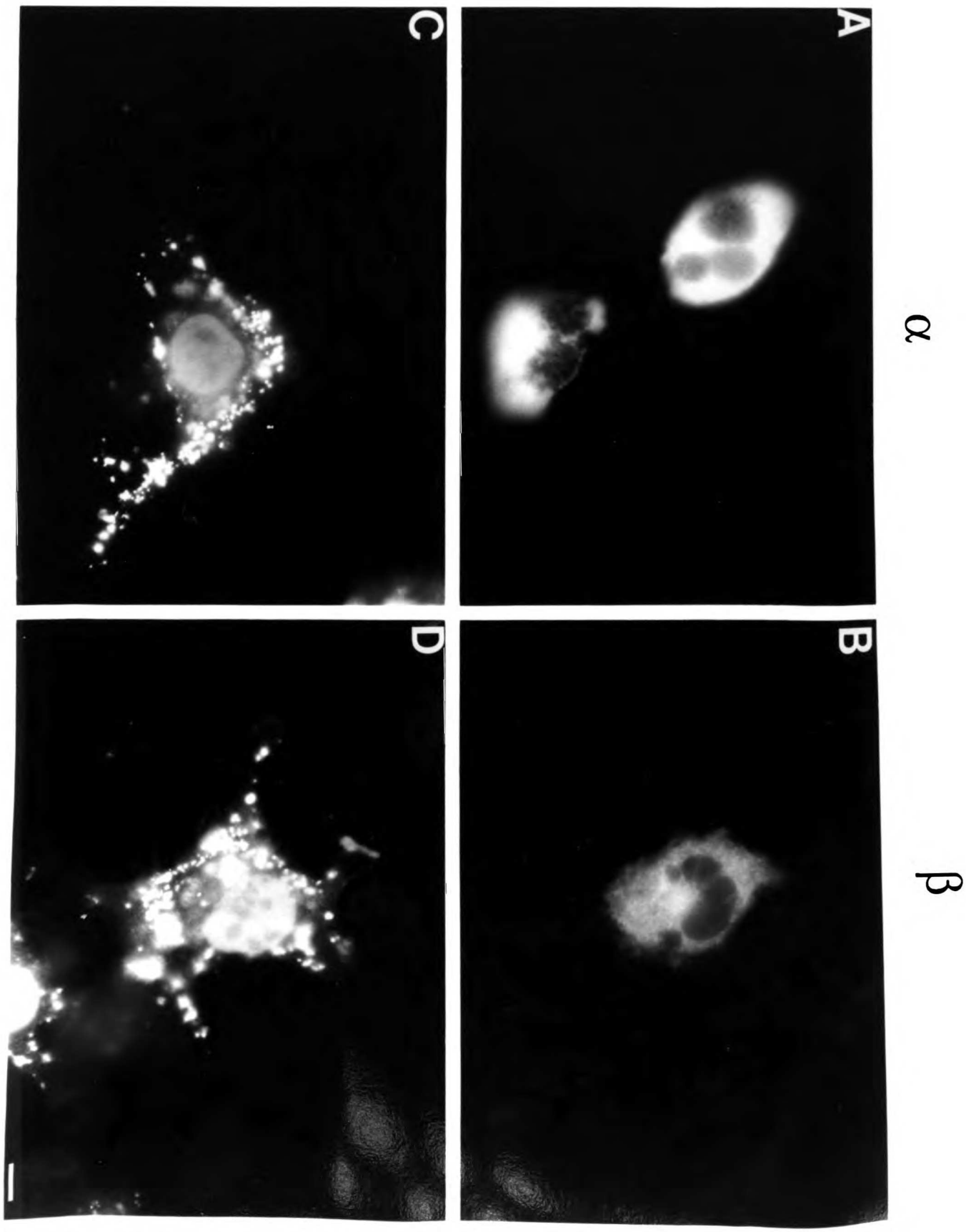


Figure 6-6. 43 kD protein-induced clustering of AChRs containing mutated α subunits. A schematic diagram of the mutated α subunits is shown along with the fraction of cells expressing AChRs that have clustered AChRs when the mutated subunit is expressed along with other AChR subunits and with the 43 kD protein.

Each mutated α subunit was constructed by in vitro mutagenesis (see Materials and Methods). cDNAs of the β, δ and ε subunits plus cDNA of each mutated α subunit were cotransfected with or without the cDNA of the 43 kD protein into COS cells. AChRs on the surface were stained and AChR-positive cells were counted in the same way as described in Table I.

Figure 6-6.

