Mutational Analysis of Muscle Nicotinic Acetylcholine Receptor Subunit Assembly

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Abstract. The structural elements required for normal maturation and assembly of the nicotinic acetylcholine receptor α subunit were investigated by expression of mutated subunits in transfected fibroblasts. Normally, the wild-type α subunit acquires high affinity α bungarotoxin binding in a time-dependent manner; however, mutation of the 128 and/or 142 cysteines to either serine or alanine, as well as deletion of the entire 14 amino acids in this region abolished all detectable high affinity binding. Nonglycosylated subunits that had a serine to glycine mutation in the consensus sequence also did not efficiently attain high affinity binding to toxin. In contrast, mutation of the proline at position 136 to glycine or alanine, or a double mutation of the cysteines at position 192 and 193 to serines had no effect on the acquisition of high affinity toxin binding. These data suggest that a disulfide bridge between cysteines 128 and 142 and oligosaccharide addition at asparagine 141 are required for the normal maturation of α subunit as assayed by high affinity

toxin binding. The unassembled wild-type α subunit expressed in fibroblasts is normally degraded with a $t_{1/2}$ of 2 h; upon assembly with the δ subunit, the degradation rate slows significantly ($t_{1/2} > 13$ h). All mutated α subunits retained the capacity to assemble with a δ subunit coexpressed in fibroblasts; however, mutated α subunits that were not glycosylated or did not acquire high affinity toxin binding were rapidly degraded ($t_{1/2} = 20$ min to 2 h) regardless of whether or not they assembled with the δ subunit. Assembly and rapid degradation of nonglycosylated acetylcholine receptor (AChR) subunits and subunit complexes were also observed in tunicamycin-treated BC3H-1 cells, a mouse musclelike cell line that normally expresses functional AChR. Hence, rapid degradation may be one form of regulation assuring that only correctly processed and assembled subunits accumulate, and ultimately make functional receptors in AChR-expressing cells.

THE muscle-type acetylcholine receptor (AChR),[1] originally isolated from *Torpedo* electric organ, is the best characterized member of the family of ligand-gated ion channels and an ideal subject for studying the structural elements required for normal assembly and expression of multimeric proteins. The AChR is composed of four different but homologous subunits with multiple transmembrane domains (Noda et al., 1983) in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Karlin, 1980). The two α subunits are not juxtaposed, having one subunit between them on one side and two on the other (Wise et al., 1981; Kistler et al., 1982; Zingsheim et al., 1982; Bon et al., 1984). The analysis of predicted protein sequences derived from cDNA clones for each of the subunits of muscle AChR from several species (Noda et al., 1983; Mishina et al., 1986), and subunits cloned thus far for the neuronal nicotinic acetylcholine (Boulter et al., 1986; Goldman et al., 1987), GABA$_A$ (Schofield et al., 1987), and glycine (Grenningloh et al., 1987) receptors indicate at least two shared structural features that may lead to common posttranslational modifications

in this family of proteins. First, 2 cysteines, 14 amino acids apart, that occur at positions 128 and 142 in the AChR α subunit, are thought to form a disulfide bridge. Second, a conserved N-linked glycosylation site, located at asparagine 141 in the AChR α subunit, is just before the second of these cysteines.

Previous studies on the kinetics of maturation and assembly of the α subunit expressed in a muscle cell line, BC3H-1, suggested that glycosylation and a time-dependent posttranslational modification that confers high affinity α bungarotoxin (BTX) binding were required for the efficient assembly and subsequent expression of the AChR (Merlie et al., 1982; Merlie and Lindstrom, 1983). Here, we have generated by site-directed mutagenesis, and transfected into fibroblasts, several AChR α subunits that are not glycosylated and/or do not achieve a mature conformation. We present data consistent with the hypothesis that a disulfide bridge between cysteines 128 and 142 is required for attainment of a mature conformation of α subunit as assayed by BTX binding. In addition, by using the association of subunits in transfected fibroblasts as an assay for assembly, we show that mutated α subunits retain the capacity to assemble with δ subunit to

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

form $\alpha\delta$ complexes; however, relative to the wild-type $\alpha\delta$ complex, complexes made from mutated α subunits that were not glycosylated or did not acquire high affinity BTX binding were quickly degraded. Thus, rapid degradation of immature subunits and subunit complexes may be a mechanism by which incorrectly assembled receptor complexes are edited to preclude surface expression of nonfunctional AChR.

