Title

A study of the topology and maturation of the subunits of the nicotinic acetylcholine receptor expressed in vitro

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A STUDY OF THE TOPOLOGY AND MATURATION OF THE SUBUNITS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR EXPRESSED IN VITRO

by

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DISSERTATION

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DOCTOR OF PHILOSOPHY

in

PHYSIOLOGY

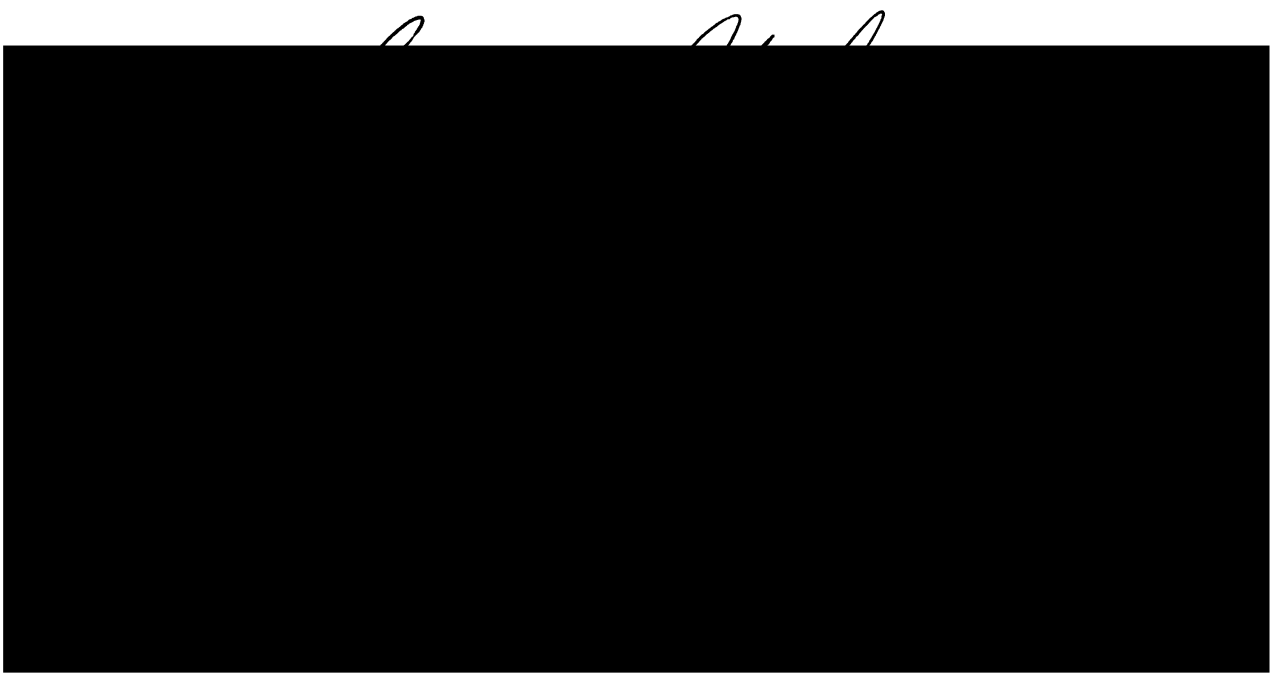
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



DEDICATION

To my parents, Eva Maria Jirón and Juan Fernando Chavez, who have made great sacrifices throughout my life to allow me to pursue my interests freely, and for whose constant love and encouragement I am profoundly grateful.

To my wife, Jill Karen Hacker, who has given me understanding, love, and support beyond my abilities to express.

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ABSTRACT

The transmembrane topology of the subunits of the mouse muscle nicotinic acetylcholine receptor (AChR) synthesized *in vitro* was investigated. The experimental system was a reticulocyte lysate translation system supplemented with pancreatic microsomes. The AChR subunits synthesized in this system were demonstrated as authentic by immunochemical criteria. To determine the topology of the amino-terminal domain of the α subunit, new glycosylation consensus sequences were introduced. Glycosylation at the new sites was taken as evidence of the transmembrane translocation of the new sites. In addition, a fragment encoding the amino-terminal domain exhibited the properties of a soluble protein. The results suggest that the amino-terminal domain of the α subunit is located in the extracellular space.

The topology of the α and δ subunits was investigated by creating fusion proteins in which a reporter domain was attached downstream of each of the putative transmembrane segments, and determining the orientation of that domain relative to the microsomal membrane by proteolysis and immunoprecipitation. Peptides that contained the reporter domain were recovered only when constructs with the reporter domain fused after either M2 or M4 were tested. These results support a model of AChR subunit topology with four transmembrane domains and an extracellular carboxy terminus.

We have used the mouse (α_M) and human α (α_H) subunits to investigate the assembly of the mammalian AChR transiently expressed in COS cells. Cells expressing hybrid receptors incorporating α_H along with other mouse subunits exhibited higher surface α-bungarotoxin (BuTx) binding than cells expressing wild-type mouse AChR. At intermediate stages in the assembly pathway, α_H acquired the BuTx-binding conformation (α_{Tx}) more efficiently than did α_M. Two residues in the amino-terminal domain were responsible for the differences between α_M and α_H. The kinetics of α_{Tx} and αδ heterodimer formation revealed that the δ subunit increased the conversion of immature forms of the α subunit into the BuTx-binding form, a previously undetected interaction in the assembly pathway. These results provide evidence of the importance of the amino-terminal domains of the AChR subunits in the assembly process.

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Chapter 1. Introduction to the Nicotinic Acetylcholine Receptor

Background

When Langley first proposed the existence of "receptive substances" to explain the different responses of skeletal muscle to nicotine and curare (Langley, 1905), he presaged a critical feature of intercellular communication in the nervous system. Since that time, many receptors have been discovered which recognize specific neurotransmitters. At the majority of central and peripheral synapses, the binding of a neurotransmitter to its receptor leads to the opening of ion channels in the membrane of the post-synaptic cell. One class of receptors, known as the ligand-gated ion channels, require no other proteins to transduce ligand binding into channel opening. They contain within their structures both the ligand binding site and the ion channel.

Ligand-gated ion channels belong to a growing family of receptors involved in the function of both excitatory and inhibitory synapses. This receptor family comprises such members as the nicotinic acetylcholine receptor (AChR; both the neuronal and skeletal muscle forms) (Numa, 1987; Patrick *et al.*, 1987; Karlin, 1989; Galzi *et al.*, 1991), the $GABA_A$ receptor (Schofield *et al.*, 1987; Stevens, 1987; Olsen and Tobin, 1990), the glycine receptor (Grenningloh *et al.*, 1987; Grenningloh *et al.*, 1988; Langosch *et al.*, 1988), and has recently been extended to include the 5-HT 3 receptor (Maricq *et al.*, 1991). The glutamate receptor is another distantly-related ligand-gated ion channel (Hollmann *et al.*, 1989). By far the best-studied of these receptors is the AChR found at the endplates of skeletal muscle cells in vertebrates and on electrocytes in the electric organs of marine elasmobranchs (*Torpedo* sp.) and freshwater teleosts

(*Electrophorus* sp.). Several different experimental approaches have been applied to the study of this receptor to understand the relationship between its structure and function. This dissertation represents a series of studies that shares this same goal.

In both skeletal muscle and the electric organ, the AChR is the primary signal transducer between the presynaptic cholinergic neuron and the postsynaptic cell. It is an allosteric protein complex that functions both as a neurotransmitter receptor and as a cation-selective channel. In skeletal muscle, the AChR is concentrated in the postsynaptic membrane beneath the synaptic terminal in a region known as the endplate. When an action potential reaches the synaptic bouton, acetylcholine is released, elevating its concentration in the synaptic cleft. Acetylcholine diffuses across the cleft, binds to the AChR, and induces a conformational change in the receptor which opens the channel, resulting in a net influx of cations across the cell membrane. The cation influx causes a depolarization of the membrane. This change in membrane potential is sensed by voltage-gated sodium channels which then open and propagate the potential change along the muscle cell surface. Sarcomeres within the myofiber are activated via the process of excitation-contraction coupling, and then contract. In the case of the electric organ, the AChRs are concentrated along one side of the electrocytes, the innervated face. The simultaneous depolarizations approximately 100 mV each in size of many thousands of electrocytes are summed into voltages large enough to stun or kill nearby prey.

The receptor oligomer

Much of the information about AChR structure is based on the analysis of the receptor from electric fish. The electric organ is an extremely rich source of AChR (approximately 2 nmol/gram of tissue) (Karlin, 1980), which has greatly facilitated its purification and study. Along with acetylcholine, the ligand of physiological importance, a wide variety of substances bind to the AChR. The most experimentally useful of these are the snake toxins. The most important, α-bungarotoxin (BuTx), is a 74 amino acid polypeptide purified from the venom of the snake *Bungarus multicinctus* (Lentz and Wilson, 1988). BuTx binds to the AChR from *Torpedo* electric organ with very high affinity (approximately 10^{-11} M) and a very slow off-rate (Lentz and Wilson, 1988). BuTx and acetylcholine are mutually competitive ligands of the AChR, and therefore it is presumed that the binding sites for these two molecules overlap.

The AChR, which can be purified from electric organ or skeletal muscle by toxin-based affinity chromatography (Karlin, 1980), is a multisubunit protein complex of approximately 250,000 Da. It is composed of four different subunits, named in order of increasing apparent molecular weight: α (approximately 40,000 Da), β (~50,000 Da), γ (~60,000 Da), and δ (~65,000 Da). The subunit stoichiometry of the purified AChR is $\alpha_2\beta\gamma\delta$, as shown by quantitative amino-terminal sequencing of purified AChR subunits (Raftery *et al.*, 1980).

Reconstitution (Epstein and Racker, 1978; Gonzalez *et al.*, 1980; Lindstrom *et al.*, 1980) and transfection experiments (Mishina *et al.*, 1984; Claudio *et al.*, 1987; Forsayeth *et al.*, 1990; Gu *et al.*, 1990) have

demonstrated that only the four subunits of the AChR are required to create a receptor with all of the physiological characteristics of the native AChR. At the endplate, as well as in the electric organ, other proteins appear to be in close association with the AChR (Carr *et al.*, 1987; Froehner *et al.*, 1987; Carr *et al.*, 1989). However, these proteins are not required for normal AChR function, and their precise role in the development of the structure of the endplate region remains unknown.

As a result of the dense packing of the receptor in the electric organ, quasi-crystalline two-dimensional arrays of the AChR oligomer *in situ* can be obtained, and these specimens can be observed directly in the electron microscope. The repetitive arrays of receptor in these preparations can then be analyzed by image processing to yield an electron density map of the receptor oligomer (Kistler *et al.*, 1982; Fairclough *et al.*, 1983; Brisson and Unwin, 1985; Mitra *et al.*, 1989; Toyoshima and Unwin, 1990). Under these conditions, the receptor appears as a rosette-shaped molecule with a diameter of 80 Å when viewed from the extracellular side of the plasma membrane (Fig. 1-1). Peaks of high electron density associated with individual subunits appear to form an elevated ridge encircling a central depression in the oligomer. This central pit is believed to represent the extracellular vestibule of the ion-conducting channel that is opened when acetylcholine binds to the receptor. The arrangement of the subunits in the AChR is often described as "pseudosymmetric," in view of the fact that an odd number of subunits, two of which are equivalent, are radially distributed around the putative ion channel. The correct order of the subunits around this central pore has not been established. Electron micrographic evidence suggests that the α subunits of the *Torpedo* AChR

do not share a common border (Karlin *et al.*, 1983; Kubalek *et al.*, 1987). Similar evidence excludes both the β and the δ subunits from the position between the two α subunits (Wise *et al.*, 1981; Karlin *et al.*, 1983; but see Kubalek *et al.*, 1987).

When observed along a plane orthogonal to the plasma membrane, the AChR in these preparations spans the membrane and has an overall length of 110 Å, with 55 Å on the extracellular side of the membrane and 15 Å projecting into the cytoplasm. The cytoplasmic projection of the AChR does not exhibit the peaks of electron density seen in the extracellular projection, although the cytoplasmic region of the AChR is not as well resolved as the extracellular region. If each of the subunits occupies an equivalent portion of this cytoplasmic region, this result suggests that all of the subunits of the AChR are transmembrane proteins. The profile of the extracellular funnel-shaped vestibule is quite marked in this view, and appears to reach its narrowest dimension (approximately 7Å) two-thirds of the way across the membrane towards the cytoplasmic side (Fig. 1-2). Permeability studies utilizing permeant species of known dimensions indicated that the channel is impermeable to compounds larger than 6.5 Å (Dwyer *et al.*, 1980). The gradual radial constriction observed within the membrane in these maps is thought to be the size filter of the receptor. The apparent radial symmetry of this constriction also suggests that the central ion channel is composed of regions contributed by each of the subunits of the receptor.

Table 1-1. The characteristics of the subunits of the AChR from mouse muscle

	α	β	γ	δ
MOL. WT. (calculated; Da)				
PRECURSOR	51,786	56,865	58,736	59,075
MATURE	46,640	54,739	56,407	56,815
LENGTH (amino acids)				
MATURE	437	478	497	496
SIGNAL	20	23	22	24
TOTAL	457	501	519	520
GLYCOSYLATION				
# SITES	1	1	4	3
LOCATION	α141	β141	γ30	δ76
			γ141	δ143
			γ306	δ169
			γ354	
OBSERVED	1	1	~2	~3
SIMPLE/COMPLEX	S	S	S/C	S/C

This table was compiled from the primary sequence data (α, (Isenberg *et al.*, 1986); β, (Buonanno *et al.*, 1986); γ, (Yu *et al.*, 1986); δ, (LaPolla *et al.*, 1984)) and a comparison of the glycosylation patterns of the *Torpedo* (Nomoto *et al.*, 1986) and mammalian muscle (Gu and Hall, 1988a) nicotinic AChR. Calculated molecular weights for the subunits were determined using the Strider DNA analysis program.

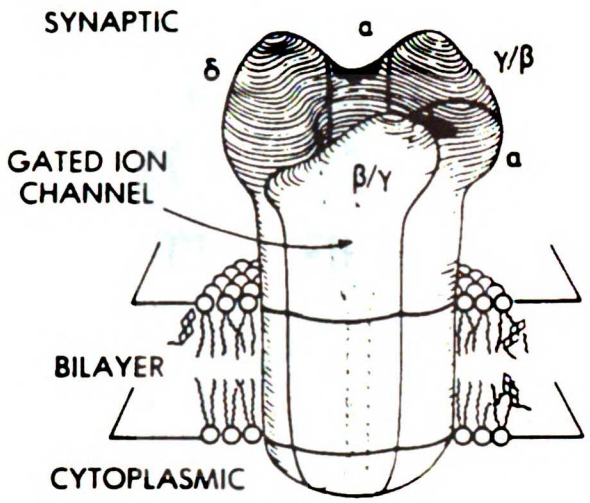


Figure 1-1. Model of the AChR oligomer

A low-resolution three-dimensional model of the *Torpedo* AChR. The positions of the subunits are indicated. Dimensions are given in the text. The precise order of the subunits around the central pore has not been established. Modified from Kistler *et al.*, 1982.

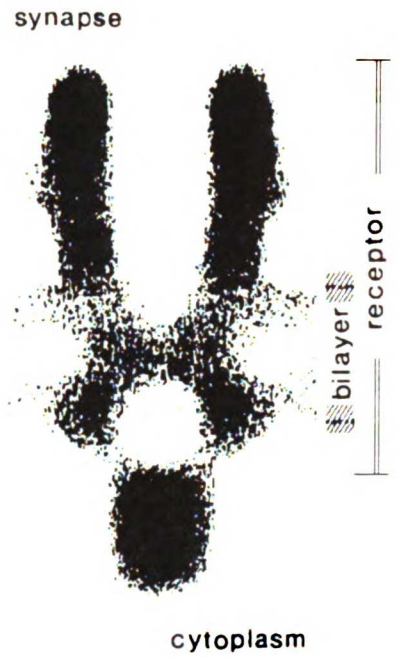


Figure 1-2. Side view of the *Torpedo* AChR at low resolution

This view, averaged cylindrically about the central axis of the receptor, was based on helical image reconstruction of electron diffraction patterns of tubular receptor-rich vesicles. Non-receptor cytoplasmic density is absent after pH 11 treatment and therefore represents a peripheral membrane protein. Taken from Toyoshima and Unwin, 1988.

The domain structure of the receptor subunits

The subunits of the AChR from a variety of organisms have been cloned and sequenced (Numa, 1987; Claudio, 1989; Galzi *et al.*, 1991). The primary sequences of the subunits are highly homologous to each other (Noda *et al.*, 1983). Generally, the α and β subunits are more closely related to each other than either subunit is to the γ or δ subunit (Noda *et al.*, 1983). The converse is true for the γ and δ subunits. The homology of a given subunit across species is greater than the homology between any two subunits in the same species. This has led to the proposal that the ancestral form of the AChR diverged along two pathways, giving rise to the α/β and γ/δ branches (Noda *et al.*, 1983).

The primary sequence homology of the AChR subunits is most obvious when the sequences are aligned. Figure 1-3 is an alignment of the subunits of the AChR from mouse muscle. A common pattern of hydrophobic and hydrophilic regions is present in all of the subunits, as revealed by hydropathic analysis (Fig. 1-4). This observation suggests that all of the subunits of the AChR share a common domain structure.

Several of the structural characteristics of the subunits of the AChR are found among the subunits of other members of the family of ligand-gated ion channels. Although the characteristics shared between the *Torpedo* and the mouse muscle AChR will be described, reference to specific details will concentrate on the mouse muscle AChR (Table 1-1), since the subunits of the receptor from this source form the basis of these studies.

The most amino-terminal region in each of the subunits is the signal sequence (Fig. 1-3). The signal sequence is cleaved co-translationally, giving rise to the primary sequence of the mature receptor subunit. In the case of the *Torpedo* δ subunit, the signal sequence interacts with the signal recognition particle (SRP) and directs the integration of the nascent chain into the membrane of the endoplasmic reticulum (ER) (Anderson *et al.*, 1982). The presence of amino-terminal signal sequences indicates that the amino termini of these subunits are translocated across the ER membrane.

A long hydrophilic amino-terminal domain that is composed of nearly half of all the amino acid residues in the mature sequence (from 210 amino acid residues for the α subunit to 224 amino acid residues for the δ subunit) follows the signal sequence in each subunit. Two features of this domain are notable. First, in all subunits a pair of cysteine residues are found separated by 13 amino acids. This pattern occurs in a highly conserved region of the amino-terminal domain of the subunits of all members of the ligand-gated ion channel family. These two cysteine residues are linked by a disulfide bond in the α subunit (Kao *et al.*, 1984; Kao and Karlin, 1986) and it is presumed that this linkage is formed in the other subunits as well.

Second, the primary sequence of each subunit possesses at least one consensus sequence for *N*-linked glycosylation (*N*-*X*-(*S*/*T*)). The amino-terminal domains of the α and β subunits have one such sequence each, the γ subunit amino-terminal domain has two, and the δ subunit domain has three (Fig. 1-3). The location of one of these sites is conserved in all of the AChR subunits, as well as the subunits of other members of the ligand-gated ion channel family. Each of the subunits of the *Torpedo* AChR is glycosylated (Table 1-1). Concanavalin A binds each of the subunits of the

Torpedo AChR (Nomoto *et al.*, 1986), and analysis reveals one, one, two, and three oligosaccharide residues on the α, β, γ, and δ subunits, respectively. In the *Torpedo* and mammalian AChR, the γ and δ subunits possess both high-mannose and complex oligosaccharides as determined by both lectin binding and specific endoglycosidase digestion (Nomoto *et al.*, 1986; Gu and Hall, 1988a). No *O*-linked sugars have been detected.

Each of the subunits of the AChR is a transmembrane protein. Electron-microscopic imaging of the receptor in *Torpedo* membranes has provided evidence for the transmembrane nature of the AChR subunits (Brisson and Unwin, 1985; Kubalek *et al.*, 1987; Mitra *et al.*, 1989; Toyoshima and Unwin, 1990), as has treatment with exogenous proteases (Strader and Raftery, 1980; Wennogle and Changeux, 1980; Wennogle *et al.*, 1981; Conti-Tronconi *et al.*, 1982).

Following the amino-terminal domain each subunit has a stretch of approximately 70 amino acids that is hydrophobic in nature (Fig. 1-3). Hydropathic analysis of this region reveals three domains (M1, M2, and M3) of sufficient length (at least 20 to 25 amino acids) to span the lipid bilayer (Fig. 1-4). These domains are highly conserved among all of the subunits of the ligand-gated ion channel family.

Two approaches have led to the conclusion that the M2 domain is part of the ion channel. In the first, non-competitive antagonists, which enter the channel and form a physical barrier to ion permeation, have been used to photoaffinity-label the receptor. Two such inhibitors, TPMP and chlorpromazine, labeled the same residues on the α, β, and δ subunits (Giraudat *et al.*, 1986; Hucho *et al.*, 1986; Giraudat *et al.*, 1987; Giraudat *et*

al., 1989), and chlorpromazine labeled residues in the γ subunit (Revah *et al.*, 1990). These residues were identified as serine, threonine, or leucine residues near the amino-terminal end of M2.

Using a molecular genetic approach, Imoto *et al.* demonstrated in microinjected *Xenopus* oocytes that hybrid *Torpedo* AChRs containing the bovine δ subunit have a bovine-type single channel conductance (Imoto *et al.*, 1986). Hybrid AChRs in which the δ subunit has the M2 region and the flanking M2-M3 loop from the bovine δ subunit substituted into the *Torpedo* δ subunit exhibited a conductance characteristic of the bovine AChR. Expression of the converse δ subunit chimera (*Torpedo* δ M2 and M2-M3 loop in a bovine δ background) in hybrid AChRs produced channels with a *Torpedo*-type conductance. These results strongly implicate the role of this region in the formation of the ion channel.

Subsequently the same investigators explored the influence of the M2 flanking domains on channel conductance by mutating negative charges on either side of the M2 domain and investigating the effects of these mutations on the inward and outward conductance of the AChR (Imoto *et al.*, 1988). They discovered that the inward single channel current was decreased when the number of negative charges in the M2-M3 domain was decreased, and that the reduction in negative charges in the M1-M2 domain caused a decrease in the outward single channel conductance. They postulated that these negative residues formed three rings of charges (two inner and one outer) whose role was to increase the local concentration of cations near the channel and thereby increase channel conductance (Adams *et al.*, 1980).

The two approaches described above, non-competitive antagonist binding and molecular genetics, were combined to investigate the binding site of the local anaesthetic QX-222 within the channel (Neher and Steinbach, 1978; Leonard *et al.*, 1988). This molecule has a quaternary ammonium group at one end and a bulky hydrophobic group at the other. Leonard *et al.* investigated the effect of mutating the serine residues near the amino-terminal end of M2 labeled by the photo-affinity reagents described above on the affinity of the blocker for the channel. As the number of serines was decreased by conversion to alanines, the affinity of the channel for QX-222 was decreased without affecting single-channel conductance or channel selectivity. Only when all of the serines were removed was a decrease in the outward conductance observed. This polar site was therefore predicted to serve as the binding site for the quaternary ammonium group of QX-222. When non-polar residues were introduced into a more carboxy-terminal region of M2 predicted to be one α-helical turn away from the "inner polar site," the binding affinity of QX-222 increased as the polarity of the new site decreased, suggesting that the hydrophobic end of the QX-222 molecule interacted with more extracellular portions of the channel. Taken together, these results support the identification of M2 as subunit segment lining the ion channel and suggest a model for its orientation in the membrane.

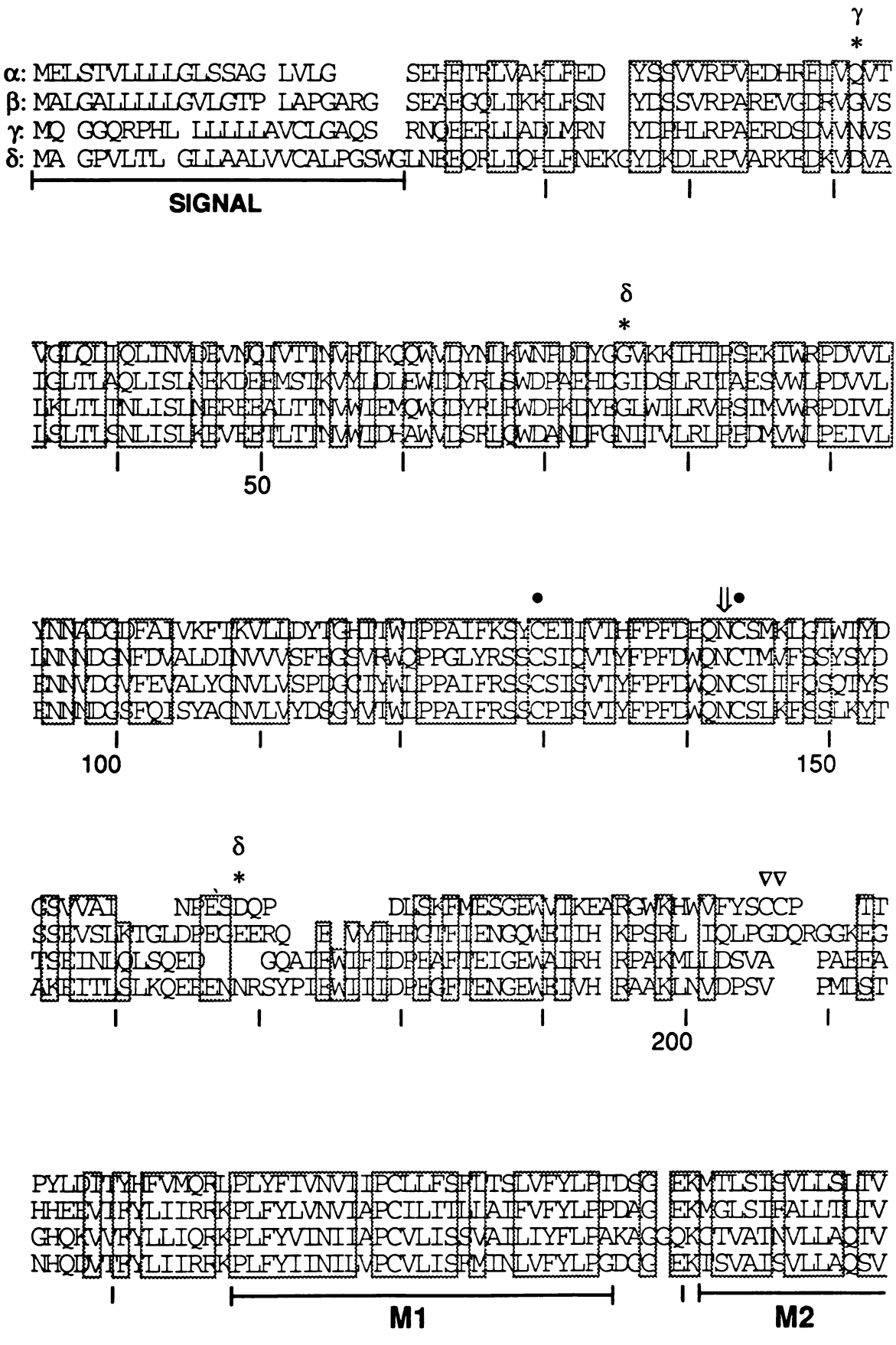
A long stretch of amino acids, approximately one-half the length of the amino-terminal domain (from 109 amino acids for the α subunit to 146 amino acids for the γ subunit), intervenes between the clustered hydrophobic region and a final hydrophobic domain. This last hydrophobic domain (M4) is the least conserved yet most hydrophobic region in the

receptor subunits and is followed by a short carboxy-terminal domain, whose length ranges from 11 amino acids (α subunit) to 27 amino acids (δ subunit) in length. The intervening region from M3 to M4 is the least homologous domain along the primary sequence of the subunits (Noda *et al.*, 1983) (Fig. 1-3); several gaps must be introduced in all the subunits in this region to attain maximal homology. In the γ subunit sequence, this domain contains two additional glycosylation consensus sequences. The M3-M4 loop also contains the phosphorylation sites of the β, γ, and δ subunits of the *Torpedo* AChR. The phosphorylation sites in this region are conserved in all other species sequenced except the mammalian γ subunit (Fig. 1-3) (Huganir and Miles, 1989; Huganir and Greengard, 1990). Each of the mammalian AChR subunits can be phosphorylated *in vivo* (Miles *et al.*, 1987; Ross *et al.*, 1987; Smith *et al.*, 1987b; Ross *et al.*, 1988). Phosphorylation of the γ and δ subunits has been implicated as an important post-translational modification in the regulation of AChR desensitization (Huganir, 1987; Huganir and Greengard, 1990) and assembly (Ross *et al.*, 1987; Ross *et al.*, 1988; Green *et al.*, 1991).

This domain structure is preserved to some extent at the level of the genes that encode the AChR subunits. The subunits of the AChR have similar gene structures and intron-exon patterns (Ballivet *et al.*, 1983; Noda *et al.*, 1983; Nef *et al.*, 1984; Shibahara *et al.*, 1985). When a comparison is made between the intron-exon boundaries and the primary sequence of the receptor, it appears that functional domains can be delineated within, but are not shared amongst, specific exons.

Figure 1-3. Alignment of the subunits of the AChR from mouse skeletal muscle

The primary sequences of the subunits of the mouse muscle AChR were aligned to maximize the intersubunit homology (Patrick *et al.*, 1987). Regions in which all of the subunits possess homologous amino acid residues are boxed. Hydrophobic domains (e.g., signal sequence, M1-M4) are indicated, as is the MA region. The conserved glycosylation consensus sequence corresponding to αN141 is indicated by a downwards arrow. Additional glycosylation consensus sequences are indicated by asterisks, with the name of the subunit with a sequence at that position indicated above the asterisk. Conserved cysteine residues are indicated by dots above the sequences; the paired cysteines at α192-α193 are marked by the downwards arrowheads. The region containing the phosphorylation sites on the receptor subunits are indicated by a black bar beneath the δ subunit sequence, and the phosphorylated residues are underlined (Huganir and Miles, 1989).


γ
*
α: MELSTVLLLLGLSSAG LVLG SEHETRLVAKLFED YSSVVRPVEDHRFLMVQVT
β: MALGALLLLLGVLGTP LAPGARG SEAEGQLIKKLFSN YDSSVRPAREVGDRVQVS
γ: MQ GGQRPHL LLLLLAVCLGAQS RNQEERLIADLMRN YDPHLRPAERDSDVVNVS
δ: MA GPVLTL GLLAALVVCALPGSWGLNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVA
SIGNAL
δ
*
VGLQLIQLINVDEVNQIVTTNVRLKQQWVDYNLKWNPDDYGGVKKIHIPSEKIWRPDVVL
IGLTLAQLISLNEKDEEMSIKVMLDLEWIDYRLSWDPAEHDGIDSLRITAESVWLPDVVL
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LSLTLSNLISLKEVEEILTTNVWIDHAWVLSRLQWDANDFGNITVLRLPFDMVWLPEIVL
50
•
⇓•
YNNADGDFAIVKFIKVLLDYTGHIIWIPPAIFKSYCEIIVTHFPFDEQNCSMKLGIWIYD
LNNNDGNFDVALDINVVVSFEGSVRWQPPGLYRSSCSIQVTYFPFDWQNCTMVFSSYSYD
ENNVDGVFEVALYGNVLVSPDGCIYWLPPAIFRSSCSISVTYFPFDWQNCSLIFQSQIYS
ENNNDGSFQISYAGNVLVYDSGYVTWLPPAIFRSSCPISVTYFPFDWQNCSLKFSSLKYT
100
150
δ
*
▽▽
GSVVAI NPESDQP DLSKFMESGEWVIKEARGWKHWVFYSCCP TIT
SSEVSLKTGLDPEGEERQ EVYTHEGTFIENGQWEIIH KPSRL IQLPGDQRGGKEG
TSEINLQLSQED GQAIEWIFIDPEAFTEIGEWAIRH RPAKMLLDSVA PAEFA
AKEITTLSLKQEEENNRSYPIEWIIIDPEGFTENGEWRIVH RAAKLNVDPSV PMDST
200
PYLDIIMHFVMQRLPLYFIVNVIIPCLLFSFLTSLVFYLPIDSG EKMTLSISVLLSLTV
HHEEVIFYLIIRRKPLFYLVNVIAPCILITLLAIFVFYLPPDAG EKMGLSIFALLTLTV
GHQKVVFYLLIQFKPLFYVINIIAPCVLISSVAILIYFLPAKAGGQKCTVATNVLLAQTV
NHQDVTFYLIIRRKPLFYIINIIVPCVLISFMINLVFYLPGDCG EKISVAISVLLAQSV
M1
M2

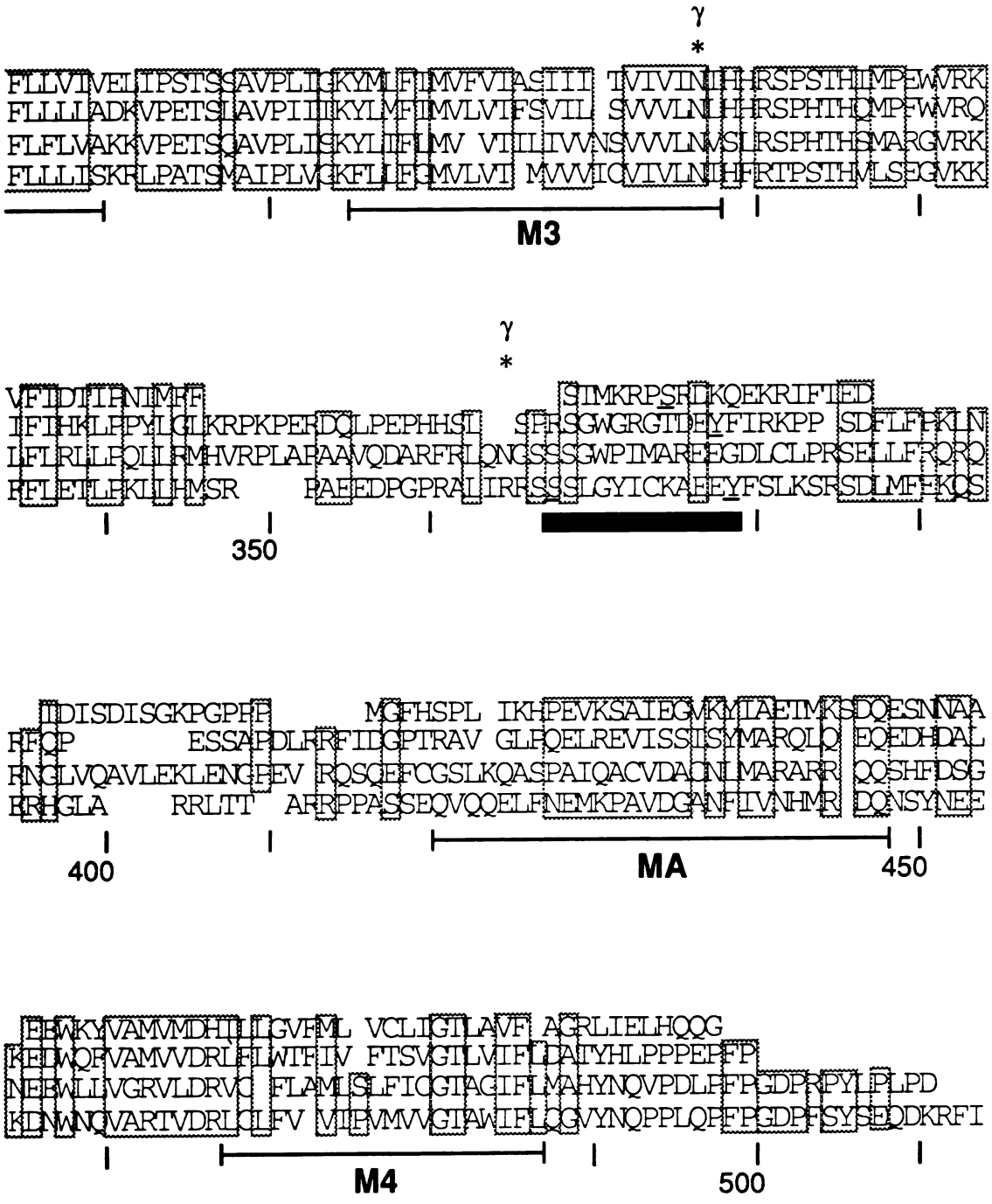
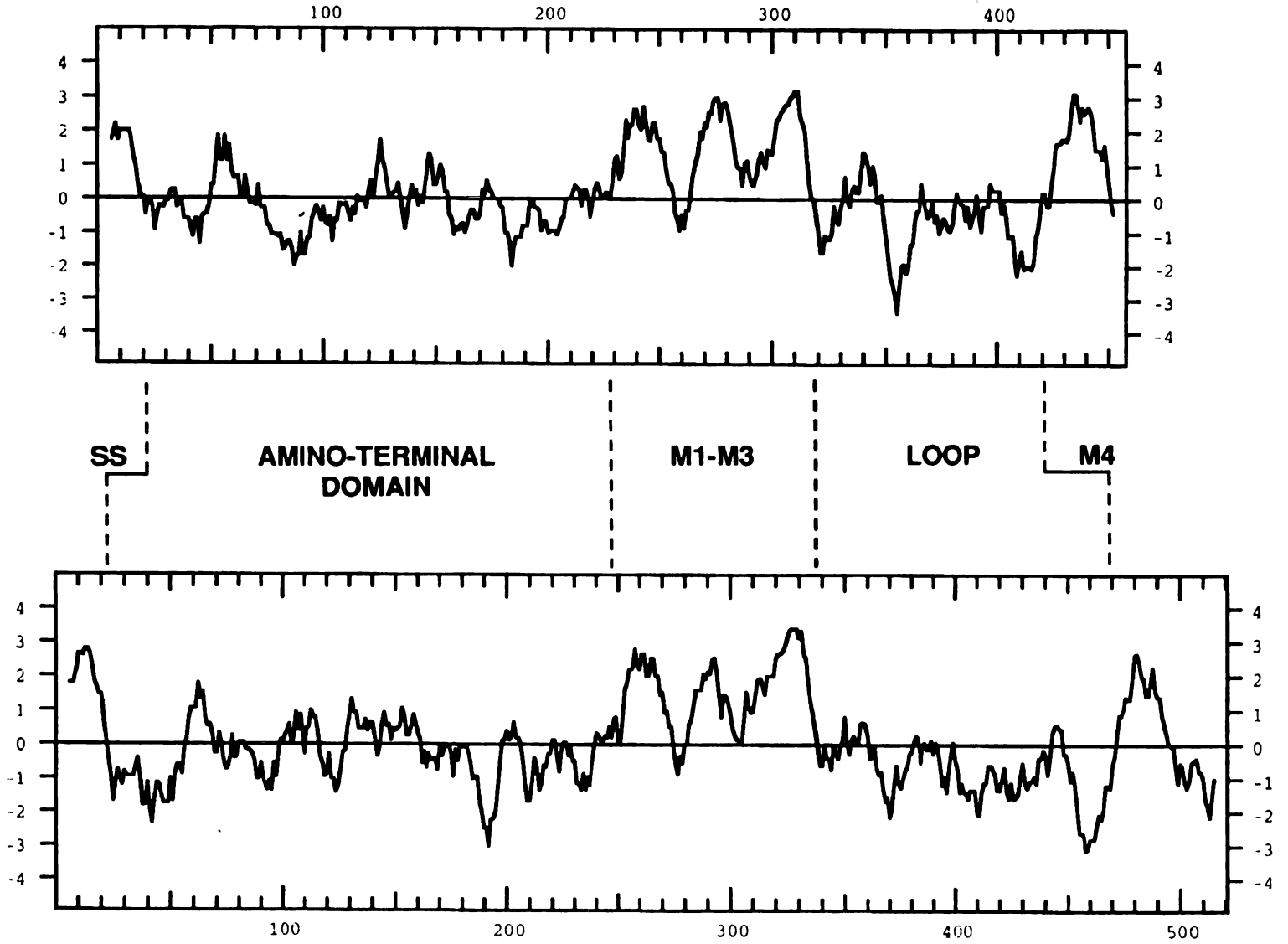

γ
*
M3
γ
*
350
400
MA
450
M4
500

Figure 1-4. Comparison of the Kyte-Doolittle plots of the mouse muscle AChR α and δ subunits

The hydropathy plots of the α (upper) and δ (lower) subunits were determined with the Strider DNA analysis program. Positive peaks indicate hydrophobic regions. The boundaries of the major domains of the subunits are indicated. The plots for the β and γ subunits are similar to the two depicted here.


100
200
300
400
4
3
2
1
0
-1
-2
-3
-4
SS
AMINO-TERMINAL DOMAIN
M1-M3
LOOP
M4
100
200
300
400
500

The subunits of the AChR

The α subunit of the AChR contains the binding site for BuTx. Unique to the α subunit from all species is a pair of cysteine residues at positions αC192-αC193. Karlin and co-workers have determined that this cysteine pair is disulfide-linked (Kao *et al.*, 1984; Kao and Karlin, 1986). The disulfide bond between these vicinal cysteines is susceptible to attack under weak reducing conditions (Kao *et al.*, 1984). After reduction this pair is also labeled by affinity alkylating reagents, such as 4-(*N*-maleimido-benzyl)trimethyl ammonium iodide (MBTA) (Kao and Karlin, 1986). When the AChR was incubated with [^{3}H]-MBTA under mildly reducing conditions (that did not affect BuTx binding or channel activation), radioactivity was recovered with the α subunit, specifically at the vicinal cysteines. Since MBTA can inhibit the binding of BuTx to the receptor, it is probable that the region around the MBTA binding site contains at least part of the BuTx binding site. The presence of paired cysteine residues has been used for the presumptive identification of the acetylcholine-binding subunits from the neuronal AChR (Patrick *et al.*, 1987).

Other approaches have been used in an attempt to determine the position of the BuTx binding site along the primary sequence of the α subunit. These methods employ BuTx binding assays to probe either synthetic (Neumann *et al.*, 1986; Wilson *et al.*, 1988; Wilson and Lentz, 1988) or recombinant (Gershoni, 1987) peptides that mimic the primary sequence of various regions of the α subunit, or proteolytic fragments derived from isolated, purified α subunit (Wilson *et al.*, 1985; Neumann *et al.*, 1986; Pedersen *et al.*, 1986). Residues within the BuTx binding site have been

mapped with photoreactive competitive antagonists (Dennis *et al.*, 1988; Langenbuch *et al.*, 1988). With these methods the binding site for BuTx has been localized between α170 and α200.