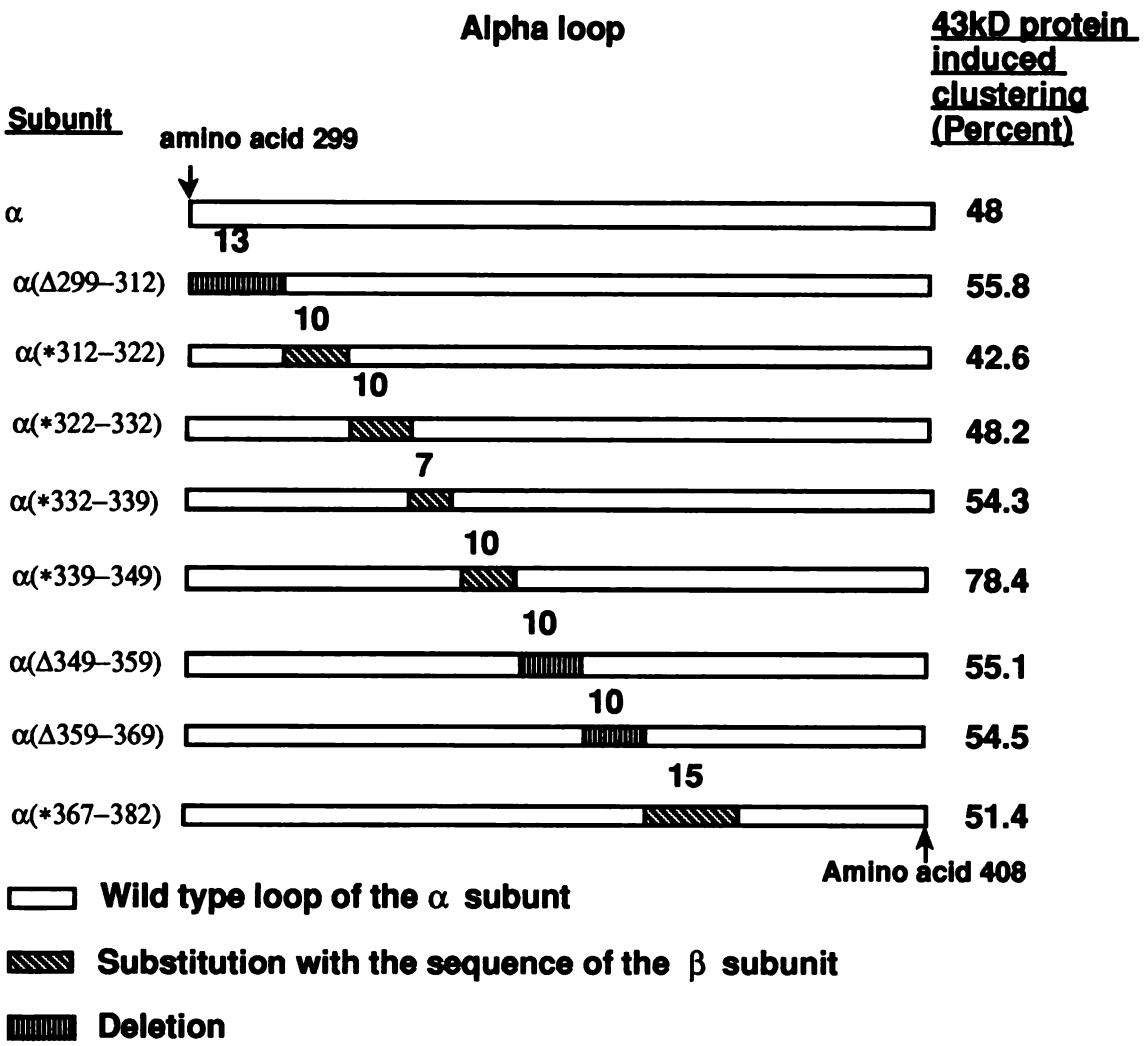
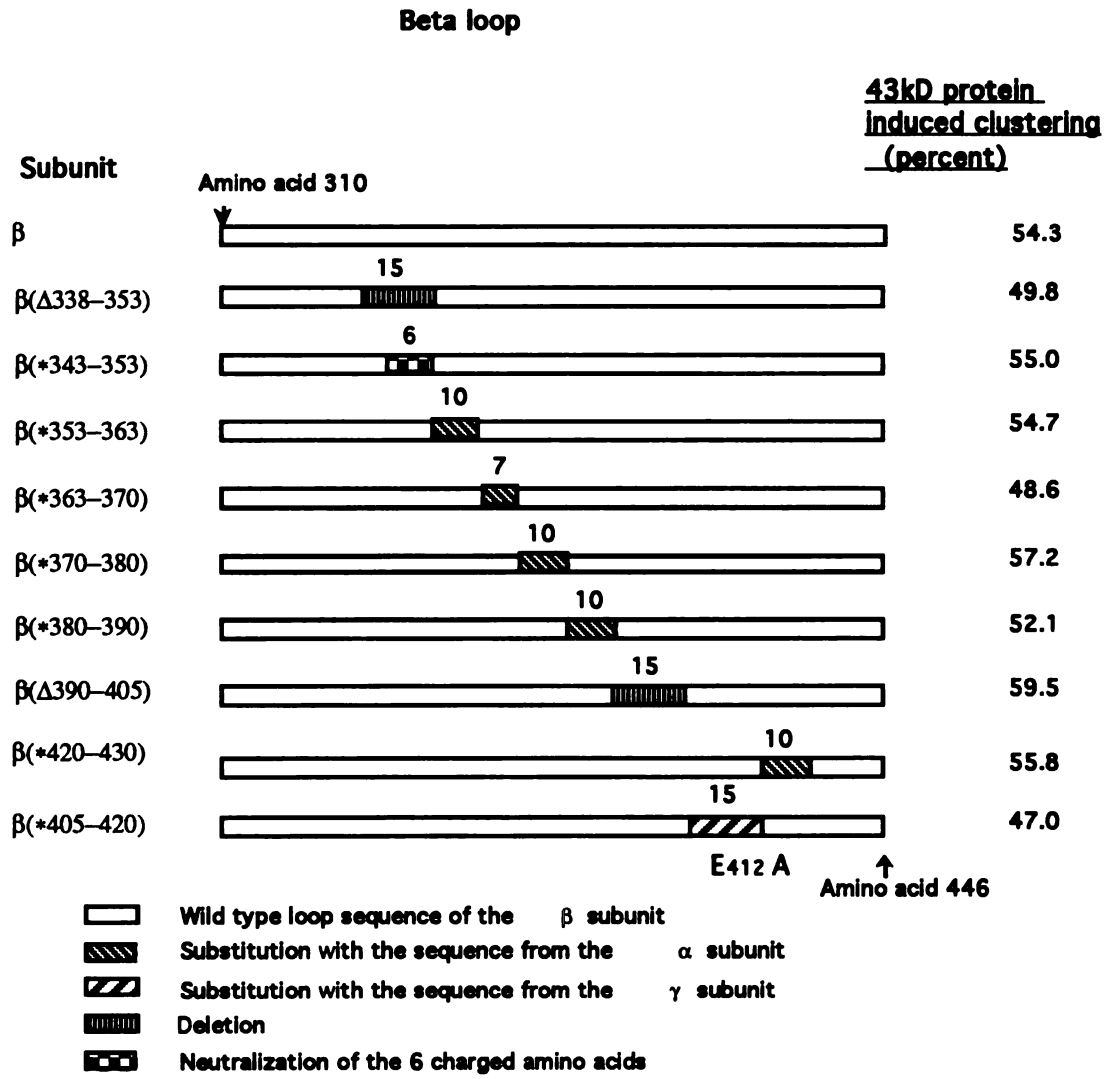