Materials and Methods

Cell Growth and Labeling

Growth conditions for BC3H-1 cells (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983) and QT-6 cells (Moscovici et al., 1977; Blount and Merlie, 1988) as well as transfection, selection and maintenance of QT-6 clones expressing α (Blount and Merlie, 1988), α and γ, and α and δ subunits (Blount and Merlie, 1989) of the mouse AChR have been described previously; all subsequent clones have been derived and maintained similarly. Pulse labeling with [^{35}S]methionine was performed at a final specific activity of ~6 Ci/mmol for long pulses or 800 Ci/mmol for 5-min pulses (Blount and Merlie, 1988). For cells that were chased, the media was removed subsequent to the pulse, and was replaced by conditioned media supplemented with 1-mM unlabeled methionine.

Construction of α Subunit Mutations and Molecular Techniques

All constructions are in M13 shuttle vectors (Blount and Merlie, 1988, 1989). Single-strand preparations were made and point mutations were induced (Burke and Olson, 1986) using mutating oligonucleotides with 10 bp flanking the mutating base(s). After transformation, M13 plaques were screened using differential hybridization of the mutating oligonucleotide. The oligonucleotides used to make the deletion mutants (128-142) and the 142C→A mutant had 18-19 bp flanking the deleted bases; these mutants were screened with oligonucleotides with 10 bp flanking the deletion. The normal sequence from 127 to 144 were changed to Tyr Asn Gly Ser Met and Tyr Ser Ser Ser Met in the glycosylated and not glycosylated deletion mutants, respectively. The 192 + 193 CC→SS mutation was made with an oligonucleotide with two mismatches. All other double mutants were made by sequential mutations. All mutations were confirmed by single stranded sequencing of the mutated area using an internal oligonucleotide primer and Sequenase obtained from United States Biochemical (Cleveland, OH). Replicative form preparations purified through two cesium chloride gradients were made from these constructions for calcium phosphate transfection. Transient transfections were harvested 2 d after transfection; stable clones were selected and screened for subunit expression (Blount and Merlie, 1988). The cell line expressing the β and δ subunits was stably transfected with the β, γ, and δ subunits; expression of the γ subunit has not been detected by coimmunoprecipitation of subunits or immunofluorescence.

Immunoprecipitations and Related Methods

Labeled cells were washed twice with PBS, 300 μM PMSF at 4°C, scraped from the plates, and pelleted by centrifugation for 10 s in a microfuge. The cell pellet from a single 10-cm dish was extracted with 1 ml PBS, 1-2% Triton X-100, 200 μM leupeptin, and 0.2 U/ml of α2 macroglobulin for 3-5 min, then the cells were centrifuged for 5 min in a microfuge and the supernatant collected. Immunoprecipitations were performed as previously described (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983) with modifications (Blount and Merlie, 1988). The antibodies used were the α subunit-specific monoclonal antibodies mAb61 (Tzartos et al., 1981) and mAb210 (Rathman et al., 1986), a rabbit polyclonal anti-BTX antibody used to precipitate α subunit that was prebound to BTX (toxin antitoxin) (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983), the β subunit-specific mAb148 (Gullick and Lindstrom, 1983), and the δ subunit-specific mAb88B (Froehner et al., 1983). After immunoprecipitation, *Staphylococcus aureus* pellets were resuspended in sample buffer and subjected to SDS-PAGE on a 10% acrylamide, 0.27% *N,N*-bis-methylene acrylamide gel and buffer system previously described (Laemmli, 1970). The gels were processed for fluorography; band intensity was proportional to radioactivity and exposure time (Laskey and Mills, 1975). The percentages of association between subunits were calculated by measuring the fluorogram intensities with a scanning densitometer (LKB Instruments, Inc., Gaithersburg, MD) and adjusting for immunoprecipitation efficiencies using ^{125}I-BTX-labeled BC3H-1 AChR as an internal standard (Blount and Merlie, 1988).