The function of the β subunit in the AChR oligomer is not known. Burden and co-workers have suggested that the β subunit interacts with the 43 kDa protein (Burden *et al.*, 1983), a peripheral membrane protein that is thought to play a role in the formation and stabilization of AChR clusters (Bloch and Froehner, 1987; Carr *et al.*, 1987). The close association of the 43 kDa protein with the AChR is visible in electron density profiles of native receptor (Toyoshima and Unwin, 1988; Mitra *et al.*, 1989). However, the 43 kDa protein is not required for any of the characteristics of AChR electrophysiology as demonstrated by reconstitution studies and the expression of the AChR in non-muscle cell lines (see above).

The γ subunit is the only one which has glycosylation consensus sequences outside the amino-terminal domain. Only two of the four potential N-linked glycosylation sites are used (Table 1-1), probably corresponding to positions γN30 and γN141 in the amino-terminal domain (Anderson and Blobel, 1981; Nomoto *et al.*, 1986). In the δ subunit, the three glycosylation consensus sequences are found in the amino-terminal half of the protein; all three sites in the *Torpedo* δ subunit are glycosylated.

The γ and δ subunits are involved in several aspects of the function of the AChR. Both subunits are important in the early stages of receptor assembly, forming the initial complexes with the α subunit (see below). In addition, the γ subunit is involved in the developmental regulation of the mammalian AChR (Schuetze and Role, 1987). The electrical properties of

the AChR differ significantly between embryonic and adult muscle. Adult muscle AChRs exhibit a shorter mean open time and a higher conductance than their embryonic counterparts (Mishina *et al.*, 1986; Gu *et al.*, 1990). Immunological studies have demonstrated the absence of the γ subunit in normal adult muscle (Gu and Hall, 1988b). The expression of a novel γ-related subunit, the ε subunit, has been implicated in these structural and functional changes in the AChR. However, the γ subunit is not repressed permanently in the adult. Upon denervation of skeletal muscle the γ subunit is expressed again at non-endplate regions (Schuetze and Role, 1987; Gu and Hall, 1988b). Both the physiological and developmental differences in the γ- and ε-type AChRs may be related specifically to the development and maintenance of the neuromuscular junction, since the AChRs in the *Torpedo* electric organ do not undergo such a change.

The δ subunit plays a role in determining the conductance properties of the AChR as well (Imoto *et al.*, 1986). Recent structural work in which an 18Å resolution map was determined for the *Torpedo* AChR in the resting and desensitized states (Unwin *et al.*, 1988) has suggested an additional function of the δ subunit. In the resting state the AChR oligomer is more radially symmetric than it is in the carbamylcholine-induced desensitized state. In the desensitized state, the δ subunit undergoes an axial shift of approximately 10°, suggesting that the δ subunit plays a role in receptor desensitization as well as the binding of carbamylcholine to the AChR (see below).

Oligomeric assembly of the AChR

The assembly of the mature AChR from its subunits appears to be a highly ordered process. Work in the last several years has revealed a specific order of subunit association in the assembly process. Of the AChR subunits, the role of the α subunit in the assembly pathway has been most clearly elucidated. In the BC_3H-1 muscle cell line, Merlie and co-workers followed the α subunit from the initiation of translation until its appearance as part of the mature AChR on the surface (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983; Merlie, 1984; Smith *et al.*, 1987a). The initial α subunit translation product (α_0) is inserted rapidly into the ER membrane where its amino-terminal signal sequence is removed and the subunit is glycosylated co-translationally. α_0 is converted ($t_{1/2}$=15-30 min) into a form that binds BuTx but is not associated with other AChR subunits (α_{Tx}). This maturation is influenced by disulfide bond formation as well as glycosylation of the α subunit (Mishina *et al.*, 1985; Blount and Merlie, 1990). After this stage, the receptor complex is formed, as reflected in the co-immunoprecipitation of the α and β subunits ($t_{1/2}$=30-90 min). This entire assembly process takes place in the ER membrane (Smith *et al.*, 1987a). The assembled AChR appears on the surface of these cells after transport through the Golgi ($t_{1/2}$=90-150 min), since oligosaccharides on both the γ subunit and the δ subunit in the mature AChR are modified to their complex (i.e., endoglycosidase H-resistant) forms (Nomoto *et al.*, 1986; Gu and Hall, 1988a).

Recent investigations have revealed that the α subunit associates with either the γ or δ subunit first to form specific heterodimers (αγ or αδ), and

that association with the β subunit is the requisite final step in the assembly process (Blount *et al.*, 1990; Gu *et al.*, 1991b; Saedi *et al.*, 1991). The signals for the initial recognition process have been localized to the amino-terminal domains of the subunits (Yu and Hall, 1991; Verrall and Hall, 1992). The γ and δ subunits were demonstrated to compete with each other for recognition by the α subunit, suggesting either that both subunits interacted with the same domain(s) on the α subunit or that the interaction of either subunit with the α subunit altered the conformation of the α subunit to prevent its interaction with the other subunit (Gu *et al.*, 1991b). Because the other subunits of the AChR are present only in single copies in the oligomer and the α subunits are non-adjacent, the α subunits by necessity have different neighbors. If the γ and δ subunits interact with the same face of the α subunit, then the β subunit cannot lie between the α subunits in the mature AChR. It appears therefore that the assembly of the AChR may specify the order of the subunits around the channel.

An additional feature of the formation of the αγ and αδ heterodimers is the creation of the binding sites for small ligands such as *d*-tubocurarine and carbamylcholine (Claudio *et al.*, 1987; Blount and Merlie, 1989; Blount *et al.*, 1990; Pedersen and Cohen, 1990; Sine and Claudio, 1991). The α subunit expressed alone lacks high-affinity binding for these ligands (Blount and Merlie, 1988). The interaction of the α subunit with the γ subunit creates a high-affinity binding site for *d*-tubocurarine (Blount and Merlie, 1989; Pedersen and Cohen, 1990). The binding affinity for carbamylcholine is the same for the complete AChR and the αδ heterodimer, suggesting that the carbamylcholine binding site is shared by the α and δ subunits (Blount and Merlie, 1989). This phenomenon may

explain the observed non-equivalence of ligand-binding affinity between the α subunits (Neubig and Cohen, 1979; Sine and Taylor, 1981; Dowding and Hall, 1987).

The subunits of the receptor are transcribed from separate genes. The expression of each transcript can be independently regulated, as is clearly the case in the developmental regulation of the receptor (see above) (Fambrough, 1983; Schuetze and Role, 1987). Several exogenous and endogenous factors appear to be involved in the regulation of AChR expression. Therefore, surface expression of functional AChR can be regulated at several steps: subunit expression, conversion of α_0 to α_{Tx}, subunit recognition and association, AChR assembly in and exit from the ER, modification of the AChR during transport through the Golgi, and the degradation rate of surface receptors.

Topological models of the receptor subunits

Several laboratories are attempting to solve the transmembrane topology of the AChR subunits by creating crystals of the receptor suitable for X-ray diffraction studies. A high resolution model of the structure of the AChR is required to understand the allosteric properties of the receptor. However, no published reports of successful crystallization of the AChR have appeared in the literature. The greatest impediment to the production of crystals of transmembrane proteins is the nature of the proteins themselves. Both the hydrophilic and the hydrophobic domains must be stabilized in a regular array. To date, the high-resolution structure of only one transmembrane protein complex, the photosynthetic reaction center from *Rhodopseudomonas viridis*, has been solved (Deisenhofer *et al.*, 1985; Deisenhofer and Michel, 1989). Alternate methods have therefore been used to develop models for the transmembrane topology of the AChR subunits. These methods include structural predictions based on an analysis of the primary sequence and the localization of specific epitopes of the AChR subunits relative to the lipid bilayer.

Experimental evidence and general consensus have fixed the location of several topological features of the receptor subunits. First, the extreme amino terminus of the subunit polypeptide faces the extracellular space, due to the presence of an amino-terminal signal sequence that is cleaved co-translationally from each of the subunit primary translation products (Anderson and Blobel, 1981; Anderson *et al.*, 1982). Since signal peptidase is a resident protein of the ER lumen, the amino terminus therefore must be translocated across the ER membrane, into a region that is topologically

equivalent to the extracellular space. Second, the presence of asparagine-linked glycosylation consensus sequences in the amino-terminal domains of the AChR subunits (α: 1, β: 1, γ: 2 (of 4), δ: 3), coupled with the fact that all of the subunits are glycoproteins, suggests that for at least the α, β, and δ subunits these regions have been translocated also, since oligosaccharyl transferase is a lumenal resident protein of the ER. Third, the disulfide bond formed between residues αC128-αC142 in the α subunit (and predicted to be present in an equivalent position in the other subunits as well) also must be extracellular since the reducing environment within the cytosol presumably would not support stable disulfide bond formation. As described above, the BuTx binding site has been identified as a 30 amino acid region of the α subunit surrounding residues αC192-αC193. Clearly this region is extracellular.

The last common feature retained by all of the topological models is based primarily on the hydropathic analysis of the subunit polypeptides. All of the models retain as transmembrane domains the regions M1-M3 described previously. This cluster of residues is predicted to traverse the membrane three times, with the amino-terminal ends of M1 and M3 oriented extracellularly and the amino-terminal end of M2 oriented intracellularly.

The differences between the various models come at either side of the M1-M3 domain. In the region upstream of M1 two additional transmembrane domains have been postulated; after M3 either zero, one, or two transmembrane domains have been predicted. Therefore the various models of the AChR possess any combination of these domains, giving from three to seven potential transmembrane domains. Each of the models will

be discussed in turn, beginning with models based on primary sequence analysis and followed by models based on epitope mapping studies.

Models based on primary sequence analysis

The four transmembrane domain model

As described earlier, the mature polypeptide chains of the AChR subunits contain four hydrophobic regions of approximately 20-25 amino acids in length that could serve as membrane-spanning alpha helices. In the four transmembrane domain model, the domains M1, M2, and M3 are clustered together and the M4 domain traverses the membrane just upstream of the carboxy terminus (Noda *et al.*, 1982; Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983). A transmembrane protein with extracellular amino terminus and an even number of membrane-spanning regions is topologically required to possess an extracellular carboxy terminus as well. Since no other proposed model predicts an odd number of membrane-spanning regions beyond M3, this is the only model in which the amino and carboxy termini of the subunits lie on the same side of the membrane (Fig. 1-5).

The method used to develop the four transmembrane domain model derives from the analysis of the primary sequence to detect regions of the polypeptide chain that could serve as α-helical transmembrane domains by calculating the relative hydrophobicity of segments of the entire chain (Kyte and Doolittle, 1982). In the α-helix the amino acid side chains extend away from the helical axis. Hydropathic analysis, therefore, is a quantitative prediction that a stretch of hydrophobic amino acids would be more thermodynamically stable if folded into an α-helix embedded within the lipid bilayer. However, the hydrophobicity scales used in these calculations are based on the partition coefficients of amino acids into solvents such as

ethanol, not lipids (Kyte and Doolittle, 1982). Therefore these scales are not suited optimally to the determination the stability of proteins within membranes. While this method has been used to develop accurate models for some membrane proteins (Henderson and Unwin, 1975), it is also true that hydropathic analysis has failed to detect subtle structural features in others (Miller, 1991). Because of our current incomplete inderstanding of the relationship between the primary and higher-order structures of proteins, the acceptance of any topological models based solely on primary sequence analysis must depend on additional experimental evidence.

Most of the evidence that supports the four transmembrane domain model is based on the biochemical determination of the transmembrane orientation of the carboxy terminus of the δ subunit from the *Torpedo* AChR. Unlike the AChR from other species, the *Torpedo* AChR occurs primarily as a dimer *in vivo*; that is, two AChR oligomers are covalently linked and form a unit. This arrangement possesses little functional significance, since the electrophysiological properties of the monomeric and the dimeric forms of the AChR are indistinguishable (Wu and Raftery, 1981). Early investigations concluded that the covalent linkage occurred between the δ subunits and was sensitive to reduction (Chang and Bock, 1977; Hamilton *et al.*, 1979). When the subunits of the *Torpedo* AChR were separated on an acrylamide gel, the δ subunit ran at twice the apparent molecular weight under non-reducing conditions than it did under reducing conditions. No other subunit demonstrated a similar change in electrophoretic mobility following reduction.

The disulfide bond linking the two receptor oligomers is predicted to lie on the extracellular side of the plasma membrane on the basis of the

inability of the reductive environment of the cytoplasm to support such a bond. Also, dimers of the AChR in native membrane vesicles digested with trypsin are converted more readily to monomers when the protease is added to the outside of the vesicle (Conti-Tronconi *et al.*, 1982). The cellular location of this disulfide bond was reported first by McCrea *et al.* (1987) using a reconstituted vesicle system to test the accessibility of the δ-δ disulfide to the hydrophilic reducing agent glutathione (GSH). Solubilized AChR was incorporated into vesicles made of asolectin and the sidedness of these vesicles was determined by ^{125}I-BuTx binding in the presence or absence of detergents. Greater than 95% of the AChR in these vesicles were oriented with the toxin binding sites exposed on the surface (right-side out). These vesicles formed a strong barrier against GSH permeation as measured by the efflux of [^{3}H]-GSH entrapped within the vesicles as well as the ability of GSH to reduce trypsin-cleaved diptheria toxin dimer incorporated within the vesicles. Using GSH, the AChR dimers in right-side out vesicles were reduced at an equal rate following freeze-thaw, incubation in the presence of 0.1% Triton X-100, or in the absence of any treatment. The degree of AChR dimer reduction at any time could not be accounted for by the rate of GSH efflux. These data demonstrate the extracellular localization of the disulfide bond that links *Torpedo* AChR monomers *in vivo*.

Dunn *et al.* (1986) obtained similar results using native *Torpedo* vesicles as the source of AChR dimers and 2-mercaptoethanesulfonic acid (2-MES) as the impermeant reducing agent. Native *Torpedo* vesicles spontaneously seal with the receptors in the right-side out orientation, and 2-MES can be loaded within the vesicles by using a freeze-thaw method to

open and reseal the vesicles. 2-MES was as effective at dimer reduction when applied only to the outside of the vesicle as it was when applied from the inside and the outside simultaneously, demonstrating the extracellular location of the disulfide bond involved in AChR dimerization. Both McCrea *et al.* and Dunn *et al.* suggested candidate cysteine residues involved in the dimerization from among the six found in the *Torpedo* δ subunit. However, neither group performed experiments to exclude or identify any possible candidate.

Subsequent experiments by DiPaola *et al.* ascertained the identity of the disulfide-bonded δ subunit cysteine residue (Czajkowski *et al.*, 1989; DiPaola *et al.*, 1989). Dimers of *Torpedo* AChR in native receptor-rich vesicles were treated with the two reducing agents previously described, 2-MES and GSH. The δ-δ disulfide bond was reduced equally in the presence and absence of membrane perturbants (e.g., saponin, Triton X-100, freeze-thaw) for right-side out vesicles, while reduction was enhanced only in the presence of such perturbation when inside-out vesicles were used. These data, taken with those described above, firmly establish the extracellular location of the δ-δ disulfide bond.

The identity of the correct cysteine residue was revealed by treating solubilized AChR with N-ethylmaleimide (NEM) to alkylate free sulfhydryl groups, followed by dithiothreitol (DTT) to reduce the δ-δ dimer and [^{3}H]-NEM to label any newly revealed sulfhydryl groups. The δ subunit was isolated by gel electrophoresis, cleaved with CNBr, and separated by high performance liquid chromatography (HPLC). The tritium peak fraction was isolated and subjected to Edman degradation. The sole CNBr fragment of the δ subunit found within this peak contained only one cysteine residue,

δC500, the penultimate residue in the subunit polypeptide. This result is consistent with proteolytic studies that concluded that the δ-δ disulfide bond was localized within the last 16 kDa (approximately 140 amino acids) of the δ subunit (Wennogle *et al.*, 1981). Since the disulfide bond in which δC500 participates is on the extracellular side of the membrane and since this residue is located in the carboxy terminus, the carboxy terminus of the *Torpedo* AChR δ subunit must be extracellular. These results are most consistent with a model in which only one transmembrane domain exists beyond the M3 domain. The only model that retains this feature is the four transmembrane domain model.

The amphipathic helix model

At the time the four transmembrane domain model was proposed, the ion channel was thought to be lined by either charged or strongly polar amino acid side chains. All of the transmembrane domains described in the four transmembrane domain model are notably apolar. None of these domains appeared to be a likely candidate to line the ion-conducting pore.

To circumvent this apparent difficulty, Finer-Moore and Stroud (1984) and Guy (1984) proposed a model containing an amphipathic helix (Fig. 1-6). In the first study, a Fourier transform analysis of the periodicity of the hydrophobicities of residues in the primary sequences of the *Torpedo* AChR subunits revealed a potential membrane-spanning α-helix that exposed charged or polar residues along one face and apolar residues on the other faces. This helix (MA) lay between M3 and M4. Guy also arrived at a structure that contained a pore-lining amphipathic helix, using a computerized predictive algorithm that considered the partition energy of

the α subunit side chains and helical packing constraints. In both cases, each subunit was predicted to contribute its MA region to the ion translocating pore, with the polar face lining the channel. Finer-Moore and Stroud also suggested that MA would co-insert into the membrane with M4 only upon assembly to prevent the exposure of the polar face to the hydrophobic interior of the plasma membrane. The distinguishing topological feature of this model, therefore, is the transmembrane nature of MA. Since M4 was retained as a transmembrane domain in this model, the carboxy-terminal tail of the α subunit was predicted to be cytoplasmic, not extracellular.

The experiments of Young *et al.* provide the best evidence in support of the amphipathic helix model (Young *et al.*, 1985). Two polyclonal antibodies were generated against subunit-specific synthetic peptides from *Torpedo* AChR. One antibody (β-350) was directed against a sequence between M3 and M4 in the β subunit and the second antibody (δ-273) was directed against the carboxy terminus of the δ subunit. The specificities of the antibodies were tested by immunoblots against affinity-purified *Torpedo* AChR and, for δ-273, by a competitive immunoassay. When the binding of δ-273 to AChR-rich vesicles was tested, only permeabilized vesicles were capable of binding δ-273, indicating that the carboxy terminus of the δ subunit was cytoplasmic. This conclusion about the transmembrane topology of the carboxy terminus is in direct opposition to the structure based on AChR dimer reduction, and it supports a model with an odd number of transmembrane domains.

Sections of *Torpedo* vesicles were used also to examine the binding of both β-350 and δ-273 to right-side out oriented AChR by electron microscopy.

The binding of the antibodies was visualized with colloidal gold-conjugated secondary antibodies. For both β-350 and δ-273, gold beads were detected on the cytoplasmic side of the membrane. Since the structures of the subunits have been proposed to be homologous, these data implied the existence of an even number of transmembrane domains between M3 and the carboxy terminus. Therefore these experiments support MA as a fifth transmembrane domain.

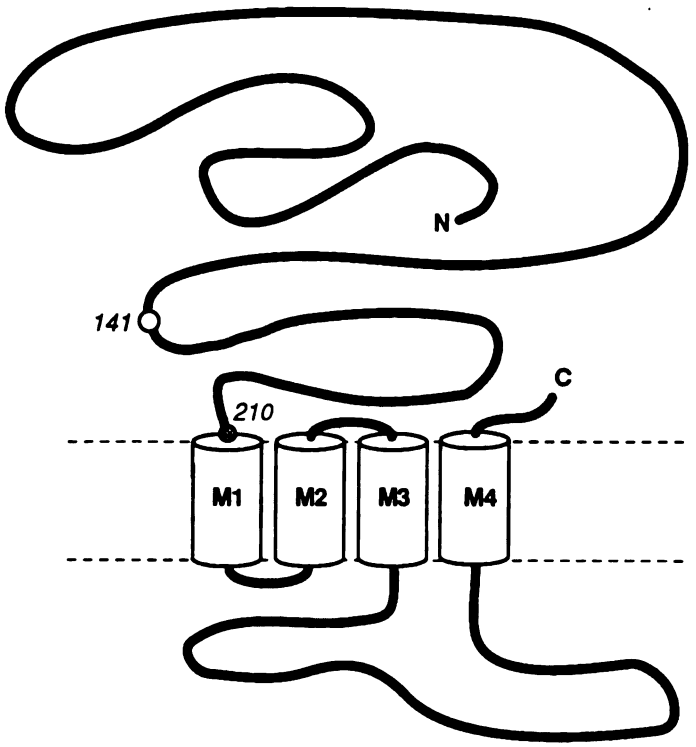


Figure 1-5. The four transmembrane domain model

A model of the transmembrane topology for the subunits of the AChR, based on the hydropathic analysis of the primary sequences of the subunits (Noda *et al.*, 1982; Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983). For this and subsequent diagrams in this chapter, the α subunit is depicted. Note that both the amino and carboxy termini are in the extracellular (upper) space, along with the glycosylation site (αN141). The start of the first transmembrane domain (M1) is given as α210. M1-M4 are given as membrane-spanning barrels.

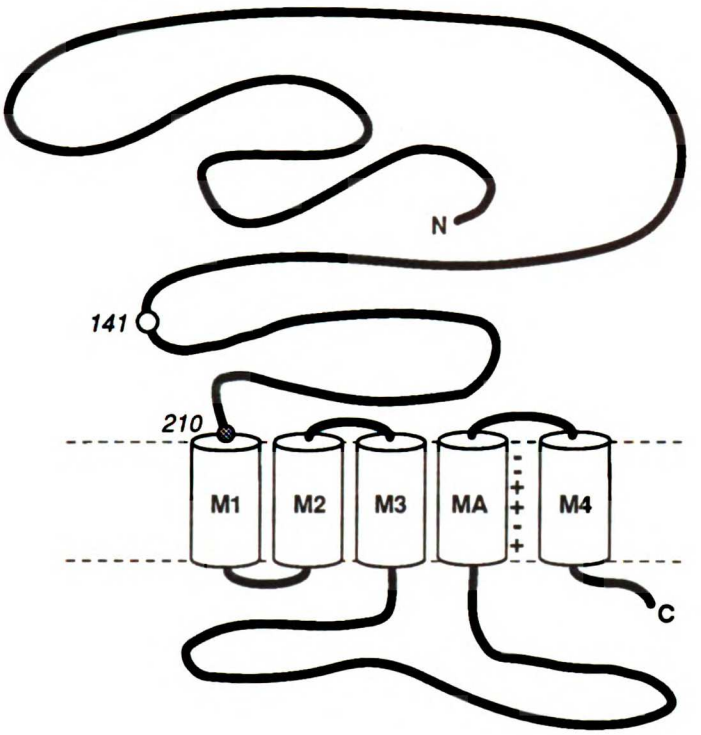


Figure 1-6. The amphipathic helix model

A model of the transmembrane topology for the subunits of the AChR, based on the amphipathic analysis of the primary sequences of the subunits (Finer-Moore and Stroud, 1984; Guy, 1984). Note that the amino and carboxy termini are on opposite sides of the membrane, with five intervening transmembrane domains. The amphipathic nature of MA is denoted by the charges alongside this domain.

Models based on epitope mapping

The accessibility of antibodies for specific epitopes on the native AChR is the basis for the other main approach used to substantiate models of AChR subunit topology. The use of mAbs to distinguish between topological models of AChR subunit structure is dependent upon the availability of antibodies that recognize specific regions of the primary sequence. Lindstrom and his colleagues have generated a library of more than 200 mAbs of which several have been used for this purpose (Gullick and Lindstrom, 1983; Lindstrom *et al.*, 1984; Ratnam and Lindstrom, 1984; Lindstrom *et al.*, 1985; Lindstrom *et al.*, 1987). Results based on an immunocompetition assay (described below), sometimes supplemented by immunoelectron microscopy, have supported structural features found in models that have been already described, e.g., the cytoplasmic loops between M1 and M2 and after M3 (Criado *et al.*, 1985b; LaRochelle *et al.*, 1985), and the cytoplasmic orientation of the carboxy terminus (Lindstrom *et al.*, 1984; Ratnam and Lindstrom, 1984). As the population of mAbs in the library grew, new features of subunit topology that contradicted both the four transmembrane domain and the amphipathic helix models were revealed.

The M6-M7 model

The locations along the primary sequence of the α subunit of the signal sequence, the intrachain disulfide bonds, the glycosylation site, and the BuTx binding site all provide strong evidence for the extracellular localization of the amino-terminal domain. However, immunochemical evidence suggests that a region between the glycosylation site and the

disulfide at α192-193 is on the cytoplasmic side of the membrane (Criado *et al.*, 1985a). Two monoclonal antibodies (mAb) against a synthetic peptide with the sequence α152-α167 of the α subunit from *Torpedo* AChR were used to investigate the topology of this region. Both mAbs bound to the synthetic peptide α159-α168 with a 10^4 lower affinity than the peptide against which they were raised, suggesting that the mAbs were directed primarily against the sequence α152-α159. Both mAbs bound to purified α subunits and solubilized AChR. Neither mAb bound to AChR in right-side out vesicles. However, both mAbs bound to liposomes into which purified AChR was reconstituted, with 50% of the AChR in the inside-out orientation. These results suggested a model in which the region α152-α167 was cytoplasmic, unlike the orientation given to this region in previous models (Fig. 1-7).

A competition ELISA approach developed by Lindstrom and others to identify the accessibility of specific antibodies for their epitopes was used to characterize the topology of the region α152-α167. Since native vesicles are impermeable to large proteins such as antibodies, the binding of an mAb to its epitope depends on the location of that epitope relative to the bilayer. If the epitope is within the lumen of the vesicle, the antibody can bind only when the membrane is disrupted and access to the lumen is provided. If the epitope lies on the extracellular side of the vesicle, the antibody should bind equally well with or without such treatments. The binding of the mAbs to immobilized AChR, in which all epitopes are accessible, was tested using native vesicles as a competitor. Detergents, alkali extraction, or freeze-thawing were used to disrupt the membrane. Only those vesicles treated with membrane-perturbing agents were effective competitors for the

binding of the mAbs to the AChR (Criado *et al.*, 1985a), indicating that the epitope to which the antibody bound was localized on the cytoplasmic side of the vesicle. Therefore Criado *et al.* concluded that two transmembrane domains were required in the amino-terminal domain of the α subunit, since a region near α152-α159 could be found on the cytoplasmic side of the membrane.

The locations of these new transmembrane domains are constrained by well-established features within the amino-terminal domain. As described above, the asparagine residue at αN141 is the sole site of glycosylation on the α subunit and therefore must be on the extracellular side of the membrane. The cysteine residue that immediately follows, αC142, has been proposed to form part of a disulfide bond with αC128 (Kao and Karlin, 1986). Therefore the first of the new transmembrane domains, M6, spans from α143 to α152 and must be only 10 or 11 amino acids long. Since the pitch of an α-helix is only 1.5Å per residue, M6 cannot cross the bilayer as an α-helix. However, M6 could span the membrane in an extended conformation (3.6Å per residue). M7 on the other hand is constrained at its carboxy-terminal end, since the cysteine pair αC192-αC193 are disulfide-linked and form part of the BuTx binding site. Between α159 and α192 lie 32 amino acids, an ample enough number to span the lipid bilayer as an α-helix. Finer-Moore and Stroud predicted the structure of this region to be an extramembranous amphipathic α-helix (Finer-Moore and Stroud, 1984), and therefore Criado *et al.* felt justified in their proposal of M7 as an amphipathic membrane-spanning region.

More recently, work by Pedersen *et al.* has given support to this model (Pedersen *et al.*, 1990). mAbs raised against SDS-denatured α subunit from

Torpedo AChR were characterized first by their ability to recognize specific proteolytic fragments of the α subunit and second by their recognition of synthetic peptides corresponding to the region between α147 and α179. A competition ELISA was employed to determine the orientation of the mAb epitope, using native and alkali-treated AChR-rich vesicles as well as reconstituted liposomes (50% of AChRs inside-out) as competitors. Only alkali-treated vesicles or reconstituted liposomes served as inhibitors of mAb binding to immobilized AChR, a result consistent with a cytoplasmic localization of the epitope. The binding of the mAbs to native AChR in osmotically-shocked and sonicated native vesicles was visualized directly by colloidal-gold labeling and freeze-etch immunoelectron microscopy. While the labeling was sparse, it was restricted to the cytoplasmic surface of the vesicle membrane. These experiments, therefore, support the existence of M6 and M7 as transmembrane domains.

The Lindstrom model

In a series of papers, Ratnam *et al.* investigated the binding of mAbs to the subunits of the *Torpedo* AChR (Ratnam *et al.*, 1986a; Ratnam *et al.*, 1986b). In the first of these investigations, the mAbs were mapped against proteolytic digests of the subunits by immunoblotting. The binding patterns of the mAbs to fragments generated by proteolysis were used to estimate the locations of the epitopes relative to the carboxy terminus (Ratnam *et al.*, 1986b). These mAbs were used to probe subunit topology. mAbs directed against the carboxy termini of the α, β, and δ subunits were mapped to the cytoplasmic side of the membrane in agreement with previous work (Lindstrom *et al.*, 1984; Ratnam and Lindstrom, 1984). mAb 125, predicted to bind the loop between MA and M4 in the β subunit, was mapped to the

cytoplasmic side of the membrane as well. Since the region after M3 as well as the carboxy terminus on the β subunit were mapped on the cytoplasmic side of the membrane, the cytoplasmic localization of the MA-M4 loop suggested that neither MA nor M4 were transmembrane domains.

The second paper extended these findings to the α subunit (Ratnam *et al.*, 1986a). An antiserum against the sequence corresponding to the loop between MA and M4 in the α subunit (α389-α408) was mapped to the cytoplasmic side of the membrane using a modified immunocompetition assay, and mAbs against sequences on the carboxy-terminal side of the MA-M4 loop were mapped to the cytoplasmic side of the membrane by a similar assay as well as by electron microscopic visualization. Taking all of their results in combination, Ratnam *et al.* proposed the following model (Fig. 1-8). From the work of Criado *et al.*, two transmembrane domains, M6 and M7, exist in the amino-terminal half of the α subunit (Criado *et al.*, 1985a). Similar evidence suggests that M1 is indeed a transmembrane domain (Criado *et al.*, 1985b) and that an even number of transmembrane domains exist between the carboxy-terminal end of M1 and the cytoplasmic domain after M3 (LaRochelle *et al.*, 1985; Young *et al.*, 1985; Ratnam *et al.*, 1986a; Ratnam *et al.*, 1986b). Finally, neither MA nor M4 are transmembrane domains in this model. This model crosses the membrane five times and possesses an amphipathic helix (M7).

The success of the imunochemical approach depends critically on a number of factors. First, the antibody used must recognize a specific epitope in the native structure that is also present in the denatured protein. The epitopes should not be conformationally dependent and should be limited to short stretches of the primary sequence to avoid the influence of

other non-contiguous regions of the polypeptide on antibody binding. Binding studies against either synthetic peptides or proteolytic fragments have been used to confirm the epitope specificity of antibodies used in topological mapping. Second, the antibodies need to be mono-specific, i.e., either monoclonal or affinity-purified. If different antibodies with a variety of affinities for the epitope are present in the antiserum, the results of mapping studies will be uninterpretable. Almost all of the antibodies used in the mapping studies described above were mAbs. Third, the antibodies used in this immunochemical approach should be tested at or near their determined affinity for the epitope. Only at this concentration can the antibody be expected to bind specifically to the epitope whose topology is being determined. If the antibody concentration is too high, secondary epitopes whose identity is unknown may be recognized.

In the topological studies of the AChR, the importance of this last requirement has been overlooked. Each of the studies supporting models other than the four transmembrane domain model has employed antibody binding to native receptor at concentrations three orders of magnitude higher than the reported antibody affinities. The potential for secondary epitope recognition was not addressed experimentally in any of these studies. Non-specific effects have been detected with some mAbs in functional assays. mAb 10, mapped to the cytoplasmic side of the α subunit (Ratnam *et al.*, 1986b), blocked AChR channel activity upon its addition to the extracellular side of the channel (Blatt *et al.*, 1986). In this study the concentration of mAb 10 used was only one order of magnitude higher than the reported affinity (200 nM vs. 15 nM). More recently, Das and Lindstrom disclosed that mAb 236, used in the initial proposal of the M6-M7 model

(Criado *et al.*, 1985a), heavily labeled the extracellular surface of the membrane after treatment with KSCN (Das and Lindstrom, 1991), a hydrophilic chaotrope that is unlikely to alter membrane protein topology (P. B. Sargent, personal communication), suggesting that the mAb 236 epitope is hidden within the amino-terminal domain. These reports suggest that secondary epitope recognition at high antibody concentration and epitope accessibility can be factors involved in improper topological mapping.

Other evidence contradicts the topological assignments based on immunolocalization experiments. The span of the proposed M6 domain has been limited not only by the existence of the glycosylation consensus sequence at αN141, but also by the agonist-sensitive labeling of residue αW149 by the competitive antagonist DDF (Dennis *et al.*, 1988). This reagent also labeled αY190, thereby shortening the extent of the M7 domain.

The M6-M7 model predicts that the toxin-binding site is located at the level of the lipid bilayer. If M6 and M7 are transmembrane domains, it is possible that the toxin-binding site extends into the lipid bilayer (J. B. Cohen, personal communication), since residue αW149 is labeled by DDF. However, fluorescence energy transfer measurements (Johnson *et al.*, 1990) and electron microscopic visualization (Kubalek *et al.*, 1987) have shown that the binding site of snake toxins is localized at a position near the apex of the AChR oligomer, at least 20 Å away from the membrane.

The glycosylation of the δ subunit may provide information about subunit topology since all three glycosylation consensus sequences on the *Torpedo* and mammalian δ subunits are localized in the amino terminus.

All of these sites are glycosylated in the *Torpedo* δ subunit (Nomoto *et al.*, 1986). Evidence suggests that the same is true of the mouse muscle δ subunit (Gu and Hall, 1988a). One of the mouse sites is located at δN169, which lies within the M6-M7 loop.

Biochemical data suggests that MA is not a transmembrane domain. To probe the location of the MA region, Dwyer labeled both native AChR polypeptides and solubilized α and β subunits with pyridoxal phosphate and tritiated $NaBH_4$ (Dwyer, 1988). The labeled proteins were digested with V8 protease and fragments containing sequences found in the MA regions of the α and β subunits were isolated by immunoabsorption. The fragments were labeled equally in the presence and absence of AChR denaturation. Roth *et al.* discovered that antisera against the MA region were unable to immunoblot trypsin-treated native α subunit but did bind both solubilized and native AChR, indicating that MA is not hidden within the membrane (Roth *et al.*, 1987).

Several independent observations also suggest that M4 is a membrane-spanning domain. Giraudat *et al.* have used photoreactive phospholipids to examine the transmembrane topology of the AChR (Giraudat *et al.*, 1985). These probes are incorporated into the lipid bilayer and therefore interact with membrane-spanning regions of the receptor subunits. One of the probes was five times more effective at labeling the α subunit from *Torpedo marmorata* than the *Torpedo californica* α subunit. The preferential incorporation of label was localized to a cysteine residue within the M4 domain of *T. marmorata*, suggesting that M4 was exposed to the lipid bilayer. White and Cohen used another hydrophobic probe, ^{125}I-TID, to label regions of the *Torpedo* α subunit (White and Cohen, 1988), using V8

protease cleavage to identify those regions of the subunit with which the probe was associated. A 20 kDa V8 cleavage fragment containing the domains M1, M2, and M3 was labelled by ^{125}I-TID, as was a 10 kDa fragment containing the M4 domain. V8 fragments that spanned portions of the amino-terminal domain were not labeled by ^{125}I-TID. Blanton and Wang, using a photoreactive phosphatidyl serine analog, obtained the same labeling pattern of V8 protease fragments (Blanton and Wang, 1990), suggesting that the hydrophobic domains M1-M4 are in contact with the membrane.

The M4 domain from the α subunit of *Torpedo* can be replaced with transmembrane domains from either VSV G protein or the human IL-2 receptor without significant loss of channel activity (Tobimatsu *et al.*, 1987), indicating that the hydrophobicity of this domain is consistent with the functioning of the AChR. Since M4 is the least conserved hydrophobic region in the AChR subunits, it is presumed that M4 interacts not with other regions of the subunit polypeptide, but with the lipid bilayer itself.

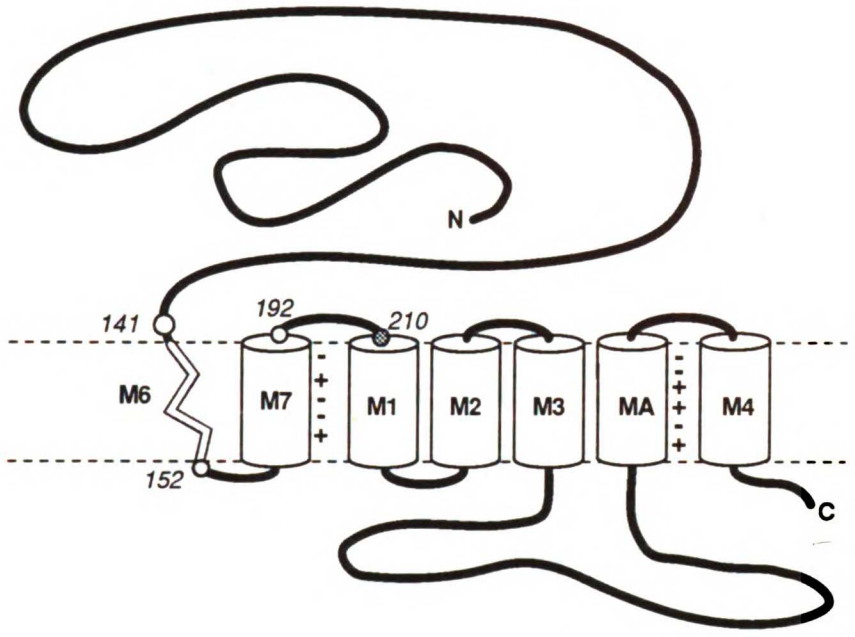


Figure 1-7. The M6-M7 model

A model of the transmembrane topology for the subunits of the AChR, first proposed on the basis of immunochemical evidence (Criado *et al.*, 1985a; Pedersen *et al.*, 1990). The amino and carboxy termini are on opposite sides of the membrane, with seven intervening transmembrane domains. Both MA and M7 are proposed to be amphipathic transmembrane domains. The locations of the carboxy-terminal end of M6 (α152) and M7 (α192) are labeled. The non-helical structure of M6 is indicated by the zig-zag line.

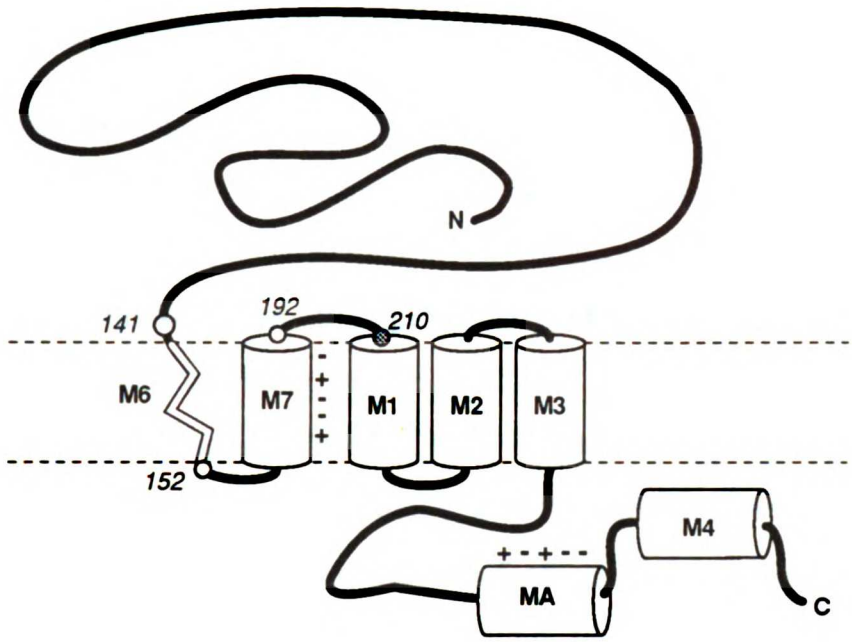


Figure 1-8. The Lindstrom model

A model of the transmembrane topology for the subunits of the AChR, based on further immunochemical evidence (Ratnam *et al.*, 1986a; Ratnam *et al.*, 1986b). Five transmembrane domains intervene between the amino and carboxy termini, placing the carboxy terminus within the cytoplasmic space. Note also that neither MA nor M4 traverse the membrane.

Summary of dissertation

The primary focus of this dissertation is the determination of the transmembrane topology of the subunits of the mammalian AChR. From the foregoing discussion, it is clear that the topologies of several regions of the subunits of the AChR are in dispute. No systematic investigations into the topology of the "consensus" regions, i.e., M1-M3, have been undertaken with either piscine or mammalian AChR. In addition to the models described above, which contain either 4, 5, or 7 transmembrane domains, two additional models can be derived with features that are consistent with the data (Fig. 1-9) (Hawrot *et al.*, 1988; Claudio, 1989).

The investigations into transmembrane topology described here were based on a method first applied to the *Torpedo* AChR in the early 1980's by Anderson and Blobel: *in vitro* translation of the AChR subunits in a heterologous translation system. In Chapter 2, the specific system used in these studies, rabbit reticulocyte lysate supplemented with canine pancreatic microsomal membranes, is described and characterized with respect to the subunits of the mouse muscle AChR. The results demonstrate that the subunits expressed *in vitro* are processed and glycosylated and can be recognized by subunit-specific antibodies. Data are also presented on the maturation of the α subunit *in vitro*.