Alpha loop
43kD protein induced clustering (Percent)
Subunit
amino acid 299
α
48
13
α(Δ299–312)
55.8
10
α(*312–322)
42.6
10
α(*322–332)
48.2
7
α(*332–339)
54.3
10
α(*339–349)
78.4
10
α(Δ349–359)
55.1
10
α(Δ359–369)
54.5
15
α(*367–382)
51.4
Amino acid 408
Wild type loop of the α subunt
Substitution with the sequence of the β subunit
Deletion

Figure 6-7. 43 kD protein-induced clustering of AChRs containing mutated β subunits. A schematic diagram of the mutated β subunits is shown along with the fraction of cells expressing AChRs that have clustered AChRs when the mutated subunit is expressed along with other AChR subunits and with the 43 kD protein.

Each mutated β subunit was constructed by in vitro mutagenesis (see Materials and Methods). cDNAs of the α, δ and ε subunits plus cDNA of each mutated β subunit were cotransfected with or without cDNA of the 43 kD protein into COS cells. AChRs on the surface were stained and AChR-positive cells were counted in the same way as described in Table I.

Figure 6-7


Beta loop
43kD protein induced clustering (percent)
Subunit
Amino acid 310
β
54.3
β(Δ338–353)
15
49.8
β(*343–353)
6
55.0
β(*353–363)
10
54.7
β(*363–370)
7
48.6
β(*370–380)
10
57.2
β(*380–390)
10
52.1
β(Δ390–405)
15
59.5
β(*420–430)
10
55.8
β(*405–420)
15
47.0
E412 A
Amino acid 446
Wild type loop sequence of the β subunit
Substitution with the sequence from the α subunit
Subetitution with the sequence from the γ subunit
Deletion
Neutralization of the 6 charged amino acids

Chapter Seven

Discussion

Many integral membrane proteins, such as ligand-gated ion channels and many receptors, are composed of several polypeptide chains. Those subunits in each molecule are independently translated by the separate mRNAs transcribed by their independent genes. The subunits are inserted into the ER membrane where assembly of the AChR takes place (Gu et al., 1989). Most of each protein is degraded in the ER unless the protein is incorporated into a mature and complete complex (Hurtley and Helenius, 1989). The fully assembled complexes are then transported to the surface membrane to exert their functions. However, how each separate subunit recognizes each other and makes a specific interaction in a precise place and in an extremely accurate order is largely unknown.

The AChR is the best understood and characterized member of oligomeric membrane proteins. The studies on AChR assembly could shed light on the mechanism of assembly of other surface polymeric proteins. A lot of experimental accessibility makes easier and simpler the studies of the molecular mechanism of AChR assembly. An abundance of the AChRs in Torpedo, easy purification with α-BTX linked chromatography, availability of cDNAs of all AChR subunits, and the wealthy information of the AChRs in biochemistry, pharmacology, electrophysiology and molecular biology have elected the AChR as one of the best protein models to study assembly of the oligomeric proteins. I thus decided to study AChR assembly by using molecular and cellular techniques as my thesis to access

understanding of assembly of a large number of oligomeric membrane proteins.