Binding Assay

The binding of ^{125}I-BTX to membranes prepared from fibroblast cells has been previously described (Blount and Merlie, 1988) with modifications (Blount and Merlie, 1989). To determine if mutant $\alpha\delta$ complexes contained carbamylcholine binding sites, an inhibition of the initial rate of ^{125}I-BTX binding assay (Blount and Merlie, 1988, 1989) was used.

Results

α Bungarotoxin Binding and Assembly of α Subunit Mutants

The α subunit of the AChR acquires high affinity BTX binding in a time-dependent manner before (Merlie and Lindstrom, 1983) and independent of (Blount and Merlie, 1988) assembly with other subunits; however, the covalent modification(s) required for the maturation of the α subunit is unknown. Merlie and Lindstrom (1983) speculated that the formation of a disulfide bridge may be the modification leading to a mature α subunit. Only four cysteines exist in the extracellular region of the AChR α subunit. Two cysteines that make a rare adjacent cysteine disulfide bridge (Kao et al., 1984) are located at position 192 and 193 of the muscle α subunit and are conserved in all α subunits of the muscle-type and neuronal nicotinic receptors (Fig. 1 *A*). Two additional cysteines are located at position 128 and 142 of the

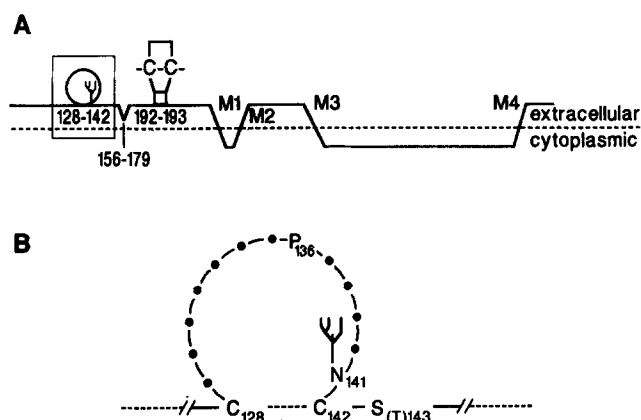*Figure 1.* Structure of the muscle nicotinic AChR α subunit. *A* shows some structural features of the α subunit. These include the four highly conserved membrane spanning domains labeled M1 to M4 and a putative loop structure formed by a disulfide bridge, depicted here as a circle, at amino acid positions 128-142. Also shown are the two cysteines located at position 192 and 193 that are unique to the α subunit; these cysteines are conserved in some subunit clones isolated for neuronal AChRs. An epitope between amino acid positions 156 and 179 has been mapped to the cytoplasmic side of the protein by antibody binding to *Torpedo* vesicles (Criado et al., 1985; Pedersen and Cohen, 1990a) and is depicted here as a "V" dipping into the cytoplasmic domain. The putative loop structure boxed in *A* is shown in greater detail in *B*. The proline approximately half way between the two cysteines and the glycosylation site just before the second cysteine are highly conserved among a family of ligand-gated channels.
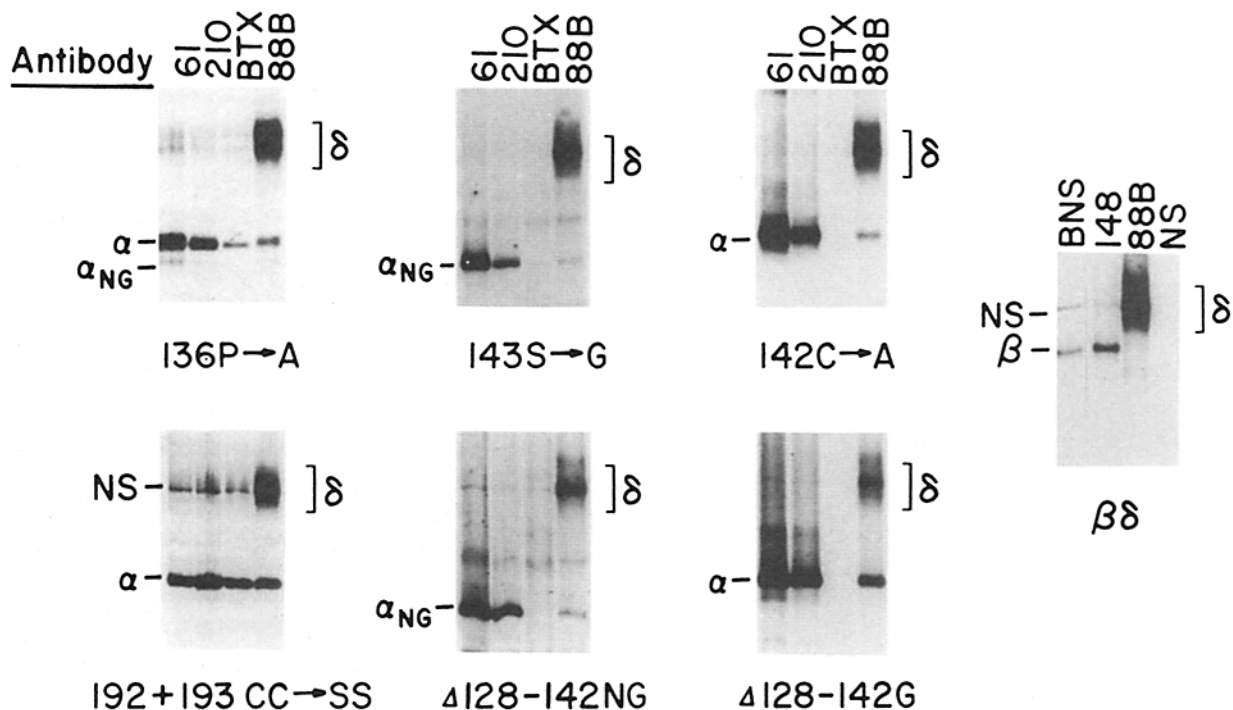
Antibody 61 210 BTX 88B   61 210 BTX 88B   61 210 BTX 88B   BNS 148 88B NS