In Chapter 3, the validity of the M6-M7 model is tested by the expression of α subunit mutants in which new glycosylation sites have been introduced. Glycosylation at a new site is demonstrative of the transmembrane translocation of that site. Additional experiments are presented that challenge the integration of the regions M6 and M7 into the

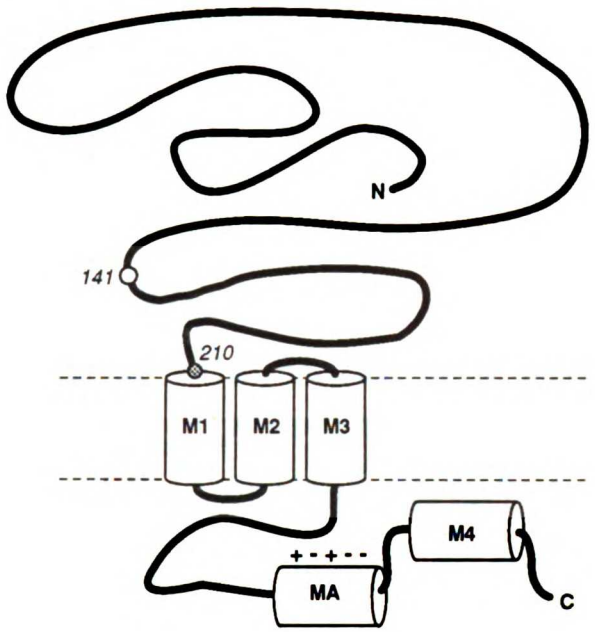
membrane. It is concluded that the entire amino-terminal domain is translocated across the membrane and is probably extracellular in the mature AChR.

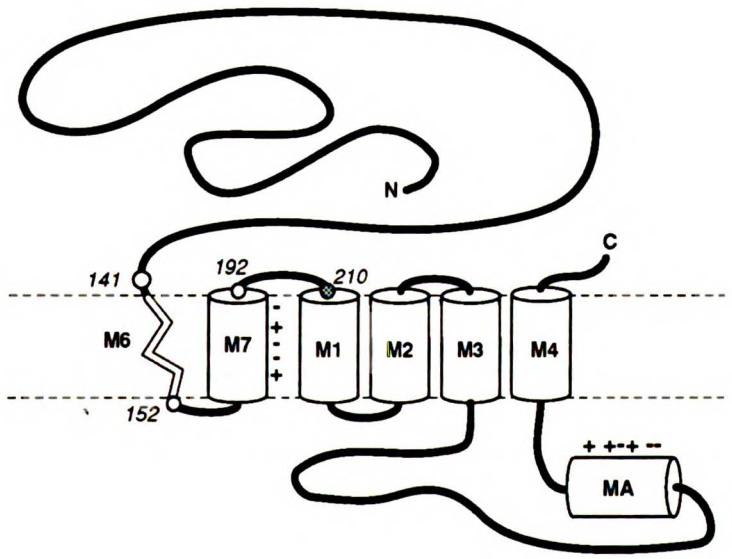
The experiments that probe the transmembrane topology of the regions M1-M4 and MA are described in Chapter 4. In these studies, chimeras of the α subunit with an antigenic tag after each of the putative transmembrane domains are expressed *in vitro* and subjected to proteolysis. Immunoprecipitation is used to determine whether the antigenic tag was accessible to protease digestion or protected by translocation into the vesicular lumen. Similar experiments using δ subunit chimeras are also described. These experiments demonstrate that the carboxy terminus is on the extracellular side of the membrane and that M1, M2, M3, and M4, but not MA, are transmembrane domains that are oriented as in the four transmembrane domain model.

The experiments described in Chapter 5 deviate from the previous chapters in that they do not deal with receptor subunit topology. These studies concentrate on the maturational differences between the mouse and human α subunits and provide new information about the early events in AChR assembly.

Figure 1-9. Two additional models of AChR subunit topology

These two models contain features found in the models given in Figs. 1-5 to 1-8, in different combinations (Lentz and Wilson, 1988; Claudio, 1989). They are, in effect, default models with no independent evidence, but they are presented here to complete the gallery of options for subunit topology, given the current evidence.


N
141
210
M1
M2
M3
M4
+ - + - -
MA
C


N
C
141
192
210
M6
M7
- + - +
M1
M2
M3
M4
152
+ +-+ --
MA

CHAPTER 2. GLYCOSYLATION AND PROCESSING OF THE SUBUNITS OF THE ACHR FROM MOUSE SKELETAL MUSCLE EXPRESSED IN A HETEROLOGOUS *IN VITRO* TRANSLATION SYSTEM

Introduction

The nicotinic AChR from electric fish, a pentameric complex of transmembrane glycoproteins with an $\alpha_2\beta\gamma\delta$ stoichiometry, has been characterized extensively with respect to its structure and function (Karlin, 1989). It is localized at the post-synaptic membrane in the electrogenic cells in the electric organ, a rich source of receptor (1-2 nmol per gram of tissue) (Karlin, 1980). The mammalian AChR found in skeletal muscle, however, has been less well characterized, primarily due to the lack of a rich source of this receptor. However, the subunits of the receptor from all species cloned thus far are highly homologous (Noda *et al.*, 1983), and it is probable that the structures of the oligomer as well as the subunits themselves are conserved.

The AChR subunits from *Torpedo* have been expressed *in vitro* to characterize the properties of their early biosynthetic forms (Anderson and Blobel, 1981; Anderson *et al.*, 1982; Anderson *et al.*, 1983). These studies demonstrated that the individual subunits expressed in a wheat germ translation system supplemented with canine pancreas microsomes can be immunoprecipitated by subunit-specific antibodies and are integrated into microsomal membranes, processed, and glycosylated (Anderson and Blobel, 1981; Anderson *et al.*, 1982). The subunits adopted a transmembrane disposition in this system similar to that seen with purified AChR in native receptor-rich vesicles (Wennogle and Changeux, 1980; Wennogle *et al.*, 1981; Anderson *et al.*, 1983).

The experiments described in subsequent chapters of this dissertation investigate the structures of the mammalian subunits in detail.

Preliminary to those studies, we have characterized the forms of the mammalian subunits expressed *in vitro*. Our experiments are in agreement with the results of those studies using the *Torpedo* subunits and demonstrate that our system of choice, the reticulocyte lysate *in vitro* translation system, is capable of expressing authentic mouse AChR subunits. In addition, we have begun the characterization of the conformational maturation of one of the subunits, the α subunit.

Materials and Methods

Preparation of reticulocyte lysate

Reticulocyte lysate was prepared from New Zealand White rabbits (Simonsen Laboratories, Gilroy, CA) as described (Jackson and Hunt, 1983). The animals were made anemic by repeated injections with 1.25% acetylphenylhydrazine (3-4 ml, subcutaneously administered; Sigma Chemical, St. Louis, MO). After the hematocrit rose to approximately 40%, the rabbits were anaesthetized with ketamine hydrochloride (35 mg/kg; Bristol Laboratories, Syracuse, NY) and xylazine hydrochloride (5 mg/kg; Haver, Shawnee, KN) and prepared for exsanguination by the intravenous administration of 2000 U of heparin (Sigma). Exsanguination was performed by cardiac puncture with a 16 ga needle attached to Tygon tubing. The chest cavity was opened to collect pooled blood within. The blood was collected into a 250 ml polycarbonate bottle on ice.

The cells were harvested by centrifugation in a Sorvall GSA rotor at 2000 rpm for 10 min at 4°C. The cells were washed 3 times by resuspension in buffered saline containing 134 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes (pH 7.2), followed by centrifugation. The packed cell volume was determined, and the cells were lysed by adding one packed-cell volume of ice-cold double-distilled water and incubating for 5 min at 4°C.

The rabbit reticulocyte lysate (RRL) was spun at 13,500 rpm for 20 min at 4°C in 30 ml Corex tubes in an SS-34 rotor. The supernatant was decanted, and hemin (1 mM solution prepared in 85% ethylene glycol;

Sigma) was added to a final concentration of 40 μM. The RRL was separated into 1 ml aliquots and stored in liquid nitrogen.

To eliminate endogenous reticulocyte-specific transcripts, the RRL was treated with micrococcal nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN) prior to the use of RRL in translation reactions. For each milliliter of thawed RRL, 10 μl of 0.1 M $CaCl_2$ and 10 μl of $15x10^3$ U/ml micrococcal nuclease [in 10 mM Tris-OAc (pH 8.0)] were added and the mixture was incubated at room temperature for 15 to 20 min. To stop the nuclease reaction, 4 μl of 500 mM EGTA was added.

To control further the composition of the nuclease-treated RRL, the lysate was desalted by passage over a G-25 Sephadex column (Pharmacia Fine Chemicals, Piscataway, NJ). The Sephadex was preswollen in 10 mM Tris-OAc (pH 7.6) and the column was poured within a 6 ml syringe barrel with nylon netting to prevent bead loss. The column was nested within a 50 ml polypropylene Falcon tube by cutting a hole in the top of the tube's cap. The RRL was added to the column and the entire assembly was spun at 1500 to 1750 rpm for 3 min at 4°C. EGTA was added to the desalted RRL to prevent re-activation of the micrococcal nuclease, and the following components were added to compensate the lysate for factors lost during the desalting process: 50 μl 4.0 M KOAc (final concentration in translation reaction: 100 mM), 4 μl 1.0 M $Mg(OAc)_2$ (final concentration: 2 mM), 20 μl 500 mM glucose (5 mM final), 20 μl 100 mM ATP (pH 7.0) (1 mM final), 20 μl 100 mM GTP (pH 7.0) (1 mM final), 100 μl 200 mM creatine phosphate (10 mM final), 40 μl 5 mg/ml creatine kinase (100 μg/ml final), 20 μl 10 mg/ml calf liver tRNA (100 μg/ml final), 20 μl 100 mM DTT (1 mM final), 96 μl RNase-free water, and 10 μl 2.0 M Tris-OAc (pH 7.6) (20 mM final). rNTPs,

creatine kinase, creatine phosphate, calf liver tRNA, and DTT were obtained from Boehringer. The levels of potassium and magnesium ions needed for optimal expression of the AChR subunits was determined empirically. 200 µl aliquots were frozen and stored in liquid nitrogen. A given aliquot of lysate was thawed only twice before discarding.

Preparation of rough microsomes

We prepared microsomal membranes from canine pancreas as described (Walter and Blobel, 1983). Several precautions were taken during the preparation of the membranes. Since the pancreas secretes digestive enzymes, the preparation of microsomal vesicles was performed as rapidly as possible to avoid degradative proteolysis. All steps were carried out in a cold room (0-4°C) to minimize degradation. PMSF (phenylmethylsulfonyl fluoride; Sigma) and EDTA were added to inhibit serine proteases and metalloendoproteases, respectively. DTT was added to protect the translocation activity of the membranes, an activity that requires free sulfhydryl groups. Since the microsomes were to be used in the *in vitro* translation reactions, manipulations of the pancreas and its homogenate were carried out in baked glassware to minimize potential RNase contamination.

The pancreas, removed from a sacrificed dog, was trimmed of fat, blood vessels and connective tissue, and minced with razor blades. The minced tissue was suspended in 4 volumes of Buffer A [250 mM sucrose, 50 mM KOAc (pH 7.5), 6 mM $Mg(OAc)_2$, 1 mM EDTA (pH 7.5), 1 mM DTT, 0.5 mM PMSF, and 50 mM triethanolamine (pH 7.5)] and homogenized in a motor-driven Dounce homogenizer, taking care to avoid generating excess

heat. The homogenate was aliquoted into 50 ml Falcon tubes, spun at 2,000 rpm for 10 min at 4°C in a Sorvall RC3B, and the supernatant was transfered into 30 ml Corex tubes. These tubes were spun at 9,500 rpm in an RC5B for 10 min at 4°C, and the resulting supernatant was layered onto a sucrose cushion containing 1.3 M sucrose, 1 mM DTT, and 50 mM triethanolamine (pH 7.5). The load-to-cushion ratio was approximately three to one. This cushion was spun at 4°C for 2.5 h at 40,000 rpm in a 50.2 Ti rotor in a Beckman ultracentrifuge. The supernatant was discarded, and the microsomal pellet was resuspended in 3 to 5 ml of Buffer B which contained 250 mM sucrose, 1 mM DTT, and 50 mM triethanolamine (pH 7.5). The concentration of the microsomes was adjusted with Buffer B to reach 50 A_{280} units per ml in 1% SDS. The microsomes were aliquoted into Eppendorf tubes (1 ml each) and stored in liquid nitrogen.

Microsomes were treated with micrococcal nuclease to destroy pancreatic mRNAs. For each milliliter of microsomes, 10 µl of 0.1 M $CaCl_2$ and 10 µl of $15x10^3$ U/ml micrococcal nuclease were added and the mixture was incubated at room temperature for 15 to 20 min. To stop the nuclease reaction, 4 µl of 500 mM EGTA was added. 200 µl aliquots of membranes were layered atop a 60 µl sucrose cushion and pelleted in an Airfuge at 4°C. The pelleted membranes were resuspended to 200 µl with Buffer B, aliquoted (approximately 20 µl) and placed in liquid nitrogen. A given aliquot of microsomal membranes was thawed only twice before discarding.

In vitro *transcription*

The cDNAs encoding the subunits of the AChR from mouse muscle (α, (Isenberg *et al.*, 1986); β, (Buonanno *et al.*, 1986); γ, (Yu *et al.*, 1986); δ, (LaPolla *et al.*, 1984)) were obtained from Drs. J. Merlie (Washington University, St. Louis, MO) and N. Davidson (California Institute of Technology, Pasadena). All of the vectors used to generate transcripts *in vitro* contained the SP6 RNA polymerase promoter upstream of the 5' end of the subunit cDNA. Therefore an SP6-based transcription system was used.

The transcription reactions were performed as described (Melton *et al.*, 1984). An rNTP stock solution was prepared that was buffered at neutral pH and contained 2.5 mM each of ATP, CTP, and UTP, and 0.25 mM GTP. The reticulocyte lysate *in vitro* translation system can efficiently express transcripts that possess modified GTP cap structures at their 5' ends. To produce capped transcripts, $m^7G(5')ppp(5')G$, a GTP cap analog (New England Biolabs, Beverly, MA), was added to the transcription from a 5 mM stock solution. A third stock solution contained the salts and buffers required for SP6 RNA polymerase activity *in vitro*. This solution contained 400 mM Tris-HCl (pH 7.5), 60 mM $MgCl_2$, and 20 mM spermidine (Sigma). The components of the transcription reaction were assembled at room temperature and added in the order given to a total of 50 µl: plasmid DNA (2.5-5.0 µg), RNase-free water, 5 µl 100 mM DTT (10 mM final), 2.5 µl 2 mg/ml BSA (100 µg/ml final), 10 µl rNTP stock (final concentrations: 500 µM each ATP, CTP, and UTP; 50 µM GTP), 5 µl GTP cap analog solution (final concentration: 500 µM), 5 µl SP6 RNA polymerase salt buffer (final concentrations: 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, and 2 mM spermidine), 0.7 µl 40 U/µl RNasin, and 2 µl SP6 RNA polymerase enzyme.

BSA was obtained from Boehringer. RNasin was obtained from Promega Biotec (Madison, WI). SP6 RNA polymerase was obtained from both Promega and New England Biolabs. The size of the reaction was scaled proportionally as dictated by need. The plasmid was not linearized for most transcription reactions. Linearization of the plasmid did not appear to affect the quality or quantity of the transcript, as determined by expression of the transcript *in vitro*. Radiolabeling of the transcript was performed by the addition of a trace amount of α-[^{32}P]-GTP to the transcription. The transcription reaction was incubated at either 37°C or 40°C for one hour. In some cases, the transcription was terminated by the addition of 2.5 U of RNase-free DNase (for a 50 µl transcription; RQ1 DNase, Promega) to the reaction to digest the template DNA. The transcript was phenol:chloroform extracted and ethanol precipitated, unless otherwise noted. Precipitated transcript was resuspended into RNase-free water (approximately 25 µl water for the product of a 50 µl transcription).

In vitro *translation*

The components of the *in vitro* translation system were thawed on ice immediately before use. Translation reactions were composed in the following manner: 0.7 volumes nuclease-treated, desalted RRL, 0.02 volumes of a 1 mM (each) 19 amino acid mixture (lacking methionine; Promega), 0.02 volumes of RNasin placental ribonuclease inhibitor (40 U/µl), 0.06 volumes labeled amino acids (tran-^{35}S-label; ICN Radiochemicals, Costa Mesa, CA), and 0.2 volumes of transcript and water. The size of the translation reaction was scaled proportionally according to need, from 10 µl to 100 µl. Translations were carried out at 30°C for one hour in sterile, siliconized Eppendorf tubes. The reactions

were terminated by transfer to ice. Aliquots of the reaction were either solubilized in sample buffer to determine the total products of translation (see below) or solubilized in a detergent solution in preparation for the precipitation of specific products (see below). Figure 2-1 is a schematic of the translation system.

Aside from the transcript, two other components were added as needed to the translations performed in these studies. The first of these was nuclease-treated canine pancreas microsomal membranes, which were added to provide membranes into which the subunits could integrate. As noted earlier, functional microsomes are capable of performing co-translational translocation, *N*-linked glycosylation, and signal sequence cleavage. For a 50 µl translation reaction, 3 µl of microsomes were used. To prevent the glycosylation of the nascent chain, the tripeptide acetyl-asparaginyl-tyrosyl-threoninyl-amide (AcNYT) was added to a final concentration of 0.5 mM. AcNYT was the generous gift of Dr. William Hansen. This compound prevents glycosylation by entering the microsome and competing with the nascent chain for the activity of the enzyme oligosaccharyl transferase, which recognizes the tripeptide *N*-glycosylation consensus sequence *N*-*X*-(*S*/*T*). In this way, the co-translational glycosylation of the nascent chain is prevented.

Immunoprecipitations

Two antibodies were used to identify the mouse α subunit. The first, mAb 61, is a rat mAb that has been mapped to residues α371-α386 (Ratnam *et al.*, 1986a). mAb 210, also a rat mAb, has been mapped in the amino-terminal domain within residues α68–α76 (Das and Lindstrom, 1989). For

characterization of the β subunit, mAb 124 was used. This rat mAb is directed against an epitope between the M3 and M4 domain (Gullick and Lindstrom, 1983). For the γ subunit, one antibody, γ-485, was used (Gu and Hall, 1988b). This antibody, directed against the carboxy-terminal decapeptide of the mouse γ subunit, was purified from rabbit serum on a peptide affinity column. Two antibodies were used to characterize the δ subunit: mAb 88B and δ-486. mAb 88B is a mouse mAb directed against the region between M3 and M4 in the δ subunit (Froehner *et al.*, 1983), and is cross-reactive against the γ subunit. δ-486 is directed against the carboxy-terminal decapeptide from the mouse δ subunit and was raised in rabbit (Gu and Hall, 1988b). mAb 61, mAb 210, and mAb 124 were the generous gifts of Dr. J. Lindstrom (University of Pennsylvania). mAb 88B was the generous gift of Dr. S. C. Froehner (Dartmouth University).

Sepharose-coupled second antibodies were prepared by activation of Sepharose 4B (Pharmacia) with CNBr (Sigma). Approximately 10 g of beads were washed with several volumes of double-distilled water and placed in 10 ml of water. A solution of CNBr (400 mg in 12 ml water) was added to the beads and the pH of the bead-CNBr suspension was maintained above pH 11 by adding 0.2 N NaOH. After CNBr activation, the beads were washed with 0.2 M Na_2CO_3. Approximately half of the CNBr-activated Sepharose was combined with 4 to 5 mg of the antibody to be coupled in 20 ml of PBS. This suspension was incubated overnight in a cold room with agitation. The coupled beads were washed with 400 ml of 50 mM Tris-HCl (pH 7.5) with 0.02% NaN_3 and stored in the same buffer. Prior to their use in immunoprecipitation reactions, an aliquot of the beads were washed several times in the appropriate wash buffer. Rabbit primary

antibodies were immunoprecipitated with protein A-Sepharose (Pharmacia).

Unless otherwise indicated, the immunoprecipitation procedure was as follows. An aliquot of the *in vitro* translation reaction to be analyzed was diluted into either a solution of PBS with 1% Triton X-100 and 2 mM PMSF or TxSwb [1% Triton X-100, 50 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl (pH 8.0)] including 2 mM PMSF. The ratio of detergent solution to translation reaction was always 9-to-1 or greater. The volume of this solution was approximately 100 µl. The appropriate primary antibody was then added, and this solution was incubated for a minimum of 2 h at 4°C with agitation. At the end of the primary antibody incubation, an aliquot of pre-washed Sepharose-coupled secondary antibody was added to the solution and the incubation was continued for an additional 2 h. The beads were pelleted by centrifugation and the supernatant was removed with a finely pulled glass pipette. Initially, this was followed by three separate washes of from 500 µl to 750 µl of TxSwb. It was subsequently determined that only one 500 µl wash was required to remove background.

Electrophoresis

A one-dimensional discontinuous gel system was used to resolve the subunits of the AChR (Laemmli, 1970). The gels were 0.75 mm thick. Both the stacking and resolving gels were made from an acrylamide:*bis*-acrylamide stock (Bio-Rad, Richmond, CA) in a 30:0.8 ratio. Both sections of the gel contained 0.1% SDS (BDH Limited, Poole, England). The stacking gel (5 ml total) contained 5% acrylamide and 0.125 M Tris-HCl (pH 6.8) and was polymerized with 2.7 µl TEMED (Bio-Rad) and 50 µl 10% ammonium

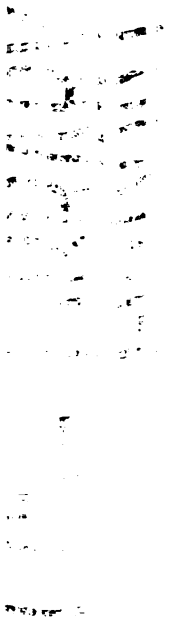
persulfate (Bio-Rad). The resolving gel (15 ml total) contained 0.37 M Tris-HCl (pH 8.8) and was polymerized with 5.2 µl TEMED and 75 µl 10% ammonium persulfate. The gels were run at constant current (15 mA through the stacking portion and 40 mA through the resolving section).

Before loading the samples, SDS loading buffer was added to the beads (or a small aliquot of the total translation reaction), and the sample was heated to 100°C for 3 min. SDS loading buffer contained 10% glycerol, 3.5% SDS, 0.25 M Tris-HCl (pH 6.8). Bromophenol blue (Sigma) was added to allow the visualization of sample migration through the gel. For reducing gels, β-mercaptoethanol was added to loading buffer to a concentration of 5%. The gels were stained using Coomassie Blue in 45% methanol: 10% acetic acid, destained in 45% methanol: 10% acetic acid, and fluorographed by immersion of the gels in 1M sodium salicylate. Subsequent to the experiments described in this chapter, fluorography was performed using the Resolution enhancer (E&M Corporation, Chestnut Hill, MA) according to the manufacturer's directions. The gels were dried under vacuum using blotter paper as a support for 45 min.

Results

In vitro *translation*

Unlabeled transcripts of each of the subunits of the mouse muscle AChR were expressed *in vitro* in the absence of added microsomes. An aliquot of the total translation product for each reaction was solubilized in SDS-PAGE loading buffer and the sample was electrophoresed through a 9% gel (Fig. 2-2A). These precursors had apparent molecular weights of 43, 49, 48, and 57 kDa for the α, β, γ, and δ subunits, respectively. These relative molecular weights are quite different from the masses predicted from the primary sequences (see Table 1-1). The subunits do not migrate in proportion relative to each other. The β subunit has a lower electrophoretic mobility than does the γ subunit (Fig. 2-2A, lanes b and c), even though the γ subunit precursor is the longer of the two (519 vs. 501 amino acids). This anomalous migration is perhaps not unexpected, since the high relative hydrophobicity of the receptor subunits could result in an increased amount of SDS binding.



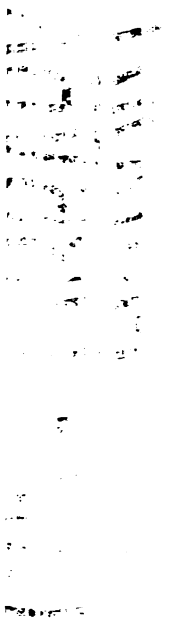
To characterize the forms of the AChR subunits synthesized *in vitro*, unlabeled transcripts were expressed in the reticulocyte lysate system in the presence and absence of microsomal membranes. In some reactions, the compound AcNYT was added to 0.5 mM to inhibit the glycosylation of the nascent chain. Samples of the translation reactions were solubilized in SDS-PAGE loading buffer and were run in a 9% acrylamide gel (Fig. 2-2B). Each of the subunit transcripts expressed in the absence of microsomes had the same mobility as in Fig. 2-2A (Fig. 2-2B, lanes a, d, g, and j). For the α, β, and γ subunits, a second species of higher mobility appeared in the

translation products expressed in the presence of microsomes and 0.5 mM AcNYT (Fig. 2-2B, lanes c, f, and i). For the δ subunit transcript expressed under these conditions, very little precursor was detected, but a higher mobility species was detected (Fig. 2-2B, lane l). These higher-mobility species were all approximately from 1 to 3 kDa smaller than the precursor species seen in the absence of microsomes. For the α, β, and δ subunit, the new species were predominant, while for the γ subunit the resolution of the bands was less clear. For the α and β subunits, from two-thirds to three-fourths of the precursor species was converted to the higher-mobility species (Fig. 2-2B, lanes c and f). These new species represent the primary translation products of the AChR subunits after cleavage of the amino-terminal signal sequence by signal peptidase, a resident protein of the ER found within the lumen of microsomal membranes. These results indicate that the extreme amino termini of the subunits of the mammalian AChR are translocated across the microsomal membrane into a region that is topologically equivalent to the extracellular space.

New species were also detected when the AChR subunit transcripts were expressed *in vitro* in the presence of microsomal membranes (Fig. 2-2B, lanes b, e, h, and k). For the α and β subunits, a fraction of the signal-cleaved species was converted to a form that migrated at or slightly above the precursor form (Fig. 2-2B, lanes b and e).[1] For the γ and δ subunits, the

[1] The different forms of the α subunit expressed *in vitro* are described in more detail in Chapter 3, "The transmembrane topology of the amino terminus of the α subunit of the nicotinic acetylcholine receptor." The different species of the α subunit described therein and their origins are essentially the same as those described for the other subunits of the mammalian AChR.

signal-cleaved forms were converted into new species that migrated from 1 to 4 kDa above the precursor form (Fig. 2-2B, lanes h and k). Band resolution was impaired due to the fluorographic enhancement method employed, immersion of the gel in 1 M sodium salicylate (unpublished observations). Since these new species were not detected in translations that included AcNYT, however, they must represent the early glycosylated forms of the subunits of the mammalian AChR. Therefore, for the mouse α, β, and δ subunits at least, a substantial portion of the amino-terminal domain must have been translocated into the lumen of the microsomal vesicle, since the glycosylation consensus sequences in these subunits are all located in this domain. These results are in agreement with those of Anderson and Blobel for the subunits of the *Torpedo* AChR (Anderson and Blobel, 1981).



Immunoprecipitation with subunit-specific antibodies

Two antibodies were used to immunoprecipitate specifically the mouse α subunit. The first of these, mAb 61, has been localized to an epitope in the region known as MA between the domains M3 and M4. The mouse α subunit is the only one of the AChR subunits for which a specific antibody to the amino-terminal domain, mAb 210, was available for use in these studies. The α subunit synthesized *in vitro* was recognized by both mAb 61 and mAb 210 (Fig. 2-3, lane a, and Fig. 4-2B, lanes a and b).

For the other subunits, very few specific antibodies were available for testing. The β subunit expressed *in vitro* was recognized by the antibody mAb 124, which is specific for the β subunit (Fig. 2-3, lane b). The γ subunit was recognized by both mAb 88B and γ-485 (Fig. 2-3, lanes c and d). mAb

88B exhibits slight cross-reactivity with the γ subunit (Froehner *et al.*, 1983), but is more effective at immunoprecipitating the δ subunit. The δ subunit was recognized by mAb 88B and δ-486, a δ subunit-specific antiserum (Gu and Hall, 1988b) (Fig. 2-3, lane e, and Fig. 4-3A, lane a). These results demonstrate that the subunits expressed in the reticulocyte lysate system are indeed the authentic subunits of the AChR.

Maturation of the α subunit in vitro

A proteolysis protection assay was used to determine if the subunits of the mammalian AChR are transmembrane proteins like the *Torpedo* subunits. Since amino-terminal-specific antibodies were available for the α subunit, we examined the transmembrane nature of this subunit. The α subunit was expressed *in vitro* in the presence of microsomal membranes, digested with varying concentrations of proteinase K, and the digests were immunoprecipitated with an antibody directed against the amino-terminal domain, mAb 210. To determine the location of the region between between M3 and M4 relative to the microsomal membrane, mAb 61 was also used.[1]

The mock-digested α subunit recognized by mAb 210 has the same mobility as the normal α subunit expressed *in vitro* (Fig. 2-4A, lane a). Digestion of the α subunit expressed *in vitro* in the presence of microsomes with proteinase K at concentrations ranging from 10 to 50 μg/ml resulted in

[1] These experiments are described in more detail in Chapter 4, "Expression of fusion proteins of the nicotinic acetylcholine receptor from mammalian muscle identifies the membrane-spanning regions in the α and δ subunits." They are described here to indicate the level of proteinase K chosen for those experiments, to demonstrate the glycosylation state of the recovered fragments, and to demonstrate the topology of the mAb 61 epitope.

the appearance of bands of higher mobility that were approximately 35 and 37 kDa in size and were recognized by mAb 210 (Fig. 2-4A, lanes b-d). Some undigested α subunit was recovered from these digests as well. At the higher concentrations of proteinase K used, the new bands gradually disappeared. These bands are greater than the size expected of the amino-terminal domain of the α subunit (see Chs. 3 and 4). From the location of the mAb 210 epitope, it is clear that these fragments represent a portion of the α subunit larger in size than the amino-terminal domain itself. These results are in agreement with similar studies by Anderson and his colleagues (Anderson and Blobel, 1981; Anderson *et al.*, 1983).

When the α subunit expressed in the presence of microsomal membranes and the glycosylation inhibitor AcNYT was digested with proteinase K, only one band, the lower of the two seen for the glycosylated α subunit, was recovered by immunoprecipitation with mAb 210 (Fig. 2-4A, lanes f-h). These results demonstrate that the amino-terminal fragment protected from proteolysis contains the site of glycosylation at αN141, since it is a glycosylated fragment of the α subunit. When mAb 61 was used to immunoprecipitate proteolytic fragments of the α subunit, no bands smaller than the full-length α subunit were detected (Fig. 2-4B, lane b). Since mAb 210-immunoreactive fragments were recovered and no mAb 61-immunoreactive fragments could be detected, the epitopes for these antibodies on the α subunit must lie on opposite sides of the microsomal membrane. Therefore, the α subunit expressed in this heterologous *in vitro* translation system must be a transmembrane protein.

Association of AChR subunits in vitro

An advantage of *in vitro* translation systems is the ease of manipulation of the reaction conditions. AChR assembly has been studied primarily in cell lines or *Xenopus* oocytes, systems with great flexibility but specific characteristics (e.g., cation concentrations, introduction of small molecules, etc.) that can be varied only with great difficulty. Therefore, we sought to demonstrate the association *in vitro* of the subunits of the AChR. Parallel reaction conditions were used for this association assay. In the first, separate translations of each of the subunits were performed. At the end of the incubation period, the translations were stopped by placing the tubes on ice, and the reaction mixtures were combined and solubilized in a Triton X-100 solution. The second set of reaction conditions differed from the first in that all of the subunits were expressed in the same translation reaction. At the end of the reaction period, the mixture was solubilized in a Triton X-100 solution. The solubilized translation mixtures were immunoprecipitated with subunit-specific antibodies as described above. Co-immunoprecipitation of subunits in this system was taken as evidence of the association of subunits.

The subunits of the AChR were expressed in the association assay in the presence of microsomal membranes (Fig. 2-5A). When the subunits were translated separately, mixed together at the end of the reaction and immunoprecipitated, no co-immunoprecipitation was detected (Fig. 2-5A, lanes a-d). Subunit association was observed, however, when the subunits that were translated in the presence of microsomal membranes in the same reaction mixture were solubilized and co-immunoprecipitated (Fig. 2-5A, lanes e-h). This result suggests that the subunits of the AChR are

capable of associating with each other *in vitro*. This result also supports the subunit-specificity of the antibodies used in this system.

To determine whether the interaction of the AChR subunits was membrane-dependent, the subunits were expressed *in vitro* as described above in the absence of microsomal membranes. Co-immunoprecipitation was not detected under either reaction condition used (Fig. 2-5B). This result suggests that the interactions of the subunits with each other require their integration into the ER membrane, since they are not associated when synthesized as soluble proteins.

When the association assay was performed in the presence of microsomes, the signal-cleaved non-glycosylated form of the α subunit could be detected by co-immunoprecipitation with mAb 124, γ-485, and δ-486 (Fig. 2-5A, lanes f-h). This result suggests that the non-glycosylated forms of the AChR subunits are able to associate with each other *in vitro*. To examine the role of glycosylation in the association of AChR subunits, we expressed the subunits in the association assay in the presence of microsomal membranes and 0.5 mM AcNYT (Fig. 2-5C). Once again, when translated separately, no subunit association was detected (Fig. 2-5C, lanes a-d). However, when the subunits were expressed simultaneously in the same reaction mixture, the association of the AChR subunits was demonstrated as evidenced by co-immunoprecipitation (Fig. 2-5C, lanes e-h). In this experiment, the δ subunit was not expressed at a detectable level. However, αβ and αγ co-immunoprecipitation was observed (Fig. 2-5C, lanes e-g). βγ association was difficult to detect because of the similar electrophoretic migration of these subunits. In a separate series of experiments, pair-wise combinations of subunits were examined in this

assay system. When expressed in the presence of microsomal membranes, all combinations of subunit pairs exhibited clear association (except the βγ pair) (data not shown). These results demonstrate that the subunits of the AChR can associate with each other in an *in vitro* translation system, and that the non-glycosylated signal-cleaved species are capable of inter-subunit association.

BuTx-binding in vitro

Anderson and colleagues were unable to detect toxin-binding species of the α subunit expressed *in vitro* (Anderson and Blobel, 1981; Anderson and Blobel, 1983b). Since the ability of the α subunit to bind BuTx depends on correct disulfide bond formation (Blount and Merlie, 1990), the α subunit was expressed in the presence of microsomal membranes with oxidized glutathione, GSSG, to decrease the level of DTT in the reticulocyte lysate without significantly impairing the efficiency of translation. This treatment has been demonstrated to promote the formation of correctly disulfide-bonded species of prolactin (Scheele and Jacoby, 1982). The α subunit expressed in the presence and absence of GSSG was precipitated by BuTx coupled to Sepharose to assay for those α subunits that attained the BuTx-binding conformation.

Expressed in the absence of GSSG, the α subunit synthesized *in vitro* was not precipitated by BuTx-Sepharose (Fig. 2-6A, lane c). When the reaction included 2 mM GSSG, however, a small fraction of the α subunit synthesized (<1%) was recognized by BuTx-Sepharose (Fig. 2-6A, lane d). The inclusion of 100 μM BuTx in the precipitation greatly decreased the amount of α subunit recovered by BuTx-Sepharose after translation in the

presence of GSSG (Fig. 2-6A, lane g). These results suggest that the BuTx-binding form of the α subunit can form *in vitro* when the translation reaction is carried out in the presence of GSSG.

Since it is known that αδ heterdimer formation leads to an increase in the amount of BuTx-binding activity in transfected cells (see Ch. 4), the α and δ subunits were co-translated *in vitro* and the products were precipitated with BuTx-Sepharose. Although the δ subunit was co-precipitated with the α subunit, the relative conversion of the α subunit to the BuTx-binding form was unchanged (data not shown).

We also investigated the ability of fragments of the α subunit to bind BuTx in this system. The three fragments included α52, α53, and α/*Hinc* II (see Ch. 3). The construct α52 encodes the entire amino-terminal domain and the adjacent M1 domain, α53 encodes the amino-terminal domain exclusively, and α/*Hinc* II terminates at a point between α53 and α52. For each construct, a BuTx-binding form could be detected both in the absence and the presence of GSSG (Fig. 2-6B). It is possible that these truncated forms are more capable of adapting their structures to the BuTx molecule since they are not restricted in their conformation by the remainder of the α subunit polypeptide. It is also clear that the most modified species of each of the constructs is most readily converted into the BuTx-binding form in the presence of GSSG (Fig. 2-6B, lanes b, d, and f). For α52 and α/*Hinc* II, the glycosylated forms exhibited the greatest difference in amount in the presence and absence of GSSG, and for α53, it is the signal-cleaved species (Fig. 2-6B, lanes b and f). These results demonstrate that the alteration of the redox potential of the reticulocyte lysate translation system can influence the maturation of the α subunit expressed *in vitro*.

Discussion

We have sought to use *in vitro* expression of the subunits of the mammalian AChR to study aspects of receptor biosynthesis in a system that is easy to manipulate. We examined the subunits expressed from the cloned genes in the rabbit reticulocyte lysate translation system. The purpose of these experiments was three-fold. First, we wished to demonstrate that the RRL system is able to synthesize authentic AChR subunits. Second, we wanted to compare the subunits of the mammalian AChR with those of the *Torpedo* AChR expressed in the wheat germ (WG) translation system to determine if the basic features of the subunits from these two species are the same. Third, we wished to extend the results of early *in vitro* expression experiments by characterizing the subunits expressed in the RRL system with respect to subunit maturation and AChR assembly.

The system chosen for the present experiments was the RRL *in vitro* translation system. The lysate retains much of the translation machinery (e.g., initiation factors, ribosomes, etc.) and can be supplemented with an ATP regeneration system to permit high level protein synthesis for an hour or more. If the endogenous mRNA species (primarily globin mRNA) are destroyed, heterologous transcripts can be expressed specifically and efficiently against a null background. Therefore this system can produce specific translation products from transcripts obtained from many exogenous sources (Jackson and Hunt, 1983).

Since the subunits of the AChR are transmembrane proteins, proper structural analysis of these proteins in a cell-free system requires target

membranes into which they can integrate. The most readily obtainable source of such membranes are pancreatic acinar cells, which produce large quantities of secretory proteins. Microsomal membranes isolated from these cells are derived from, and retain the orientation of, the rough ER. They contain the components necessary to translocate, integrate, and process nascent transmembrane proteins produced in cell-free translation systems (Walter and Blobel, 1983). These microsome-supplemented heterologous translation systems have been used to study a variety of bitopic and polytopic membrane proteins (Lingappa *et al.*, 1978; Anderson and Blobel, 1981; Anderson *et al.*, 1983; Yost *et al.*, 1983; Friedlander and Blobel, 1985; Audigier *et al.*, 1987; Rothman *et al.*, 1988; Kobilka, 1990; Sawyer and Doyle, 1990). Thus this system is a logical choice to investigate the topologies of individual subunits.

We have used subunit-specific antibodies to identify the products of the expression of the subunit mRNAs translated *in vitro*. This analysis has demonstrated that the polypeptides that we have synthesized in the RRL translation system are indeed the subunits of the mammalian AChR. Each of these subunits is synthesized as a precursor that is co-translationally cleaved by signal peptidase to remove the signal sequence. All of the subunits are glycoproteins, as demonstrated by the membrane-dependent appearance of new species that are not synthesized when glycosylation is inhibited by the presence of AcNYT in the translation reaction. The α subunit expressed in this system is a transmembrane protein, as demonstrated by the membrane-dependent cleavage of the signal sequence, the appearance of a glycosylated species, and the differential accessibility of proteinase K to two epitopes, of which one is in the amino-terminal domain

and the second lies in the region between M3 and M4 (see Ch. 1 and Ch. 4). The results of our studies with the α subunit of the mammalian AChR are entirely consistent with the work of Merlie and his co-workers, who used the RRL translation system to express the α subunit from the BC3H-1 muscle cell line using membrane-bound polyribosomes and poly-(A^+) RNA as the expression template (Merlie *et al.*, 1981; Sebbane *et al.*, 1983). Our results are also consistent with information obtained from the analysis of the native mammalian AChR (Gu and Hall, 1988b; Gu and Hall, 1988a), and they suggest that the RRL translation system can synthesize authentic mammalian AChR subunits. Therefore, the RRL translation system is appropriate for the study of AChR subunit biosynthesis.

The early work of Anderson and Blobel demonstrated the ability of the WG translation system to express the subunits of the *Torpedo* AChR *in vitro* (Anderson and Blobel, 1981; Anderson *et al.*, 1982; Anderson *et al.*, 1983). They were able to characterize the initial steps in subunit biosynthesis, including SRP-dependent integration into the microsomal membrane (Anderson *et al.*, 1982), co-translational processing and glycosylation (Anderson and Blobel, 1981), and general transmembrane structure (Anderson and Blobel, 1981; Anderson *et al.*, 1983) by expressing the AChR subunits from total mRNA purified from the *Torpedo* electric organ. Several of the features of the *Torpedo* subunits revealed by this analysis were seen in the native *Torpedo* AChR, suggesting that the WG *in vitro* translation system is capable of expressing authentic subunits. Our results are consistent with the findings of Anderson and Blobel as well. The early investigations into the expression of mammalian and *Torpedo* AChR subunits were unable, however, to reproduce two important features of

AChR biosynthesis *in vitro*: acquisition of toxin binding by the α subunit, and AChR subunit assembly. In view of our initial results, we can provide several possible explanations for their early (and our more recent) difficulties.

Since toxin-binding depends on proper disulfide bond formation (Mishina *et al.*, 1985; Blount and Merlie, 1990), and since both the WG and RRL translation systems require high levels of DTT (≥ 1 mM) (Erickson and Blobel, 1983; Jackson and Hunt, 1983), it is perhaps not surprising that neither Anderson and Blobel, nor Merlie and his colleagues were able to detect toxin-binding species of the α subunit expressed *in vitro* (Anderson and Blobel, 1981; Merlie *et al.*, 1981; Anderson and Blobel, 1983b; Sebbane *et al.*, 1983). The inability of the α subunit (and for reasons of homology the other subunits) to achieve the mature conformation *in vitro* could certainly impede the process of AChR assembly (see below). In our system, however, we have detected the presence of a small percentage of total α subunit that has acquired the toxin-binding conformation when expressed in the presence of 2 mM GSSG. The addition of GSSG serves to reduce the effective level of DTT by acting as an oxidant, thereby altering the redox potential of the system to allow intra-chain disulfide bonds to form. The low (~1%) level of conversion to a BuTx-binding conformation is similar to the amount observed in transfected COS cells (see Ch. 5), a system in which high-level expression of the AChR has been demonstrated (Gu *et al.*, 1990).

