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Topical Review

Synthesis and Assembly of Acetylcholine Receptor, a Multisubunit Membrane Glycoprotein

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Introduction

Ion channels and transport proteins are frequently composed of transmembrane oligomers (*see* Klingenberg, 1981, for a review). Some of these proteins, like the nicotinic acetylcholine receptor (AChR), are composed of two or more different subunits. In addition to having significant consequences for channel and transport protein function (Jardetzky, 1966; Singer, 1974; *see also* Singer, 1977), an oligomeric transmembrane structure raises interesting questions concerning the mechanisms of synthesis, assembly and intraorganellar transport of the subunits. For example: How is the synthesis of different subunits coordinated? In which organellar compartment(s) does assembly occur? How do post-translational modifications such as glycosylation, phosphorylation, fatty acylation, and disulfide bond formation contribute to the assembly process, and conversely, how does assembly affect such modifications? Because of the rapidly increasing wealth of structural information available for the AChR, studies of the biosynthesis of this protein are beginning to offer answers to these and related questions. In this review we would like to briefly summarize the relevant structural information (reviewed more completely elsewhere) (Changeux, Devillers-Thiery & Chemouilli, 1984; Popot & Changeux, 1984) and present our current ideas about receptor biosynthesis.

Structure of the Receptor Protein

The AChR of the electric organ of *Torpedo* rays is the best characterized of the nicotinic AChRs. It has been prepared in sufficient quantities for many types of structural and functional investigations, including: topological mapping with monoclonal antibodies, partial protein sequencing, reconstitution of ACh-responsive membranes, and image analysis by electron microscopy and X-ray diffraction (*see* Changeux et al., 1984; Popot & Changeux, 1984).

Analyses of the polypeptide composition by SDS polyacrylamide gel electrophoresis (Reynolds & Karlin, 1978; Lindstrom, Merlie & Yogeeswaran, 1979) and by cosequencing of the N-termini (Raftery, Hunkapiller, Strader & Hood, 1980) of the purified receptor suggested a subunit stoichiometry of $2\alpha \cdot 1\beta \cdot 1\gamma \cdot 1\delta$. Microsequence determination of the N-termini of the isolated subunits was additionally important in establishing that the four subunits are homologous and in preparing synthetic oligonucleotide probes for the identification of recombinant clones containing subunit cDNAs (Numa et al., 1983). Thus, the complete primary structure predicted from the sequences of four separate cDNAs confirms the existence of four distinct but highly homogous subunits, containing approximately 500 amino acids each. The homology relationships among the four suggest that all were derived from a common ancestral gene.

In the electron microscope, the AChR appears as a rosette of 80–90 Å in diameter (Changeux et al., 1984). Analyses of images with α-bungarotoxin (or derivatives of α-bungarotoxin) bound to receptor have indicated the presence of two toxin binding sites per rosette (reviewed in Changeux et al., 1984). Decoration with anti-α subunit Fab fragments has identified two α subunits per rosette (Fairclough et al., 1983). Since the α subunit contains all or part of the α-bungarotoxin binding site (Haggerty & Froehner, 1981; Tzartos & Changeux, 1983), the imaging data and the biochemical determinations of subunit stoichiometry are all consistent with a receptor oligomer composed of $2\alpha \cdot 1\beta \cdot 1\gamma \cdot 1\delta$ (Fig. 1).

Key Words synthesis · assembly · transmembrane protein · multisubunit · receptor · ion channel

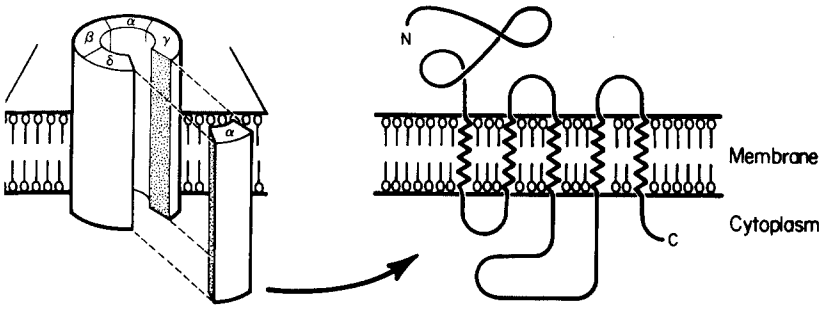

Fig. 1. These models depict a possible transmembrane orientation of a single subunit polypeptide chain, on the right, and on the left a representation of how the individual subunits may be organized into the "stave"-like walls of a cylinder. The positions of the individual subunits are, in fact, controversial (*see* Changeux et al., 1984; Popot & Changeux, 1984, for review)