Molecular mechanism of assembly of the T-cell receptor

Recent literature described assembly of the T-cell receptor very attractively, but knowledge of the T-cell receptor assembly was not very useful to study AChR assembly.

The T-cell receptor-CD3 complex is an octomer with six different subunits and expresses in most lymphocytes. It is composed of a ζ2 homodimer and a heterodimer of α and β chains, connected with the CD3 complex (CD3-ε in combination with either CD3-γ or CD3-δ) (Alarcon et al., 1991). Only a single transmembrane domain has been identified with an extensive extracellular domain and a smaller cytoplasmic domain. A recent study demonstrated that the transmembrane domain, not the extracellular or intracellular domain, of the α and CD3-δ subunits of the T-cell receptor provided recognition for the association of those subunits (Lippincott-Schwartz et al., 1988). The cytoplasmic domain-truncated α subunit can interact with the cytoplasmic domain truncated δ subunit. Even when the extracellular domain of the α subunit was substituted by that from another protein, the chimeric α subunit was still able to associate with the CD3-δ subunit. These experiments indicate that the transmembrane domains of these two subunits participate the association. Each of the transmembrane domains has been analyzed, and contains highly charged residues. The transmembrane domain of the α chain contains an arginine and a lysine residues spaced by

five amino acids and that of the CD3-δ chain contains an aspartic acid residue. When either the two basic amino acids from the α chain or the negatively charged aspartate from the CD3-δ chain was mutated by in vitro mutagenesis, association between the two chains was eliminated (Berkhout et al., 1988). The introduction of a single positively charged residue into the uncharged transmembrane domain of the interleukin 2 receptor enable it to interact to the CD3-δ chain. These results clearly imply that charge interaction within the transmembrane domain not only results in association between the subunits, but also specify it.

Although charge neutralization within the transmembrane region of the T-cell receptor is the first case to be identified as a driving force to mediate association between subunits, the other mechanisms of subunit association exist as well even in the T-cell receptor, because the α and β subunits can associate each other to form a dimer not by charge interaction.

Molecular mechanism of AChR assembly

The AChR obviously uses a different mechanism to finish its assembly. Although the five subunits in the AChR are also non-covalently associated together, they have four transmembrane domains, instead of one transmembrane domain in the TCR subunits. Certainly no evidence has so far found that charge neutralization within the transmembrane domains exists in the AChR complex. A large number of mutations constructed in the transmembrane

domains only demonstrated that M2 of all the subunits are lined up to form a channel wall and negatively charged residues in either the end of M2 form rings to determine ion specificity and to influence the rate of ion movement (Imoto et al., 1988; Imoto et al., 1986).

Our studies found that both the extracellular and intracellular domains are of importance for AChR assembly. The studies on assembly of the AChR, as another independent example, apparently turn to be more useful for understanding assembly on a superfamily of ligand-gated ion channels and surface oligomeric membrane proteins. At least K^+ channel, a voltage-gated ion channel, is assembled by an interaction in the N-terminal domains, not transmembrane domains (Li et al., 1992).

It has been demonstrated that AChR assembly is processed by a defined pathway. The α subunit, as well as the other subunits, has to undergo several posttranslational modifications such as glycosylation and disulfide bond formation to acquire correct comformational changes. After this process, the α subunit typically obtains α-BTX binding activity and also an ability to associate with δ and γ/ε subunits. These two features of the α subunit have been used as standards to test accuracy of fold process of the α subunit. The assembly studies (Blount et al., 1990; Gu et al., 1991b; Saedi et al., 1991) defined the first step of AChR assembly as the heterodimer formation of α subunit with δ or γ/ε subunit. Then both heterodimers subsequently associate with the β subunit to complete pentamer assembly.

By using powerful in vitro mutagenesis and expression systems, the domains of each subunit containing recognition sites to mediate interaction between the subunits have been identified. In chapter two, three, four and five, I have provided a great deal of evidence to identify several associations between the different subunits and demonstrated that these associations mediate the different steps of AChR assembly.

Initially, we found that only the extracellular domains of the ε subunit are required for AChR assembly. This was concluded by the work on the ε_β subunit. The chimeric ε_β subunit was constructed in such a way that it contains the extracellular domains (N- and C-termini) derived from the ε and the mid-region from the β subunit. This chimeric subunit, ε_β, sufficiently substitutes the wild type ε subunit to complete assembly of the AChR. The AChR incorporated with the ε_β subunit apparently expresses on the surface with toxin-binding characteristics and exhibits the similar properties to those of the normal AChR. The normal turnover rate, α-BTX association rate and the same sedimentation coefficient between ε_β-AChR and ε-AChR indicate the functional AChR is fully assembled with four different subunits, α, β, δ and ε_β. The N- and C-terminal domains of the ε subunit contain all necessary recognition domains to accomplish associations between the subunits. This work initiated AChR assembly at the amino acid sequence level by providing novel information that only the half a ε-subunit gives rise to specificity of the associations between the ε and the other subunits. Arrangement

of the M2 transmembrane domains of the subunits are not the initial contact although they form the inner surface of the ion pore.