]δ   ]δ   ]δ

α−   αNG−   α−
αNG−   NS−   β−   ]δ

136P→A   143S→G   142C→A

NS−   ]δ   ]δ   ]δ   βδ

α−   αNG−   α−

192+193 CC→SS   Δ128-142NG   Δ128-142G

Figure 2. Immunoprecipitation of AChR subunits from fibroblast cells expressing mutated α and wild-type δ subunits. Mutations were made as described in experimental procedures; stably transfected fibroblasts coexpressing mutated α and wild-type δ subunits of the AChR were labeled with [^{35}S]methionine for 1 h, harvested, and extracted as described in Materials and Methods. The mutated α subunits were immunoprecipitated with the α-specific monoclonal antibodies mAb61 and mAb210. Toxin antitoxin was used to immunoprecipitate α subunit that had acquired high affinity BTX binding (α$_{Tx}$). The δ subunit specific monoclonal antibody mAb88B was used to immunoprecipitate δ subunit, and α subunit associated with δ subunit. Precipitates were analyzed by SDS-PAGE and the fluorograms are shown. Amino acid changes resulting from the mutations are designated by position, wild-type amino acid→mutant amino acid; each panel is labeled at the bottom. The δ subunit is marked by a bracket because of its heterogeneity in SDS-PAGE, and the α subunit is labeled with a tick. As seen in *136P→A*, δ subunit is sometimes observed as a doublet. We suspect this to be a partially glycosylated state of the δ subunit; note the previously observed (Blount and Merlie, 1988) nonglycosylated α subunit in the same panel. The ratio of α precipitated by BTX to total α varied between experiments; no consistent difference was observed in the efficiency of α$_{Tx}$ formation between the wild-type α subunit, 136P→(G or A), or 192 + 193 CC→SS. Sequences for the deletion mutants Δ128-142, glycosylated and not glycosylated, are presented in Materials and Methods. βδ shows an immunoprecipitation of a cell line expressing only the β, or expressing the β and δ (βδ) AChR subunits. The lane *BNS* shows immunoprecipitation of a cell line expressing only the β subunit with the β-specific mAb148. The βδ cell line was immunoprecipitated with mAb148 and mAb88B; the lane *NS* is an immunoprecipitation where only the second antibody for mAb88B and *S. aureus* were added. The mutated α, the wild-type δ, and the nonglycosylated mutated α subunits (α$_{NG}$) are labeled. Note that because the bands observed in the BNS lane are derived from a cell line that does not produce the δ subunit, the band comigrating with δ in the BNS and 148 lanes must be a nonspecific protein. This discrete nonspecific band has been previously observed (Blount and Merlie, 1989; Blount et al., 1990), and is also labeled in the 192 + 193 CC→SS mutant.