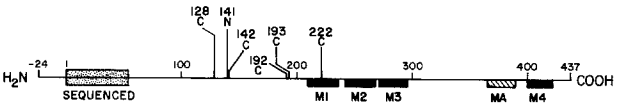

Fig. 2. This linear map of the amino acid sequence of an "archetypical" subunit shows the approximate locations of the predicted transmembrane segments, cysteines 128, 142, 192, and 193 and 222, and asparagine 141. The subunit cDNA sequences were identified by comparison with the NH_2-terminal amino acid sequence (stippled box), determined by Raftery et al. (1980)

Neubig, Krodel, Boyd and Cohen (1979) showed that membrane vesicles substantially stripped of all but α, β, γ and δ polypeptides were functional for ACh-regulated Na^+ flux, providing evidence that the four subunits are sufficient to assemble a functional channel. Reconstitution of functional membranes from affinity purified AChR and synthetic lipid mixtures supported this conclusion (Nelson, Anholt, Lindstrom & Montal, 1980). It is satisfying that the biochemical data are entirely consistent with the conclusions arrived at by recombinant DNA techniques. Mishina et al. (1985) (*see also* White, Mayne, Lester & Davidson, 1985) demonstrated that injection of the mRNAs derived from the four cloned subunit cDNAs was sufficient for the expression of an ACh-responsive ion channel on the surface of *Xenopus* oocytes. Furthermore, no functional AChR was detectable in oocytes injected with RNA preparations lacking one of the four mRNAs. One exception was the low but measurable sensitivity to ACh achieved in oocytes that were injected with α, β and γ mRNAs only. This may mean that other subunits can substitute, albeit poorly, for δ (Mishina et al., 1984; White et al., 1985).

The low resolution model (Fig. 1) which emerges from these varied structural analyses is that of a cylindrical protein complex, the walls of which are made up of five subunit staves, oriented perpendicular to the membrane plane. It is attractive to hypothesize that the central core of the cylinder is the hydrophilic ion channel whose opening is regulated by agonist binding to sites on each of the 2 α subunits (*see* Popot & Changeux, 1984; Changeux et al., 1984).

The topology of the individual receptor subunits with respect to the lipid bilayer is a complex issue which is being investigated at many levels. Because sealed membrane vesicles containing high concentrations of AChR were readily available, it was possible to determine that each subunit traversed the membrane and had large domains exposed at both the cytoplasmic and extracellular surfaces (Fig. 1, reviewed in Changeux et al., 1984). Analyses of the predicted primary amino acid sequences suggested that each subunit contained four hydrophobic α helical sequences (M1–M4, Fig. 2), which were of sufficient length to span the lipid bilayer. A postulated fifth α helical sequence (MA) has charged and hydrophobic residues aligned on opposite faces of the helix and, therefore, may form an interface between the hydrophobic environment of the interior of the protein (or lipid bilayer) and the hydrophilic wall of the ion channel (Finer-More & Stroud, 1984; Guy, 1984). Thus five hydrophobic or amphipathic α helical sequences located at homologous positions within the primary sequence of each subunit have been predicted.

Determination of which of the predicted transmembrane (M) segments actually cross the bilayer will depend upon topological mapping of several different domains. Although a systematic approach to this question has not been undertaken yet, several important facts are available. The NH_2-terminus of newly synthesized δ subunit was found to be sequestered within microsomal vesicles, suggesting an extracellular orientation (Anderson, Walter & Blobel, 1982). Since the NH_2-termini of native AChR subunits were inaccessible to antibodies prepared against synthetic peptides, it was concluded that the termini were buried within the core of the large extracellular domain (Ratnam & Lindstrom, 1984; Neumann, Gershoni, Fridkin & Fuchs, 1985). The COOH terminus of δ subunit (Young et al., 1985), residues β350–358 (Young et al., 1985) and γ360–377 (LaRochelle, Wray, Sealock & Froehner, 1985) have been localized by immunogold electron microscopy to the cytoplasmic side of the bilayer. Thus, some tentative conclusions may be drawn concerning the number and location of transmembrane regions. If the NH_2 and COOH ter-

Table. Effects of site directed-mutagenesis on AChR expression

Group name	Btx binding (fractional)	Permeability (fractional)	Immunoprecipitation $\alpha\ \beta\ \gamma\ \delta$
Wild type	1.0	1.0	++++
Point Mutations			
Glycosylation			
Asn 141	0	0	+++−
Cyst(e)ines			
Cys 222	0.8	1.1	++++
Cys 192 or 193	0.3 to 0.4[c]	0	++++
Cys 128 or 142	0	0	−+−−
Deletions[a]			
Membrane spanning			
M1 224–37	0	0	+++−
M2 249–57	0.2	0	++++
M3 279–84	0.1	0	++++
M4 409–20	0.2	0	++++
Cytoplasmic regions			
Seven from 316 to 371	0.6 to 1.0	0.3 to 1.4	++++
MA[b] 355–89	0.5	0.03	++++
MA Five from 366 to 389	0.3 to 0.4	0	++++
389–95	0.5	0.7	+++−
394–401	0.4[d]	0	+++−
C-terminus			
428–34	0.8	0.2	++++

[a] With the insertion of between 1 and 3 extraneous amino acids to the sequence.