AChR assembly in an *in vitro* translation system is dependent on the co-insertion of the nascent polypeptides for all of the subunits in the same vesicle. Therefore, high expression levels of the subunits are needed to increase the probability of multi-subunit insertion within the same vesicle.

Anderson and Blobel estimated that each subunit chain expressed from total electric organ mRNA accounts for only 0.5% of total protein synthesis in the WG system (Anderson and Blobel, 1981). While they were able to detect homo-oligomeric structures that they characterized as potential assembly intermediates on the basis of sucrose gradient centrifugation of the products of *in vitro* translation (Anderson and Blobel, 1983a), no such homo-oligomers have been observed either in transfected cells or in oocytes. In the experiments of Merlie and his co-workers, for example, only 5% of the total BC_3H-1 RNA was recovered as poly-(A^+) RNA, of which only a small fraction would code for any one of the subunits (Sebbane *et al.*, 1983). Because the mRNA for the subunits from these sources are scarce, it is not likely that more than one subunit would be integrated into the same vesicle. Therefore, the association of AChR subunits expressed *in vitro* from the mRNA obtained from these sources is improbable. Our system, on the other hand, uses as template the mRNA derived from *in vitro* transcription of the subunit cDNAs. Therefore, the proportion of AChR subunit transcripts obtained from *in vitro* transcription that are translated in RRL is higher than that from the cellular sources, and it is probable that the resultant increase in the probability of multi-subunit insertion is the basis for our ability to detect inter-subunit association.

Several caveats must be mentioned about the association process we have observed. First, we were unable to determine whether all of the subunits immunoprecipitated with a given subunit-specific antibody were present within the same vesicle. For example, we were able to co-immunoprecipitate the α and β subunits with the antibody δ-486 when the α, β, and δ subunits were translated *in vitro* (data not shown). We were

unable to determine, however, if this was the result of the immunoprecipitation of αβδ heterotrimers or the αδ and βδ pairs. Second, we were unable to determine the specificity of the associations we observed. It is possible that any transmembrane proteins could become associated with the subunit polypeptides as a result of non-specific (perhaps hydrophobic) interactions. We attempted to express other transmembrane proteins (e.g., opsin, β-adrenergic receptor, and *Shaker* B and C) along with the receptor subunits as controls for the specificity of the association between the subunits of the AChR (data not shown). We could not express these non-receptor polypeptides in our translation system, however, and therefore we were unable to investigate the specificity of the observed associations. Third, the association we have detected may be the result of the non-specific aggregation of the misfolded subunit polypeptides. Since the degree of subunit maturation in this system is quite low (even in the presence of GSSG), it is likely that a large proportion of the subunits expressed *in vitro* are misfolded. Lastly, it appears that the temperature of the expression system may have an effect on the association of the AChR subunits. The assembly of the *Torpedo* AChR, an efficient process at 26°C, is considerably less efficient at 37°C (Paulson and Claudio, 1990). The association between the mammalian α and β subunits that we have observed does not occur in transfected cells incubated at 37°C (Blount *et al.*, 1990; Gu *et al.*, 1991b; Verrall and Hall, 1992). The β subunit does not appear to enter the AChR assembly pathway until the α, γ, and δ subunits have associated (see Chs. 1 and 5). Therefore, one possible reason for the association we have observed is the enhanced stability of the αβ heterodimer at the translation temperature (30°C).

It should be noted, however, that cell-free heterologous translation systems have been shown to assemble hetero-oligomeric membrane proteins (Sawyer and Doyle, 1990). High-efficiency assembly of the rat asialoglycoprotein receptor (RHL), a trimeric membrane protein complex, was observed in the RRL translation system with dog pancreas microsomes by using an association assay virtually identical with the one described above. The subunits of the RHL were cross-immunoprecipitated with specific antibodies for either subunit only when the subunits were expressed simultaneously in the presence of microsomal membranes; an unrelated membrane protein was not co-immunoprecipitated with the RHL subunits under the same translation conditions. It is possible, therefore, to assemble multimeric membrane proteins in *in vitro* translation systems under the proper conditions. At this time, however, the conditions under which AChR assembly *in vitro* will occur remain to be discovered.

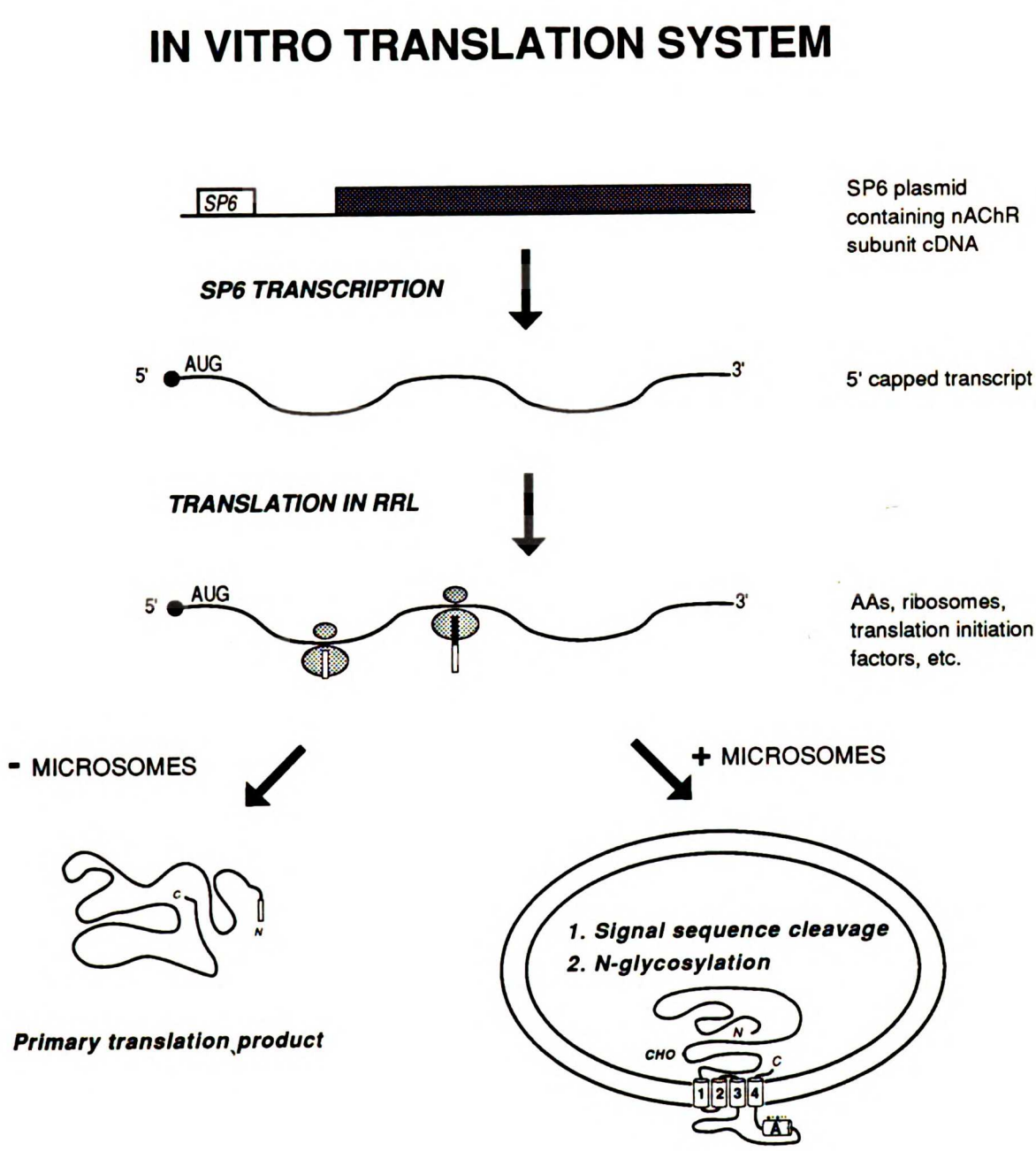


Figure 2-1. Schematic of the heterologous cell-free translation system

For discussion, see text.

Figure 2-2. Expression of the subunits of the mouse muscle AChR

The cDNAs encoding the subunits of the AChR were transcribed and translated *in vitro* as described in the Methods section above.

A, Translation of the subunits of the AChR in the absence of microsomal membranes.

B, Glycosylation and processing of the AChR subunits. The indicated transcript (**Tr**) was expressed *in vitro* in the presence or absence of microsomal membranes (**RM**) or glycosylation inhibitor (**AcNYT**; 0.5 mM). An aliquot of the translation mixture was solubilized in SDS loading buffer and electrophoresed as described.

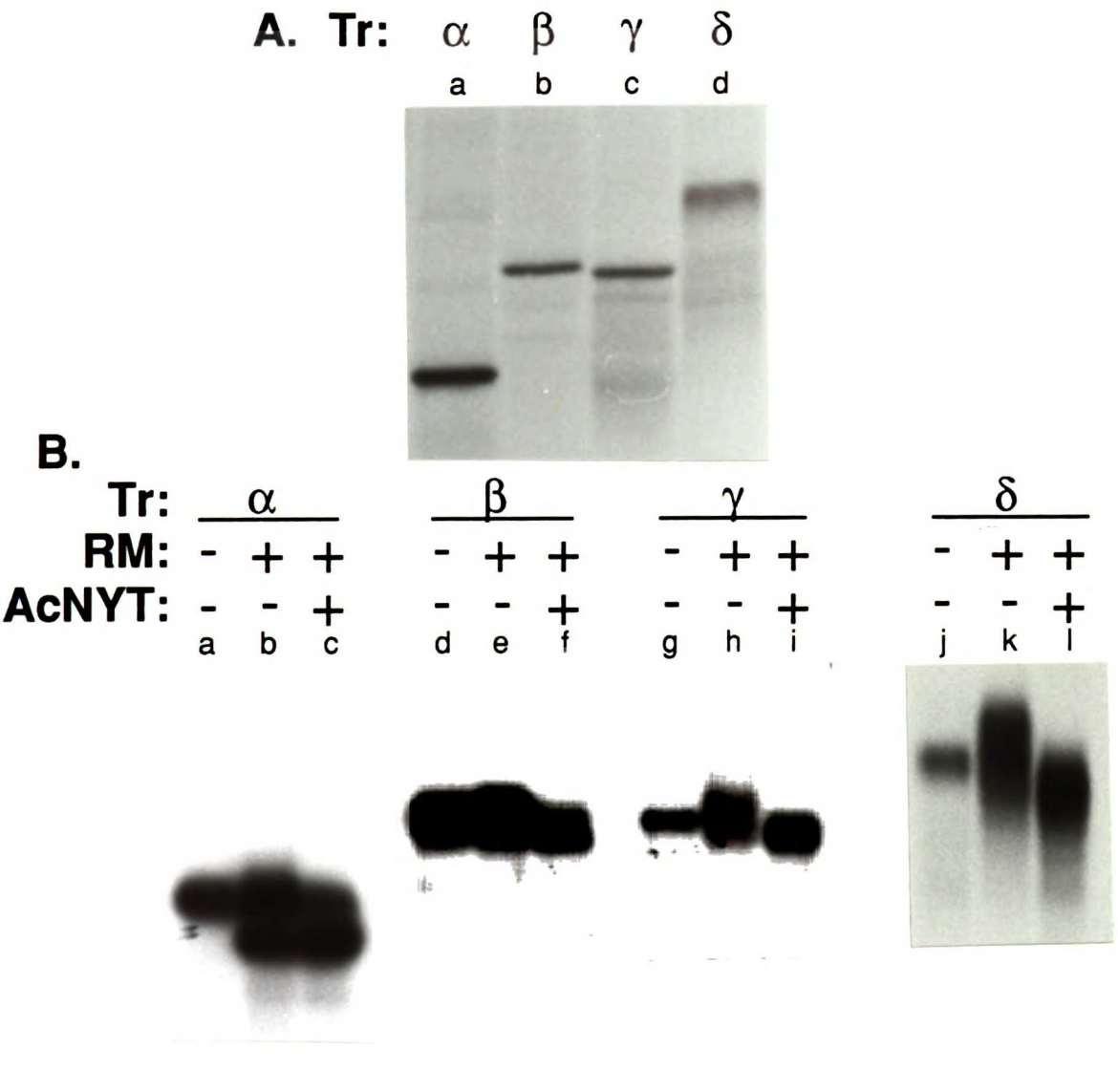

A. Tr: α β γ δ
a b c d
B.
Tr: α β γ δ
RM: - + + - + + - + + - + +
AcNYT: - - + - - + - - + - - +
a b c d e f g h i j k l

Figure 2-3. Subunit-specific immunoprecipitation

The indicated transcripts (**Tr**) were expressed *in vitro* without added microsomal membranes. An aliquot of the translation reaction was solubilized in a Triton X-100 buffer and immunoprecipitated with the appropriate antibody (**Ab**) as described. The immunoprecipitated was solubilized in SDS loading buffer and electrophoresed as described.

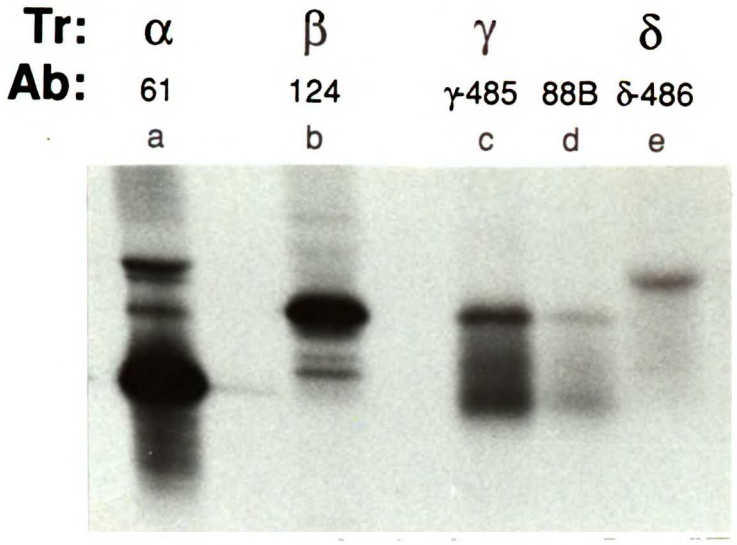
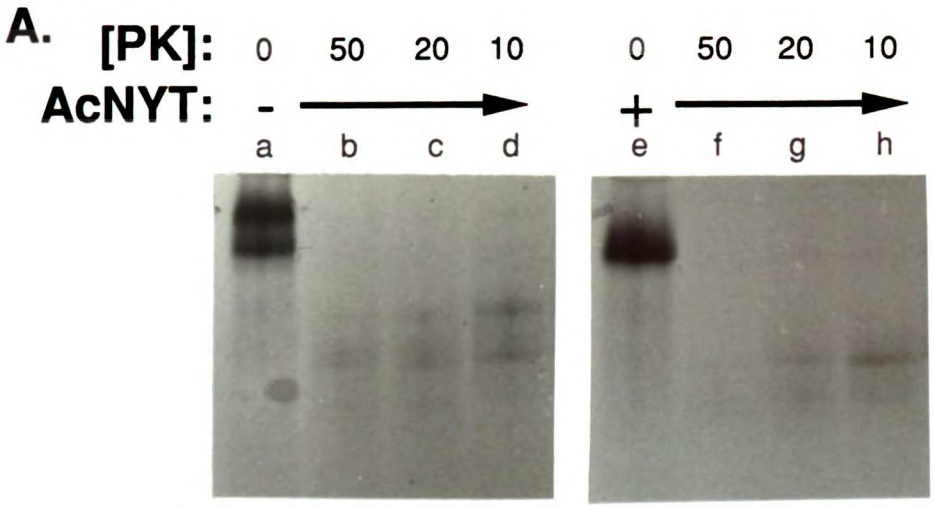

Tr: α β γ δ
Ab: 61 124 γ-485 88B δ-486
a b c d e

Figure 2-4. Proteolysis of the α subunit of the AChR

The α subunit transcript was expressed in the presence of microsomal membranes, with and without 0.5 mM **AcNYT**, and an aliquot of the translation mixture was digested with varying amounts of proteinase K (**PK**; values are μg/ml final concentration) as described. The proteolysate was then immunoprecipitated, and the samples electrophoresed.

A, Immunoprecipitation with mAb 210; **B**, immunoprecipitation with mAb 61. Refer to Fig. 4-4 for details of proteolysis.


A.
[PK]:
0
50
20
10
0
50
20
10
AcNYT:
-
+
a
b
c
d
e
f
g
h


B.
PK:
-
+
+
DET:
-
-
+
a
b
c

Figure 2-5. Association of the subunits of the AChR *in vitro*

The subunits of the AChR were translated either in separate reactions or together in the same reaction. At the end of the translation, the reaction mixtures containing the separately-translated subunits were mixed together and solubilized in a Triton X-100 buffer. The reaction containing the subunits translated together was also solubilized. Aliquots from each mixture were then immunoprecipitated with subunit-specific antibodies (**Ab**), and the resulting samples were electrophoresed as described.

A, Subunits translated in the presence of microsomes (**+RM**); **B**, subunits translated in the absence of microsomes (**-RM**); **C**, subunits translated in the presence of microsomes and 0.5 mM AcNYT (**+RM+AcNYT**)

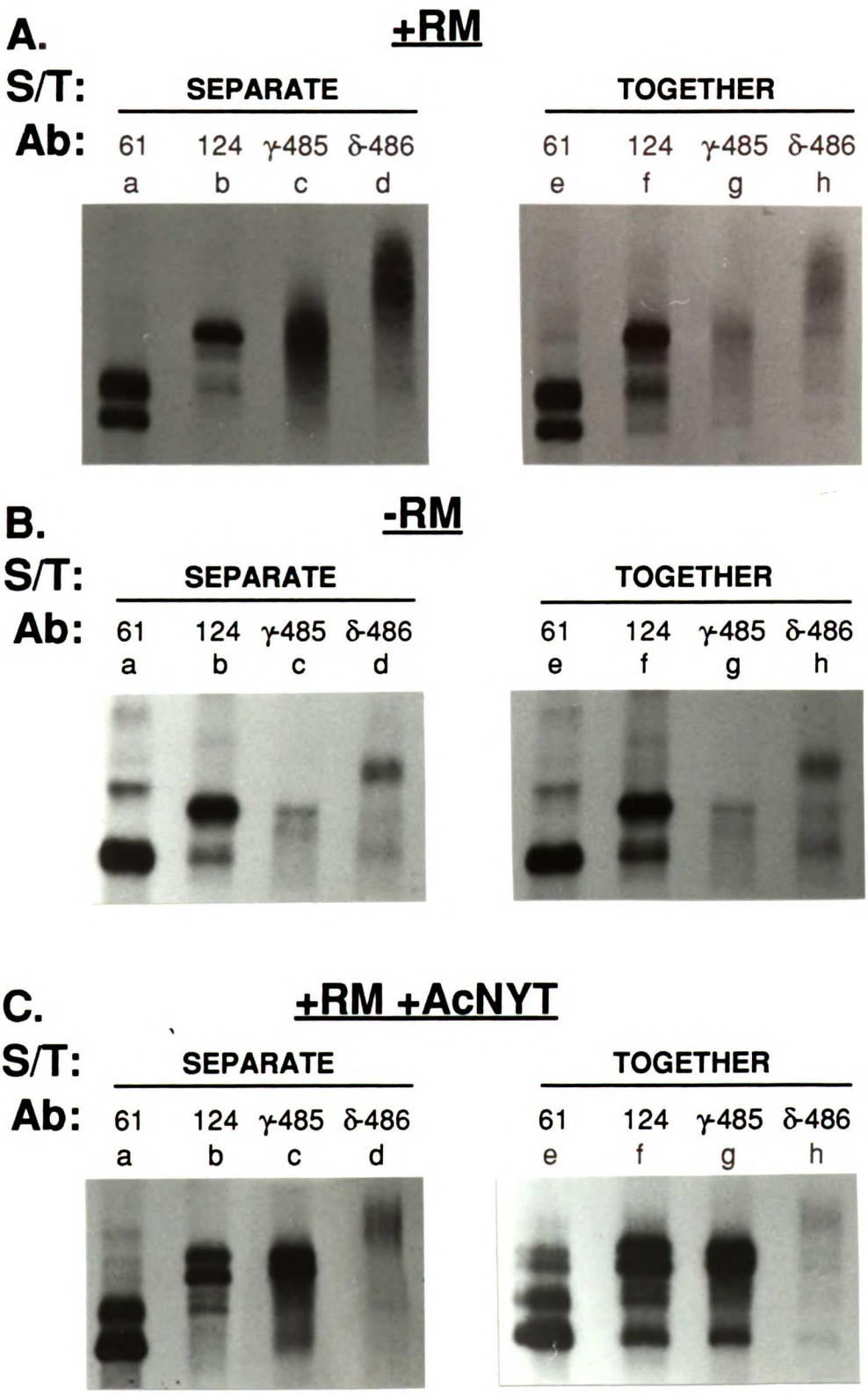
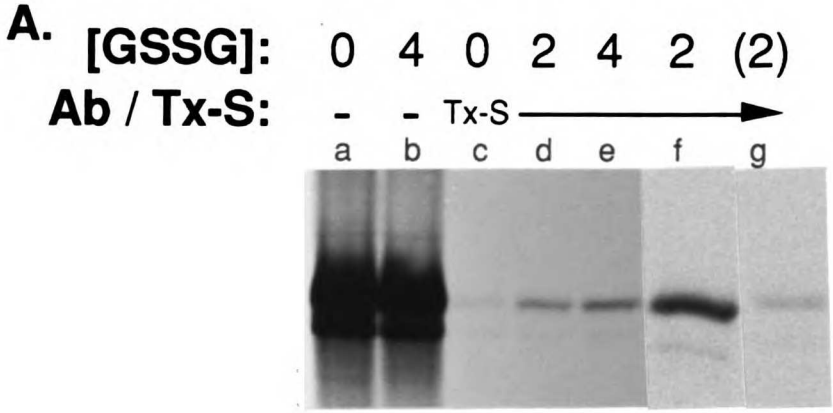

A.
+RM
S/T:
SEPARATE
TOGETHER
Ab:
61 124 γ-485 δ-486
a b c d
61 124 γ-485 δ-486
e f g h
B.
-RM
S/T:
SEPARATE
TOGETHER
Ab:
61 124 γ-485 δ-486
a b c d
61 124 γ-485 δ-486
e f g h
C.
+RM +AcNYT
S/T:
SEPARATE
TOGETHER
Ab:
61 124 γ-485 δ-486
a b c d
61 124 γ-485 δ-486
e f g h

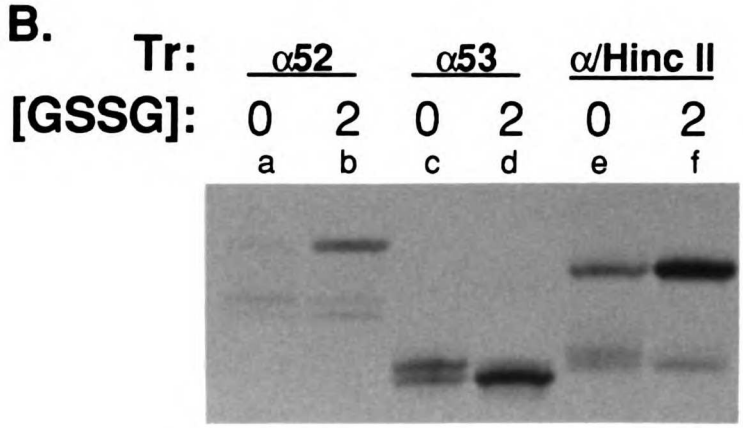
Figure 2-6. Maturation of the BuTx-binding form of the α subunit *in vitro*

The α subunit (panel **A**) or amino-terminal fragments of the α subunit (panel **B**) were expressed *in vitro* in the absence or presence of oxidized glutathione (GSSG; values are mM). The translation mixture was solubilized and BuTx-binding forms of the α subunit were precipitated using BuTx coupled to Sepharose (Tx-S). In panel **A**, the parenthesis around the GSSG concentration in lane g represents the recovery of the BuTx-binding form of the α subunit when the precipitation reaction was performed in the presence of 100 μM BuTx. See Fig. 3-7A for a description of the constructs used in panel **B**.

A.


[GSSG]: 0 4 0 2 4 2 (2)
Ab / Tx-S: - - Tx-S
a b c d e f g

B.


Tr: α52 α53 α/Hinc II
[GSSG]: 0 2 0 2 0 2
a b c d e f

CHAPTER 3. THE TRANSMEMBRANE TOPOLOGY OF THE AMINO TERMINUS OF THE α SUBUNIT OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

Introduction

The nicotinic acetylcholine receptor (AChR) in mammalian skeletal muscle is a hetero-oligomeric complex of four transmembrane polypeptides whose stoichiometry is $\alpha_2\beta\gamma\delta$ (Claudio, 1989). Upon binding the neurotransmitter acetylcholine (ACh) to the two α subunits, the receptor undergoes a conformational change that allows the passage of ions into the muscle cell, depolarizing the membrane and eventually leading to muscular contraction. The nicotinic AChR from muscle and the closely related receptor from the *Torpedo* electric organ are the best-studied members of a super-family of ligand-gated ion channels that includes the glycine and $GABA_A$ receptors (Grenningloh *et al.*, 1987; Schofield *et al.*, 1987; Langosch *et al.*, 1988). The elucidation of the mechanism that links ligand binding to channel opening for these receptors depends upon detailed knowledge of the structure of each of the subunits.

The subunits of all of the ligand-gated receptors in this super-family have highly homologous primary sequences and share a stereotyped structure based on hydropathic analysis. In each case, a long, hydrophilic amino-terminal region is followed by four hydrophobic domains whose location within the sequence is highly conserved. The amino-terminal region in each case contains at least one N-linked glycosylation consensus sequence. These findings have led to the proposal (Noda *et al.*, 1983; Numa *et al.*, 1983; Boulter *et al.*, 1985) of a model in which a long amino-terminal extracellular domain (Fig. 3-1A) is followed by four transmembrane segments (Fig. 1-5).

An alternative model of the amino-terminal domain (Fig. 3-1B) arises from experiments with monoclonal antibodies (mAbs) raised against a synthetic peptide whose sequence corresponds to residues α152-167 of the α subunit of the *Torpedo* AChR. Because these antibodies, which recognize predominantly the sequence α152-159 (Criado *et al.*, 1985a), bind the AChR in *Torpedo* vesicles only after treatments that permeabilize the membrane, Criado *et al.* have proposed that residues α152-159 are cytoplasmic and connect two new transmembrane domains extending from residues α142 to α152 and from α159 to α192, respectively. Recently, more detailed experiments using immunoelectron microscopy (Pedersen *et al.*, 1990) have strongly supported these findings, by identifying a region between α156-179 as a cytoplasmic domain.

We have used two strategies to investigate the transmembrane topology of the amino-terminal domain of the α subunit of the nicotinic acetylcholine receptor from mouse skeletal muscle. By oligonucleotide-directed mutagenesis we have altered the primary sequence to introduce consensus sequences for N-linked glycosylation into two novel sites: α154 and α200. Constructs containing these new sites were then expressed either in an *in vitro* translation system or in transfected mammalian cells. In all cases, the new sites were glycosylated. In addition, cDNAs which encode fragments of the amino-terminal domain of the α subunit were transcribed and translated *in vitro*. The expressed proteins were subjected to carbonate extraction to determine if the fragments were integral membrane proteins (Fujiki *et al.*, 1982). The results of both series of experiments lead to the conclusion that the amino-terminal domain of the α subunit up to amino

acid residue α207 is completely translocated across the endoplasmic reticulum (ER) membrane.

Materials and Methods

Mutagenesis

The α subunit cDNA, derived from the mouse muscle cell line BC3H-1 (Isenberg *et al.*, 1986), was obtained from Dr. J. Merlie, and was cloned as an *Eco R*I fragment into the vector pSM, generously provided by M. Brodsky and Dr. D. Littman (Brodsky *et al.*, 1990). This vector contains the M13 origin of replication for generation of single-stranded DNA, as well as SV40 origin/promoter elements. *E. coli* CJ236 cells were a gift from Dr. R. Myers. Strain HB101 and T4 DNA ligase were obtained from BRL (Gaithersburg, MD). T4 DNA polymerase and dNTPs were from Boehringer Mannheim Biochemicals (Indianapolis, IN). DNA sequencing was performed following manufacturer's instructions using the Sequenase™ kit from US Biochemical Corporation (Cleveland, OH).

Oligonucleotide-directed mutagenesis was performed essentially by published methods (Kunkel, 1985; Geisselsoder *et al.*, 1987). *E. coli* HB101 cells were transformed with the resulting double-stranded DNA. Colonies were screened either by restriction digests or by sequencing.

The oligonucleotides used to generate the mutants were obtained from the UCSF Biomolecular Resource Center (Fig. 3-2). The original N-glycosylation consensus sequence at α141-143 (NCS) was rendered non-functional by mutagenesis with the oligonucleotide N141K, which changed the primary sequence from α141-NCS to α141-KCS. The unique *Pst* I restriction site in the α subunit cDNA sequence is altered as well, allowing for selection of mutants by restriction digest. The other oligonucleotides

(g154, g200) were used to introduce new consensus sequences. Mutagenesis with g154 converted the sequence α154-SVV to α154-NVS, and mutagenesis with g200 converted the sequence α200-DIT to α200-NIT, creating a new *Ssp* I site. In all, five new α subunit constructs were made, with either one (αX g154, αX g200), two (α g154, α g200), or zero (αX) N-linked consensus sequences.

In order to investigate further the translocation of the α subunit amino-terminal domain into the lumen of the ER, two additional α subunit constructs were expressed *in vitro* in the presence of microsomes and subjected to carbonate extraction (see below). Both constructs were generously provided by Drs. P. Blount and J. Merlie, and were derived from the construct PBα1 (Blount and Merlie, 1988) by oligonucleotide-directed mutagenesis (Burke and Olson, 1986). The first construct, PBα53, expresses an α subunit fragment with a termination codon before the putative first transmembrane domain M1, replacing residue αQ208 (Fig. 3-7A). The second construct, PBα52, expresses an α subunit fragment with a termination codon in the loop between M1 and M2, replacing residue αE241 (Fig. 3-7A). Restriction fragments isolated from both PBα52 and PBα53 were inserted into pGEM2 to generate transcripts *in vitro*. The translation products of both pGEM2-α52- and pGEM2-α53-derived transcripts are recognized by mAb210, a rat mAb directed against an epitope between residues α46-127 (Ratnam *et al.*, 1986b) (Chavez and Hall, unpublished experiments).

In vitro *transcription*

The normal and mutant α subunits were cloned into the vector pGEM2 so that RNA transcripts could be generated. Capped transcripts were synthesized with the SP6 RNA polymerase system described by Melton *et al.* (1984), using as template SP6 expression plasmids containing the cloned cDNA encoding normal or mutant α subunits. The SP6 expression vector pGEM2 was obtained from Promega Biotec (Madison, WI). SP6 RNA polymerase and GTP cap analog ($m^7G5'ppp5'G$) were obtained from New England Biolabs (Beverly, MA), placental RNase inhibitor (RNasin) and RNase-free DNase from Promega Biotec, and ribonucleotide triphosphates from Boehringer Mannheim Biochemicals (Indianapolis, IN). Following incubation of the transcription mixture at 37°C for one hour, the DNA template was removed by incubation with RNase-free DNase for 15 minutes at 37°C. The synthesized transcript was extracted once with phenol: chloroform, ethanol precipitated, and resuspended in RNAse-free water for use in the *in vitro* translation reactions. For the experiments described in Figure 3-7, the plasmid encoding the normal α subunit was digested with *Hinc* II, and the linearized plasmid was used for *in vitro* transcription. This construct was designated α/*Hinc* II (Fig. 3-7A).

In vitro *translation*

Micrococcal nuclease-treated rabbit reticulocyte lysate and canine pancreatic microsomes were prepared as described (Jackson and Hunt, 1983; Walter and Blobel, 1983). Ac-Asn-Tyr-Thr-NH_2 (AcNYT) was generously provided by Dr. W. Hansen. AcNYT is an acetylated tripeptide containing an N-linked glycosylation consensus sequence and prevents N-

glycosylation by competing with the nascent chain for glycosylation (Lau *et al.*, 1983; Welply *et al.*, 1983; Wieland *et al.*, 1987). Tran^{35}S-label was obtained from ICN Radiochemicals (Irvine, CA). The translation mixtures, containing reticulocyte lysate, RNasin, tran^{35}S-label, an amino acid supplement lacking methionine, *in vitro*-synthesized transcript, and pancreatic microsomes (as indicated) were incubated for one hour at 30°C. Where indicated, AcNYT was added to a final concentration of 0.5 mM to prevent co-translational glycosylation of the nascent polypeptide. At the end of the incubation, an aliquot of the translation was prepared for immunoprecipitation by solubilization in a buffer containing 1% Triton X-100 and 2 mM PMSF in phosphate-buffered saline (PBS).

COS cell transfection

A modified DEAE-dextran protocol (Seed and Aruffo, 1987) was used for transient transfection of COS 3 cells, which were plated and grown in DME-H16 containing 10% fetal bovine serum (Gu *et al.*, 1990). The cDNAs encoding the normal and mutant α subunits used in the transfection were in the pSM vector (see above). The transfected cells were radiolabeled by incubating them for 30 minutes in cysteine-and-methionine-free DME-H16 supplemented with 250 μCi/ml tran^{35}S-label. After scraping them from the plate, the cells were pelleted and solubilized for immunoprecipitation in a Triton X-100 buffer according to published procedures (Gu *et al.*, 1989).

Immunoprecipitation and electrophoresis

The rat monoclonal antibodies, mAb61, mapped to the region between M3 and M4 in the α subunit (residues α371-386) (Tzartos *et al.*, 1981; Ratnam *et al.*, 1986b), and mAb35, mapped to a conformation-specific

domain in the amino terminus (Tzartos *et al.*, 1981; Merlie and Lindstrom, 1983), were generously provided by Dr. J. Lindstrom. Rat IgG was obtained from Organon Teknika (West Chester, PA). Antibody was added to the solubilized translation or COS cell extract, and the mixture incubated for at least 2 hours at 4°C with agitation. At the end of this period, 20 µl of a 1:1 slurry of Sepharose-coupled rabbit anti-rat IgG (prewashed with wash buffer containing 1% Triton X-100, 500 mM NaCl, 10 mM EDTA, 10 mM EGTA, and 100 mM Tris-HCl, pH 7.5) was added to the mixture, and the incubation was continued at 4°C for at least 2 hours with continuous agitation. The Sepharose pellet was washed three times with 500 µl of wash buffer containing 2 mM PMSF at room temperature and aspirated to dryness. For immunoprecipitations from COS cells, the Sepharose wash buffer contained 1 M NaCl, 1% Triton X-100, 2 mM PMSF, and 50 mM Tris-HCl, pH 7.5. PAGE gel loading buffer with 10% β-mercaptoethanol was added, the sample heated to 100°C for 3 minutes, and electrophoresed through an SDS-polyacrylamide gel (Laemmli, 1970). The gels were stained, fluorographed, and exposed to Kodak X-AR film.

Carbonate extraction

Transcripts synthesized from the constructs pGEM2-α52 and pGEM2-α53 were expressed in the *in vitro* translation system in the presence of microsomes and subjected to carbonate extraction. Parallel translations were performed, expressing the normal mouse AChR α subunit and the truncated α subunits. The translations were pooled and the pooled translation mixture was separated into two aliquots. One aliquot was added to 1.0 ml of ice-cold 250 mM sucrose/100 mM Tris-HCl, pH 7.4, and the second aliquot was added to 1.0 ml of ice-cold 100 mM Na_2CO_3, pH 11.5.

Both mixtures were incubated for 30 minutes on ice, and then were centrifuged at 40,000 rpm in a Beckman Ti 70.1 rotor for 3 hours at 4°C. At the end of the centrifugation, the supernatant was carefully removed from the pellet. This pellet was solubilized in 1% SDS/100 mM Tris-HCl, pH 8.0. The pH of the Na_2CO_3 supernatant was adjusted between 7 and 8 with glacial acetic acid. Both supernatants were then precipitated by the addition of ice-cold TCA to 15% and incubation for 15 minutes on ice, followed by a 15 minute spin in a microfuge. The TCA pellets were washed once with 1 ml of a 1:1 mixture of ethanol and ether, respun in the microfuge, and solubilized in 1% SDS/100 mM Tris-HCl, pH 8.0. SDS-PAGE loading buffer containing β-mercaptoethanol was added, and the samples were electrophoresed as indicated in the figure legends.

Results

Novel glycosylation sites can be processed in in vitro *translations*

Initial experiments were carried out to characterize the translation products of the normal mammalian muscle α subunit. mRNA was synthesized *in vitro* from α subunit cDNA using SP6 RNA polymerase and was then used to prime a rabbit reticulocyte *in vitro* translation system.

Translation of α subunit mRNA in the absence of added microsomes produced a single 43 kDa polypeptide that was immunoprecipitated by a monoclonal antibody (mAb61) specific for the α subunit (Fig. 3-3, lane a), and is thus the mouse α subunit precursor. When translation was carried out in the presence of microsomes and an acetylated tripeptide (AcNYT) that prevents N-glycosylation (Fig. 3-3, lane c), a higher mobility band appeared (M_r ca. 41,000) in addition to the α subunit precursor. This band represents the product of cleavage of the 20 amino acid signal sequence from the α subunit precursor. When α subunit mRNA was translated in the presence of canine pancreatic microsomes alone (Fig. 3-3, lane b), a fraction of the signal-cleaved species was converted to a new species that had a slightly lower mobility than the α subunit precursor (M_r ca. 44,000). Since it was not made when N-linked glycosylation was prevented, this new species must represent the fully processed, N-glycosylated form of the α subunit. The presence of these two modifications, cleavage of the signal sequence and N-linked glycosylation, establishes that the extreme amino terminus and amino acid residues α141-143 of the α subunit are transported across the membrane of the microsomal vesicle. These results are in agreement with the previous findings of Anderson and Blobel (1981)

for the *Torpedo* AChR α subunit, and of Merlie *et al.* (1981) for the mouse AChR α subunit.

Messenger RNA was then prepared from a construct (αX) in which the asparagine at α141 was replaced by lysine, thus eliminating the predicted site of N-glycosylation of the native α subunit (Boulter *et al.*, 1985; Isenberg *et al.*, 1986). When αX transcript was translated without membranes, a product identical to that seen with normal α subunit mRNA was seen (Fig. 3-3, lane d); in the presence of membranes, however, a new band of higher mobility was seen (Fig. 3-3, lane e). Addition of AcNYT to the translation mixture, along with microsomes, caused no change in this pattern (Fig. 3-3, lane f), indicating that none of the bands in lane e represents a glycosylated product. These results provide direct experimental evidence that α141 is the site of N-linked glycosylation of the mouse muscle AChR α subunit.

Translation experiments were then carried out with mRNA prepared from constructs containing new glycosylation sites either at position α154 or at position α200, in each case with or without the original site. In all cases, translation without microsomes produced a single polypeptide chain that was immunoprecipitated by mAb61 and was identical to that seen with normal α subunit mRNA (Fig. 3-3, lanes g, j, m, q). When microsomes were added, both old and new sites were glycosylated. For example, translation of α g154 mRNA yielded two glycosylated products (Fig. 3-3, lane h). One of these, presumably the monoglycosylated polypeptide, migrated at the same position as the α subunit precursor (compare the precursor bands in lanes h and i, Fig. 3-3); the second (M_r ca. 46,000) had a lower mobility than the glycosylation product of the original α subunit.

Neither product was seen after translation in the presence of the glycosylation inhibitor, AcNYT (Fig. 3-3, lane i). Since neither band represents the normal glycosylated species, the band of lowest mobility must be the diglycosylated product.

To visualize glycosylation at the new site alone, we tested a construct containing the new site at α154, but with the old site eliminated (αX g154). In this case, after translation in the presence of microsomes, a single glycosylated species appeared, which comigrated with the primary translation product as seen with α g154 (Fig. 3-3, lane k). This glycosylation was prevented by the addition of AcNYT to the translation mixture (compare lowest mobility bands, lanes k and l). Thus, residue α154 is glycosylated in both constructs.

Similar results were obtained with the constructs containing a new site at α200, which should be extracellular according to both models in Fig. 3-1. When translated with microsomes, α g200 was clearly glycosylated twice (Fig. 3-3, lane n). Four bands were detected following immunoprecipitation of the translation mixture containing microsomes. Two were identified as the precursor and signal-cleaved bands. The third had a mobility slightly less than that of the normally glycosylated α subunit. The fourth was a new band that appeared at M_r ca. 47,000. As both of the latter bands were eliminated by co-translational addition of AcNYT (Fig. 3-3, lane p), they represent glycosylated products. In αX g200, which lacks the original glycosylation site but retains the novel site at α200, a single glycosylated species was detected (Fig. 3-3, compare lanes r and s). Thus, mutated sites at both α154 and α200 can be glycosylated, indicating that these residues are translocated across the microsomal membrane.

Transfected COS cells can efficiently process novel glycosylation sites

In order to determine whether the novel glycosylation sites could also be recognized *in vivo*, the native and mutant α subunit cDNA constructs were transiently transfected into COS cells. COS cells, which are modified simian kidney cells, support high efficiency replication and transcription of SV40-based plasmids, as well as expression of foreign proteins under the control of the SV40 promoter (Ghosh *et al.*, 1981; Gluzman, 1981). When transfected with the four mouse AChR subunit cDNAs, each under the control of the SV40 promoter, COS cells express functional AChR on the surface, as measured by ^{125}I-α-bungarotoxin binding and by standard pharmacological and electrophysiological assays (Gu *et al.*, 1990).

cDNAs encoding the normal α subunit and the five mutants were transfected individually into COS cells. In each case, the cells were radioactively labeled, solubilized as described, and immunoprecipitated with mAb61 (Fig. 3-4). Immunoprecipitation from COS cells transfected with normal α subunit cDNA (Fig. 3-4, lane A) revealed three bands: a major band of the same mobility as the processed and glycosylated α subunit translated *in vitro* (data not shown), a minor band of the same mobility as the signal-cleaved α subunit produced by *in vitro* translation, and a third, non-specific band which appeared in all lanes (see also Fig. 3-5, lanes N). For the construct αX (Fig. 3-4, lane B), the predominant species had the same mobility as the signal-cleaved unglycosylated α subunit species. For each of the constructs with two potential N-glycosylation consensus sequences (α g154 and α g200), a diglycosylated species was clearly detected (Fig. 3-4, lanes C and E, respectively). In the case of α g200, a monoglycosylated species was also found. For the

constructs αX g154 and αX g200, possessing only a single N-glycosylation consensus sequence, only a monoglycosylated form was seen (Fig. 3-4, lanes D and F, respectively). αX g200 was incompletely converted to a monoglycosylated species, since a fraction of the signal-cleaved species was not glycosylated (Fig. 3-4, lane F). These results indicate that the glycosylation consensus sequences at α154 and at α200 are both functional substrates for N-glycosylation in COS cells. Therefore, *in vivo* as well as *in vitro*, the novel sites at α154 and α200 are translocated across the membrane of the ER and into the lumen.