α subunit, and are conserved in all ligand-gated channel subunits cloned thus far with the exception of glutamate receptor (Hollmann et al., 1989). These two cysteines are thought to form a disulfide bridge with a proline approximately midway, and an N-linked glycosylation site one amino acid before the second cysteine (Fig. 1, *A* and *B*). To determine if these conserved elements are required for the normal maturation of the AChR α subunit, a series of mutations were made in the α subunit by site directed mutagenesis. Expression vectors encoding mutated proteins were transiently or stably transfected into QT-6 fibroblasts. These cells were then metabolically labeled for 1 h with [^{35}S]methionine, harvested, immunoprecipitated, analyzed by SDS-PAGE, and visualized by fluorography. To assay for high affinity BTX binding, an antitoxin antibody was used to immunoprecipitate BTX prebound to α subunit (toxin antitoxin) (Merlie and Sebbane, 1982; Merlie and Lindstrom, 1983). As shown

in Fig. 2, mutation of the proline at position 136 to alanine or mutation of the cysteines at positions 192 and 193 to serines had no detectable effect on the expression of α subunits acquiring high affinity BTX binding in this assay, suggesting that the posttranslational modification leading to the formation of a high affinity BTX binding site is not dependent upon the 136 proline and cannot be the disulfide bridging of one or both of the cysteines located at 192 or 193. In contrast, high affinity BTX binding was significantly reduced when the glycosylation consensus sequence was mutated (143S→ G), and no BTX binding was detected when cysteine at position 142 was changed to alanine. Deletion of the amino acids between position 128 and 142 in the α subunit resulted in no detectable high affinity BTX binding whether or not the glycosylation site was preserved.

From results of several mutations reviewed in Table I, some generalizations can be made. First, glycosylation is

Table I. The Effect of Amino Acid Mutation and Deletion on the Ability of the α Subunit to Bind BTX and Associate with δ Subunit

Mutation*	BTX binding‡	α/δ association§
Wild type	+(6/6)	+(2/2)
192 + 193 CC→SS	+(7/7)	+(2/2)
Glycosylation		
143S→G	−(7/8)‖	+(5/5)
143S→G + 148T→N	−	+
Loop mutations		
Cysteines		
128C→S	−(3/3)	+
128C→A	−(5/5)	+(4/4)
142C→S	−(3/3)	+
142C→A	−(5/5)	+(1/1)
128C→S + 142C→S	−(6/6)	+(3/3)
Proline		
136P→G	+(10/10)	+(6/6)
136P→A	+(5/5)	+(3/3)
Deletions		
Δ128–142 glycosylated	−(6/6)	+(5/5)
Δ128–142 not glycosylated	−(3/3)	+(2/2)

* Mutations were made as described in Materials and Methods. Amino acid changes resulting from these mutations are designated by position, wild-type amino acid→mutant amino acid. The sequence of the deletion mutants are presented in Materials and Methods.
‡ High affinity BTX binding was assayed by toxin antitoxin immunoprecipitation of extracts from cells pulse labeled with [^{35}S]methionine for 1 h. Precipitates were analyzed by SDS-PAGE and fluorography as in Fig. 1 and densitometric scanning. In some cases results were confirmed with a ^{125}I-BTX binding assay previously described (Blount and Merlie, 1988). Positive results (+) ranged from ∼15 to 40% of total α subunit with signals 3–40-fold above background determined by nonspecific immunoprecipitation. Negative results were less than or equal to background. All results were originally observed in transient transfections. Where applicable, the number of clones expressing the mutation showing the same properties/the total number of clones expressing the mutation are shown in brackets.
§ Association of α and δ subunits was assayed by coimmunoprecipitation of the α subunit by the δ subunit specific mAb88B from extracts of cells pulse labeled with [^{35}S]methionine for 1 h. Precipitates were analyzed by fluorography and densitometric scanning. Positive results (+) ranged from ∼7 to 35% of total α subunit. All results were originally observed in transient transfections of the mutant α subunits into a QT-6 clonal cell line transfected with and stably expressing the δ subunit. Stably transfected cell lines expressing the mutant α subunits and the wild-type δ subunit were made by cotransfection of the two constructs and selection and screening of clones as described in Materials and Methods. Where applicable, the number of clones expressing the mutation showing the same properties/the total number of clones expressing the mutation are shown in brackets.
‖ In one high expressing stably transfected clone expressing this mutation, a long fluorographic exposure suggested that <2% of the α subunit was precipitated with toxin antitoxin as determined by densimetric scanning.