[b] The membrane-spanning amphipathic helix (MA), as predicted by Guy, 1984; (*see also* Finer-Moore & Stroud, 1984) extends from 371 to 389.

[c] 10- to 30-fold lower affinity for the agonist, carbamylcholine, than control.

[d] Threefold higher affinity for carbamylcholine than control.

The effects of various mutations of α subunit mRNA on the expression of AChR in frog oocyte. These data are summarized from Mishina et al., 1985. Site-specific mutations were made in a cDNA clone of the α subunit. Wild type, or mutagenized cDNA clones were transcribed in vitro, and the mRNA injected, along with wild type, β, γ, and δ subunit mRNAs, into frog oocytes. Binding of ^{125}I-α bungarotoxin (Btx Binding) to oocyte extracts, the transmembrane current induced by ionophoretically applied ACh (Permeability), and the amount of each of the [^{35}S]methionine-labeled subunits which could be immunoprecipitated (Immunoprecipitation) were measured. The presence of wild-type amounts of a subunit is denoted by (+), diminished amounts by (−). Unfortunately, the antiserum used by Mishina et al. (1985) has not been described, and it is not known if it contains antibodies capable of binding to all unassembled subunits. If, for example, the antiserum is composed largely of anti α subunit MIR (*see* Tzartos, 1984) then other subunits will be precipitated only if they are assembled into the complex. Mutations were induced to alter the coding for a single amino acid residue (Point Mutations), or to delete long stretches of codons (Deletions). In the latter case, the method used for construction of mutations resulted in the insertion of from 1 to 3 extraneous codons.

mini of all subunits are oriented on opposite sides of the membrane, then there must be an odd number of total membrane-spanning (M) segments. Also, there must be an even number (or 0) of membrane segments between residues 350 and the COOH terminus so that both of these domains are on the cytoplasmic side. The minimum number of M segments which fit the current topological data is one; the maximum number for which models have been proposed is seven (Criado et al., 1985*a*,*b*). Clearly, more topological mapping is required.

The functional significance of membrane spanning (M) segments has been studied by site-directed mutagenesis. Deletion mutations have been studied in a *Xenopus* oocyte expression system which employed mRNA for wild type β, γ and δ and mutated α subunit (Mishina et al., 1985). The results of this study, summarized in the Table (*see also* Fig. 2), show that deletions within the large cytoplasmic domain surrounding amino acid 350 have little effect on receptor function, whereas deletions within any of the predicted M segments M1–M4 and MA dramatically reduce function. Two potential criticisms of the conclusions drawn from this work are that: (1) Some mutations may affect the biogenesis of receptor, and therefore, conclusions concerning function may not be relevant (*see below*); and (2) the deletions, in fact, were substitutions of a natu-

rally occurring amino acid sequence with a shorter extraneous amino acid sequence. Nevertheless, the results of mutational analyses support an important role for these hydrophobic and amphipathic helices.

Several other structural or functional characteristics have been localized to the large extracellular domain between the NH_2-terminus and the M1 segment of the subunit polypeptides (Fig. 2). One of these is a pair of cysteines at positions 128 and 142, which is highly conserved among all subunits thus far examined. From the analyses of effects of point mutations (replacing cys with ser) within the α subunit, Mishina et al.(1985) concluded that these residues formed a disulfide bond which was absolutely essential for the formation of α-bungarotoxin binding sites and a functional channel (*see* Table). It is important to note that a mutation of cys 222 was without effect, showing that not all cysteines are essential. Similarly, cys 192 and cys 193 (which are unique to α subunits) were identified as a possible disulfide-bonded pair. However, in this case, the effects of mutation were more subtle; α-bungarotoxin binding sites with 10- to 30-fold lower agonist binding affinity were formed, while channel function was completely blocked (Mishina et al., 1985; Fig. 2). Recently, cysteines 192 and 193 have been identified as the residues alkylated by the irreversible antagonist, 4-(-N-maleimidobenzyltrimethyl ammonium) (MBTA) (Kao et al., 1984). Therefore, the results of mutational analysis and chemical modification provide strong evidence that α cys 192 and 193 participate in a disulfide bond(s) located near the agonist/antagonist binding site on the extracellular side of the membrane.