To examine the further maturation of the translated mutant α subunit polypeptides in COS cells, we examined their ability to be recognized by a conformation-specific antibody. Merlie and Lindstrom (1983) have demonstrated that in the mouse muscle cell line BC3H-1, the α subunit undergoes a conformational transition prior to assembly with other subunits. MAb61 recognizes the α subunit both before and after the transition, but mAb35, which recognizes an epitope between α46 and α127 (Tzartos *et al.*, 1981), is unable to recognize the α subunit prior to the transition. COS cells were transfected with the cDNA encoding either the native α subunit, or the constructs αX or αX g154. The transfected cells were labeled, solubilized, and an aliquot from each transfection immunoprecipitated with either mAb61, mAb35, or rat IgG. In all three cases, mAb35 immunoprecipitated approximately the same fraction of the α subunit recognized by mAb61, suggesting that all three polypeptides must have undergone the pre-assembly conformational transition to approximately the same extent (Fig. 3-5). These results indicate that the

structure of each of the mutant α subunits is not grossly different from that of the normal α subunit.

The amino-terminal domain of the α subunit is not an integral membrane protein

A prediction of the model of Criado *et al.* (1985a) is that the amino-terminal domain should be an integral membrane protein. One of the standard *in vitro* assays for the integration of proteins into the microsomal membrane is carbonate extraction (Fujiki *et al.*, 1982). Treatment of microsomal vesicles with sodium carbonate at pH 11.5 opens the vesicles into phospholipid sheets and strips the membrane of peripheral proteins. After ultracentrifugation, integral membrane proteins are found primarily in the pelleted membrane fraction, while peripheral and translocated proteins are found in the supernatant. This experimental protocol was used to compare the distribution relative to the complete α subunit of two fragments: α52, which contains the amino terminus, the first transmembrane domain (M1), and the loop between M1 and M2 up to αG240; and α53, which contains the amino terminus up to residue αM207. Separate translations of *in vitro*-derived transcripts of the α subunit and α52 or α53 were combined and solubilized in either a buffered sucrose solution (microsomes pellet intact) or an alkaline carbonate solution (microsomes pellet as extracted sheets).

After the combined translation mix (α subunit plus α52) had been solubilized in the sucrose solution and was centrifuged, the precursors of each of the translated polypeptides were distributed between the supernatant and the pellet, while the processed forms appeared exclusively

in the pellet (Fig. 3-6A, lanes a and b). For α52, these pelleted forms included the precursor, the signal-cleaved form, and a glycosylated form (see Fig. 3-7B, lane b). Following carbonate treatment, the precursors of both the normal α subunit and α52 were more completely released into the supernatant, while the processed and glycosylated forms of both polypeptides remained in the pellet (Fig. 3-6A, lanes c and d). Therefore, both the α subunit and α52 behave like integral membrane proteins. For α53, the sucrose solubilization separated the precursor and processed forms of α subunit and α53 in a similar fashion (i.e., the precursor was found in both pellet and supernatant, and the processed form was found in the pellet (Fig. 3-6B, lanes a and b)). However, when solubilized in the carbonate solution, approximately 50% of the signal-cleaved α53 band was released into the supernatant, while neither the signal-cleaved nor the glycosylated α subunit species were released from the membrane pellet (Fig. 3-6B, lanes c and d). Little or no glycosylation of α53 was detected (see Fig. 3-7B, lanes h and i). Since the signal-cleaved form of α53 was extractable by carbonate treatment, it must not be an integral membrane protein.

Glycosylation of the amino-terminal domain is increased by its attachment to the membrane

The apparent lack of glycosylation of the α53 construct was a surprising result, since it does contain the normal glycosylation consensus sequence at αN141. When transiently expressed in COS cells, α53 also failed to be glycosylated (data not shown). In some *in vitro* translation experiments, a faint band of M_r ca. 30,000, which was not present in translations with 0.5 mM AcNYT, was detected. However, this

glycosylated species represented a far smaller proportion of the total translation product than the glycosylated species for either the normal α subunit or the α52 construct. Because both the α subunit and α52 are integral membrane proteins and α53 is not, it appeared possible that the inefficient glycosylation of α53 was due to its lack of membrane attachment. To test this hypothesis, cDNA for the α subunit was linearized with the restriction enzyme *Hinc* II, which cleaves the α subunit coding region before the termination codon at a site after residue αV216 of the mature amino acid sequence, six amino acids carboxyl to the start of M1. This truncated cDNA was then transcribed *in vitro* and expressed in the reticulocyte lysate system. Previous experiments (Perara *et al.*, 1986) have demonstrated that ribosomes do not disengage from such a transcript, and therefore the translocated chain is anchored in the membrane, not by a typical transmembrane domain or stop-transfer sequence, but by the attached ribosome on the extravesicular surface of the microsome. The nascent chain must pass through the large ribosomal subunit, across the microsomal membrane, and into the microsomal lumen.

When the transcripts for α52 and α/*Hinc* II were expressed *in vitro* in the presence of microsomes, the nascent chains were both cleaved and glycosylated (Fig. 3-7B, lanes b and e). Abundant glycosylated species were formed, and co-translational treatment with AcNYT prevented the glycosylation of these species (Fig. 3-7B, lanes c and f). Expression *in vitro* of α53 transcripts, however, produced no glycosylated species (Fig. 3-7B, lanes h and i). Since only six residues from M1 are present in the α/*Hinc* II polypeptide, and since the pore in the large ribosomal subunit is longer than the span of these six amino acids, residues which lie amino-terminal

to M1 comprise the apparent transmembrane domain of α/*Hinc* II. Anchoring to the membrane may therefore promote high-efficiency glycosylation at the normal amino-terminal consensus sequence of the α subunit. In this context, the lack of glycosylation of α53 further indicates that this amino-terminal fragment of the α subunit is not anchored to the membrane.

Discussion

We have analyzed the transmembrane topology of the amino-terminal domain of the mouse muscle nicotinic AChR, and conclude on the basis of several criteria that this domain is entirely translocated across the ER membrane. Our first approach was based on a specific post-translational modification, N-linked glycosylation, that occurs only in the lumen of the ER. For glycosylation to take place, a tripeptide consensus sequence for glycosylation, N-X-(S/T), must be translocated across the ER membrane into the lumen, where the enzyme oligosaccharyl transferase is localized (Kornfeld and Kornfeld, 1985). Our method was to introduce novel glycosylation consensus sequences into the primary sequence, and to determine the extent of glycosylation both in an *in vitro* translation system and in an *in vivo* expression system.

We used this method to test a topological model of the α subunit amino terminus proposed by Criado *et al.* (1985a). These investigators raised mAbs against synthetic peptides based on the *Torpedo* α subunit sequence α152-167, a subset of which were primarily immunoreactive against the sequence α152-159. The mAbs did not bind the *Torpedo* AChR in right-side out receptor-rich vesicles, nor did they bind to saponin-permeabilized vesicles. However, mAb binding was observed when the membranes were treated with either pH 11 or 10 mM lithium diiodosalycilate. Based on these findings, Criado *et al.* (1985) proposed two novel transmembrane domains in the amino terminus of the AChR α subunit. The first of these (M6) spans the membrane between residues α142 and α152. Because this segment is too short to form an α-helix that would extend across the

membrane, an alternative structure must be formed. M7, the second postulated membrane-spanning region in their model, forms an amphipathic helix between residues α159 and α192. Recent experiments by Pedersen *et al.* (1990) support and extend the earlier findings. In this work, mAbs raised against the *Torpedo* α subunit were screened against fragments generated by protease digestion, and were mapped to the region between α156-179 using synthetic peptides. Although the staining was sparse and visible only at high mAb concentrations, these mAbs bound the cytoplasmic surface of receptor-rich vesicles after osmotic shock and sonication, as demonstrated by colloidal gold staining and immunoelectron microscopy. These findings imply that there are two transmembrane domains in the region between α141 and α192.

To test the proposed model, we altered the primary sequence of the mouse muscle α subunit to create a glycosylation consensus sequence in the putative cytoplasmic loop (α154), as well as in a region predicted to be extracellular (α200). In the *in vitro* translation system, glycosylation was demonstrated by the appearance of a new species following translation in the presence of microsomes, and the disappearance of this species following translation with microsomes and AcNYT. For both novel sites tested, each of these criteria was met. A glycosylated species was retained even when the original site at α141-143 was mutated. Therefore, each site must have been translocated into the lumen of the microsomal vesicle.

When the constructs were transiently transfected into COS cells, all extant glycosylation sites served as substrates for glycosylation. Compared to the *in vitro* translation system, the COS cells were more efficient in processing the α subunit constructs into mature, glycosylated forms. Since

little or no precursor forms were detected following immunoprecipitation of COS cells transfected with the α subunit mutants, each glycosylated species could be clearly distinguished. Analysis of the α subunit mutants in both systems thus leads to the same conclusion: the glycosylation consensus sequences at α154 and α200 can be recognized by the lumenal glycosylation machinery, and must therefore have been translocated across the ER membrane into the lumen. Glycosylation of a new glycosylation consensus sequence at α148 has also recently been reported (Blount and Merlie, 1990), showing that this site is also translocated across the membrane. Since the enzymatic machinery for glycosylation is in the lumen of the ER, glycosylation at these sites can only occur if the sites are transported across the membrane.

Is it possible that the changes in primary sequence used to create the novel glycosylation sites caused an abnormal translocation of segments M6 and M7? We believe this to be unlikely. For each mutagenesis reaction, only one (N141K, g200) or two (g154) amino acid residues were changed, with at most one non-conservative substitution (Fig. 2). In other systems the insertion or deletion of large hydrophobic domains (10-20 amino acids) is usually required to alter transmembrane topology (Yost *et al.*, 1983; Davis and Model, 1985). In addition, a fraction of the αX g154 protein comparable to that seen with the normal α subunit was converted to a form that is recognized by mAb35. If the change in primary sequence of this mutant had grossly altered the transmembrane topology of the amino-terminal domain, the correct conformational transition would be unlikely to occur, and mAb35 would not recognize the mutant α subunits.

Although transfection of COS cells with the cDNAs for all four subunits of the mammalian AChR results in surface expression of functional AChR (Gu *et al.*, 1990), substitution of αX g154 for the normal α subunit cDNA in preliminary experiments did not yield detectable binding of ^{125}I-α-bungarotoxin to the surface of transfected cells (Chavez and Hall, unpublished experiments). Further investigation will be required to determine whether the failure to detect toxin-binding on the surface results from inability of the modified α subunit to bind toxin (Blount and Merlie, 1990), to assemble with the other subunits (Mishina *et al.*, 1985), or to reach the cell surface (Sumikawa and Miledi, 1989). In the case of αx g154, the additional glycosylation at α154 may also block access of the toxin to its binding site. The amino-terminal domain of the *Natrix tessellata* skeletal muscle AChR α subunit cloned from a snake cDNA library, for example, possesses two potential N-glycosylation sites (α111 and α189) not found in non-snake muscle AChRs, and apparently does not bind α-bungarotoxin (Neumann *et al.*, 1989).

The analysis of the amino-terminal constructs α52 and α53 provides additional support for the topological assignment based on the glycosylation mutants. Here, normal α subunit fragments were used to study the transmembrane integration of the amino terminus; no primary sequence changes, apart from the introduction of a termination codon, occured. The construct α53, which lacks a classical, hydrophobic transmembrane domain (or stop-transfer sequence), was extracted by carbonate treament, unlike either the normal α subunit or the construct α52, both of which possess well-defined hydrophobic transmembrane domains. In addition, the efficient glycosylation of the membrane-bound forms of the amino

terminus (α52 and α/*Hinc* II) contrasts sharply with the lack of glycosylation of α53, and suggests that membrane anchoring is at least one of the prerequisites for efficient glycosylation at αN141. At present, we are unable to determine the mechanism whereby anchoring to the membrane affects glycosylation at αN141. Membrane anchoring could either restrict the amino-terminal conformation to favor glycosylation, or merely allow a lengthening of the time window within which oligosaccharyl transferase can act on the nascent chain.

Our results, taken with those of others, suggest that the entire amino-terminal domain, at least up to α207, is translocated. First, cleavage of the signal sequence indicates that the amino terminus itself is translocated. Second, we have found that a glycosylated fragment of the mouse muscle α subunit that is recognized by mAb210 is translocated into the microsomal lumen *in vitro* (Chavez and Hall, unpublished experiments). This epitope, which lies between α66 and α76, has been shown to be extracellular in *Torpedo* membranes (Ratnam *et al.*, 1986b; Das and Lindstrom, 1989). Third, αN141 is normally glycosylated, and when the appropriate sites are present, residues α154 and α200 can be glycosylated. Fourth, treatment of intact *Torpedo* vesicles with an impermeable affinity reagent, MBTA, alkylates residues α192 and α193 (Kao and Karlin, 1986). Finally, a mouse muscle α subunit construct terminating before M1 (α53) is translocated into the lumen of the ER, but is not an integral membrane protein.

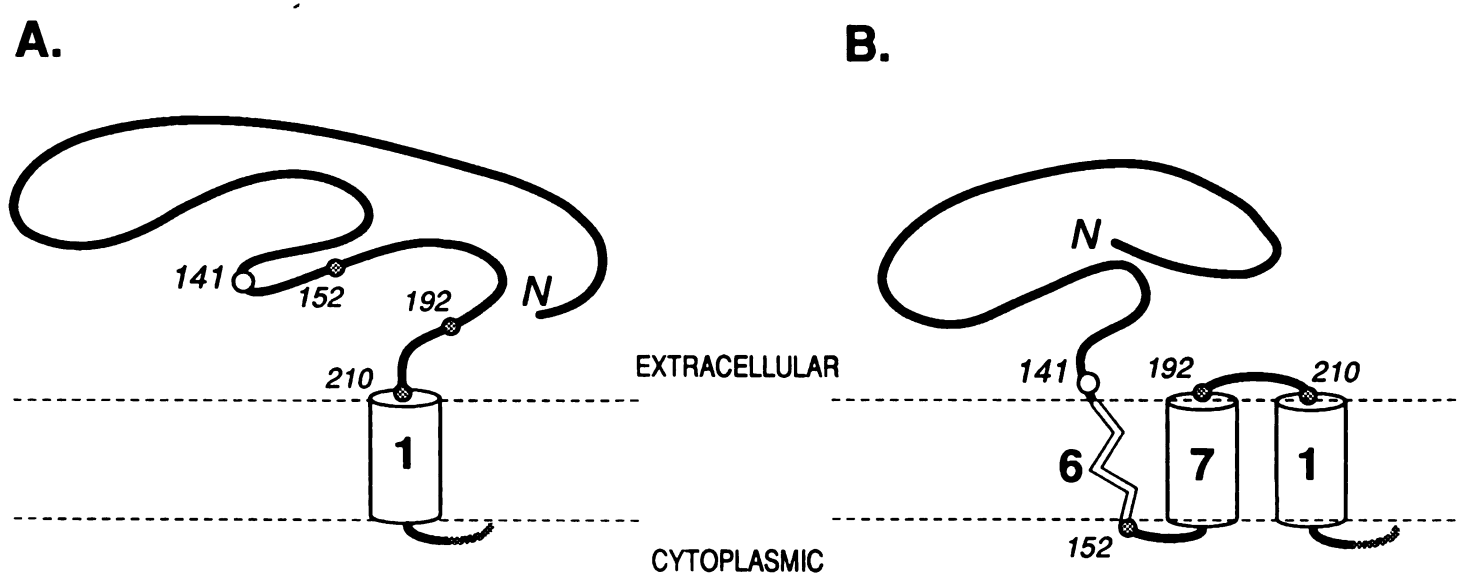
If residues α1-207 are translocated into the ER lumen, the most straightforward interpretation of these combined results is that residues α154 and α200 in the assembled AChR are in an extracellular location on the surface membrane. Our results thus appear to be incompatible with

those of Criado *et al.* (1985a), and of Pedersen *et al.* (1990), and to constitute strong evidence against their model. Our experiments demonstrate that the introduction of new glycosylation sites can provide valuable information about the topographical location of particular sites in the primary sequence of transmembrane proteins. This method should be generally applicable to the study of receptors and other membrane proteins.

Figure 3-1. Topological models of the α subunit of the *Torpedo* AChR

A. Model of the transmembrane topology of the amino-terminal domain and M1 based on primary sequence hydrophobicity (Claudio *et al.*, 1983; Numa *et al.*, 1983; Devillers-Thiery *et al.*, 1983). The balance of the subunit has been omitted for clarity.

B. Model of the transmembrane topology of the amino-terminal domain and M1 based on Criado *et al.* (1985a).


A.
B.
141
152
192
N
210
1
EXTRACELLULAR
CYTOPLASMIC
N
141
192
210
6
7
1
152

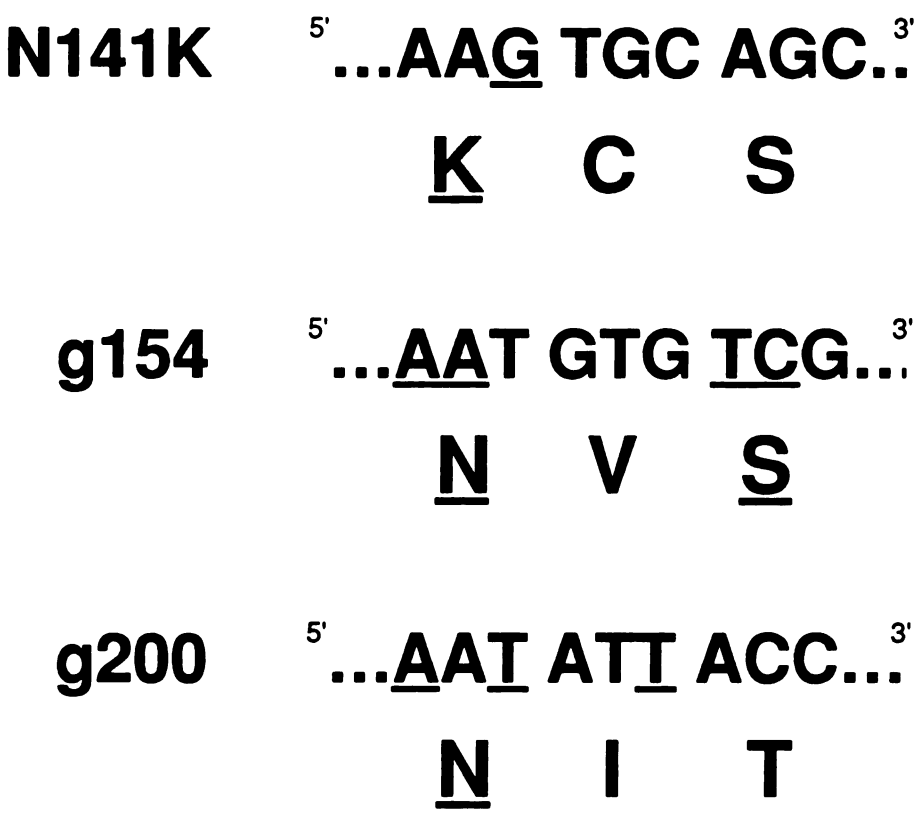


Figure 3-2. Oligonucleotides used in the construction of the α subunit glycosylation mutants

Underlined bases in the nucleotide sequence indicate differences from the wild-type sequence. The new amino acids (underlined) are given below the nucleotide sequence. Flanking sequences in the oligonucleotide (indicated by ellipses) are identical to the native nucleotide sequence and are omitted for clarity. The oligonucleotides were between 21 and 28 bases in length.

Figure 3-3. Glycosylation of *in vitro*-translated native and mutant α subunits.

The indicated transcript (**Tr**) was incubated in a reticulocyte lysate *in vitro* translation system alone, or in lysate containing either microsomes (**RM**), or microsomes and glycosylation inhibitor (**AcNYT**). After translation, an aliquot was solubilized and immunoprecipitated with mAb61, and then electrophoresed through a 10% SDS-PAGE gel.

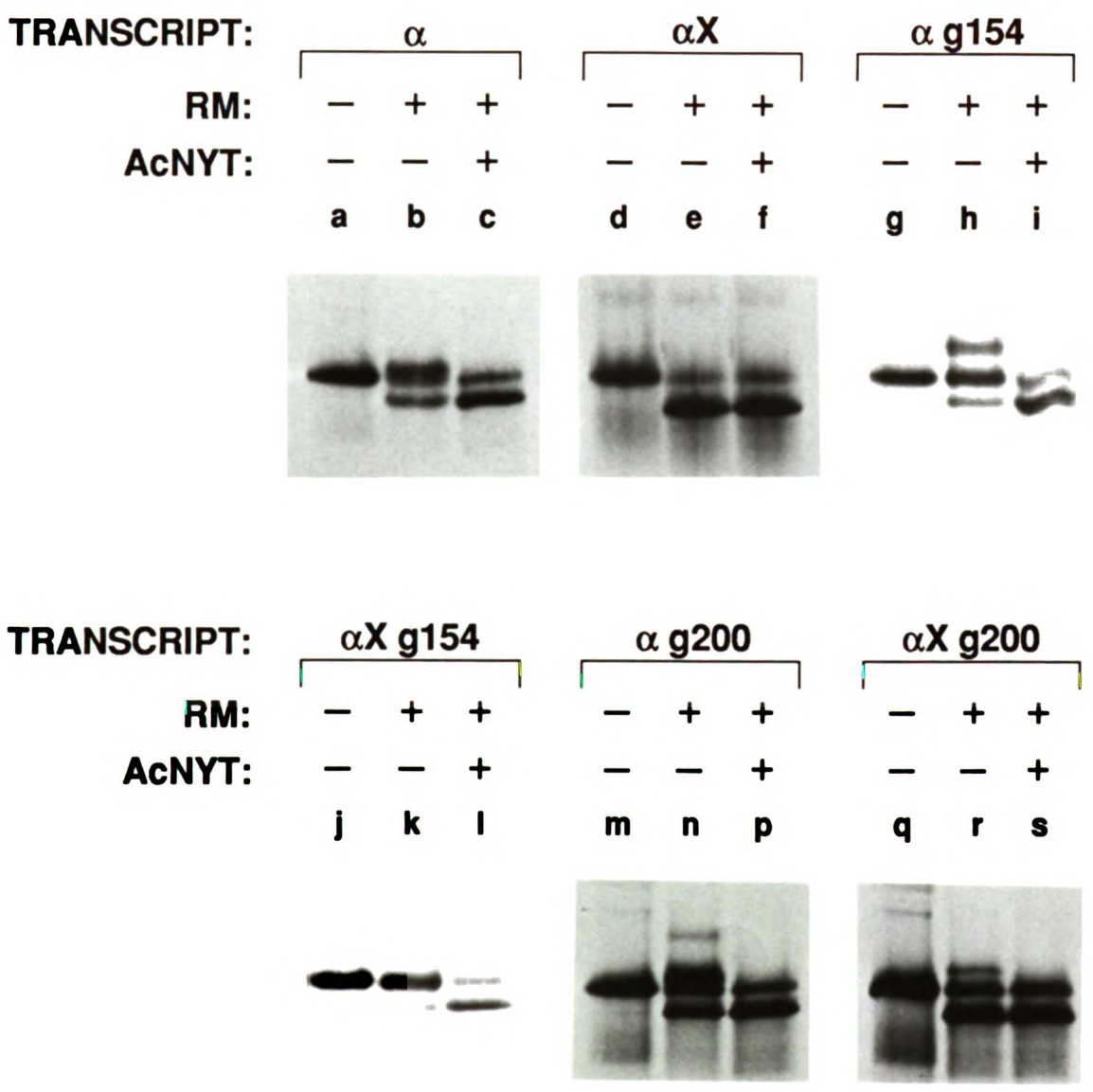

TRANSCRIPT:
α
αX
α g154
RM:
AcNYT:
a b c d e f g h i
TRANSCRIPT:
αX g154
α g200
αX g200
RM:
AcNYT:
j k l m n p q r s

Figure 3-4. Expression of native and mutant α subunits from COS cells.

COS cells were transiently transfected as described with the appropriate pSM-α subunit plasmid. Twenty-four hours after trypsinization and replating, the cells were solubilized, immunoprecipitated with mAb61, and electrophoresed through a 10% SDS-PAGE gel. The positions of fully processed, glycosylated α subunit (α) and processed, unglycosylated α subunit (α_{ng}) immunoprecipitated from an *in vitro* translation are indicated. **A**, pSM-α; **B**, pSM-αX; **C**, pSM-α g154; **D**, pSM-αX g154; **E**, pSM-α g200; **F**, pSM-αX g200.

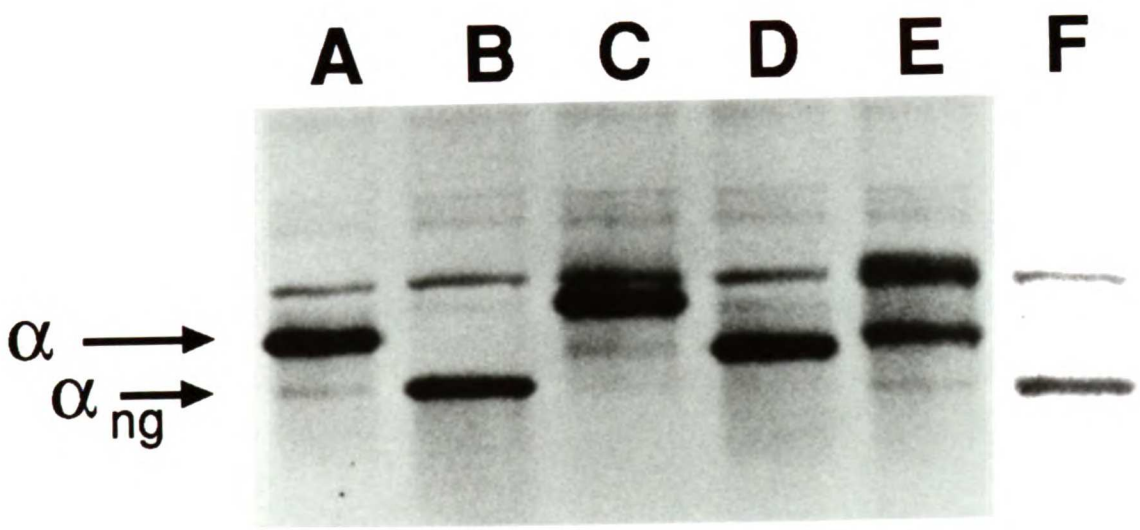

A
B
C
D
E
F
α
α ng

Figure 3-5. Immunoprecipitation of α subunit mutants with mAb35 and mAb61.

COS cells were transiently transfected with the indicated construct as described (see Fig. 3-4) and radiolabeled. The cells were solubilized, and aliquots of the solubilized cells were immunoprecipitated with either mAb**35**, mAb**61**, or rat IgG (**N**), followed by rabbit anti-rat-Sepharose. Immunoprecipitates were electrophoresed as described.

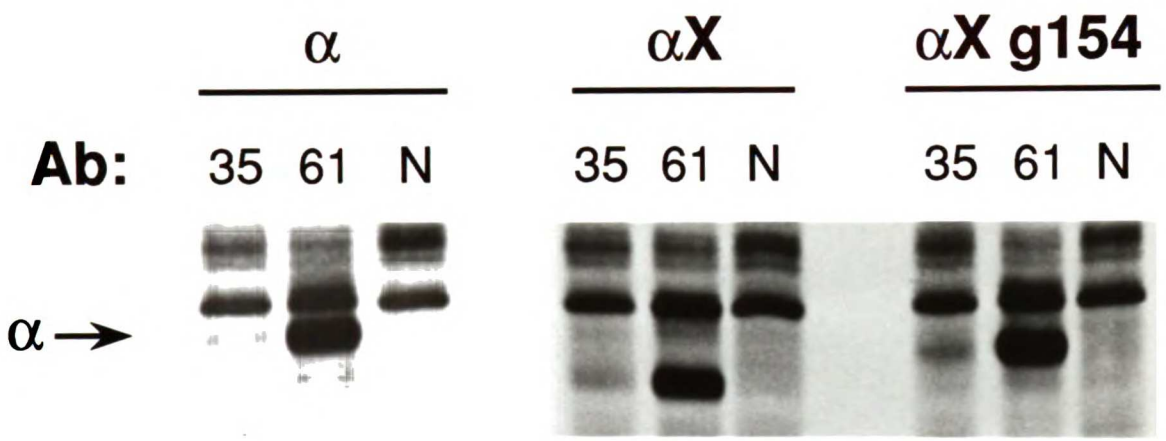

α
αX
αX g154
Ab:
35 61 N
35 61 N
35 61 N
α

Figure 3-6. Carbonate extraction.

Aliquots from separate translations of normal α subunit and α52 (**A**) or normal α subunit and α53 (**B**), were mixed and solubilized in either 0.25 M sucrose/0.1 M Tris-HCl (pH 7.5) (**SUCR**) or 0.1 M sodium carbonate (pH 11.5) (**CARB**). After incubation and centrifugation as described (see Methods), supernatants (**S**) and pellets (**P**) were separated and TCA-precipitated, and electrophoresed as described.

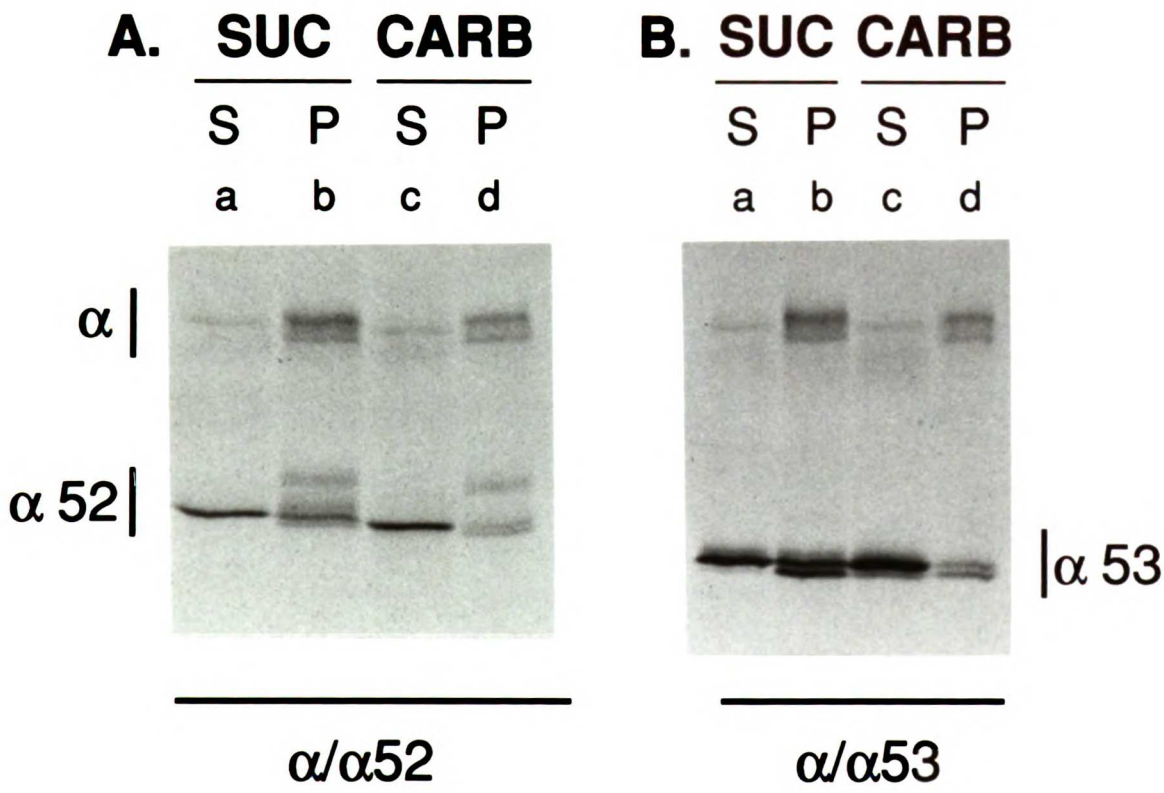
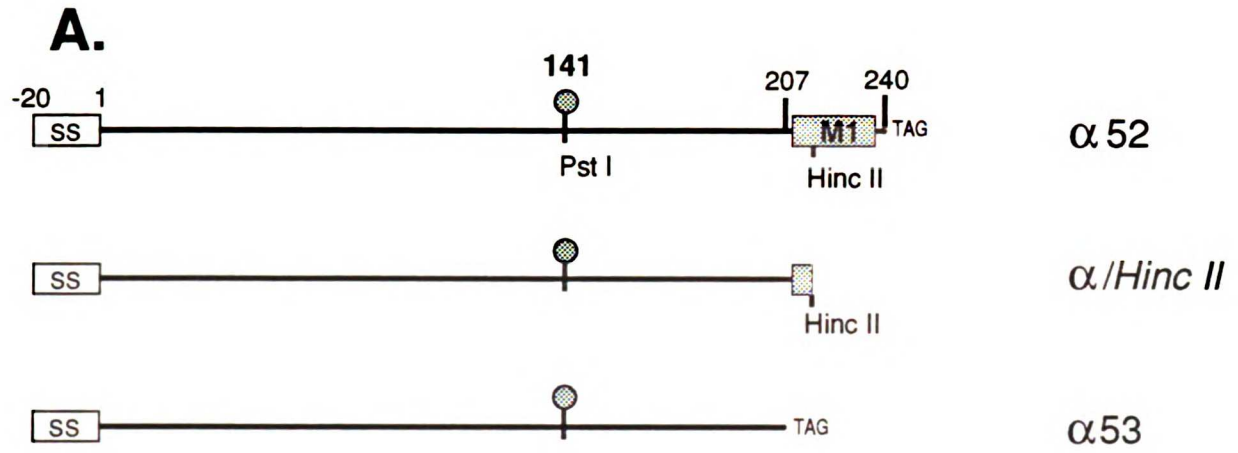
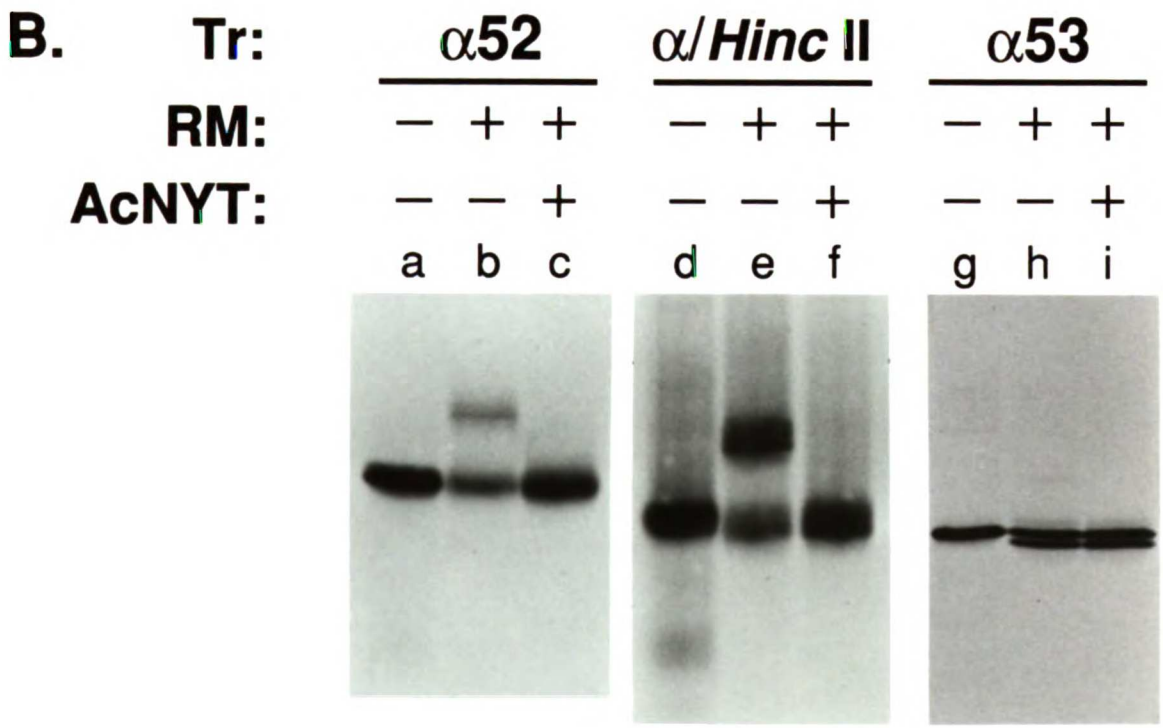

A.
SUC
CARB
S
P
S
P
a
b
c
d
α
α 52
α/α52
B.
SUC
CARB
S
P
S
P
a
b
c
d
α 53
α/α53

Figure 3-7. Glycosylation of *in vitro*-translated amino-terminal fragments of the α subunit.

A. Diagram of the amino-terminal constructs α52, α/*Hinc* II and α53. The signal sequence (open box), transmembrane domain M1 (stippled box), and the glycosylation consensus sequence (αN141) are indicated. The relative positions of the *Pst* I and *Hinc* II restriction sites along the primary sequence are indicated.

B. The indicated transcript was incubated in a reticulocyte lysate *in vitro* translation system as described in the legend to Fig. 3-3. An aliquot of the total translation products was solubilized in SDS-PAGE loading buffer and electrophoresed as described.


A.
141
-20
1
207
240
SS
M1
TAG
Pst I
Hinc II
α 52
SS
Hinc II
α/Hinc II
SS
TAG
α53


B.
Tr:
α52
α/Hinc II
α53
RM:
– + + – + + – + +
AcNYT:
– – + – – + – – +
a b c d e f g h i

CHAPTER 4. EXPRESSION OF FUSION PROTEINS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR FROM MAMMALIAN MUSCLE IDENTIFIES THE MEMBRANE-SPANNING REGIONS IN THE α AND δ SUBUNITS

Introduction

The nicotinic acetylcholine receptor (AChR), which is found at the neuromuscular junction in vertebrate skeletal muscle and at the endplates of electric organs, is a pentameric complex composed of four glycoprotein subunits in an $\alpha_2\beta\gamma\delta$ stoichiometry (Claudio, 1989). The subunits are arranged pseudosymmetrically around a central ion channel (Toyoshima and Unwin, 1988; Mitra *et al.*, 1989). A comparison of the primary sequences of the subunits of the AChR reveals a high degree of homology both within and across species boundaries (Takai *et al.*, 1985). The AChR is the best-studied member of a super-family of ligand-gated ion channels which includes the $GABA_A$, glycine, 5HT-3, and neuronal nicotinic acetylcholine receptors (Grenningloh *et al.*, 1987; Patrick *et al.*, 1987; Schofield *et al.*, 1987). All of these channels are hetero-oligomers whose subunits are highly homologous, suggesting that they are derived from a common evolutionary precursor.

The subunits of the AChR and related channels also share a common domain structure. Hydropathic analysis of the primary sequence reveals five regions of high hydrophobicity comprised of an amino-terminal signal sequence (Anderson and Blobel, 1981; Anderson *et al.*, 1982; Anderson and Blobel, 1983b) and four regions of sufficient length to form α-helical transmembrane domains (M1 through M4) (Noda *et al.*, 1982; Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983). This domain pattern has led to a structural model of the AChR subunits in which the amino and carboxy termini are on the extracellular side of the membrane separated by four

transmembrane domains (M1-M4) and a long cytoplasmic loop between M3 and M4 (Fig. 1-5).

Although this model is commonly accepted, experiments on the topological arrangement of the AChR subunits have yielded conflicting results, leading to several alternative models of subunit structure. An amphipathic transmembrane domain between M3 and M4 (designated MA) was proposed on the basis of primary sequence analysis (Finer-Moore and Stroud, 1984; Guy, 1984), placing the carboxy terminus on the cytoplasmic side of the membrane. Epitope mapping experiments support models with a pair of transmembrane domains in the region before M1 (Criado *et al.*, 1985a; Ratnam *et al.*, 1986b; Pedersen *et al.*, 1990), and other immunochemical and biochemical experiments support models with zero (Ratnam *et al.*, 1986b), one (McCrea *et al.*, 1987; DiPaola *et al.*, 1989), or two (Finer-Moore and Stroud, 1984; Guy, 1984; Young *et al.*, 1985) transmembrane domains beyond M3. Thus, models with from three to seven transmembrane domains can be constructed.

As an approach to this problem, we have investigated the transmembrane orientation of the AChR subunits synthesized in an *in vitro* translation system from rabbit reticulocytes supplemented with dog pancreatic microsomes. In earlier experiments, we investigated the topology of the amino-terminal domain of the α subunit using glycosylation consensus sequences as reporter domains (Chavez and Hall, 1991). In the present study, we fused a reporter domain derived from bovine prolactin to the α and δ subunits of the AChR after each of the proposed transmembrane domains beyond M1 (M1-M4 and MA) and expressed these constructs in the *in vitro* translation system. The accessibility of this

reporter domain to proteolytic enzymes was used to determine its orientation relative to the microsomal membrane. The results of these experiments, taken with our earlier findings, demonstrate that the amino and carboxy termini of the subunit polypeptides are sequestered within the vesicle and are separated by four transmembrane domains.