Subsequently, a similar mechanism found in the δ subunit. The δ subunit likewise associates with the α subunit to generate the other intermediate of the assembly as the ε subunit does. To wonder whether the extracellular domains of the δ subunit also mediate all its associations with the other subunits, we engineered several chimeric δ subunits, δ_β, δ_γ, and δ_ε which all contain the N- and C-termini of the δ subunit. The intervening region, the M1-M4, was derived from the corresponding region from the β, γ or ε, respectively. When the δ subunit was replaced by each of those chimeric δ subunits in the transfection mixture of in vitro expression system, they all sufficiently substitute the δ subunit to support AChR assembly. The expression of the surface ^{125}I-α-BTX binding complexes with each of the three chimeric δ proteins strongly indicates that only these domains contain specific recognition sites to mediate the associations. Because the β subunit does not associate with the α subunit, the δ_β subunit becomes the best representative of the three chimeric δ proteins.

Does the δ_β subunit resemble the normal δ subunit? Experimental results revealed that they are alike. The chimeric δ_β subunit, like δ subunit, not only incorporates with the α, β and γ to express the γ-AChR, but also with the α, β and ε to express the ε-AChR. These AChRs have the same sedimentation coefficients as that of the normal AChR and contain all the signs of the other subunits,

indicating that the AChRs with the δ_β are the completely assembled pentamers, not just oligomer. The AChRs with either ε_β or δ_β seem to be fully assembled receptors with toxin-binding activity which was competitively inhibited by a small ligand, dTC. This experiment certainly confirms a conception that the ligand binding sites of the AChR are localized in the extracellular domains of the AChR. Only the N- and C-terminal domains of δ and ε subunits, not the transmembrane domains and cytoplasmic domains, contribute to local environments of the two α subunits and differ the response of the α subunits to small ligands.

Finally, only the extracellular (N- and C-terminal) domains of the δ ans ε subunits required for AChR assembly were furthermore confirmed by successful expression of the AChR in the presence of both chimeric ε_β and δ_β subunits, instead of ε and δ, respectively. Its same sedimentation coefficient of 9 S and other characteristic properties as those of the normal AChRs indicate that they are the completely assembled receptors regardless of the cytoplasmic and transmembrane domains of the δ and ε subunits.

Not-surprisingly, attempts to detect the channel on the AChRs with either ε_β, δ_β or both were not successful because the transmembrane domains of the δ and ε subunits are the componant of the channel and are certainly not replaceable.

Heterodimer formation is mediated by the N-terminal domains of subunits

One of the most important accomplishments in this thesis is to demonstrate that the heterodimer formation is clearly mediated by the N-terminal domain of the α and the δ or ε subunit. Several lines of strong evidence have supported this conclusion.

1. Both ε_β and δ_β were able to associate with α subunit to form heterodimers. The heterodimers can be determined by both sucrose gradient and immunoprecipitation with β subunit specific antibody. Obviously, ε and δ subunits use their extracellular side of the molecule to associate with α to form heterodimers, proceeding the late step of assembly.

2. A truncated α subunit, αM1, whose C-terminal part after M1 has been deleted, thus only contains the N-terminal half part of the molecule. The αM1 can fold correctly and acquire α-BTX binding. It is able to associate with δ to form a heterodimer determined by immunoprecipitation and sucrose gradient. The αM1, further, succeeds in association with δ and β subunits to form a trimer. All experimental data suggest that the N-terminal domain of the α subunit is enough to process heterodimer and heterotrimer formation.

3. More interestingly, αM1 is able to interact with δ_β to form a heterodimer, $(\alpha M1)(\delta_\beta)$. The successful detection of this heterodimer by immunoprecipitation with β subunit specific antibody, MAb 124

strongly convey a message that the heterodimer formation, the first step of assembly, is mediated by association through the N-terminal domain of the α subunit and the extracellular domains of the δ and ε subunits.

4. Another line of independent evidence comes from a chimeric α subunit, α–β1, whose main cytoplasmic domain, loop, has been substituted by the correspoding loop from the β subunit. The α–β1 has the normal level of α-BTX binding activity, suggesting that it must process routine and regular conformational changes. Its correct folding process is further confirmed by its ability to associate with δ subunit. The toxin-binding activity of the α–β1 is increased by its association with the δ subunit. The heterodimer, (α–β1)δ, was also immunoprecipitated by δ subunit specific antibody, MAb 88B. These results clearly support the above conclusion that the N-terminal domain of the α subunit is sufficient for heterodimer formation.

5. Moreover, the α–β1 can associate with ε_β and δ_β to generate heterodimers. The toxin-binding activity of the α–β1 is substantially elevated by its coexpression with either the ε_β or δ_β subunit. The αM1 and α–β1 (data not shown) can inhibit the normal heterodimer formation as well as surface AChR expression at 1:1 ratio of normal α and αM1 or α–β1. Of course, the mechanism of these phenomena is the association via the N-terminal domain and the extracellular domains of the subunits.