From the amino acid sequence it was predicted that the N-terminal one-half of the each subunit contains one or more sites of N-glycosylation: one for α and β subunits, three sites for δ subunit and two of the four possible sites for γ subunit (Numa et al., 1983). All four subunits have been shown to be cotranslationally N-glycosylated (Anderson & Blobel, 1981). However, whereas the single oligosaccharide chains of the α and β subunits remain sensitive to endogylcosidase H (and are thus of the "high mannose" or "hybrid" type) even in the mature receptor (Merlie, Sebbane, Tzartos & Lindstrom, 1982), γ and δ may have at least one oligosaccharide which contains complex modifications (Lindstrom et al., 1979). An asn to asp mutation at position α 141, a site of N-glycosylation conserved among all subunits, resulted in the complete block in the formation of α-bungarotoxin binding sites or functional channels when expressed in *Xenopus* oocytes (Table; Mishina et al., 1984), thus identifying another critical post-translational modification.

Another conserved structural feature of the AChR is the main immunogenic region (MIR), a highly immunogenic group of determinants which has been defined with the aid of a panel of monoclonal antibodies (Tzartos & Lindstrom, 1980). This domain, which is outside the agonist binding site on an extracellular portion of the α subunit, though highly conserved among many species, is not known to participate in AChR function (Tzartos, 1984). However, the degree of immunologic conservation suggests that MIR may play an important role in the tertiary conformation, and possibly in interactions with the extracellular environment. Interestingly, MIR antibodies represent greater than 60% of the AChR antibodies in experimental autoimmune myasthenia gravis as well as in humans with this disease (Tzartos, 1984).

Although the structural information reviewed above has been derived, in large part, from studies of the *Torpedo* AChR, it is highly likely that it applies as well to mammalian muscle AChR. Microsequence determination has shown that the AChR from fetal bovine skeletal muscle is formed from four closely related subunits (Conti-Tronconi, Gotti, Hunkapiller & Raftery, 1982). Indeed, the complete nucleotide sequences of four subunit cDNA's from a fetal bovine muscle cDNA library have been reported (Noda et al., 1983; Takai et al., 1984; Tanabe et al., 1984; Kubo et al., 1985). Comparison of the predicted amino acid sequences as well as predicted secondary structures derived from the bovine subunit cDNA clones with those of the corresponding subunits of *Torpedo* indicates that the AChR subunits have been conserved throughout a long evolutionary period (*see,* for example, Kubo et al., 1985). Recently, an important new finding has emerged from the effort to clone and characterize the bovine muscle AChR subunits. A fifth subunit cDNA (ε) has been discovered (Takai et al., 1985), and although it is not known yet whether this cDNA sequence is normally expressed into a polypeptide, it is highly likely. The new subunit is most homologous with γ subunit and will substitute for *Torpedo* γ in the oocyte expression system. This report heralds an exciting new phase of discovery which promises to clarify the structural basis for different nicotinic pharmacologies (*see* Patrick & Heinemann, 1982; Barnard & Dolly, 1982, for a review).

Biosynthesis of AChR

The earliest events in the biogenesis of the AChR take place in the cell nucleus: transcription, RNA processing, and RNA export to the cytoplasm. At present very little is known concerning these processes beyond what can be inferred from the structure of subunit genes. The human muscle α and γ subunit genes (Noda et al., 1983; Shibahara et al.,

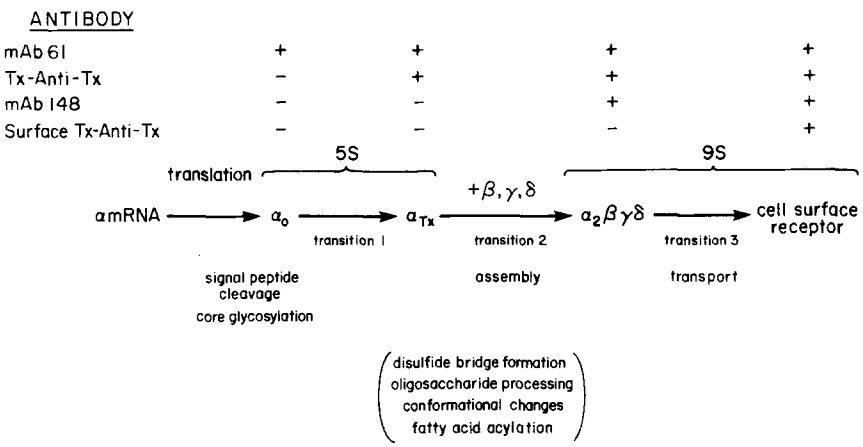

Fig. 3. Immunoprecipitation of α subunit forms. This model summarizes the conformational transitions and covalent modifications involved in the translation and processing of AChR subunits. Above the flow diagram is a summary of the reactivity of a panel of antibodies used to identify forms of the α subunit

1985) as well as the chicken muscle α (Klarsfeld & Changeux, 1985) γ and δ (Nef et al., 1984) have been characterized. These data indicate that primary transcripts will range from ~10 kB (γ and δ) to 30 kB (α) and include from 12 to 9 exons, respectively. Therefore, a significant amount of RNA processing is required for expression of a functional cytoplasmic mRNA.