Materials and Methods

Subunit clones and molecular biology reagents

The clones for the α and δ subunit cDNAs of the mouse muscle AChR (LaPolla *et al.*, 1984; Isenberg *et al.*, 1986) were obtained from Drs. J. P. Merlie (Washington University, St. Louis, MO) and N. Davidson (California Institute of Technology). The plasmids pSP BPI, which contains the cDNA for bovine prolactin, and pSP P^{tr}, a truncated form of pSP BPI which contains the cDNA for the carboxy-terminal 142 amino acid residues of bovine prolactin, were generous gifts of Dr. V. R. Lingappa (UC San Francisco) and have been characterized elsewhere (Lingappa *et al.*, 1977; Rothman *et al.*, 1988). Several vectors (pGEM 1, pGEM 2, pSP72, pSP73) were obtained commercially (Promega Biotec). The vector pSM was provided generously by M. Brodsky and Dr. D. Littman (UC San Francisco) (Brodsky *et al.*, 1990). All enzymes were obtained commercially from either Promega Biotec, New England Biolabs, Boehringer Mannheim Biochemicals, or Bethesda Research Laboratories. Sequencing of constructs was performed with the Sequenase kit (US Biochemicals, Cleveland, OH). Oligonucleotides used in the creation of new restriction sites in the α and δ subunit sequences were obtained from either the Biomolecular Resource Center at UC San Francisco or were synthesized by Dr. P. Garcia (UC San Francisco). Table 4-1 details the amino acid sequences at the subunit-prolactin fusion points.

pGEM P1: The plasmid pGEM P1, which has several restriction sites upstream of the coding region for the carboxy-terminal 142 amino acid residues of bovine prolactin, was constructed to facilitate the cloning of the

prolactin fragment into the subunit coding regions. The vector pGEM1 was digested with *Sma* I and calf intestinal phosphatase (CIP), and was ligated to the *Hinc* II/ *Pvu* II fragment from pSP BPI overnight at 16°C with T4 DNA ligase.

α subunit-prolactin fusion proteins

αPM1: The construct pSM α_m was altered by oligonucleotide-directed mutagenesis (Kunkel, 1985; Geisselsoder *et al.*, 1987; Chavez and Hall, 1991) to create a *Sma* I site in the region between the first and second transmembrane domains. The resulting plasmid, pSM αA869, was digested with *Sma* I and CIP. pGEM P1 was digested with *Hinc* II and *Eco* RI, and the fragment was blunted with the Klenow fragment of DNA polymerase. This fragment was ligated with the *Sma* I-digested pSM αA869 to yield pSM αPM1. To create pSP73 αPM1, pSP73 was first digested with *Sph* I and *Eco* RI, and treated with CIP. pSM αPM1 was digested with *Sph* I and *Eco* RI, and the fragment was ligated into the digested pSP73.

αPM2: The construct pSM α_m was altered to create an *Hpa* I site in the region between the second and third transmembrane domains. This construct, pSM αA967, was digested with *Eco* RI and *Xmn* I to excise the modified α subunit coding region, and this fragment was ligated into pGEM2 cut with *Eco* RI and *Sma* I and treated with CIP. The construct pGEM2 αPM2 was created by digestion of pGEM2 αA967 with *Hpa* I and CIP, into which the *Hinc* II/ *Eco* RI/ Klenow fragment from pGEM P1 was ligated (see above).

αPM3: A new *Hpa* I site was created in the region between the third transmembrane domain and the putative amphipathic helix in the

construct pSM α_m. This construct, pSM αA1158, was digested with *Eco* RI/ *Xmn* I, and ligated into pGEM2 digested with *Sma* I/ *Eco* RI/ CIP. pGEM2 αPM3 was prepared by digestion of pGEM2 αA1158 with *Hpa* I and CIP, into which the *Hinc* II/ *Eco* RI/ Klenow fragment from pGEM P1 was ligated.

αPMA: A new *Bam* HI site was created in the region between the putative amphipathic helix and fourth transmembrane domain of pSM α_m. This construct, pSM αA1346, was digested with *Eco* RI/ *Bam* HI and the fragment was inserted into the plasmid vector pSP73 prepared by digestion with *Eco* RI/ *Bam* HI/ CIP. To create pSP73 αPMA, pSP73 αA1346 was digested with *Bam* HI/ *Sph* I/ CIP, and a *Bam* HI/ *Sph* I fragment from pGEM P1 was ligated into the prepared vector.

αPM4: The construct pSM α_m was altered to create an *Hpa* I site in the carboxy-terminal tail of the α subunit beyond the fourth transmembrane domain. The modified α subunit coding region was excised from the resulting construct, pSM αA1453, by digestion with *Eco* RI and *Xmn* I, and was ligated into pGEM2 which had been digested with *Sma* I/ *Eco* RI/ CIP. pGEM2 αA1453 was digested with *Bam* HI/ *Hpa* I/ CIP. pSP BPI was digested with *Pvu* II and *Bam* HI, and the fragment was ligated into the digested pGEM2 αA1453 to create pGEM2 αPM4.

δ subunit-prolactin fusion proteins

δPM2: pSP73 δ_m was prepared as a vector by complete digestion with *Nco* I, followed by Klenow treatment and digestion with *Pst* I and CIP. A *Hinc* II/ *Pst* I fragment from pGEM P1 was ligated into this site to create pSP73 δPM2.

δPM3: pSP73 δ_m was digested with *Stu* I and *Pst* I and treated with CIP. A *Hinc* II/ *Pst* I fragment of pGEM P1 was ligated into the prepared pSP δ_m vector to create pSP δPM3.

δPMA: The construct pSM δ_m was modified to create an *Hpa* I site in the region between the putative amphipathic helix and the fourth transmembrane domain. The altered δ subunit coding region (δD1411), excised from this construct by digestion with *Eco* RI and *Xba* I, was ligated into pSP73 which had been digested with *Eco* RI/ *Xba* I/ CIP. pSP73 δD1411 was digested with *Hpa* I and CIP, and a *Hinc* II/ *Eco* RI/ Klenow fragment from pGEM P1 was inserted into the prepared vector. This construct was designated pSP73 δPMA.

δPM4: The construct pSP73 δ_m was prepared by digestion with *Pst* I, *Sph* I, and CIP. A *Pst* I/ *Sph* I fragment from pGEM P1 was ligated into the prepared vector. This construct was designated pSP73 δPM4.

In vitro *transcription and translation*

Capped transcripts were synthesized from unlinearized plasmid DNA as described previously (Melton *et al.*, 1984; Chavez and Hall, 1991) except that the digestion with RNAse-free DNAse at the end of the transcription was omitted. The synthesized transcript was extracted with phenol: chloroform, precipitated with ethanol, and resuspended in RNAse-free water. Micrococcal nuclease-treated rabbit reticulocyte lysate and canine pancreatic microsomes were prepared as described (Jackson and Hunt, 1983; Walter and Blobel, 1983). The translations were performed as described (Chavez and Hall, 1991). All translations included canine pancreatic microsomes, except those used in Fig. 4-2. At the end of the

incubation the tubes were placed on ice, and a solution of $CaCl_2$ was added to a final concentration of 10 mM.

Proteolysis and immunoprecipitation

Aliquots from the *in vitro* translation were prepared for digestion with proteinase K (PK; Sigma). To 9 µl of the translation mixture, either 1 µl water, or 1 µl of 100 µg/ml PK (predigested for 30 minutes at 37°C), or 1 µl of 100 µg/ml PK plus 1.2 µl 10% Triton X-100 was added, and the digests were incubated on ice for 1 hour. At the end of the digestion PMSF was added quickly to the reaction mixture to a concentration of 2 mM, and the entire mixture was plunged into 100 µl of 1% SDS in 100 mM Tris-HCl (8.0) in a boiling water bath. After 10 minutes of boiling, a 50 µl aliquot of the sample was diluted with a solubilization buffer containing 1.25% Triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM Tris-HCl (7.4), 0.02% NaN_3 to make the Triton:SDS ratio 5 to 1 (1% Triton/0.2% SDS).

Prolactin antiserum (US Biochemicals) was diluted 1:10 with 50 mM Tris-HCl (7.4), 0.02% NaN_3. 2 µl of this antiserum was added to 250 µl of the Triton-solubilized proteolysis mixture and incubated for 2 hours on ice. 15 µl of a 1:1 slurry of prewashed protein A-Sepharose (Pharmacia) was added, and incubated for 1 to 2 hours with agitation at 4°C. At the end of the incubation, the beads were pelleted and washed twice with 500 µl 1% Triton X-100, 100 mM NaCl, 10 mM EDTA, 10 mM EGTA, 100 mM Tris-HCl, pH 8.0. PAGE gel loading buffer with 5% β-mercaptoethanol was added, the sample was heated to 100°C for 3 minutes and was subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The gels were stained, fluorographed, and exposed to Hyperfilm MP (Amersham).

Three different antibodies were used to characterize the α subunit-prolactin constructs: mAb 210 (a rat mAb which has been mapped to α68-76 (Das and Lindstrom, 1989)), mAb 61 (a rat mAb which has been mapped to α371-386 (Ratnam *et al.*, 1986b)), and the prolactin antiserum described above. mAbs 210 and 61 were the generous gifts of Dr. J. Lindstrom (University of Pennsylvania). Two antibodies were used to characterize the δ subunit-prolactin constructs: mAb 88B (a mouse mAb which has been mapped to the cytoplasmic loop between M3 and M4 (Froehner *et al.*, 1983)) and the prolactin antiserum. mAb 88B was the generous gift of Dr. S. C. Froehner (Dartmouth University). Figure 4-1 shows the location of the mAb epitopes on the primary sequences of the α and δ subunits. Aliquots (5 μl) were taken from the *in vitro* translation reactions and diluted to 50 μl with 2 mM PMSF in PBS. This sample was added to 200 μl of solubilization buffer and immunoprecipitated as described above, except that either rabbit-anti-rat-Sepharose (for mAb 210 and mAb 61) or mAb 88B-Sepharose was used instead of protein A-Sepharose.

Results

We investigated the transmembrane topology of the α and δ subunits of the AChR in a rabbit reticulocyte lysate *in vitro* translation system supplemented with dog pancreas microsomes. Fusion proteins were constructed in which a fragment of prolactin used as an antigenic marker (reporter domain) was placed after each of the postulated membrane-spanning domains of the AChR subunits. After synthesis of these constructs *in vitro*, the position of the marker with respect to the microsomal membrane was determined by proteolysis of the intact microsomes. The rationale of these experiments is that if the prolactin marker is translocated into the lumen of the microsome (topologically equivalent to the extracellular space) it will be protected from proteolysis, whereas if the marker remains outside the vesicle (i.e., the cytoplasmic surface) it will be susceptible. Once the location of the prolactin marker domain is determined for each construct in the series, we could then determine whether each of the postulated domains spans the membrane or not.

Construction of the fusion proteins

A schematic diagram of the fusion proteins that we constructed is given in Fig. 4-1. The prolactin fragment (M_r=18 kD) was introduced after each of the segments M1-M4 and MA of the α subunit, and after M2-M4 and MA of the δ subunit. The specificities of the antibodies that we used and the structures of the polypeptides encoded by our constructs were verified by immunoprecipitation after *in vitro* translation. The prolactin fragment, expressed alone, was recognized by the antiserum to prolactin, but not by

antibodies to the α or δ subunits (Fig. 4-2A); conversely, the prolactin antiserum recognized neither the intact α subunit (Fig. 4-2B, lane c) nor δ subunit (Fig. 4-3A, lane d).

The fusion proteins were then tested. All of the α subunit-prolactin constructs were immunoprecipitated with the antiserum to prolactin and with an antibody (mAb 210) that recognizes an epitope in the amino-terminal domain of the α subunit (Figs. 4-1A and 4-2B). Only those with the prolactin fragment inserted after MA (αPMA and αPM4) were immunoprecipitated with mAb 61, an antibody that recognizes an epitope in the segment between M3 and M4 (Fig. 4-1A; Fig. 4-2B, lanes n and q). Similarly, all of the δ subunit-prolactin constructs were immunoprecipitated with the antiserum to prolactin, but only δPM3, δPMA, and δPM4 were immunoprecipitated with mAb 88B (Fig. 4-3B, lanes e, g and i). The mAb 88B epitope is known to be located in the loop between M3 and M4 (Fig. 4-1B) (Froehner *et al.*, 1983); since the prolactin domain in δPM3 was inserted approximately 85 amino acids after the end of M3, the epitope must lie within this 85 amino acid stretch. These results show that the α subunit- and δ subunit-prolactin fusion proteins were synthesized correctly, with a prolactin-immunoreactive domain located downstream from each of the transmembrane domains in the mouse α and δ subunits.

Protease digestion of the α subunit-prolactin fusion proteins

We then investigated whether the prolactin domain in each of the subunit chimeras was translocated into the microsomal lumen or remained outside the vesicle. The polypeptides were expressed *in vitro* and the microsomes containing the newly-synthesized polypeptides were

subjected to proteolytic digestion with proteinase K (PK) and immunoprecipitated with the prolactin antiserum. When the five α subunit-prolactin constructs were tested, prolactin-immunoreactive peptides were recovered from αPM2 and αPM4 (Fig. 4-4A, lanes e and n), but not from αPM1, αPM3, or αPMA (Fig. 4-4A, lanes b, h, and k). When Triton X-100 was added prior to the digestion, no prolactin-immunoreactive peptides were recovered in any case (Fig. 4-4A). Thus, the prolactin domains in αPM2 and αPM4 were protected and must lie within the lumen of the microsomes, whereas the prolactin domains in αPM1, αPM3, and αPMA are exposed on the cytoplasmic surface of the microsomes. These results indicate that M1, M2, M3, and M4 traverse the membrane, but that MA does not.

Comparison of the size of the fragments recovered from proteolysis of the α subunit-prolactin constructs with those resulting from digestion of the native α subunit (Fig. 4-4B) are consistent with this conclusion. Synthesis of the α subunit *in vitro* gave rise to two bands, representing the glycosylated and unglycosylated forms of the α subunit (Chavez and Hall, 1991). Immunoprecipitation with mAb 210 after proteolysis of microsomes containing the α subunit yielded two additional bands of 38 and 36 kD (Fig. 4-4B, lane b). Protected fragments of the same size were obtained when microsomes containing either αPM3 or αPM4 were immunoprecipitated with mAb 210 after PK digestion (Fig. 4-4B, lanes k and n). These results suggest that the cytoplasmic segment between M3 and M4 is the principal site of PK attack of the α subunit, and that little proteolysis occurs between M1 and M2. The segment between M1 and M2 may not be susceptible because of its short length, which is predicted to be less than 10 amino acids

long. The results obtained after mAb 210 immunoprecipitation of the proteolytic products of PK digestion of αPM1 and αPM2 demonstrate that the short loop between M1 and M2 is not accessible to PK. The size of αPM2 was unaffected by PK digestion, whereas digestion of αPM1 yielded a smaller fragment (approximately 32 kD) which is recognized by mAb 210 (Fig. 4-4B, lane e) and which is similar in size to the primary translation product of a truncated α subunit cDNA that terminates just after M1 at αG240 (Chavez and Hall, 1991). Thus the prolactin fragment is removed in αPM1, but not αPM2, and the segment between M1 and M2 is not cleaved.

This analysis of the proteolytic products immunoprecipitated by mAb 210 allows rationalization of the sizes of the protected prolactin-immunoreactive fragments seen with αPM2 and αPM4 (Fig. 4-4A, lanes e and n). Proteolysis of αPM2 yielded a full-length prolactin-immunoreactive peptide, indicating that the polypeptide chain of αPM2 was not accessible to PK attack. For αPM4, in contrast, a single prolactin-immunoreactive fragment of approximately 22 kD was obtained (Fig. 4-4A, lane n). This peptide is presumably slightly larger than the prolactin peptide (Fig. 4-2A) because of the additional amino acids that comprise M4.

Protease digestion of the δ subunit-prolactin fusion proteins

The pattern of protection seen with the δ subunit-prolactin fusion proteins after PK digestion and immunoprecipitation with the prolactin antiserum was similar to that of the α subunit-prolactin fusions (Fig. 4-5). The entire δPM2 polypeptide was immunoprecipitated with the prolactin antiserum after digestion with PK (Fig. 4-5, lane b), whereas a small fragment of δPM4 slightly larger than the prolactin peptide (23 kD) was

recovered (Fig. 4-5, lane k). No fragments of the normal δ subunit were recovered after digestion with PK, whether immunoprecipitated by mAb 88B or the prolactin antiserum (Fig. 4-3A). As with αPM4, the prolactin-immunoreactive fragment recovered after PK digestion of δPM4 was slightly larger than the prolactin polypeptide (Fig. 4-5, lane k). No prolactin-immunoreactive fragments were recovered after the proteolysis of either δPM3 or δPMA (Fig. 4-5, lanes e and h). Therefore, for analogous α- or δ-subunit-prolactin chimeras, the location of the prolactin domain is identical. For either subunit, when placed after M1, M3, or MA, the prolactin domain remained accessible to proteolysis, indicating that the regions that follow these domains are oriented outside of the microsome (cytoplasmic *in vivo*). When placed after either M2 or M4, however, the prolactin domain was inaccessible to proteolysis, indicating that the carboxy-terminal ends of M2 and M4 are oriented within the microsome (extracytoplasmic *in vivo*).

Discussion

We have made a systematic investigation of the transmembrane topology of the subunits of the mammalian muscle AChR in an *in vitro* translation system by inserting a prolactin reporter domain after each of the major proposed transmembrane domains. Our experiments demonstrate that the carboxy termini of the newly translated, unassembled α and δ subunits of the AChR reside in the lumen of the endoplasmic reticulum indicating that the subunit polypeptides span the membrane an even number of times. Further, our results are consistent with a topological model in which the AChR subunits span the membrane at least four times and in which M1-M4 traverse the membrane but MA does not. Thus prolactin domains placed to the carboxy-terminal side of M1, M3, and MA are susceptible to proteolysis, whereas those placed after M2 and M4 are protected.

Most previous experiments on the AChR have used antibodies to determine the location of specific regions on particular subunits in the intact receptor (Froehner *et al.*, 1983; Lindstrom *et al.*, 1984; Ratnam and Lindstrom, 1984; Sargent *et al.*, 1984; Criado *et al.*, 1985a; Criado *et al.*, 1985b; LaRochelle *et al.*, 1985; Lindstrom *et al.*, 1985; Young *et al.*, 1985; Ratnam *et al.*, 1986a; Ratnam *et al.*, 1986b; Lindstrom *et al.*, 1987; Pedersen *et al.*, 1990). Such studies have been limited to regions for which antibodies are available and in some cases by the specificity of the antibodies used. Our approach differs from these experiments in two ways. First, we have identified transmembrane regions by determining the orientation of a neutral reporter domain whose susceptibility to proteolysis we can detect

reliably using specific antibodies. Reporter domains have been used widely and successfully to study the topology of other polytopic membrane proteins (Manoil and Beckwith, 1985; Boquet *et al.*, 1987; Boyd *et al.*, 1987; Gutierrez *et al.*, 1987; Chepuri and Gennis, 1990; Yun *et al.*, 1991). A possible disadvantage of this use of a reporter domain is that its introduction could disrupt the normal topology of the subunits. To minimize this possibility, we chose for our experiments a cDNA fragment encoding a carboxy-terminal region of bovine prolactin of approximately 142 amino acids in length (predicted molecular weight 16,786 Da). This fragment lacks the codons for the signal sequence found in full-length prolactin and has been shown to be topogenically neutral when used as a reporter in other experiments (Yost *et al.*, 1983; Perara and Lingappa, 1985; Rothman *et al.*, 1988). Thus its transmembrane orientation in our experiments presumably is determined solely by topogenic elements within the AChR subunit sequences. The addition of the prolactin domain is also unlikely to disturb the topology of the AChR subunit in the regions examined as experiments in other systems have shown that the removal of downstream topogenic domains does not affect the topology of upstream regions (Manoil and Beckwith, 1985; Boyd *et al.*, 1987) and that topogenic domains exert their function on non-native domains in predictable ways (Yost *et al.*, 1983; Rothman *et al.*, 1988).

A second feature of our experiments is the use of an *in vitro* translation system to give information about transmembrane orientation. Although assembly of the intact receptor has not been achieved *in vitro*, subunits in an *in vitro* translation system supplemented with microsomes are faithfully synthesized, inserted into the membrane, glycosylated, and

their signal sequences cleaved (Anderson and Blobel, 1981; Anderson *et al.*, 1983; Chavez and Hall, 1991). *In vitro* experiments have the advantage that the translocation of domains in subunits can be investigated in constructs that may not undergo assembly and transport to the cell surface and may not form functional AChR. Transmembrane domains that arise from subsequent rearrangements during assembly, however, might not be detected (see below).

The amino termini of AChR subunits expressed *in vitro* are translocated into the lumen of microsomal vesicles (equivalent topologically to the extracellular space) as are the amino termini of the native AChR subunits (Fig. 4-4) (Anderson and Blobel, 1981; Anderson *et al.*, 1983; Chavez and Hall, 1991). Although the experiments described here offer no information on the position and number of transmembrane regions on the amino-terminal side of M1, we have previously described experiments *in vitro* showing that an α subunit fragment terminating at position α207 is not an integral membrane protein and that novel glycosylation sites introduced at positions α154 and α200 are on the lumenal side of the membrane (Chavez and Hall, 1991). These findings, which are consistent with previous data on the amino-terminal signal sequence, the glycosylation sites, and the location of the binding site for α-bungarotoxin in the intact AChR (Claudio, 1989), suggest that the entire amino-terminal domain preceding M1 is translocated into the microsomal lumen.

Our experiments show that in the newly synthesized α subunit both M1 and M2 span the membrane and do so in opposite directions. The peptide containing the amino-terminal domain, M1, and a carboxy-terminal prolactin domain (αPM1) is, in contrast to the α207 fragment, an

integral membrane protein and has the prolactin domain on the cytoplasmic side; when placed after M2 (in both αPM2 and δPM2), the prolactin domain is lumenal. By a similar argument, M3 and M4 are also transmembrane domains. The assignment of M1-M4 as transmembrane domains is consistent with experiments in oocytes showing that after deletion of any of the segments M1-M4 functional AChRs are not expressed (Mishina *et al.*, 1985).

Our observation that the carboxy termini of the α and δ subunits are located on the lumenal side of the membrane are supported by previous experiments demonstrating that the AChR oligomers of *Torpedo* are linked as dimers via an extracellular disulfide bond between cysteine residues near the carboxy terminus of neighboring δ subunits (Hamilton *et al.*, 1979; McCrea *et al.*, 1987; DiPaola *et al.*, 1989). Earlier reports based on immunocytochemical localization (Young *et al.*, 1985; Ratnam *et al.*, 1986a; Ratnam *et al.*, 1986b) placed the carboxy terminus on the cytoplasmic side of the membrane.

Our results clearly indicate that MA is cytoplasmic, consistent with earlier immunochemical studies (Ratnam *et al.*, 1986a; Ratnam *et al.*, 1986b). Thus prolactin domains placed on either side of MA are susceptible to proteolysis. Because MA is amphipathic, one might argue that MA does not adopt a transmembrane orientation until the AChR oligomer is assembled. To be compatible with a placement of the carboxy terminus on the extracellular side of the membrane, however, this hypothesis would require either that the polypeptide chain traverse the membrane twice between M3 and M4 or that M4, the most hydrophobic of all the putative

transmembrane domains in the AChR subunits, become extracellular in the assembled receptor. Neither possibility seems likely.

An important question is whether the orientation assumed by the newly synthesized subunits is identical to that of the subunits in the mature AChR. Although a rearrangement of transmembrane domains is theoretically possible, the domain structure of the AChR subunits makes this unlikely. All of the transmembrane domains, even M2 which lines the aqueous pore (Imoto *et al.*, 1986; Imoto *et al.*, 1988; Oiki *et al.*, 1988), are highly hydrophobic, and are likely to be quite stable as transmembrane segments. We cannot rule out the possibility that assembly might cause other segments to insert into the membrane, giving rise to additional transmembrane regions, but with the possible exception of the M6 and M7 segments on the amino-terminal side of M1, postulated by Criado *et al.* (1985a) to span the membrane, no other segments of the subunit polypeptides are plausible candidates for such a role. We have argued previously that M6 and M7 are also unlikely to insert during assembly (Chavez and Hall, 1991).

Because of the high degree of primary sequence homology between the AChR subunits and other members of the family of ligand-gated ion channels, our conclusions can be extended to these molecules as well. The characterization of specific conformational changes that accompany ligand binding and desensitization await a more detailed analysis involving the determination of the crystal structure of the receptor in a variety of functional states. However, our studies show that alternative strategies such as the use of reporter domains can be very useful in the elucidation of the basic topology of polytopic membrane proteins.

Table 4-1. Sequences at the junction points in the subunit-prolactin fusion proteins

CONSTRUCT	SEQUENCE
αPM1*	...[234]YLPTDSDSRGSP**CHTSSL**...
αPM2	...[266]STSSAVDSRGSP**CHTSSL**...
αPM3*	...[329]MKRPSRVDSRGSP**CHTSSL**...
αPMA	...[391]ESNNAAEGSP**CHTSSL**...
αPM4*	...[426]FAGRLIEF**CHTSSL**...
δPM2*	...[278]LPATSMDSRGSP**CHTSSL**...
δPM3	...[392]RLTTARRDSRGSP**CHTSSL**...
δPMA	...[443]QVARTVDSRGSP**CHTSSL**...
δPM4*	...[465]AWIFLQVDSRGSP**CHTSSL**...

Amino acid sequences at the fusion points of the subunit-prolactin constructs, given in single-letter code. Amino acids derived from the AChR subunit coding sequences are in PLAIN text, amino acids derived from the prolactin coding sequence are **BOLD**, and amino acids derived from vector sequences are UNDERLINED. Number to the right of the sequence indicates the position along the mature subunit sequence of the first amino acid residue given.

* Constructs sequenced for confirmation. All others predicted from construction protocol as well as immunoprecipitation results (Figs. 4-2 and 4-3).

Figure 4-1. Diagram of the AChR subunit-prolactin fusion proteins.

A, α subunit-prolactin fusions; **B**, δ subunit-prolactin fusions. Chimera names are given at the left. For each construct, the AChR subunit sequences are denoted by the open box (with the putative transmembrane domains M1-M4 and MA given as stippled boxes), and the prolactin sequences denoted by the black box. Details of the amino acid sequences at the junction points are given in Table 4-1. The locations of the epitopes for monoclonal antibodies mAb 210, mAb 61, and mAb 88B are indicated.

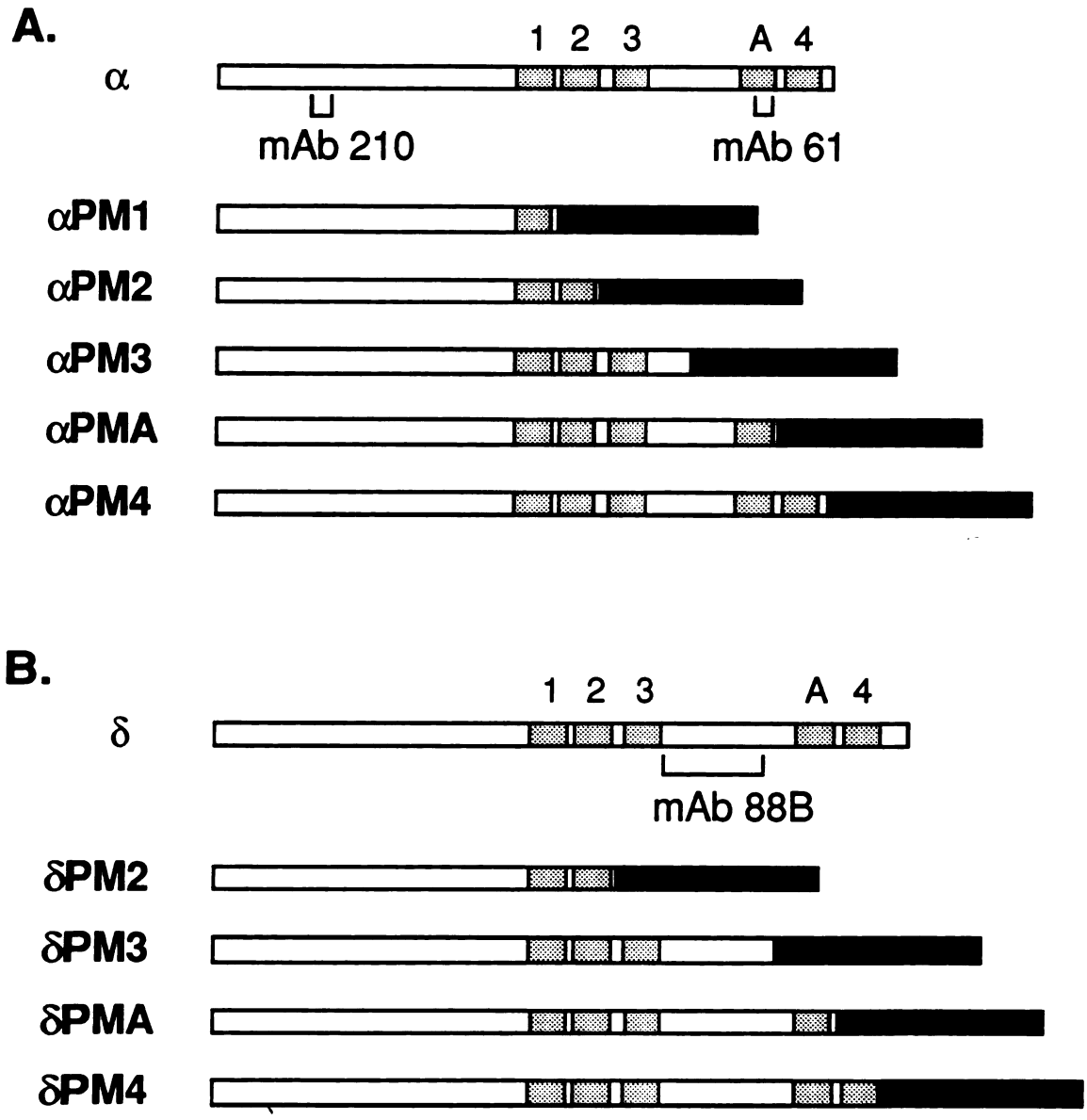
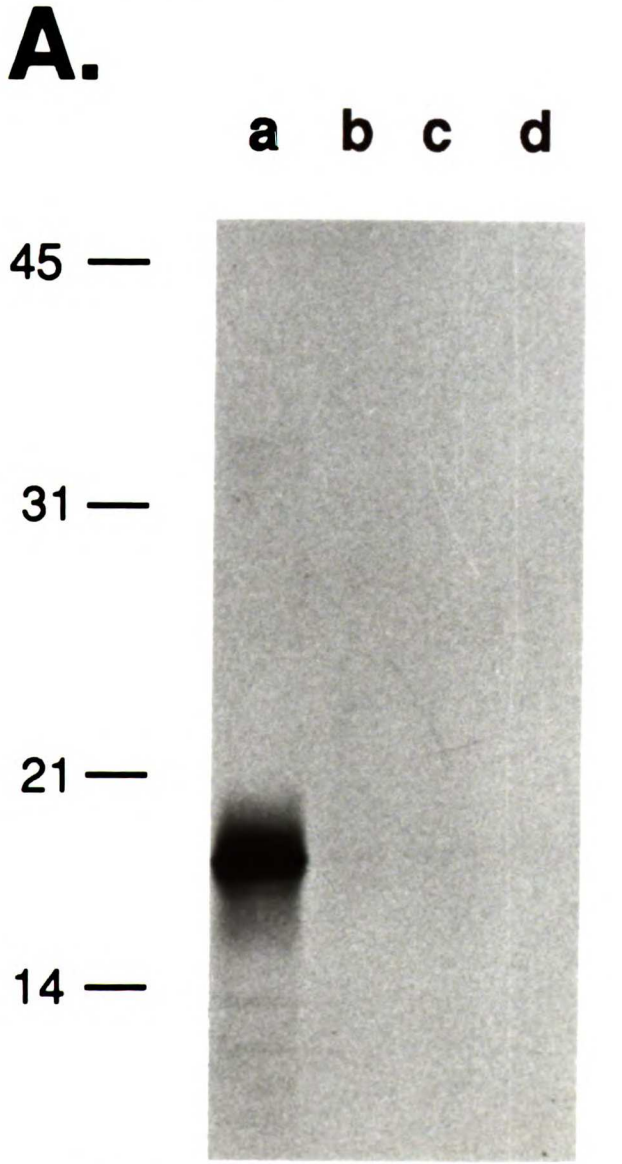

A.
1 2 3 A 4
α
mAb 210
mAb 61
αPM1
αPM2
αPM3
αPMA
αPM4
B.
1 2 3 A 4
δ
mAb 88B
δPM2
δPM3
δPMA
δPM4

Figure 4-2. Characterization of prolactin fragment and α subunit-prolactin fusion proteins.

A, Immunoprecipitation of truncated prolactin fragment. A fragment of bovine prolactin encoding the carboxy-terminal 142 amino acids was expressed *in vitro*, solubilized in a Triton X-100 buffer, and immunoprecipitated with either the prolactin antiserum (lane a), α subunit-specific mAbs 210 (lane b) and 61 (lane c), or δ subunit-specific mAb 88B (lane d).



B, Immunoprecipitation of α subunit-prolactin fusion proteins. The indicated transcripts (**Tr**) were expressed *in vitro*, solubilized, and immunoprecipitated with either mAb 210 (**2**), mAb 61 (**6**), or the prolactin antiserum (**P**).

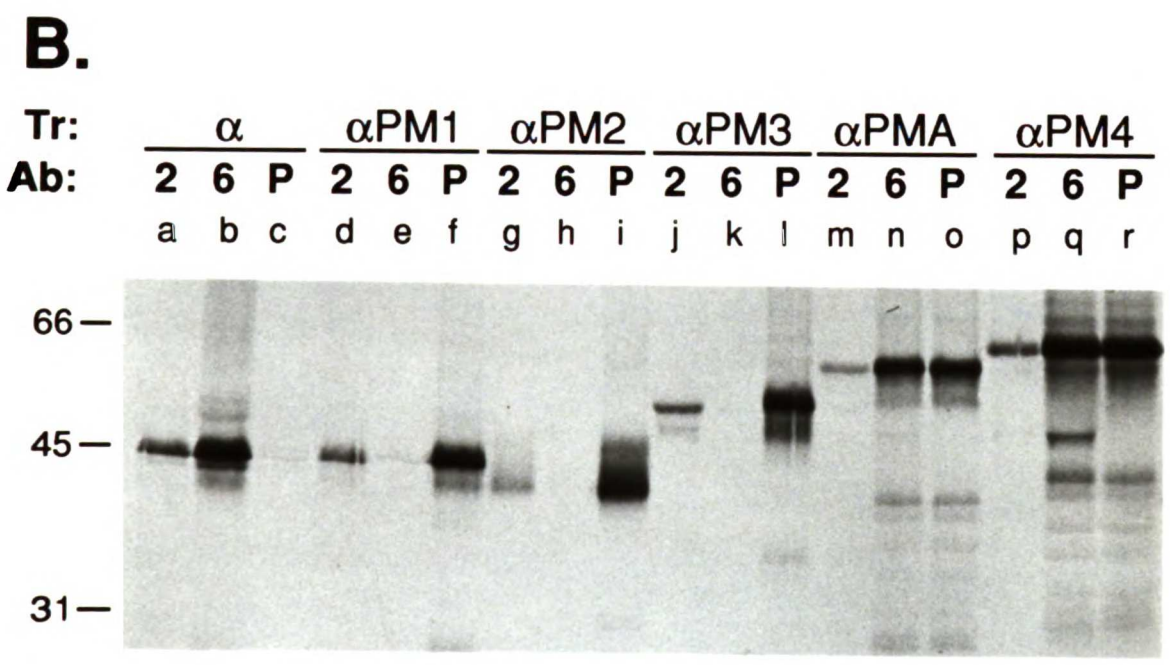
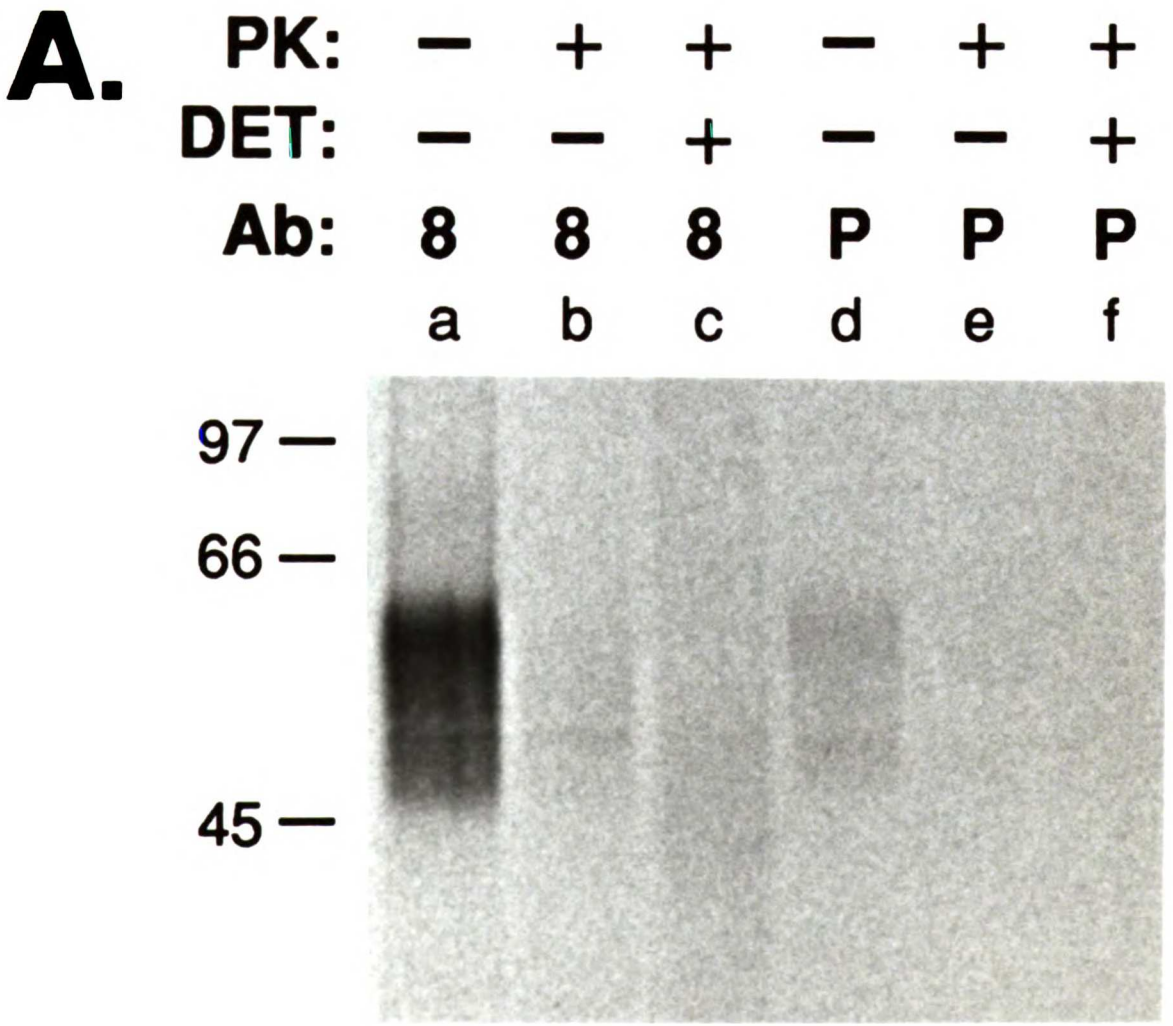


Figure 4-3. Characterization of δ subunit and δ subunit-prolactin fusion proteins.

A, Proteolysis and immunoprecipitation of normal δ subunit. The normal δ subunit was expressed in the presence of microsomes. The translation mixture was either left untreated or digested with proteinase K (**PK**) in the absence or presence of Triton X-100 (**DET**) as described in Methods. The solubilized mixture was immunoprecipitated with either mAb 88B (lanes a-c), or the prolactin antiserum (lanes d-f).



B, Immunoprecipitation of δ subunit-prolactin fusion proteins. The indicated transcripts (**Tr**) were expressed *in vitro* in the presence of microsomes, solubilized, and immunoprecipitated with either mAb 88B (**8**) or the prolactin antiserum (**P**).