Taken together, all the evidence strongly manifests that the association ocurring in the N-terminal and extracellular domains of two subunits controls the heterodimer formation, the first step of assembly. This conclusion is consistent with the results from dominant negative inhibition experiments (Verrall and Hall, 1992) which demonstrated that the N-terminal partial proteins, αM1, γM 1 and δM1, were able to inhibit surface AChR expression and heterodimer formation, presumably through their forming abortive complexes with the normal subunits. Chapter V has provided strong evidence to demonstrate that the C-terminal domains of the α, δ, ε and γ are evidently involved in late assembly. These results lead to a final conclusion that the N-terminal domain contains recognition signals that mediate initial AChR assembly.

<u>The association between the cytoplasmic loops of the α and β subunit participates the late step of AChR assembly</u>

Do the cytoplasmic domains contain any recognition sites, and involve in any association during AChR assembly? Answer is yes. Although the cytoplasmic domains of δ and ε subunits are not required to complete AChR assembly, and the N-terminal domains of the subunits mediate the first step of assembly, the cytoplasmic domains in the α and β subunits are definitely indispensable and may directly participate the late step of assembly. The first evidence to indicate their significance in assembly comes from chimeric α and chimeric β subunits. By using the same approach as making δ_β and ε_β, we also constructed the chimeric α_β and β_α, β_δ, β_ε, and β_γ subunits. The α_β subunit consists of the N- and C-termini from the α

subunit and the M1-M4 region from the β subunit. When the α_β coexpressed with the other required subunit, β, δ and ε, it does not support AChR assembly, suggesting that transmembrane and cytoplasmic domains of α subunit are required for AChR assembly. The same results obtained to the chimeric β_α, β_δ, β_ε, and β_γ, which all include the same N- and C-termini from the β and the diverse mid-region from α, δ, ε and γ, respectively and correspondingly. Apparently, the transmembrane and cytoplasmic domains of the β subunit are also important. Since the transmembrane domains are highly homologous and cytoplasmic domains are largely diversified, the main loop were chosen to be investigated further. The loops between α and β subunits are restrictly switched, resulting two chimeric subunits, α–β1 and β–α1. The α–β1 has been described to some extent above. Although it folds, acquires toxin-binding activity, associates with δ and ε, it does not support and complete AChR assembly. Incompetence of the α–β1 to accomplish AChR assembly is resulted from missing necessary recognition domains in the cytoplasmic loops of the α and β subunits, not from any other possibilities. Therefore only one conclusion left, i.e. the loops of α and β subunits are involved in the late step of AChR assembly.

The assembly significance of the loops of the α and β subunits motivates a deeper investigation: systematic mutational analysis throughout the loops. Sequentially mutating or deleting 7-15 amino acid fragment discovered that only two regions near M3 or M4 in either α or β loop are essential for AChR assembly. Mutating or deleting these areas completely destroyed surface expression. In

contrast, mutating or deleting the other regions in either α or β loop has no effects on AChR assembly. Inability of the loop mutants of the α subunit to support AChR assembly is not resulted from misfolding, but missing structurally critical, necessary recognition domain to mediate the late step of assembly. All these mutated α subunits bore mutation or deletion in the loop near M3 or M4 were able to bind α-BTX and could associate with the δ. Their acquisition of α-BTX binding, the elevated toxin-binding activity of each mutated α subunit in the presence of the δ subunit, the successful immunoprecipitation of the heterodimers and heterotrimers suggest that the mutated α subunits are folded correctly and process the first step of assembly as the normal α subunit does. Their incompetences to finish the late step of assembly to produce the fully assembled pentamers are due to destruction of recognition sites located in the loop. Defects must occur in the process from trimers to pentamers.

Because the cytoplasmic domains of δ and ε subunts are not required and the cytoplasmic loops of only the α and β subunits are not substitutable, both loops must interact each other to mediate pentamer formation. Identification of this interaction ocurring in the cytoplasmic loops is another accomplishment in the thesis. This interaction is critical to complete AChR assembly.

Two features have been notified. The interaction taking place in the cytoplasmic loops of the α and β subunits is a secondary induced interaction; the recognition sites in the loop of α subunit may be hidden until the association of the N-terminal domain of the α

subunit with the δ and ε subunits because αβ heterodimer formation has not been detected so far by the conventional experimental approaches such as sucrose gradient and coimmunoprecipitation. The alternative explanation is also possible. The recognition sites of the α loop may be exposed normally and the α subunit is able to associate with the β subunit to form a heterodimer. Since this interaction is weak and happens in the loops which may not influence the toxin-binding domain located right before M1 and stability of the α subunit, then conventional experimental approaches can not detect this interaction. This situation is rare because the evidence for the αβ heterodimer formation is not strong and the αβ heterodimer has only been described in one case (Green and Claudio, 1993).