Work in progress in several laboratories is directed toward elucidating the mechanisms involved in the alteration of transcriptional rates of AChR genes during muscle development and in response to muscle denervation or injury. The questions which are being explored currently are based largely upon observations of changes in the steady-state levels of receptor subunit mRNAs. Thus, studies of mRNA levels by "northern blot" hybridization have shown that the increase in AChR levels observed after denervation correlate well with the increase in steady-state levels of α and δ subunit mRNA (Merlie, Isenberg, Russell & Sanes, 1984; Klarsfeld & Changeux, 1985; Couvalt et al., 1986). More recently, preliminary experiments have demonstrated that the increase in AChR mRNA levels during development of muscle cells in tissue culture correlate well with increased rates of transcription of α and δ subunit genes (A. Buonanno and J.P. Merlie, *in preparation*). These studies represent an important beginning in our understanding of the mechanisms that regulate AChR expression. However, in order to provide a quantitative explanation of AChR regulation, the measurements of transcription rates and steady-state mRNA levels must be extended to all four subunits. Eventually, these approaches are expected to lead to an understanding of the regulation of transcription at the level of defining the interaction between gene-specific regulatory sequences and proteins which bind to them.

Studies of translational and post-translational events in AChR biogenesis, though far from complete, have been abundant. The overall view of these processes is that of a highly complex series of steps leading from polypeptide synthesis to assembly of a mature functional receptor complex and its insertion into the plasma membrane (Fig. 3). The detailed analysis of this pathway may provide useful information concerning the ways by which channel and transport proteins are regulated.

Anderson and Blobel (1981) demonstrated that total RNA fractions of *Torpedo* electric organ could be translated in a cell-free system to produce α, β, γ and δ subunit polypeptides. They demonstrated that initiator methionine was incorporated into each of the subunits and, thus, provided the first definitive evidence that the four subunits were the products of discrete mRNA's and were not the result of cleavage of a polyprotein. This important result immediately raised the question of how four independently synthesized subunits were brought together into a functional complex, a question whose answer remains incomplete. The intuitive appreciation of the efficiency of the cellular processes involved in AChR assembly was re-inforced by the finding that in these experiments, as in all cell-free translation experiments involving AChR (*see also* Sebbane et al., 1983; J.P. Merlie, *unpublished*), the polypeptide products were immunoprecipitated using antibodies prepared against SDS denatured subunits. In general, antisera or monoclonal antibodies (mAb's) prepared against native, nonionic detergent solubilized AChR do not recognize the cell-free translation products (*see* Fig. 3; Anderson & Blobel, 1981; Sebbane et al., 1983). The implication of this result is that receptor assembly requires additional processing of the newly synthesized subunit polypeptides, thereby piquing interest in post-translational modifications and the cellular processes involved.

Translation of AChR subunits occurs on membrane-bound polysomes (Merlie, Hofler & Sebanne, 1981). The techniques available for reconstituting functional rough endoplasmic reticulum in vitro have allowed many aspects of co-translational pro-

cessing of receptor subunits to be examined. Using cell free translation systems supplemented with dog pancreas microsomes (Anderson et al., 1982), it was possible to demonstrate that membrane-polysome assembly is mediated by an interaction between signal recognition particle (SRP) (Walter & Blobel, 1982), AChR synthesizing polysomes, and a receptor that is an integral membrane protein of the rough endoplasmic reticulum (Gilmore, Walter & Blobel, 1982; Meyer, Krause & Dobberstein, 1982). Furthermore, the nascent, membrane-inserted polypeptides were found to be covalently modified. The signal peptides were cleaved (Anderson et al., 1982) and N-linked oligosaccharides were added, one each in the case of α and β subunits and two or more in the case of γ and δ (Anderson & Blobel, 1983). Although the microsome-supplemented cell-free translation system appeared to mediate normal membrane insertion (Anderson et al., 1983), co-translational signal peptide cleavage, and glycosylation, it is significant that no receptor complexes appeared to be assembled and indeed no α subunits with α-bungarotoxin binding were formed (*see* Anderson & Blobel, 1983; Sebbane et al., 1983). As was found for translation without added microsomes, the cell-free products of the microsome-coupled system were immunoprecipitated only with antibodies reactive with denatured subunits.