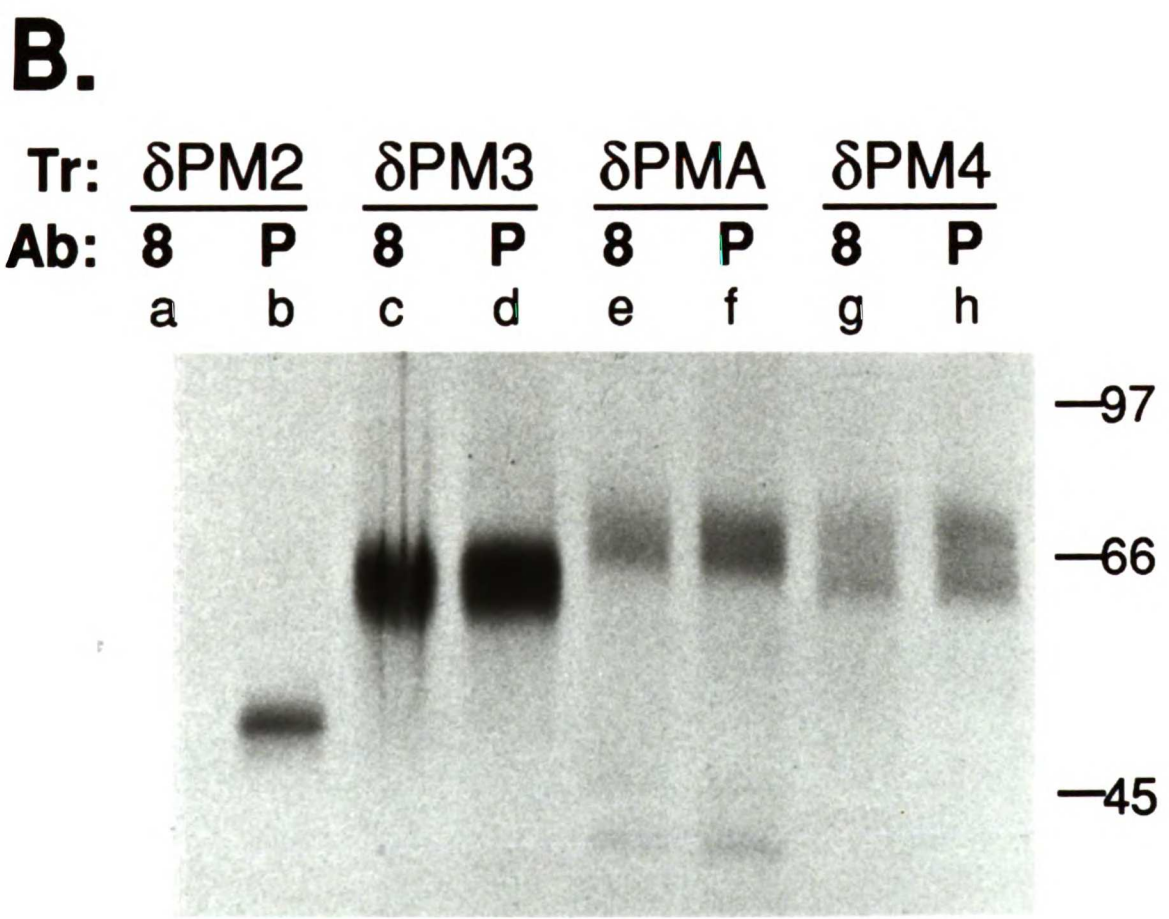
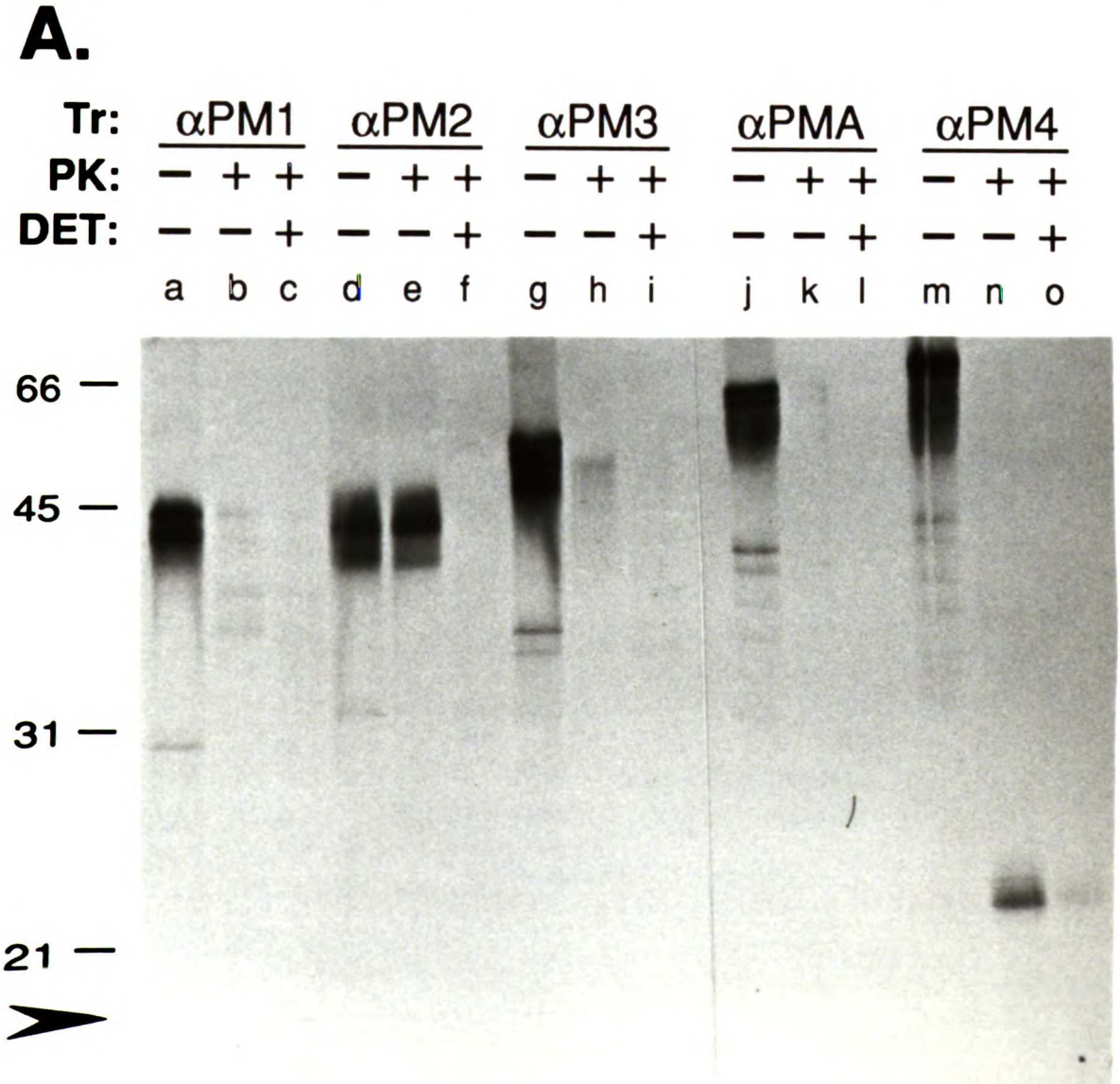


Figure 4-4. Proteolysis and immunoprecipitation of α subunit-prolactin fusion proteins.

The indicated transcripts (**Tr**) were expressed *in vitro* in the presence of microsomes. The translation mixtures were either left untreated or digested with proteinase K (**PK**) in the absence or presence of Triton X-100 (**DET**) as described in Methods.

A, Immunoprecipitation with the prolactin antiserum. Large arrowhead at left indicates the migration of the prolactin fragment.



B, Immunoprecipitation with mAb 210. Note reversal of lanes n and o. Small upwards arrowhead in lane e indicates migration of smallest mAb 210-immunoreactive fragment derived from αPM1.

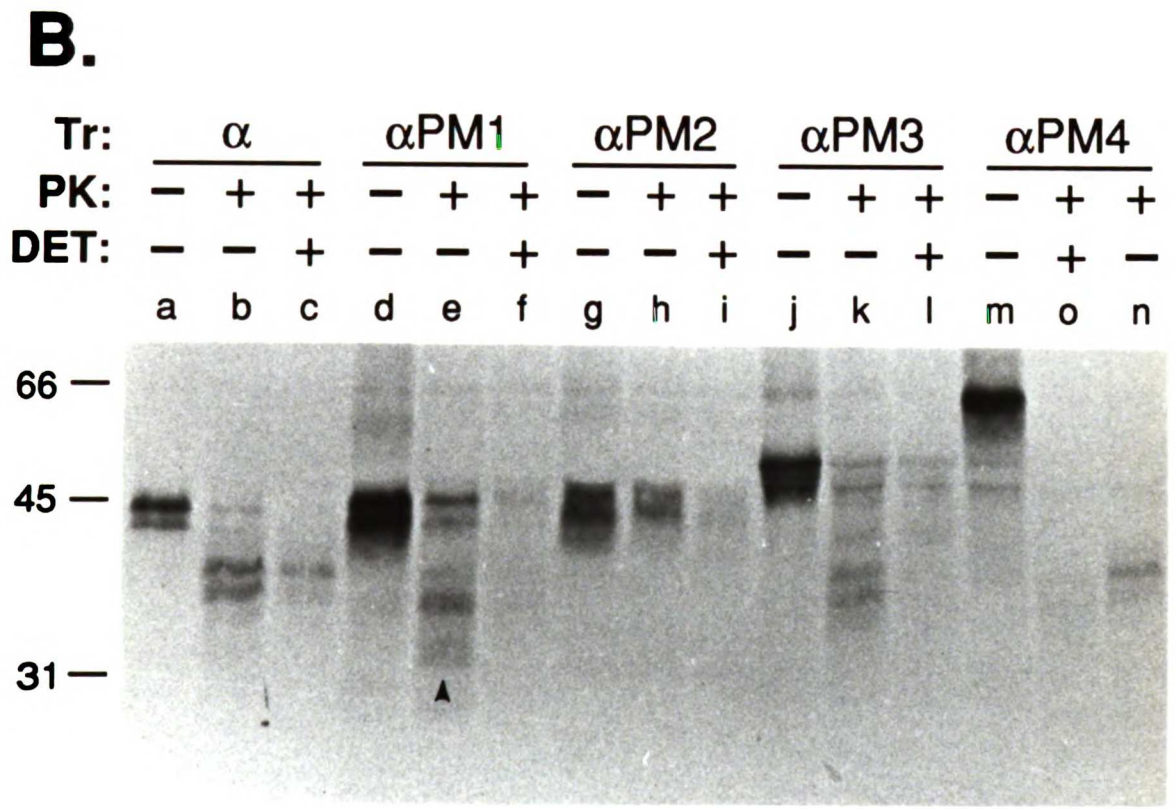
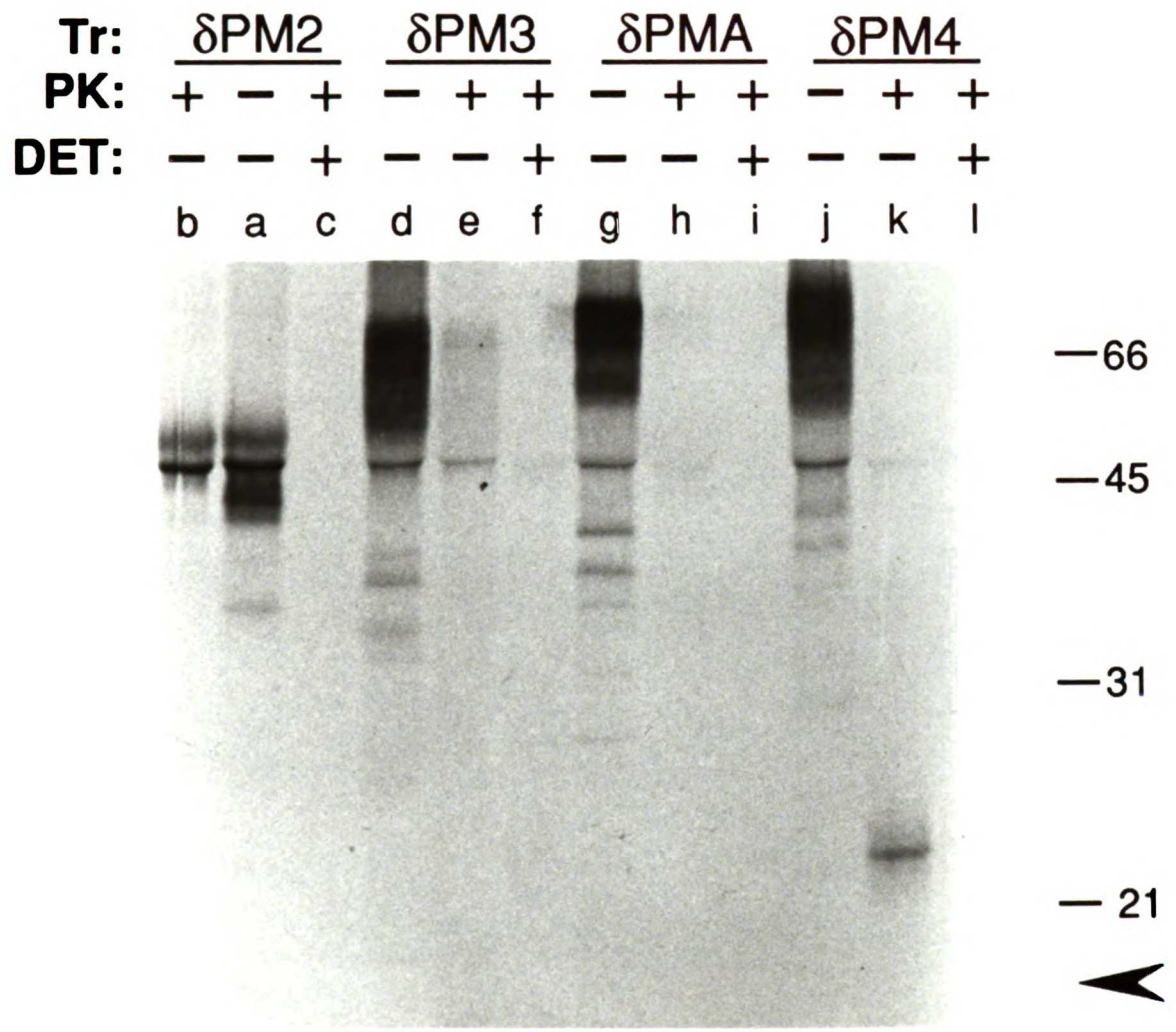


Figure 4-5. Proteolysis and immunoprecipitation of δ subunit-prolactin fusion proteins.

The indicated transcripts (**Tr**) were expressed *in vitro* and treated as described in Fig. 4-4. The solubilized mixture was immunoprecipitated with the prolactin antiserum. Note reversal of lanes a and b. Large arrowhead at right indicates the migration of the prolactin fragment.


Tr:
δPM2
δPM3
δPMA
δPM4
PK: + − + − + + − + + − + +
DET: − − + − − + − − + − − +
b a c d e f g h i j k l
66
45
31
21

Chapter 5. Subunit Folding and αδ Heterodimer Formation in the Nicotinic Acetylcholine Receptor: Comparison of the Mouse and Human α Subunits

Introduction

The nicotinic acetylcholine receptor (AChR) at the neuromuscular junction of skeletal muscle is a pentamer that consists of four subunits in an $\alpha_2\beta\gamma\delta$ stoichiometry (Karlin, 1989). A fifth subunit, the ε subunit, replaces the γ subunit in adult muscle (Takai *et al.*, 1985; Mishina *et al.*, 1986; Gu and Hall, 1988b). The subunit polypeptides exhibit a high level of primary sequence homology, suggesting that they are structurally related (Noda *et al.*, 1983). All of the subunits are transmembrane glycoproteins, possessing an extracellular amino-terminal domain and a long cytoplasmic loop separated by three clustered transmembrane domains; a fourth transmembrane domain follows the cytoplasmic loop (Noda *et al.*, 1982; Claudio *et al.*, 1983; Devillers-Thiery *et al.*, 1983; Chavez and Hall, 1991; Chavez and Hall, 1992). The binding site for α-bungarotoxin (BuTx), a competitive antagonist, has been mapped to the amino-terminal domain of the α subunit near a disulfide-bonded pair of cysteine residues at positions α192 and α193 (Karlin, 1989).

Each of the AChR subunits are synthesized from separate mRNAs and co-translationally inserted into the membrane of the endoplasmic reticulum, the site of receptor assembly (Anderson and Blobel, 1981; Smith *et al.*, 1987a; Gu *et al.*, 1989). The assembly of the receptor is preceded by a series of intermediate stages that reflect changes in the conformation of the individual subunits and in the interactions between the subunits. The conformational maturation of the α subunit has been characterized in detail by Merlie and his co-workers who demonstrated that shortly after its synthesis, the subunit adopts a conformation in which it binds BuTx

(Merlie and Lindstrom, 1983). Their work has shown that the formation of the BuTx-binding species (α_{Tx}) can occur independently of the association with other AChR subunits (Blount and Merlie, 1988). Recent work has suggested that α_{Tx} first associates with the γ and δ subunits (or ε and δ subunits in the adult) to form heterodimers; the αγ and αδ pairs then are linked by the β subunit to form the AChR oligomer (Blount *et al.*, 1990; Gu *et al.*, 1991b; Saedi *et al.*, 1991).

Our laboratory has attempted to define regions of the subunit polypeptides that participate in the interactions leading to assembly. Data obtained from transient expression of AChR subunits in COS cells suggests that the extracellular regions of the subunits, particularly the amino-terminal region, mediate inter-subunit recognition and association (Hall, 1992). A chimeric ε subunit, in which the transmembrane domains and cytoplasmic loop of the β subunit replaced those of the ε subunit, was able to substitute for the ε subunit, but not the β subunit, to create a hybrid AChR oligomer when expressed with other AChR subunits in transiently transfected COS cells (Yu and Hall, 1991). The results of a dominant negative assay used to study interactions during AChR assembly further demonstrated that amino-terminal fragments of the α and δ subunits were capable of interfering with assembly and that an amino-terminal fragment of the α subunit could bind specifically to the δ subunit (Verrall and Hall, 1992).

As part of our investigations on AChR assembly we have also investigated a striking (10-fold) difference between the abilities of rat and mouse ε subunits to support the assembly of the AChR, independent of whether the other subunits were from rat or mouse (Gu *et al.*, 1991a). We

found that the mouse ε subunit was more efficient than the rat subunit in the formation of the αε heterodimer, and that this difference could be entirely accounted for by two amino acid differences in one small region of the amino-terminal domain. Although these results offered further support for the importance of the amino-terminal domain, we were unable in these experiments to determine whether the changed amino acids caused more efficient folding of the ε subunit or a higher affinity for the α subunit.

We describe here experiments using a similar approach to investigate species differences in the α subunit, a subunit for which folding and heterodimer formation can be separately determined. Using the COS cell transient transfection system, we have expressed AChRs containing either the human α subunit (α_H) or the mouse α subunit (α_M), which also differ in their ability to support AChR assembly. We find that the difference can be ascribed to two amino acid residues near the toxin binding site in the amino terminus, and that substitution of the residues from the human α subunit into the mouse α subunit increases the efficiency of both subunit maturation and of association with the δ subunit.

Methods

Plasmids

The mouse AChR subunits used in this study (α_M, (Isenberg *et al.*, 1986); β_M, (Buonanno *et al.*, 1986); γ_M, (Yu *et al.*, 1986); δ_M, (LaPolla *et al.*, 1984)) were obtained from Drs. J. Merlie and N. Davidson. The human α subunit cDNA (α_H) was derived from human leg muscle poly-(A^+) RNA (Beeson *et al.*, 1990). The subunit cDNAs were cloned into the pSM vector described previously (Brodsky *et al.*, 1990; Gu *et al.*, 1990) for expression in COS cells.

Standard oligonucleotide-directed mutagenesis protocols (Kunkel, 1985; Geisselsoder *et al.*, 1987) were used to convert specific residues of the mouse AChR primary sequence into their human counterparts. The oligonucleotides used contained 10 bases flanking the region being mutagenized. The mutant α subunits were sequenced using the Sequenase kit (US Biochemicals, Cleveland, OH).

COS cell transfection

The AChR subunits were expressed in a COS cell transient transfection system (Seed and Aruffo, 1987) modified as described in Gu *et al.* (1990). COS cells were grown on tissue culture plates to 30% to 60% confluence. The cDNAs encoding the subunits to be expressed were combined in Dulbecco's modified Eagle's medium H-16 (DMEM) containing 1% heat-inactivated fetal calf serum, 400 μg/ml DEAE dextran, 100 μM chloroquine and added to the cells for 3 to 4 hr at 37°C. The cells were treated with 10% DMSO in phosphate-buffered saline (PBS) for 2 min,

washed with PBS, and incubated overnight with DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U penicillin G and 100 μg/ml streptomycin sulfate. The next day, the cells were replated and assays were performed 24 hr later.

Except where noted, the amounts of the plasmids used were as given in Gu *et al.* (1990). For expression of all four subunits, the amounts per 21 cm^2 surface area were: α_M or α_H, 1.3 μg; β_M, 0.6 μg; γ_M, 0.7 μg; δ_M, 0.25 μg. For experiments in which only one or two subunits were expressed, 1 μg of each plasmid was used.

Toxin binding assays

COS cells that had been transfected with all four AChR subunits were assayed for the expression of surface toxin-binding as described (Gu *et al.*, 1990). The cells were incubated with 200 μl of DMEM supplemented with 10% fetal calf serum containing 20 nM ^{125}I-BuTx (Amersham, Arlington Heights, IL) for 2 hr at 37°C and were then washed 3 times with 500 μl PBS, removed from the dishes with 0.2 N NaOH, and counted in a gamma counter. The assays were performed in triplicate and non-specific binding was determined using sham-transfected cells.

The turnover of surface toxin binding was determined as described previously (Gu *et al.*, 1990). ^{125}I-BuTx was bound to the cells and the appearance of ^{125}I-labeled breakdown products was monitored by counting samples of the medium at specified times. At the end of the assay period, the cells were stripped from the plate with 0.2N NaOH to determine the ^{125}I-BuTx remaining associated with the cells.

Saponin assay

To measure the expression of toxin-binding species that did not reach the surface of the COS cells (i.e., single α subunits or heterodimer species), toxin-binding assays were performed on cells permeabilized with saponin (Blount *et al.*, 1990). ^{125}I-BuTx (20 nM) was applied to the cells in 200 µl of a buffer containing 0.5% saponin, 0.25% BSA, 0.02% NaN_3 and 10 mM Hepes (pH 7.4) for 2 hr on ice. The cells were washed three times with 500 µl saponin buffer lacking BuTx, scraped from the dishes, and pelleted in a microfuge. The cell pellets were counted in a gamma counter. Non-specific binding was determined using sham-transfected cells. Each assay was performed in triplicate.

Metabolic labeling

To determine the relative amounts of expressed α subunit that were converted to the toxin-binding conformation, COS cells transfected with α subunit cDNA were labeled metabolically. In separate experiments, cells were co-transfected with mouse α and δ subunit cDNA. After a 10 min incubation at 37°C in cysteine- and methionine-free DMEM, transfected COS cells were labeled for 5 min with cysteine- and methionine-free DMEM containing 250 µCi/ml tran-[^{35}S] label (ICN Radiochemicals, Costa Mesa, CA). The cells were washed with DMEM supplemented with 2 mg/ml each of cysteine and methionine, and then chased for the times specified with DMEM containing 10% fetal calf serum. At the end of the chase period, the cells were removed from the dish with ice-cold PBS, pelleted, and solubilized in a Triton X-100-containing buffer as described (Gu *et al.*, 1990; Chavez and Hall, 1991). The cleared lysate was assayed for expressed α

subunit by immunoprecipitation with mAb 61 (Tzartos *et al.*, 1981; Ratnam *et al.*, 1986a) and precipitation with BuTx coupled to Sepharose. mAb 61 was the generous gift of Dr. J. Lindstrom (University of Pennsylvania). The human α subunit was not detected by mAb 61 in immunoblots (data not shown), and therefore it was not used to immunoprecipitate the lysates from cells transfected with the α_H cDNA. The precipitation reactions were performed for 2 hr at 4°C with agitation. A rabbit-anti-rat second antibody coupled to Sepharose was used to precipitate the mAb-containing samples. The Sepharose pellets were washed twice with 0.75 ml of a buffer containing 1 M NaCl, 1% Triton X-100, 0.02% NaN_3, and 50 mM Tris-HCl (pH 7.4). The samples were heated for 3 min at 100°C in SDS-PAGE loading buffer containing 5% β-mercaptoethanol and electrophoresed through SDS-polyacrylamide gels (10%). The gels were dried, fluorographed, and exposed to Kodak X-Omat film. Quantitation of the bands was performed using the Molecular Dynamics Phosphor Imaging system (Molecular Dynamics, Sunnyvale, CA).

Results

Surface AChR expression

COS cells transfected with the cDNAs for the β, γ, and δ subunits along with the cDNA encoding either the α_M or the α_H subunit expressed assembled AChR on their surface as determined by a surface BuTx-binding assay. The level of toxin binding was approximately 2-fold higher in transfected cells expressing the α_H-AChR than the level seen in cells expressing α_M-AChR (Fig. 5-1), and showed the same dependence on cDNA concentration for both α subunit types. The two receptor types did not differ in their affinity for BuTx as both exhibited half-maximal saturation at 1-2 nM BuTx (data not shown). The higher steady-state level of surface receptor in cells expressing α_H-AChR could result either from a higher rate of addition of AChR to the surface or from a decreased rate of surface turnover relative to the α_M-AChR. To distinguish between these alternatives, the surface turnover for both receptor types was determined by binding ^{125}I-BuTx to transfected cells and measuring the rate at which ^{125}I-labeled breakdown products were released into the medium (Fig. 5-2). The half-lives of the α_M-AChR and α_H-AChR were essentially equivalent. The α_H-AChR must therefore be more efficiently assembled and transported to the surface than the α_M-AChR.

Amino acid differences

We then carried out experiments to determine which amino acids in the α subunit are responsible for the greater surface expression of AChR containing α_H as compared to α_M. The mature α_M and the α_H subunits

differ in only 21 of 437 amino acid residues (Table 5-1). These differences are about equally divided between the amino-terminal domain and the long cytoplasmic loop. To determine which domain of the α_H subunit contains the responsible amino acids, we constructed a chimeric α subunit, αHN, in which the first 216 residues of the human α subunit were attached to the last 221 residues of the mouse α subunit (Fig. 5-3A). When the cDNA encoding αHN was co-transfected with the β, γ, and δ subunit cDNAs and assayed for surface toxin binding, the amount of surface expression of the αHN-AChR was equivalent to that seen with α_H-AChR (Fig. 5-3B). Therefore the amino-terminal domain of the α_H subunit is the region that contains the elements responsible for the assembly differences. A second chimera, in which a fragment containing the first 4 non-identical residues (positions 13, 27, 28, and 30) from the mouse α subunit was substituted for the equivalent region in the α_H subunit, was also tested. When assayed for surface toxin binding, this construct was indistinguishable from the normal α_H subunit (data not shown). Of the remaining 7 amino acid differences in the amino-terminal domain, three (positions 187, 189, and 195) are clustered around the toxin-binding site. Therefore we concentrated further efforts on these residues.

The three amino acid differences in the toxin-binding region are non-conservative (Table 5-1). The residues in the α_M subunit are αW187, αF189, and αA195; the α_H counterparts are αS187, αT189, and αD195. We used oligonucleotide-directed mutagenesis to convert each of the three residues near the toxin-binding site of the α_M subunit to the equivalent α_H subunit residue either singly, in pairs, or all together. Each α_M mutant cDNA was transfected together with the β, γ, and δ subunits and assayed for surface

toxin binding. Of the seven constructs tested, two α_M mutant-AChRs expressed surface toxin binding at levels similar to α_H-AChR (Fig. 5-4). The first, α_MSTD, possessed all three mouse-to-human residue changes (Fig. 5-4A) and the second, α_MST, was altered at positions α187 and α189 (Fig. 5-4B). When the amino acid at each position was mutated separately and and the mutant subunit expressed with other subunits, neither the tryptophan-to-serine mutant (α_MS) nor the phenylalanine-to-threonine mutant (α_MT) produced surface receptor at the same level as that seen with α_H (Fig. 5-4B). Thus the αS187 and αT189 residues together confer on the mouse α subunit the ability to support expression of surface toxin binding at the same level as α_H-AChR.

Steps in assembly

We next examined the early steps in AChR assembly to determine whether α_M or α_H differed in their ability to form αδ heterodimer. The formation of αδ heterodimers was measured by the increase in the toxin-binding activity of α subunits co-transfected with the δ subunit (Blount and Merlie, 1990; Gu *et al.*, 1991b). COS cells were transfected with equal amounts of the α and δ subunit cDNAs, and the toxin-binding assay performed in the presence of 0.5% saponin to permeabilize the cells (Blount *et al.*, 1990). When the α_H subunit was co-expressed with the δ subunit, the toxin-binding level attained was approximately two-fold greater than that seen with the α_M/δ heterodimer (Fig. 5-5A). This disparity between the level of α_M/δ and α_H/δ heterodimer formation could account for the difference in the surface expression of α_M- and α_H-AChR. The increased level of α_H/δ heterodimer formed relative to α_M/δ heterodimer could be due

to a difference either in the conversion of the α subunits to α_{Tx} or in the affinity of the α_{Tx} forms for the δ subunit.

We investigated whether the mouse and human α subunits were equally efficient at acquiring the α_{Tx} conformation by expressing each of them in transfected COS cells without other subunits. COS cells transfected with 1 μg of α_H cDNA exhibited a level of toxin binding approximately 1.5-fold higher than cells transfected with the same amount of α_M cDNA (Fig. 5-5B). Thus the α_H subunit is either expressed at a higher level or folded more efficiently into the α_{Tx} form than the α_M subunit.

To compare directly the ability of the α_{Tx} form to interact with the δ subunit in the two cases, we repeated the co-transfection of α and δ cDNAs, but reduced the amount of α_H cDNA to one-half that used for α_M so that the amount of α_{Tx} expressed in each case was the same. Under these conditions, cells transfected with α_H and δ cDNAs still expressed more toxin-binding activity than cells transfected with α_M and δ cDNAs (Fig. 5-5C). Thus even when the difference in toxin-binding activity between the α subunits was equalized, the level of αδ heterodimer formed by α_H is greater than that formed by α_M. We tentatively conclude that α_H is more efficient than α_M both in the formation of α_{Tx} and in the interaction between α_{Tx} and δ subunits.

Kinetics of maturation and heterodimer formation

We further investigated the differences between α_H and α_M by examining the kinetics of α_{Tx} formation and αδ heterodimer formation. A direct comparison of the kinetics in the two cases is not possible because

antibodies that recognize α_M and α_H equally well are not available. The mutant mouse α subunit, α_MST, however, behaves like the α_H subunit, and is precipitated just as well as α_M by mAb 61, an antibody that recognizes an epitope on the long cytoplasmic loop (Tzartos *et al.*, 1981; Ratnam *et al.*, 1986a). We therefore carried out further studies using α_M and α_MST.

We first examined the kinetics of formation and degradation of the total α subunit in COS cells transfected only with the α subunit cDNA. Either α_M or α_MST was expressed in COS cells, and the cells were labeled with radioactive amino acids for 10 min followed by chase periods of up to 8 hours incubation with unlabeled amino acids. At the end of the chase, cell extracts were immunoprecipitated with mAb 61 and analyzed by SDS-polyacrylamide electrophoresis. Immediately following the pulse, the total amount of radioactive α subunit immunoprecipitated by mAb 61 was approximately the same in the two cases, indicating that the initial level of expression of the two subunits was similar for α_M and α_MST (Fig. 5-6A). Following the pulse, the level of radioactivity declined with a half-time of approximately 2 hr in both cases. This value for the half-time of the α subunit is similar to the value obtained for the mouse α subunit expressed in quail fibroblasts (Blount and Merlie, 1988; Blount and Merlie, 1990).

The rates of formation and degradation of α_{Tx} was examined by precipitation of the labeled α subunit with BuTx-Sepharose. Immediately following the pulse, more α_MST was detected than α_M (Fig. 5-6B). A comparison of the amount of α_{Tx} with the total α subunit showed that the percentage of the mAb 61-precipitable α subunit (α_0) converted to the α_{Tx} form was 0.6% and 1.2% for the α_M and α_MST subunits, respectively. During the first hour after the pulse, the amount of the BuTx-binding form

of α_M first increased and then decreased, whereas the amount of the α_{Tx} form of α_MST continued to increase. At the end of the first hour, the amount of the toxin-binding form relative to the total α subunit was 0.8% for the α_M subunit and 2.8% for α_MST subunit. After the first hour, the amount of the α_{Tx} form decreased for both α subunits with a half-life that was approximately 1.5-2 hours, the same as that found for the total α subunit. These results show that α_{Tx} is formed more rapidly for α_MST than for α_M and are consistent with the higher steady-state accumulation of α_{Tx} seen in cells transfected with α_H than in those transfected with α_M. The increased accumulation is thus due to increased efficiency of formation of α_{Tx}, and not to a difference in α_{Tx} degradation.

To examine the influence of the δ subunit on these processes, COS cells were co-transfected with α_M or α_MST cDNA plus δ cDNA, and the same pulse-chase experiment performed. When mAb 61 was used to immunoprecipitate the total α subunit, no difference in the half-life of the α subunit was seen between cells expressing the α subunit alone (Fig. 5-6A) as compared to those expressing α plus δ subunits (Fig. 5-6C). When α_{Tx} was examined, the amount of α_{Tx} found at the end of the pulse was again higher for α_MST than for α_M (1.2% of the total subunit for α_M versus 9.0% for α_MST; Fig. 5-6C and 5-6D). Interestingly, the percentage of α subunit that was converted to α_{Tx} in both cases was higher when cells were transfected with both α and δ subunit cDNAs than with the α subunit cDNA alone. These results thus demonstrate directly that the δ subunit can facilitate the conversion of α_0 to α_{Tx}.

When the time course of α_{Tx} accumulation was further examined, both labeled α_M and α_MST continued to increase immediately following the

pulse, then declined. As in cells transfected with the α subunit cDNA alone, the increase in α_{Tx} lasted longer for α_MST than for α_M. At one hour after the pulse, the amount of α_{Tx} was 2.1% of the total for α_M, and 29% for α_MST. After the first hour, the amount of α_{Tx} then declined for both α subunits. In both cases, the rate of loss of α_{Tx} was considerably slower in cells co-transfected with δ subunit cDNA (Fig. 5-6D) than in cells transfected with the α subunit cDNA alone (Fig. 5-6B). For cells co-expressing α_M and δ subunits, the half-life of α_{Tx} was increased to greater than 8 hr; the corresponding value for cells expressing α_MST and δ subunits was approximately 4 hr. Thus in both cases, the presence of the δ subunit clearly increased the stability of the α_{Tx} subunit, presumably due to the stabilizing effect of the αδ heterodimer. We conclude that the δ subunit increases the accumulation of α_{Tx} in two ways: it facilitates the folding of immature α subunit to α_{Tx}, and it stabilizes the α_{Tx} that is formed. Changing the two amino acids found at positions α187 and α189 in the mouse α subunit to those in the human subunit enhances the first of these processes but not the second. This enhancement results in a more efficient formation of the relatively stable α_{Tx}/δ heterodimer.

Discussion

The primary sequence and structural homologies between AChR subunits of different species allow them to assemble to form functional AChRs. Heterospecific AChRs have been expressed both in *Xenopus* oocytes and in transfected mammalian cells (Sakmann *et al.*, 1985; White *et al.*, 1985; Imoto *et al.*, 1986; Mayne *et al.*, 1987; Conroy *et al.*, 1990; Gu *et al.*, 1991a). Comparison of the properties of AChRs containing subunits from different species can be an effective tool for identifying sequences that determine particular properties. This approach, for example, first directed attention to the M2 region as the segment lining the channel pore of the AChR (Imoto *et al.*, 1986). Subunits from different species thus provide a ready-made pool of mutations for the study of particular properties. We have previously used this approach by exploiting differences in the ε subunits from rat and mouse to investigate the assembly of the AChR (Gu *et al.*, 1991a). In this paper, we have performed similar experiments on the mouse and human α subunits. Our aim has been to identify regions of the α subunit that participate in the steps of folding and subunit assembly that lead to the formation of the intact AChR.

Our initial finding was that substitution of human for mouse α subunit cDNA resulted in an approximately two-fold increase in toxin binding on the cell surface. The AChRs expressed using human or mouse α subunits have similar binding constants for BuTx and similar half-lives in the surface membrane. The higher level of surface AChR expression appears to arise from a higher efficiency of αδ heterodimer formation with α_H than with α_M. This is consistent with previous results from our

laboratory (Gu *et al.*, 1991b; Yu and Hall, 1991) in which the extent of heterodimer formation is correlated with the extent of assembly and surface expression of the AChR.

Although α_M and α_H have 21 amino acid differences between them, the difference in their ability to promote assembly is principally due to the amino acid residues at positions α187 and α189. These residues are within a region of the α subunit (α170-203) that has been identified as being part of the binding site for BuTx (Wilson *et al.*, 1988), and are close to the paired cysteine residues at positions α192-α193 which are known to be within 1 nm of the binding site for acetylcholine and other agonists in the intact AChR (Karlin, 1969). The residues responsible for the differences in assembly between α_M and α_H are thus in a different region from those responsible for a similar difference in ε subunits (ε106 and ε115) (Gu *et al.*, 1991a). The residues at α187 and α189, which also differ in human and *Torpedo* α subunits, have been suggested in the latter case to contribute to differences in the affinity of *Torpedo* and human AChRs for BuTx (Neumann *et al.*, 1986; Wilson and Lentz, 1988). The differences at these positions in mouse and human α subunits, in contrast, do not affect the affinity for toxin binding.

Our experiments show that the discrepancy between the extent of heterodimer formation for α_H and α_M has two sources. First, α_H (or its equivalent, α_MST) folds more efficiently to form the toxin-binding form of the α subunit, α_{Tx}. This increased efficiency is seen both when the α subunit is expressed alone, and when the α subunit is expressed with the δ subunit. Second, α_{Tx} interacts more efficiently with the δ subunit to form a stable heterodimer.

The increased efficiency of α_{Tx} formation seen in our experiments is similar to that seen by Conroy *et al.*, who compared the human α subunit with that of *Torpedo* in a *Xenopus* expression system (Conroy *et al.*, 1990). They found that α_H, when expressed with the *Torpedo* β, γ, and δ subunits, gave 5- to 10-fold higher levels of surface AChR expression than did the *Torpedo* α subunit. α_H, expressed alone in *Xenopus* oocytes, also produced a higher level of BuTx-binding activity than did the *Torpedo* α subunit. Our results suggest that the differences between the amino acids at positions α187 and α189 may also have been responsible for these findings.

When expressed alone, the α_{Tx} forms of α_H and α_M have half-lives that are similar to each other and to the unfolded forms of the α subunit. Thus the increased toxin-binding activity seen with α_H is due to an increase in the rate of formation of the α_{Tx} form. Because the rate of degradation is relatively slow (approximately 2 hr), the increased α_{Tx} seen at the end of the 10 min pulse may be taken as a measure of the difference in the rate of conversion of α_0 to α_{Tx}. The precise steps involved in this conversion are unknown, but disulfide bond formation is probably an important part of the process (Blount and Merlie, 1990).

A similar, but larger, difference in the rate of formation of α_{Tx} for the two α subunits is also seen when the α subunit is co-expressed with the δ subunit. In the case of both α_H and α_M, the amount of α_{Tx} at the end of the pulse is increased when the δ subunit is also expressed (Fig. 5-6D). These results suggest that the δ subunit must interact directly with α_0 and must facilitate the conversion of α_0 to α_{Tx}. The increase in α_{Tx} seen with the addition of the δ subunit is relatively greater (10.4-fold) for α_H than for α_M (2.6-fold), suggesting that α_H folds more efficiently than α_M both when

expressed alone, and when folding is facilitated by interaction with the δ subunit.

The idea that the δ subunit can interact directly with the unfolded α subunit is a novel one, and has not been seen in previous experiments. The finding in muscle cells that α_{Tx} is formed before association of the α subunit with other subunits (Blount and Merlie, 1988; Blount and Merlie, 1990; Gu *et al.*, 1991b), and the ability of α_0 to form α_{Tx} when expressed alone has led to the idea that the δ subunit interacts only with α_{Tx}. Our experiments suggest otherwise. Whether the interaction with the δ subunit is a significant factor in facilitating the conversion of α_0 to α_{Tx} in muscle cells is not known. The low efficiency of this conversion in COS cells may allow an otherwise small effect of the δ subunit to be seen more clearly.

In the case of both α_H and α_M, the formation of the αδ heterodimer stabilizes the α subunit, increasing its half-life 2- to 4-fold. The combination of these two effects, the facilitation of α subunit folding by the δ subunit and its stabilization as the result of heterodimer formation, presumably accounts for the increased toxin-binding activity seen in cells transfected with α subunit cDNA along with γ, δ, and ε subunit cDNAs, as compared to cells transfected with α subunit cDNA alone (Blount and Merlie, 1988; Gu *et al.*, 1991a; Gu *et al.*, 1991b; Verrall and Hall, 1992).

These studies demonstrate the utility of heterospecific expression of the subunits of the AChR as a tool to obtain insight into the function and assembly of the receptor. We have characterized the role of species differences at several stages of the assembly pathway and have revealed a novel interaction between the α and δ subunits. Comparable information

about the maturation of the other subunits will depend on the availability of probes that will delineate specific steps in their maturation.

Table 5-1. Amino acid differences: mouse and human α subunits

POSITION	α_M	α_H
13	E	K
27	E	Q
28	I	V
30	Q	E
90	V	L
111	D	Q
169	K	N
181	A	S
187	W	S
189	F	T
195	T	D
230	S	G
307	I	V
310	E	N
335	D	E
338	E	D
340	R	K
379	V	I
397	E	A
419	L	I
434	H	N

The positions at which differences occur in the primary sequences of the mouse and human α subunits, and the single-letter code for the residues at those positions, are listed. The line between residues α195 and α230 indicate the regions exchanged to form the construct αHN.

Figure 5-1. Surface expression of α_M- and α_H-AChRs

COS cells were transiently transfected as described with increasing amounts (0 to 2 μg) of either the mouse or human α subunit plasmid and a fixed amount of the cDNAs for the other subunits. Twenty-four hours after trypsinization and replating, the cells were incubated with 20 nM ^{125}I-BuTx in COS cell medium for 2 hr at 37°C, washed with PBS, removed from the culture dish, and counted in a gamma counter. Non-specific binding was determined using sham-transfected cells. Filled circles, human α subunit; open circles, mouse α subunit. All values represent the mean ± SEM of three determinations.

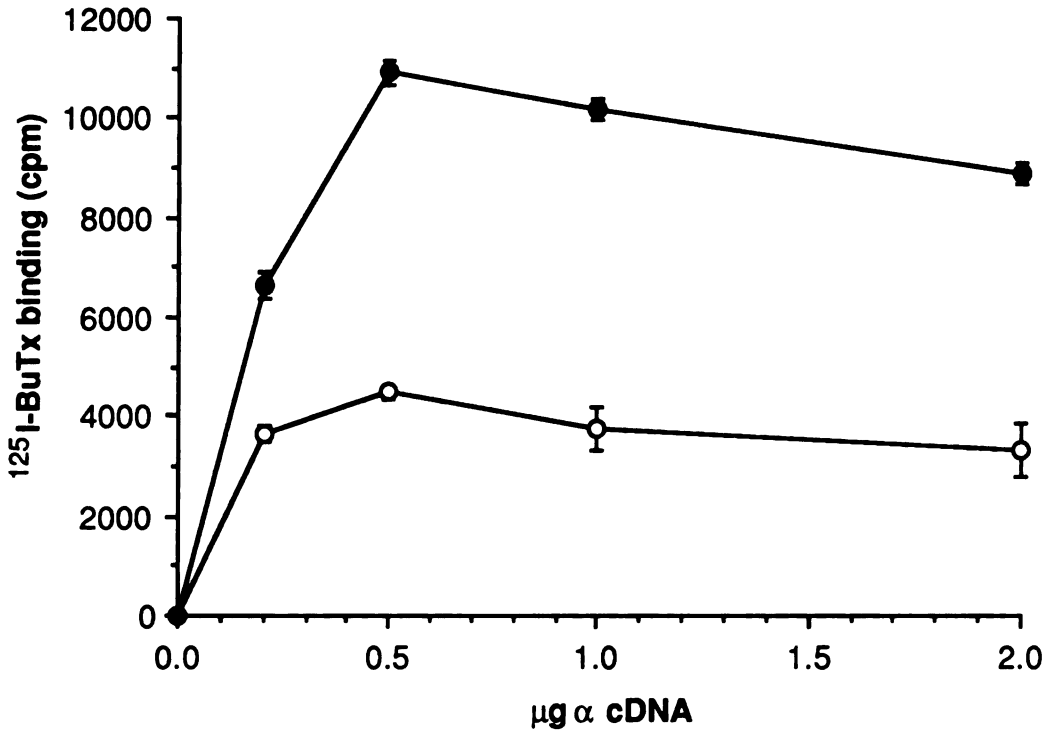

12000
10000
8000
6000
4000
2000
0
^{125}I-BuTx binding (cpm)
0.0
0.5
1.0
1.5
2.0
μg α cDNA

Figure 5-2. Turnover of surface α_M- and α_H-AChRs

COS cells were transfected as described in Fig. 5-1, except that the same amount of α subunit cDNA from both species was used. After replating, the cells were labeled with ^{125}I-BuTx as described. At the times indicated, the medium was removed and the breakdown products of ^{125}I-BuTx were monitored by gamma counting. Fresh medium was added. At the end of the time course, the cells were also removed to determine the remaining ^{125}I-BuTx counts bound. Each point represents the mean ± SEM of three determinations.