Another feature of the interaction occurred in the loops is its weakness. It is estimated about 10-fold weaker than the heterodimer interaction occurring in the N-terminal domains. This result leads to another speculation that the heterotrimer formation is reversible. At an instantaneous moment, a heterotrimer meeting a heterodimer forms a pentamer which transports to the surface. This process forces the equation to go the heterotrimer formation.

<u>The C-terminal domains of α, δ, γ, ε, but β subunits are required for AChR assembly</u>

The relationship of structure-function of the C-terminal domains of AChR subunits has extensively analysed by in vitro mutagenesis (Chapter V and unshown observations). The C-terminal domains are

short and vary in the different subunits. The fact that none of the C-terminal domains, except for the β C-terminus, can be removed or substituted to maintain AChR assembly clearly suggests that they are structurally required for AChR assembly. Experimental results revealed that the C-terminus-substituted subunits and the C-terminus-truncated subunits are not misfolded because they are expressed in COS cells and can be immunoprecipitated by their subunit specific antibodies and that they formed the heterodimers with the α subunit.

The representative example that the δ C-terminal domain rescued the C-terminus-truncated α subunit to finish assembly clearly exhibits the involvement of both C-termini in late assembly (Chapter V). The truncated α subunit acquires toxin-binding activity and associates with the δ and ε subunits to form the hterodimers, but fails to support AChR assembly in the late step. These results suggest that the C-terminal domain of the α subunit is not required for heterodimer formation, but is essential for late assembly. Likewise, C-terminus-truncated δ subunit did not generate the surface AChR while the truncated δ subunit with deletion of the last 17 amino acids forms a heterodimer with the α subunit, but not whole receptor. The chimeric δ subunit with the γ C-terminus, but not with the other C-tails, directs AChR assembly because of homology between the C-termini of the γ and δ subunits which contain an identical proline-rich fragment that turned out to be undeletable and required for assembly. The work carried out with the chimeric β_{δ}^{s} subunit which did not associate with the α subunit

obviously shows that C-terminus of the δ subunit does not , if any, not sufficient, play a role in the first step of assembly. The study that the chimeric subunit α_{δ}^{s}, not α_{β}^{s}, supports AChR assembly, gives us an example that the C-termini of the α and δ, not β subunit participate the late step of assembly. This example sheds light to understanding the importance of the C-termini of the γ and ε subunit.

Removal of C-termini of either the ε or γ subunit abolishes AChR assembly as well, suggesting that the C-termini of the ε and γ subunits are important in assembly. Heterodimer formation of the C-terminus-substituted ε subunit with the α subunit, but not subsequent assembly of this subunit with the other subunits suggests that the C-terminal domain of the ε subunit is as well involved in the late step of assembly. The C-terminal domain of the γ subunit mediating the late part of assembly is shown as well by the same approaches.

The C-terminal domain (11 amino acids) of the α subunit may be just a partial region of the putative leucine zipper (28 amino acids). The leucine zipper dimerization could be a mechanism to mediate late assembly of AChR which needs experimentally confirmed. The 9 amino acids in the δ C-terminal domain happens to be proline-rich domain which have been suggested to mediate protein-protein interaction in the transcriptional machinary. This could be another mechanism responsible for late assemby of AChR,

Because all the C-termini, except for the β C-terminus, are required for AChR assembly, I would certainly imagine that the late assembly must establish extremely massive and extensive interactions among the subunits which are most likely induced because those interactions would not take place without the early association between the α and δ or γ/ε. Among all these interactions, the interactions through the N-terminal domains are ones of the most important associations which trigger all interactions for the late step of assembly.

Identification of several associations occuring in the extracellular, and intracellular domains and of the amino acids in the C-termini does not imply that these interactions are all interactions in the AChR assembly process. Many other interaction existing in the assembly process may not be easy or possible to discover because they could be conformationally induced, or could be weak, or could be too many, forming a cascade where disrupting one screws up the other. All these problems complex the study of the molecule mechanism of AChR assembly. In confronting these challenges, I am glad that the several interactions discussed above make their ways to be conclusive.

<u>AChR may provide multiple alternative binding sites for association with the 43 kD protein</u>

Coexpression of the AChR and 43 kD protein causes clustering of the AChR in COS cells expression system, in an accordance to the results reported in fibroblast cells and Xenopus oocytes. Expression

of the 43 kD protein by itself leads to 43 kD protein aggregation whereas the AChR, expressed alone, are uniformly distributed over the cell surface, suggesting that the AChR must directly or indirectly associate with the 43 kD protein and aggregation of the 43 kD protein causes clustering of the AChR. Although this result was not confirmed in muscle cells where the 43 kD protein does not aggregate in AChR-deficient cell line, the conclusion is quietly consistent in non-muscle cell lines.