ACh receptors are synthesized by muscle cells in tissue culture, including primary cultures of embryonic myoblasts from many different species, as well as several cell lines (Pearson, 1980). One of these, the mouse cell line BC3H-1, overproduces ACh receptor, and it has been used extensively for studies of biosynthesis. The availability of such a tissue culture system has made possible studies of subunit metabolism under a variety of experimental conditions. Pulse-chase experiments employing brief labeling periods with high specific activity [^{35}S]methionine, followed by detergent solubilization and immunoprecipitation, have demonstrated that the newly synthesized α subunit (like that synthesized in a cell free lysate) can be immunoprecipitated only by antibodies directed at the denatured structure of the polypeptide (Merlie & Sebbane, 1981). Neither main immunogenic region (MIR) antibodies nor α-bungarotoxin bind to newly synthesized α subunit (Fig. 3).

Not surprisingly, the newly synthesized subunits do acquire the ability to bind both α-bungarotoxin and MIR antibody within a short time after synthesis in vivo (Merlie & Sebbane, 1981; Merlie & Lindstrom, 1983). The process of acquisition of α-bungarotoxin binding has been the most extensively studied, while a careful comparison of the time course of the appearance of the many epitopes for which monoclonal antibody probes exist (Tzartos, 1984) has not been completed. Thus, the first discrete change (transition 1 in Fig. 3) which can be detected for α subunit by pulse-chase experiments is a conversion from a form immunoprecipitable only with mAb61 (anti-denatured α subunit) to a form which can bind α-bungarotoxin with high affinity and which is immunoprecipitable with anti-bungarotoxin.

Since transition 1 does not occur in a cell-free system, it has been suggested that it may involve a conformational change which is dependent upon a covalent modification (Merlie & Lindstrom, 1983). Evidence is accumulating to suggest that disulfide bond formation, which may not proceed efficiently in cell-free translation, is one such covalent modification. First, the subcellular site of acquisition of α-bungarotoxin binding by newly synthesized α subunit has been localized to the endoplasmic reticulum (M.M. Smith, J. Lindstrom and J.P. Merlie, *in preparation*). These experiments have been performed by the immunoprecipitation techniques, described above, combined with fractionation of cellular organelles after a pulse-chase labeling. Thus, in vivo, transition 1 occurs in the ER, even though this step does not proceed with rough ER reconstituted in vitro. These results implicate a modification which is normally carried out in the ER but which is not performed optimally by the cell-free preparation, characteristics consistent with what is known about disulfide formation (*see* Freedman, 1984). Second, as discussed above, mutations in the *Torpedo* α subunit 128 and 142 cysteines, when expressed in *Xenopus* oocytes with wild type β, γ and δ subunits, resulted in a complete block in AChR expression and absence of detectable toxin binding sites (Mishina et al., 1985). The final and most recent indication that disulfide bond formation may accompany transition 1 derives from experiments which show that the newly synthesized α subunit immunoprecipitated with mAb61 from BC3H-1 cell extracts resolves into two species on SDS polyacrylamide gels run under nonreducing conditions, even though it behaves as a single species when fully reduced. The distribution between the two unreduced species changes with time after pulse labeling, and only the more rapidly migrating of the two is immunoprecipitated with α-bungarotoxin (M.M. Smith, *unpublished*). Thus, a strong correlation exits between an alteration in migration in nonreducing SDS-polyacrylamide gels and ability to bind α-bungarotoxin. It remains to be conclusively demonstrated that the behavior of α on nonreducing SDS gels is due to differences in disulfide formation and whether such bonds are required before toxin binding is acquired.

Transition 1 does not occur if N-linked glycosylation is inhibited with tunicamycin (Merlie et al., 1982). This finding is consistent with the result of in vitro mutagenesis of the α subunit glycosylation site (Mishina et al., 1985; *see* Table). Inhibition of glycosylation has different effects on the biosynthesis of different proteins. For example, immunoglobulin M molecules are assembled normally in B cells treated with tunicamycin (*see* Carlin & Merlie (1986) for a discussion). A reasonable explanation of the tunicamycin effect on AChR biosynthesis (as well as on other glycoproteins which are affected) is that the protein conformation depends upon the presence of oligosaccharide. In some proteins the alteration in conformation due to absence of oligosaccharide may be severe and result in a defect in further processing. It is probably significant that the site of glycosylation on α subunit is position 141 (conserved on all subunits), between the 128 and 142 cysteines, mutations of which have similarly dramatic effects on the formation of α-bungarotoxin binding sites in the oocyte expression experiments. Although it seems clear that core N-glycosylation is required for transition 1 to proceed, this modification cannot be sufficient, since the microsome supplemented cell-free system is active in this regard. A reasonable hypothesis would be that both α 128-142 disulfide formation and N-glycosylation are required for the correct folding of the extracellular domain involved in α-bungarotoxin binding.