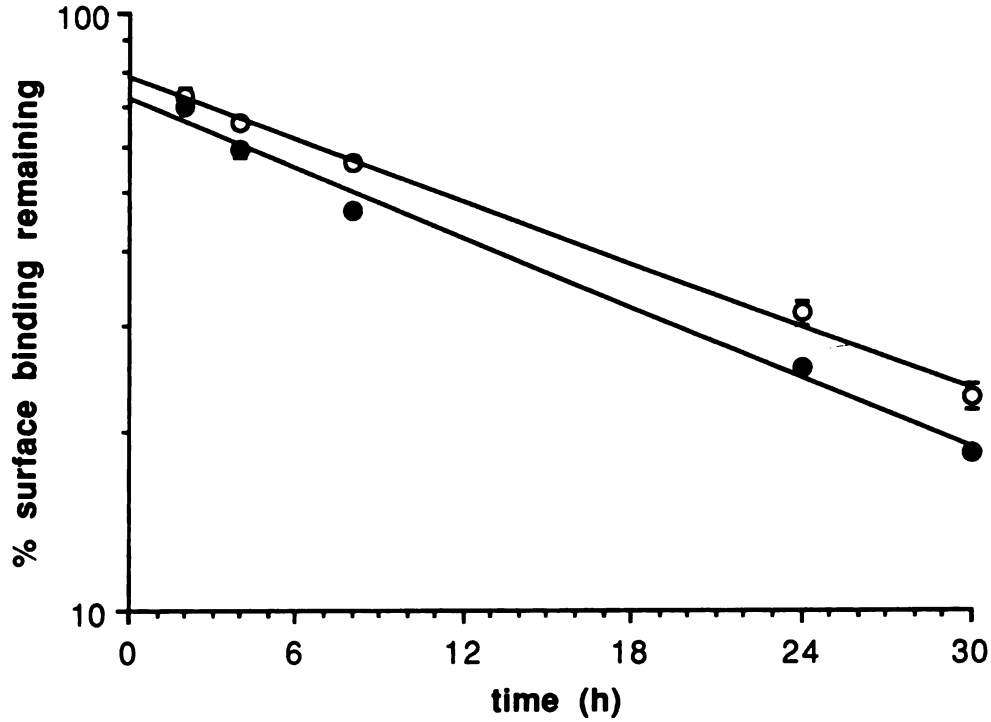
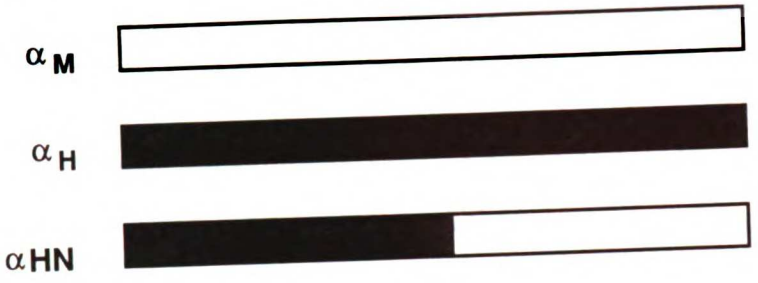

100
10
% surface binding remaining
0
6
12
18
24
30
time (h)

Figure 5-3. Localization of the domain responsible for the difference in surface expression of α_M- and α_H-AChRs

A, Diagram of the mouse and human α subunits and the chimeric subunit αHN.

B, Surface toxin-binding activity of hybrid α_H- and αHN-AChRs. COS cells were transfected with α_H and αHN cDNAs in the presence of the other subunits, and surface toxin-binding activity was determined as described. Each value represents the mean ± SEM of three determinations. Non-specific binding was determined using sham-transfected cells.

A.


αM
αH
αHN

B.

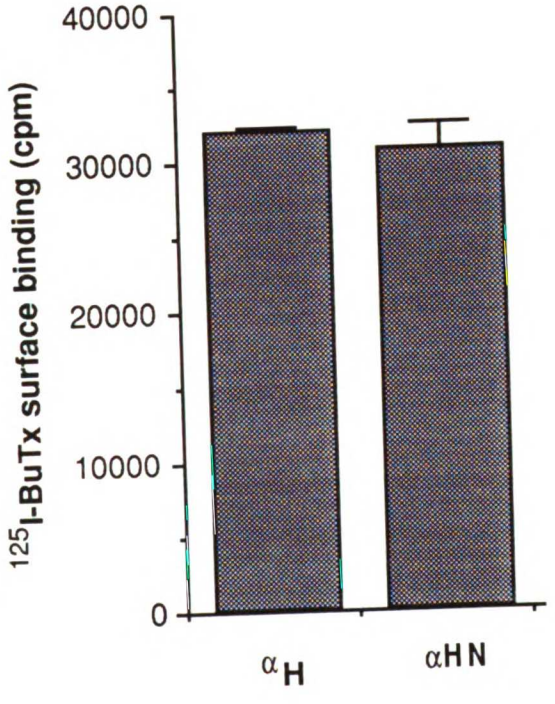
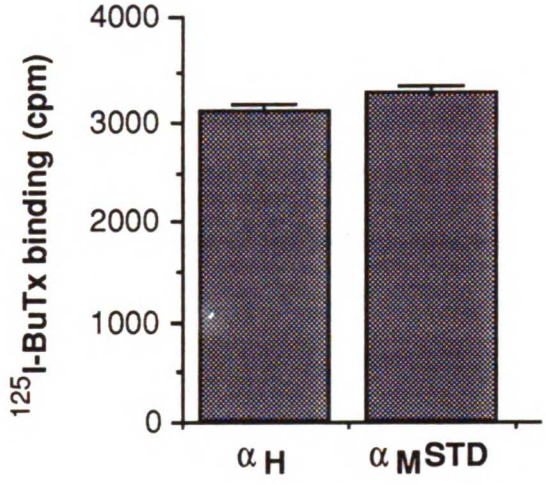

40000
30000
20000
10000
0
125I-BuTx surface binding (cpm)
αH
αHN

Figure 5-4. Surface toxin binding of hybrid receptors containing mouse α subunit mutants

COS cells were transfected and analyzed as described in Fig. 5-1, except that the mouse α subunit mutants α_MSTD (panel **A**) and α_MS, α_MT, and α_MST (panel **B**) were substituted in the indicated cases for the mouse or human α subunit. Each value represents the mean ± SEM of three determinations. Non-specific binding was determined using sham-transfected cells.

A.


4000
3000
2000
1000
0
^{125}I-BuTx binding (cpm)
α_H
α_MSTD

B.

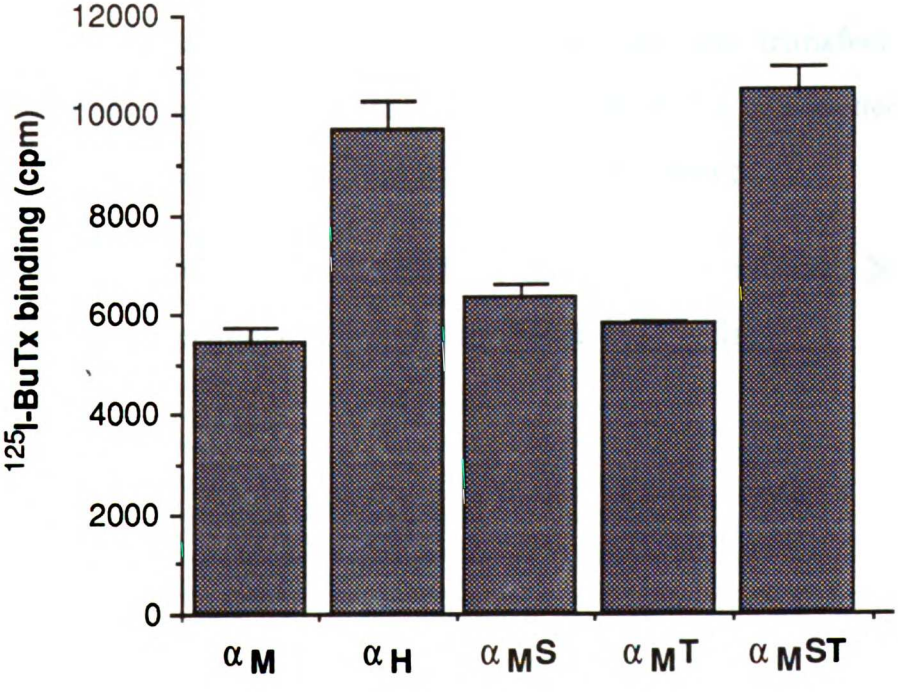

12000
10000
8000
6000
4000
2000
0
^{125}I-BuTx binding (cpm)
α_M
α_H
α_MS
α_MT
α_MST

Figure 5-5. Toxin-binding activity of permeabilized COS cells transfected with the mouse and human α subunits.

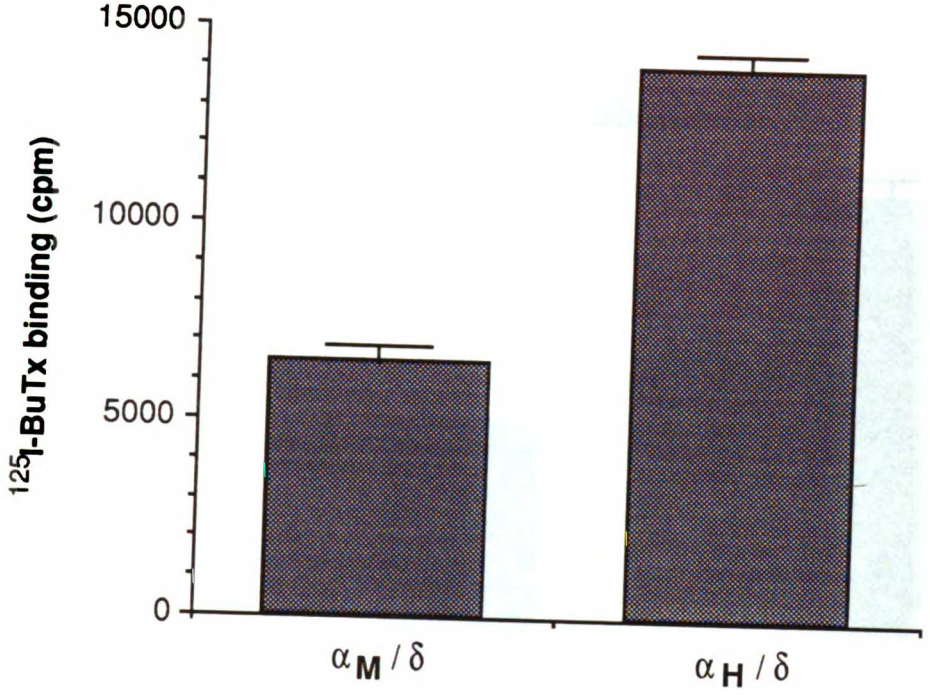
A, Heterodimer formation. The total toxin-binding activity of COS cells transfected with equal amounts of the α and δ subunit cDNAs was determined in the presence of 0.5% saponin as described in Methods. The cells were scraped from the dish, pelleted in a microfuge, and counted in a gamma counter.

B, BuTx-binding activity of mouse and human α subunits expressed alone. COS cells were transfected with the α subunit cDNAs only (1 μg each), and BuTx-binding activity was determined as described.

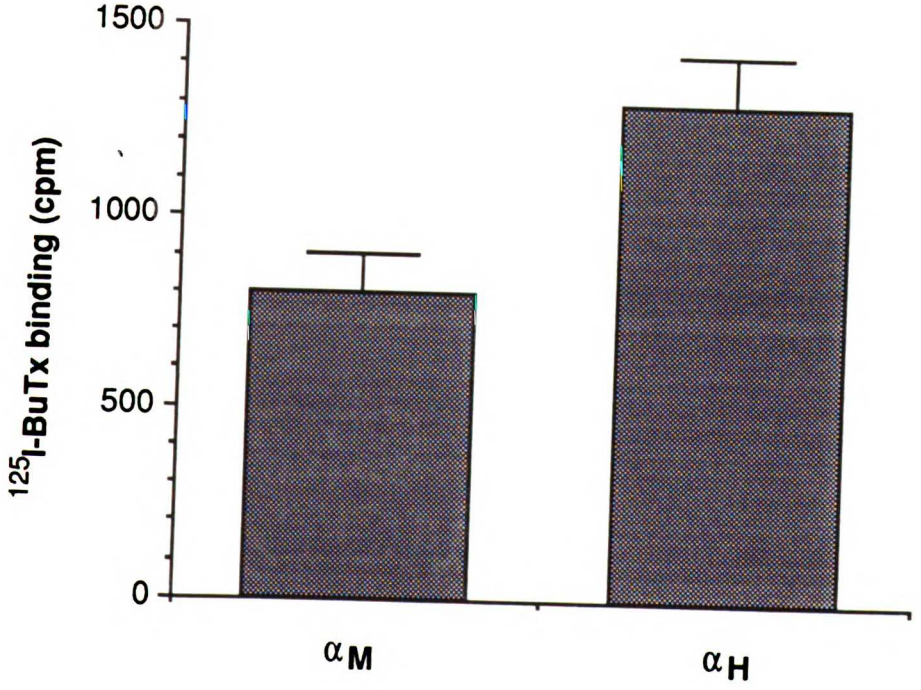
C, Heterodimer formation, with compensation for the level of initial toxin-binding activity of the human α subunit. COS cells were transfected with the indicated amount of α subunit cDNA in the presence or absence of 1 μg δ subunit cDNA, and the cells were analyzed as described.

Each value represents the mean ± SEM of three determinations. Non-specific binding was determined using sham-transfected cells.

A.


15000
10000
5000
0
^{125}I-BuTx binding (cpm)
α_M / δ
α_H / δ

B.


1500
1000
500
0
^{125}I-BuTx binding (cpm)
α_M
α_H

C.

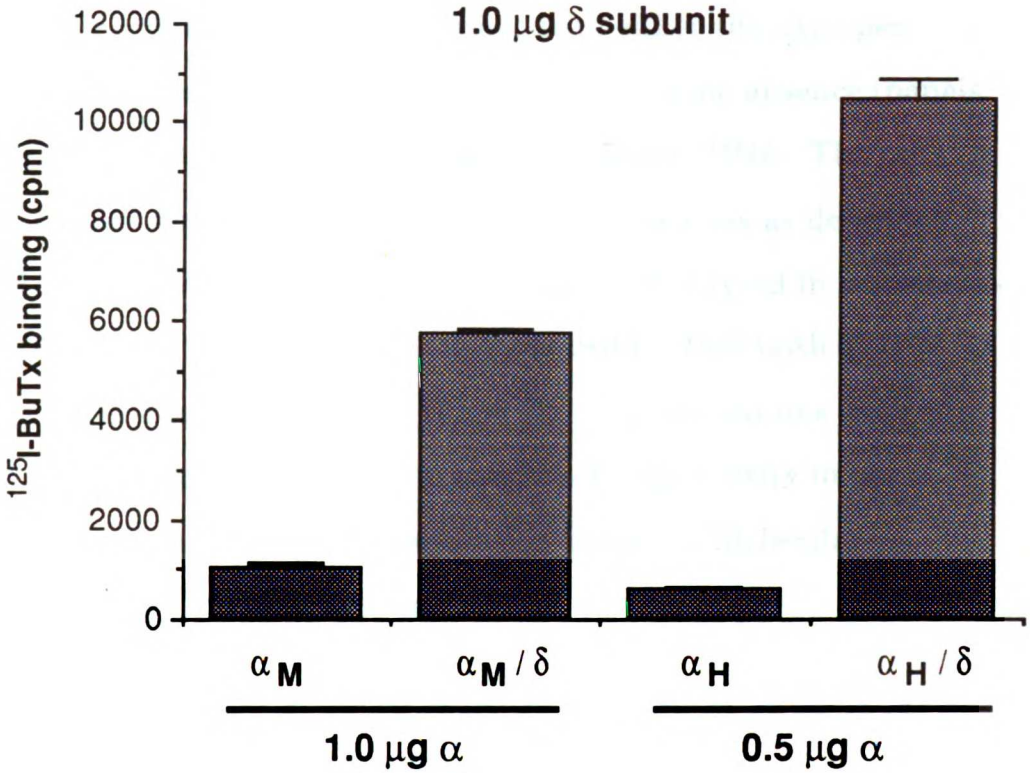
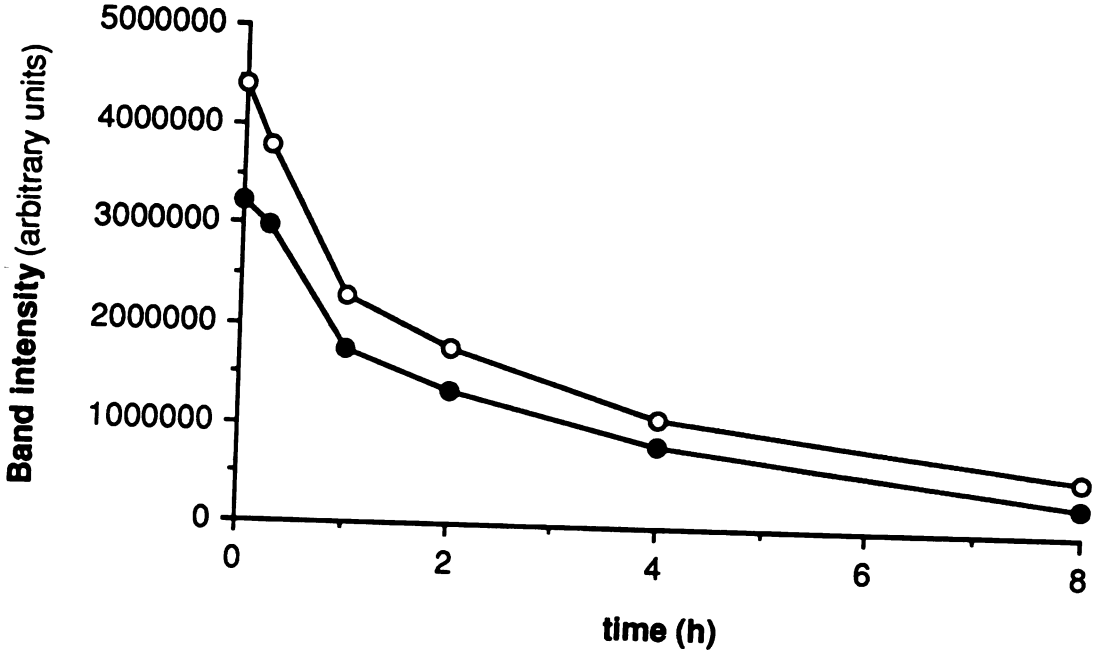

1.0 μg δ subunit
12000
10000
8000
6000
4000
2000
0
125I-BuTx binding (cpm)
αM
αM / δ
αH
αH / δ
1.0 μg α
0.5 μg α

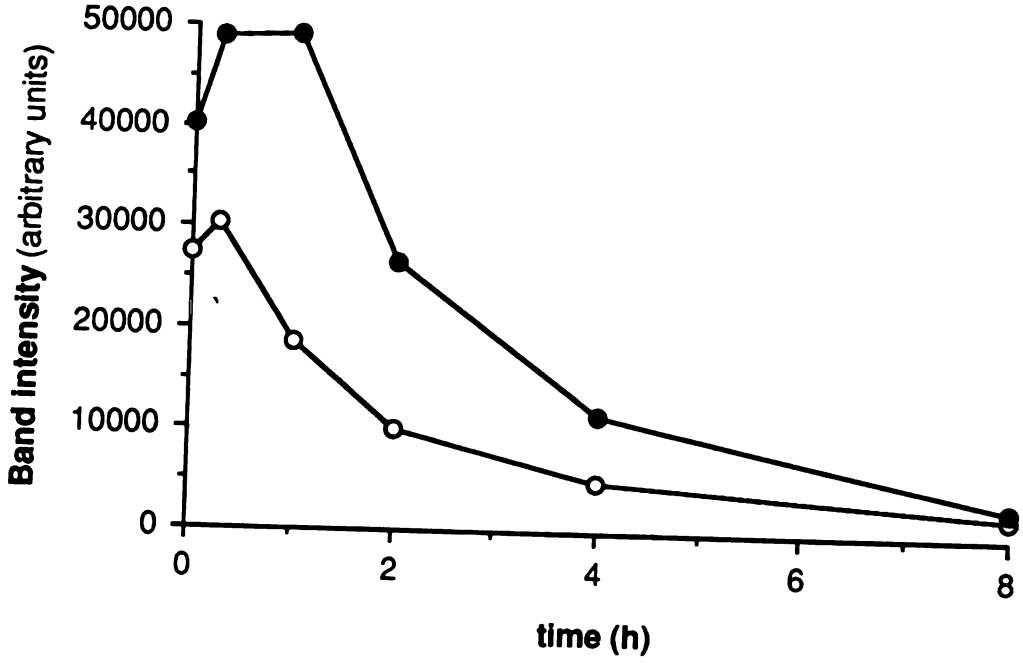
Figure 5-6. Kinetics of metabolically-labeled α_M and α_MST subunits expressed in COS cells in the presence and absence of the δ subunit.

COS cells were transfected as described with either the α_M (open symbols) or α_MST (closed symbols) subunit cDNAs, in the absence (panels **A** and **B**) or the presence (panels **C** and **D**) of δ subunit cDNA. The cells were pulse-labeled for 10 min with radioactive amino acids as described and chased for the indicated times. The cells were then lysed in a Triton X-100 buffer, and the α subunit was precipitated using either mAb 61 (panels **A** and **C**) or BuTx-Sepharose (panels **B** and **D**). The precipitates were run on an SDS polyacrylamide gel, and the amount of radioactivity in the α subunit band was determined by quantitation using the Molecular Dynamics Phosphor Imaging system.

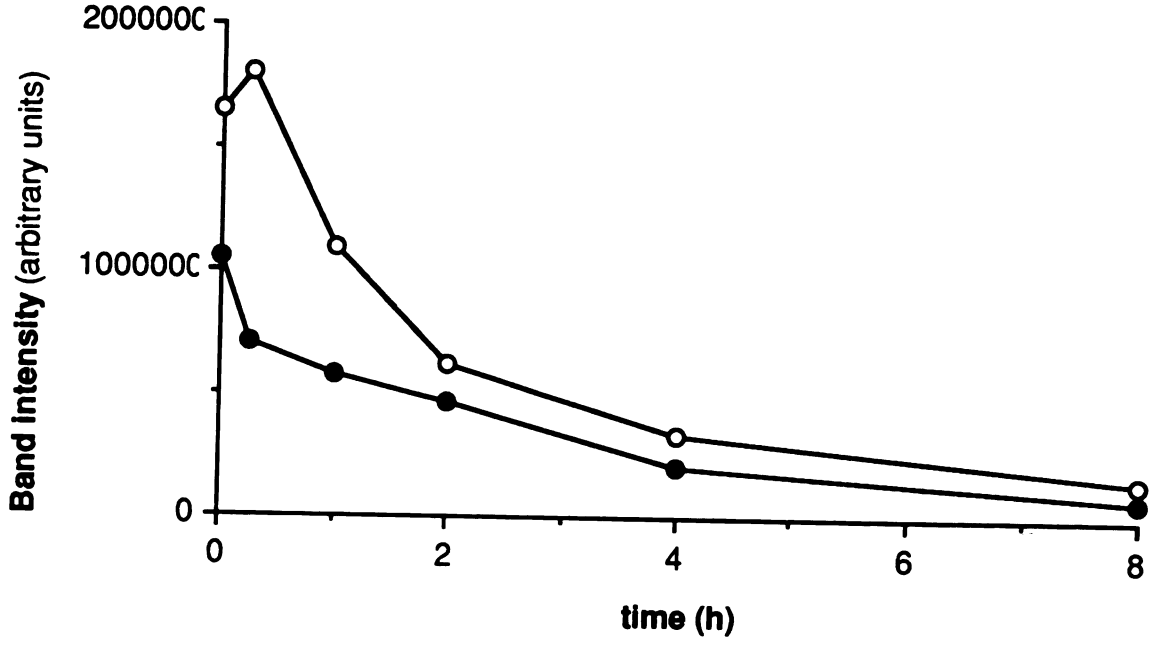
A.


5000000
4000000
3000000
2000000
1000000
0
Band intensity (arbitrary units)
0
2
4
6
8
time (h)

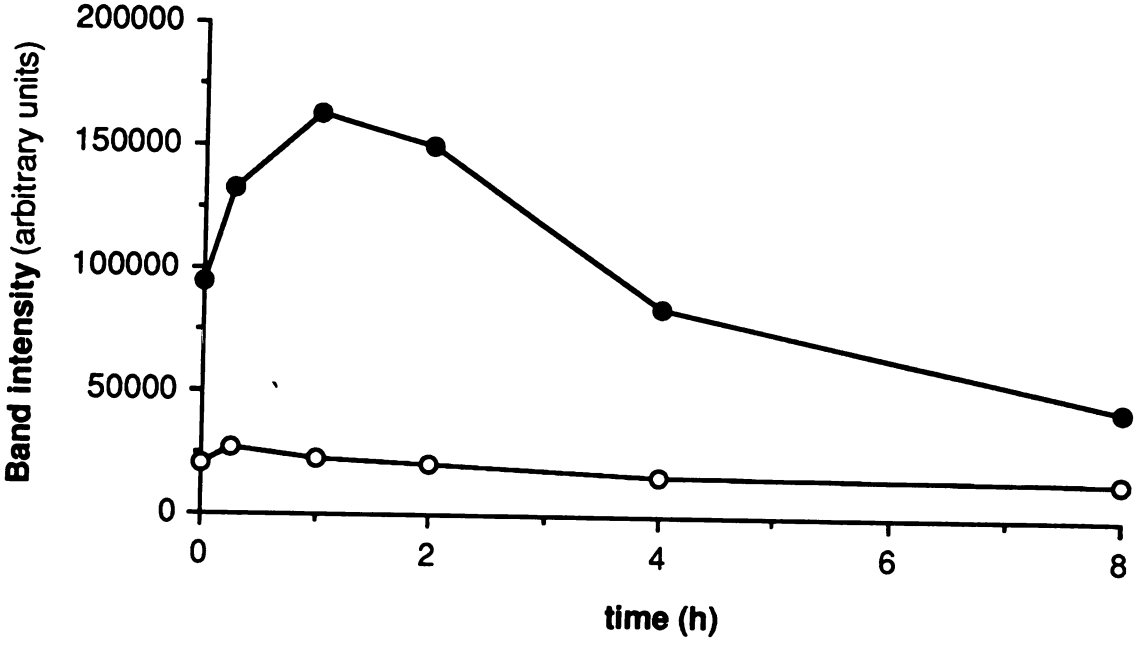
B.


50000
40000
30000
20000
10000
0
Band intensity (arbitrary units)
0
2
4
6
8
time (h)

C.


200000C
100000C
0
Band Intensity (arbitrary units)
0
2
4
6
8
time (h)

D.


200000
150000
100000
50000
0
Band Intensity (arbitrary units)
0
2
4
6
8
time (h)

Chapter 6. Conclusions

Summary and Discussion

The precise structure of the AChR oligomer is, at present, not known. While many features of the receptor are well-characterized, such as its pharmacology, electrophysiological properties, and biochemistry, little information about the molecular mechanisms by which the AChR carries out its various functions has been acquired. It is not known, for example, how ligand binding leads to channel opening or how prolonged exposure to ligand desensitizes the receptor and causes closure of the ion channel. In addition, assembly of the AChR depends upon the interaction of specific domains in the subunits, and yet we have identified neither the precise regions involved nor their structural interactions with the other subunits. Molecular genetics, biochemical analysis, and other approaches have provided insights into some important structural and functional features of the AChR. Precise mechanisms that are based on the solution of the structure of the AChR oligomer and its component subunits to a high-resolution are required to define the structure-function relationship for this well-studied membrane protein. These mechanisms would contribute not only to the study of the AChR, but also to the analysis of other ion channels, most especially (but not exclusively) the members of the family of ligand-gated ion channels. The best information about AChR structure would consist of X-ray crystallographic maps of the receptor in a variety of functional states. These maps are not yet available. Low resolution maps of the AChR oligomer have provided a framework within which we can examine the mechanism of AChR function (Unwin, 1989), but they leave a considerable amount of information out of reach.

One aspect of the structure of the AChR that has not been determined at even a low level of resolution is the transmembrane topology of the subunits of the receptor. Basic information about subunit topology derived from non-crystallographic methods can not only help direct investigations into AChR function, but could also provide important parameters in crystallographic analyses (see below). We have seen how two basic approaches to this problem have led to the proposal of structural models with from three to seven transmembrane domains. The experiments we have performed have attempted to elucidate the transmembrane topology of the subunits of the AChR. The results of our experiments are in agreement with the four transmembrane domain model of AChR subunit topology.

In Chapter 2, we described the system we chose to pursue the study of AChR subunit topology, the expression of receptor subunits from mammalian muscle *in vitro*, and we demonstrated that the subunits of the AChR synthesized in this fashion were in many respects similar to the native subunits. We used the mouse AChR subunits for two reasons. First, we have expressed the mouse AChR in non-muscle cells to investigate the assembly of the receptor in the absence of muscle-specific factors (Forsayeth *et al.*, 1990; Gu *et al.*, 1990; Gu *et al.*, 1991b; Yu and Hall, 1991; Verrall and Hall, 1992). It was therefore logical to pursue the assembly of the AChR at a further level of reduction. Some of the experiments described in Chapter 2 represent our efforts in this direction. Second, most previous topological information was derived from the analysis of the piscine AChR. We could therefore provide new information about the mammalian AChR which could be compared to the pre-existing data on the piscine AChR. On the

basis of our results, we conclude that the subunits of the *Torpedo* and mammalian AChRs are structurally similar.

The topology of the amino-terminal domain of the α subunit is the focus of the experiments described in Chapter 3 (Chavez and Hall, 1991). We used molecular genetics to introduce new glycosylation consensus sequences in the primary sequence of the α subunit to investigate the topological model of the region between α142 and α200 proposed by Criado *et al.* (1985a). Our experiments demonstrated not only that the region between α142 and α200 was translocated across the membrane, but also that a fragment of the α subunit that encodes the amino-terminal domain and includes the region in dispute is not a transmembrane protein. These results, therefore, argue against the model proposed by Criado and others, and support models in which the entire amino-terminal domain of the α subunit, and by analogy the other subunits as well, is translocated across the membrane and faces the extracellular space in the mature receptor.

The topology revealed by our use of novel glycosylation consensus sequences could possibly represent an intermediate stage in α subunit biosynthesis. For example, the M6-M7 domain may be inserted into the membrane during the integration of the α subunit into the ER. The interaction of the α subunit with either the γ or δ subunit may stabilize this structure. The failure of the α subunit to assemble with other subunits within a given time interval would result in the ejection of the thermodynamically unfavorable M6-M7 domain from the membrane, perhaps as a signal marking the unassembled α subunit for degradation. At this point, the site that we introduced at α154 would be post-translationally glycosylated, resulting in the intermediate form we

detected. Based on this model, one would predict that the α subunit expressed alone would have a shorter half-life than the α subunit co-expressed with other subunits. However, as we (Ch. 5) and others (Blount and Merlie, 1988; Blount and Merlie, 1990) have demonstrated, the α subunit in both these cases has an equivalent half-life (2 hr). Alternatively, the region between α142 and α200 could be initially translocated across the membrane, only to be reinserted into the ER membrane from the lumenal side upon assembly with other subunits. Therefore, we would have prevented this reinsertion from occuring by introducing a functional glycosylation site at residue α154. Insertion of a translocated polypeptide chain into the lipid bilayer from the lumenal side of the ER, however, has not been observed. Neither of these alternatives, therefore, satisfactorily reconciles the M6-M7 model and our results.

Several other observations support the model in which the amino-terminal domain is translocated completely (see Ch. 1 and Ch. 3). For example, the oligosaccharide analysis of the *Torpedo* δ subunit (Nomoto *et al.*, 1986) and mouse AChR (Gu and Hall, 1988a) suggests that the mouse δ subunit is glycosylated three times. One of these sites is in the δ subunit region that is homologous to the α142-α200 region (δ169; see Fig. 1-3). Glycosylation site deletion analysis could be used to determine whether the site at δ169 is glycosylated. Glycosylation at this site could be demonstrated by expressing a mutant δ subunit in which the δ169 consensus sequence was eliminated and comparing the mobility of this mutant with the mobility of the wild-type δ subunit. If the pattern of glycosylation were different, this result would demonstrate that not all subunits possess the M6-M7 topology. In addition, the other sites could be eliminated, creating a δ subunit with

only one site, δ169. For the α subunit, a more precise analysis of the identity of the residues involved in the BuTx-binding site and location of this site relative to the lipid bilayer could eliminate the M6-M7 model from consideration (Kubalek *et al.*, 1987; Dennis *et al.*, 1988; Johnson *et al.*, 1990). We are confident, however, that sufficient evidence exists to support our claim that the entire amino-terminal domain for each of the subunits is translocated across the ER membrane and appears on the extracellular face of the mature receptor.

We then discussed experiments designed to differentiate among the structural models of the carboxy-terminal half of the receptor subunits (Chapter 4) (Chavez and Hall, 1992). We introduced a fragment of the peptide hormone prolactin into several regions in the primary sequences of the α and δ subunits as an antigenic marker. We used the accessibility of the prolactin domain to proteolytic digestion as an indicator for the orientation of the site of prolactin insertion relative to the membrane, and found that M1, M2, M3, and M4 are true transmembrane domains and that the carboxy termini of the α and δ subunits are extracellular. The prolactin "tag" is large enough that its presence or absence can be verified by simple electrophoretic analysis. We chose the α and δ subunits for this analysis because while most of the evidence in support of the four transmembrane domain model was based on the analysis of the δ subunit, the other topological models found their support in analyses based primarily on the α subunit. Therefore, it was formally possible that, in spite of the considerable homologies shared by the subunits of the AChR, the topologies of the subunits could differ. Our results demonstrate that this is not the case.

Apart from the evidence discussed in Ch. 1 and presented in Ch. 4, an additional observation can be made that supports the extracellular localization of the carboxy terminus of the subunits of the AChR. Yu and Hall demonstrated that an ε subunit chimera, formed by the substitution of the entire region between M1 and M4 of the ε subunit with the homologous regions of the β subunit, replaced the ε subunit in the AChR oligomer when the chimera was expressed with other AChR subunits in the COS cell expression system (Yu and Hall, 1991). Since the amino-terminal domain is extracellular and the presence of both the amino- and carboxy-terminal domains of the ε subunit is required for the efficient incorporation of the chimeric ε subunit in the AChR oligomer, these results support a topological model in which the amino- and carboxy-terminal domains interact with each other on the same side of the membrane. Therefore, the carboxy terminus of the ε subunit must lie on the extracellular side of the membrane.

From the foregoing, it is clear that we reject the topological models based on epitope mapping experiments of the subunits of the AChR. The main objection to these experiments is the lack of appropriate controls for the epitope specificity of the antibodies at the concentrations utilized in these studies. The antibodies used in the majority of these experiments were not characterized completely. One assumption of these studies is that the antibodies are recognizing only short (7 to 10) contiguous amino acid sequences in the primary sequence. Structural analysis of crystallized antigen-antibody complexes, however, has revealed that epitopes consist of from 15 to 25 non-contiguous amino acids (Davies *et al.*, 1988; Laver *et al.*, 1990). Additional regions are thus likely to be involved in the binding of the

antibodies used in AChR structural analyses than just the epitope defined by such methods as synthetic peptide binding. When used at high concentrations, therefore, the antibodies used in the epitope mapping of the AChR probably recognize secondary low-affinity sites if their primary epitopes are not accessible in the native receptor. We suggest, therefore, that the epitopes recognized by these antibodies remain hidden within the native structure of the AChR and that the treatments used to disrupt the lipid bilayer either denature the receptor sufficiently to allow access of the antibody to the correct primary epitope or provide access to secondary epitopes which are recognized at the elevated concentrations of antibody used in these experiments. In at least two instances, further analysis of antibodies used to support alternative models of AChR subunit topology cast severe doubt on the use of antibodies in topological analysis (Blatt *et al.*, 1986; Das and Lindstrom, 1991), even when the disparity between the antibody concentration used and its affinity for the receptor was small (Blatt *et al.*, 1986).

It would appear, therefore, that the hydropathic analysis of the AChR subunits, as well as other transmembrane proteins, is useful in the prediction of the basic structure. On the other hand, it is true that models based solely on hydropathic analysis require strong experimental support for confirmation. In fact, in view of their high hydrophobicity, if any of the transmembrane domains (M1, M2, M3, or M4) are found not to span the membrane, one would have evidence to question seriously the utility of hydropathy plots as predictive tools for membrane protein structure. Even without these observations, however, our results and the data from the

variety of biochemical approaches discussed earlier combine to provide strong evidence in support of the four transmembrane domain model.

In Chapter 5, heterospecific AChR assembly in transiently transfected COS cells was used to investigate the assembly characteristics of the mouse and human α subunits when expressed with mouse non-α subunits. Exploiting the sequence differences between these two subunits, we identified two amino acids responsible for α_H subunit's increased efficiency of assembly when compared to the α_M subunit. While these residues, at positions α187 and α189, lie within the BuTx-binding site as identified by DDF binding (Dennis *et al.*, 1988), they do not affect the affinity of the AChR for toxin, and therefore are presumably involved in the conformational maturation of the α subunit. The expression of the modified mouse α subunit, α_MST, allowed us to study the kinetics of formation of the α_{Tx} species. We discovered a novel interaction between the δ subunit and the immature form of the α subunit, α_0, but we were unable to determine the degree to which this interaction affects the normal pathway of AChR assembly. Previous investigations into the differences between the mouse and rat ε subunits (Gu *et al.*, 1991a), taken together with the present work, demonstrate the advantages of the use of heterospecific assembly to study the mechanism of AChR subunit biosynthesis and assembly.

Future directions

Our investigations into the topology of the subunits of the mammalian AChR demonstrate the importance of alternate methods in the search to discover the transmembrane orientation of polytopic membrane proteins, and provide further evidence that cell-free expression systems can be useful

in this search. These expression systems have been widely used to study the mechanism of protein translocation and integration into the membrane of the ER, and therefore it is not surprising that they can be applied successfully to the problem of membrane protein topology. Further work is required, however, to realize the potential of these systems in the study of multisubunit assembly. At present, the association of the subunits of only one multimeric membrane protein complex has been described in an *in vitro* expression system (Sawyer and Doyle, 1990), but the assembly of this complex has not been definitively demonstrated. The assembly of the AChR in a cell-free expression system would be more difficult to achieve (see Ch. 2) yet relatively more easy to demonstrate, because of the information known about the pharmacology, biochemistry, and electrical properties of the receptor. For example, the binding of BuTx to AChRs expressed *in vitro* could be compared in the presence and absence of *d*-tubocurarine and carbamylcholine to demonstrate the ability of subunit complexes to bind small ligands. The size of complexes formed *in vitro* could be determined by sucrose gradient centrifugation. In addition, since it is possible to fuse microsomal vesicles with planar lipid bilayers (Simon and Blobel, 1991), it should be possible to determine whether ACh-gated channels can be created by *in vitro* translation of the receptor subunits. All of these approaches, however, require higher levels of subunit maturation and assembly than we have achieved in our preliminary investigations. It remains to be seen whether high-efficiency conversion of α_0 to α_{Tx} ($\geq$ 20%) can be observed *in vitro*, and whether intersubunit association in the RRL translation system can be improved. One possible approach involves the use of a different source of membranes for the integration of the subunits of the AChR. For example, Kobilka has demonstrated the maturation of the

human β-2 adrenergic receptor in a cell-free system consisting of RRL and microsomal membranes derived from *Xenopus* oocytes (Kobilka, 1990). The expression of mature β-2 adrenergic receptor, a polytopic membrane protein, depends on correct integration and disulfide bond formation. It should be possible to express the AChR subunits in this system as well.

Our identification of the transmembrane domains in a well-defined protein like the AChR can be used to help dissect the mechanism by which the topology of the receptor is achieved. We now know that the M1 and M3 domains have the same orientation within the membrane, while M2 and M4 are oriented in the opposite direction. How is this topology achieved? Molecular genetics approaches can be used to determine the topogenic functions of the different transmembrane domains (e.g., classical stop-transfer sequences or internal uncleaved signal sequences). Only minor modifications of the α subunit-prolactin chimeras described in Ch. 4 would be required to begin to investigate these questions.

It should also be possible to exploit our conclusions about the basic structure of the subunits to provide more detailed structural information. Since the entire amino-terminal domain of the α subunit is extracellular, it should be possible to use high-efficiency expression systems (e.g., bacterial expression, baculovirus, etc.) to produce quantities of this soluble domain sufficient for crystallographic studies. While our own studies suggest that the conformation of this fragment is different from its structure in the native protein (i.e., lack of glycosylation; Ch. 3), it seems possible that much of the core of this domain would not differ greatly from the native structure. These studies may provide useful information about the structure of this region and provide diffraction "fingerprints" to direct future

crystallographic efforts. When the crystallographic structure of the entire AChR is determined, we are confident that the subunits will possess the basic transmembrane topology predicted from the primary sequences whose characteristics have found strong support in the observations we have made.

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