It seems plausible to identify the interaction regions of both the 43 kD protein and AChR. The region of the 43 kD protein to mediate interaction between the AChR and the 43 kD protein has been identified and is localized in the central part of the molecule (Phillips et al., 1991b). We are interested in identifying the regions of the AChR that mediate AChR clustering. The cytoplasmic domains of the AChR subunits are very likely to associate with the 43 kD protein because the 43 kD protein is a peripheral cytoplasmic protein. The cytoplasmic domains of the δ and ε subunits were first excluded, based on the result that the AChRs incorporated either ε_β, δ_β or both subunits were clustered in the presence of the 43 kD protein. The cytoplasmic loops of the α and β subunit were subsequently analysed. We found that the α and β subunits without their own loop do not support AChR assembly, suggesting that the loops of both α and β subunit may be important for AChR assembly and clustering. The potential significance of the loops motivated us systematically to analyze them. Most areas can be mutated or deleted without

influencing AChR clustering. The only region near M4 can not be mutated or deleted to maintain AChR assembly. These region may also mediate AChR clustering, but we were unable to test them. It is also possible that the multiple alternative sites, not specific sequences of the AChR are provided for interaction with the 43 kD protein. A recent study showed that the 43 kD protein can interact all the AChR subunits (Maimone and Merlie, 1993).

Although coexpression of the AChR and 43 kD in nonmuscle cell lines causes AChR clustering, the mechanism of AChR clustering in muscle cell lines appears different. Cytoskeleton proteins may also be involved in clustering. In the future, the mechanism of AChR clustering needs to be studied in more detail in muscle cell lines.

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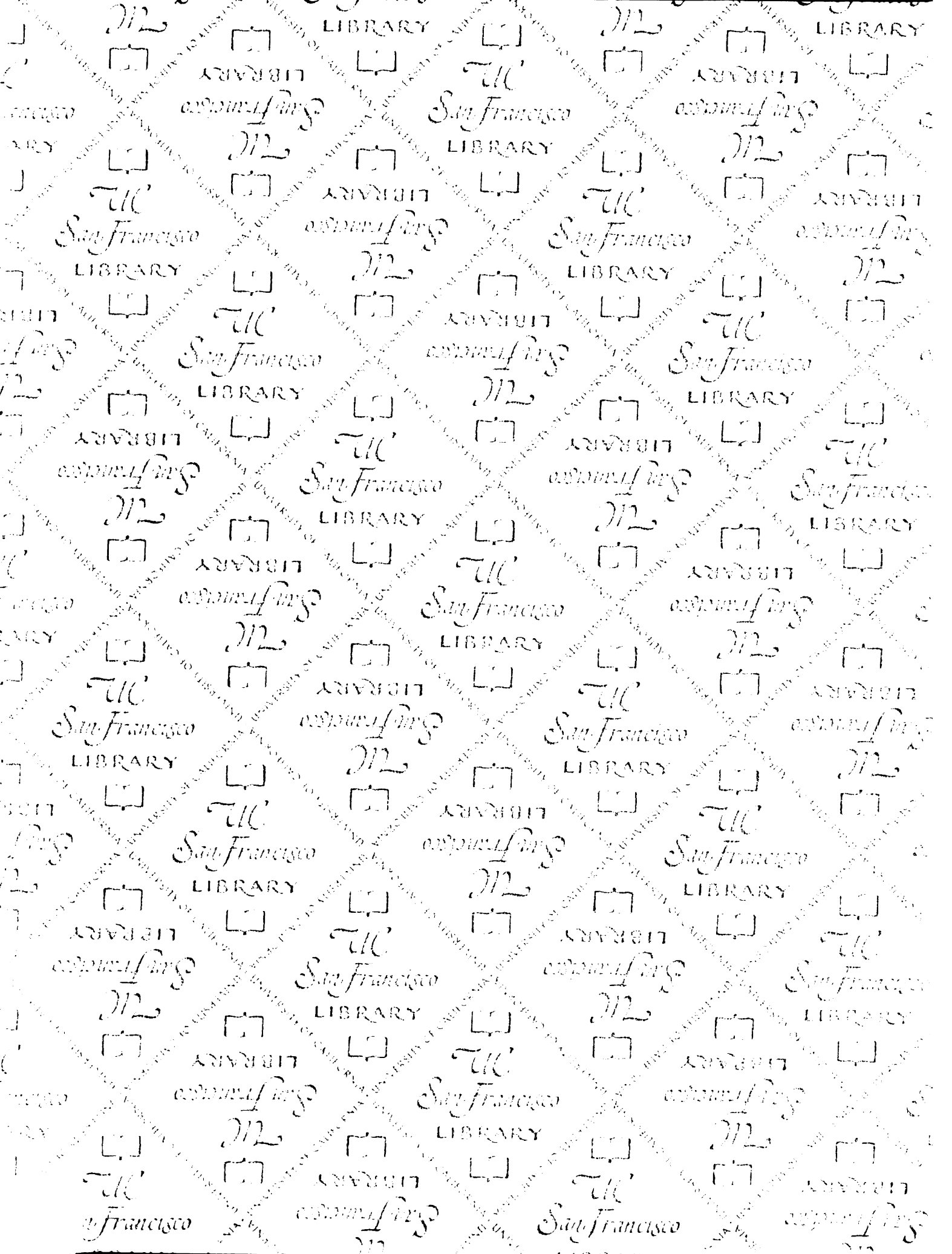
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