Newly synthesized subunits are subject to other covalent post-translational modifications. Olson, Glaser and Merlie (1984) have shown that ^{3}H-palmitate is covalently bound by an alkali resistant linkage to α and β subunits. Although the requirement for acylation is not fully understood, the effects of the inhibitor, cerulenin, suggest that inhibition of acylation may interfere with subunit assembly (Olson et al., 1984). Unfortunately, cerulenin has pleiotrophic effects (Omura, 1976; Schlesinger & Malfer, 1982), which make interpretation of this kind of experiment difficult. Site-directed mutagenesis may provide a powerful approach to this particular question.

Finally, oligosaccharide trimming and addition of some complex-type sugars are presumed to occur, based upon sugar analyses (Lindstrom et al., 1979) and binding to phytohemagglutinin agarose (Meunier, Sealock, Olsen & Changeux, 1974; confirmed for BC3H-1 AChR, J.P. Merlie, *unpublished*). At present little is known concerning the specific aspects of these modifications of AChR, or whether they are required for expression. Several inhibitors of oligosaccharide processing are currently available (Schwarz & Datema, 1984), which may help to determine the role of such modifications in AChR biogenesis.

The species which we call α_{TX}, the product of transition 1, was initially thought to represent mature AChR (Merlie & Sebbane, 1981). However, we discovered that this species, formed within 15–30 min after completion of the primary translation product α_o, was not co-immunoprecipitated with an anti-β subunit monoclonal antibody, whereas α subunit in mature AChR could be co-immunoprecipitated in this manner (Merlie & Lindstrom, 1983). Subsequently, we found that α_{TX} and mature AChR could be separated by velocity sedimentation, AChR having an *s* value of 9 and α_{TX} an *s* value of 5 (Merlie & Lindstrom, 1983). Combining velocity sedimentation analysis with pulse-chase immunoprecipitation experiments has permitted the characterization of the relative kinetics of transitions 1 and 2, Fig. 3. At time zero after a 5-min pulse of [^{35}S] methionine, most of the α subunit was in a 5*S* form, α_o, immunoprecipitable only with mAb61. By 15–30 min, a significant amount of 5*S* α_{TX} had been formed (transition 1, therefore, is not accompanied by a change in *s* value) along with some 9*S* AChR, and by 60–90 min α_{TX} had disappeared and formation of 9*S* AChR was maximal (Merlie & Lindstrom, 1983). This precursor product relationship was even more clearly evident when the experiment was repeated in primary cultures of embryonic rat muscle cells (Carlin et al., 1986*a*,*b*), in which the α_o and α_{TX} species appear to be more stable than those found in BC3H-1.

α_{TX} can be distinguished from α subunit in mature receptor by another criterion, small ligand binding. The binding of α-bungarotoxin to AChR can be blocked by small ligand agonists or antagonists (*see* Popot & Changeux, 1984; Changeux et al., 1984, for review). Suprisingly, the α_{TX} precursor, although it bound toxin specifically and with high affinity, was not at all blocked by curare or decamethonium, at concentrations of up to 10 mM (Carlin, Lawrence, Lindstrom & Merlie, 1986*a*). In pulse chase experiments, it was impossible to identify a 5*S* α subunit to which toxin binding was sensitive to curare or decamethonium inhibition; inhibition of toxin binding by such small ligands was unique to 9*S* mature AChR (Carlin et al., 1986*a*). Several laboratories have reported the reconstitution of both toxin and cholinergic ligand binding to SDS-denatured, purified α subunit (Haggerty & Froehner, 1981; Gershoni, Hawrot & Lentz, 1983; Oblas, Boyd & Singer, 1983; Tzartos & Changeux, 1983), a result in apparent contradiction to those just cited. However, these results taken together might argue that once α_{TX} has been processed and assembled in vivo into mature AChR, renaturation

of the ligand binding site in vitro does not require assembly with the other subunits. In any case, the binding properties of α_{TX} as well as other pre-receptor subunit complexes remain to be fully explored.

The process of subunit assembly (transition 2 in Fig. 3) has thus far been defined in two ways: immunoprecipitation of α and β subunit with a heterologous subunit specific antibody, or by a shift in sedimentation coefficient from 5*S* to 9*S*. These two independent assays give entirely consistent results. Thus, only the homologous subunit was immunoprecipitated from the 5*S* region of the gradient whereas both subunits were precipitated in constant proportions in the 9*S* region when either subunit specific antibody or α-bungarotoxin and anti-bungarotoxin were used (Merlie & Lindstrom, 1983; Carlin et al., 1986*a*,*b*). In fact, the assembly process may be more complicated than we have been able to observe, thus far. For example, it is possible that an ordered process of subunit assembly may take place with γ and δ being involved in the early intermediates. We have not been successful in detecting γ and δ subunits, probably because specific monoclonal antibodies which recognize mouse γ and δ subunits are rare and because γ and δ subunits are particularly prone to proteolysis during immunoprecipitation. Therefore, $\alpha\gamma$ and/or $\alpha\delta$ subunit dimers (as well as others) may form and escape detection. Such a possibility should be tested when adequate techniques for working with γ and δ subunits are developed. Since specific polypeptides corresponding to γ and δ have been detected by anti-bungarotoxin immunoprecipitation (Merlie & Sebbane, 1981), and *Torpedo* AChR is known to retain its sedimentation coefficient as well as some γ and δ subunit specific epitopes even after extensive endo-proteolytic nicking (Lindstrom, Gullick, Conti-Tronconi & Ellisman, 1980), we believe that the 9*S* complex observed in pulse-chase experiments represents the mature $\alpha_2\beta\gamma\delta$, AChR. Thus the kinetics of formation of the 9*S* complex represent formation of what we feel is the fully mature AChR, while the existence and rates of formation of heterodimer, trimer and other intermediates are not known.

The subcellular compartment in which assembly occurs has not been identified. However, other multisubunit membrane proteins for which this information is available assemble in the endoplasmic reticulum (*see* Carlin and Merlie (1986) for a discussion). Although we suggested that the long time course of assembly might indicate that transport to the golgi was required (Merlie et al., 1982), it is clear now that a significant fraction of the time required for assembly is, in fact, required for α_{TX} formation. Since α_{TX} formation occurs within the ER, assembly may also occur there. The solution to this question should be possible with the application of subcellular fractionation techniques.

Transport of newly synthesized AChR from the ER to the Golgi apparatus can be inferred from the fact that γ and/or δ subunits have complex type N-linked oligosaccharides, as discussed above. Such modifications are known to be carried out by enzymes resident within the Golgi (*see* Rothman (1985) for review). Furthermore, Fambrough and Devreotes (1978), employing autoradiographic methods, detected a population of α-bungarotoxin binding sites with properties of newly synthesized AChR's within the Golgi. Bursztajn and Fischbach (1984) have shown that coated vesicles are involved in transport of newly synthesized AChR to the cell surface, referred to as *transition 3* in Fig. 3. Thus, studies of AChR transport, are presently at a very early stage; the problem of how receptor transport is targeted to the plasma membrane is unexplored.

Examples of possible regulation of post-translational processing of AChR have been observed. Olson et al. (1983) showed that α subunit synthesis was stimulated when differentiated BC3H-1 cells were forced back into the cell cycle by refeeding with 20% fetal calf serum. However, AChR expression at the cell surface was completely blocked under these conditions. The dramatic decrease in surface AChR was accounted for by an inhibition of assembly of α subunits into mature receptor and an inhibition of transport of assembled AChR to the cell surface. In another example, primary cultures of embryonic chick myotubes, the level of AChR falls dramatically with the onset of spontaneous contractile activity (Shainberg et al., 1976; Birnbaum, Reis & Shainberg, 1980), and inhibition of activity with the sodium channel blocker, tetrodotoxin, results in an increase in AChR levels. Recently, analyses employing a cloned fragment of the chick α subunit gene to quantitate mRNA levels have shown that tetrodotoxin treatment of active chick myotubes resulted in a 17-fold increase in α subunit mRNA but only a twofold increase in AChR levels (Klarsfeld & Changeux, 1985). From these two examples, it is clear that regulation of α subunit mRNA levels and polypeptide synthesis do not adequately reflect the degree to which the level of the functional protein is altered. Finally, in a similar experimental system employing spontaneously active embryonic rat myotubes (Carlin et al., 1986*b*) observed by pulse-chase labeling that the relative efficiency of assembly of α_{TX} into a 9*S* complex increased after tetrodotoxin treatment. All of the above observations may be explained by one of two general mechanisms: (1) one of the post-translational processes involved in AChR expression is

modulated by cell cycle and/or contractile-electrical activity, or (2) the rate of β, γ or δ synthesis is not regulated coordinately with that of α subunit and that AChR assembly is limited by the availability of β, γ or δ subunit. Data on the mRNA levels and rates of synthesis of all four subunits under similar experimental conditions should help resolve these uncertainties.

In the near future several interesting issues concerning AChR biosynthesis should be resolved. A more quantitative evaluation of subunit gene transcription and mRNA levels should provide an idea of whether the coordinate synthesis of the four subunit polypeptides is determined by pre- or post-translational mechanisms. Information about regulatory sequences involved in transcription should provide insight into further work on developmental and activity linked control of gene expression. Continuing experiments to determine the requirement and the involvement of covalent post-translational modifications should become more definitive as progress is made in reconstituting the post-translational processes in vitro. And finally a major emphasis can be placed on the study of gross protein folding patterns, including the insertion across the membrane of the several transmembrane spanning segments, which accompany translation and transitions 1 and 2. All of these issues will rely heavily on application of recombinant DNA and monoclonal antibody techniques.

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