necessary for efficient acquisition of BTX binding; however, in long exposures of fluorograms from high expressing clones, ∼2% of the nonglycosylated α subunit made was detected using toxin antitoxin suggesting that glycosylation is not an absolute requirement. A mutation that reconstituted a glycosylation consensus sequence seven amino acids downstream from the normal glycosylation site (143S→G + 148T→N) resulted in an α subunit with electrophoretic migration identical to that of the glycosylated form, but did not restore the efficiency of acquisition of BTX binding; thus, the requirement for glycosylation is site specific. Second, cysteines at positions 128 and 142, but not at 192 and 193, are required for acquisition of high affinity BTX binding. Deletion of the amino acids between 128 and 142 (Δ 128–142, glycosylated or not glycosylated) confirmed that preservation of secondary structure in this region is a requirement for normal expression of high affinity BTX binding. Finally, mutation of proline at position 136 had no effect, suggesting that this amino acid is not critical for acquisition of high affinity binding of toxin. All of these data are consistent with the hypothesis that a disulfide bridge between cysteines 128 and 142 and glycosylation at asparagine 141 are required for the normal maturation of α subunit of the AChR.

Previously, we demonstrated that membranes derived from stably transfected fibroblasts expressing the wild-type α subunit had a K_{app} of 9.5 × 10^{-11} M for ^{125}I-BTX in a 22-h binding assay (Blount and Merlie, 1988). In an attempt to determine the extent of alteration of the binding properties of α subunit mutants, we measured the apparent affinity of ^{125}I-BTX to membrane preparations from some of the fibroblast cell lines expressing mutant α subunits. The 192 + 193 CC→SS mutant had a K_{app} for ^{125}I-BTX binding of 9.2 × 10^{-10} M, an affinity approximately 10-fold less than the wild type. However, no specific binding was observed for the 128C→S + 142C→S mutant in this assay at concentrations of ^{125}I-BTX up to 1 × 10^{-8} M. Thus, mutations of the cysteines at position 128 and 142 had dramatic effects (>1,000-fold) on affinity of BTX for AChR α subunit. In contrast, mutations of cysteines at position 192 and 193 reduced affinity of BTX by 10-fold.

As an assay for assembly, shuttle vector constructions encoding mutant α subunits were transiently transfected into, and expressed in a fibroblast cell line stably expressing only wild-type δ subunit; alternatively, stably transfected fibroblasts expressing both α and δ subunits were isolated. The coimmunoprecipitation of subunits using subunit specific antibodies has been shown to be a sensitive and specific assay for assembly of these subunits (Blount and Merlie, 1990). However, the δ subunit is often difficult to detect because it migrates on SDS-PAGE as a heterogeneous band, comigrates with a nonspecifically precipitated protein, and is extremely sensitive to proteolysis. Therefore, the coimmunoprecipitation of α with δ specific mAb88B (Froehner et al., 1983), a more sensitive and reliable assay than coimmunoprecipitation of δ with α specific antibodies, was used to routinely detect α/δ association. One caveat to these experiments is that high levels of expression or nonstoichiometric expression of subunits in transfected fibroblasts may lead to nonspecific subunit association. However, characterization of several clones expressing varying stoichiometries of α and δ subunits as well as consistent observations made in transiently transfected fibroblasts decreases the likelihood of artifactual subunit association. Although many of the mutated α subunits did not achieve a mature conformation as assayed by their ability to bind BTX with high affinity, they all assembled with δ subunit in a coimmunoprecipitation assay (Fig. 2 and Table I). Coimmunoprecipitation of subunits was also tested in a cell line stably coexpressing the β and δ subunits (Fig. 2). Although the β subunit shares 40% identity with α subunit, and shares many of the same secondary structural features, β did not efficiently assemble with the δ subunit. These data suggest that assembly of β with δ subunit may be a late event in normal assembly of AChR subunits. Hence, we conclude that structural domains present in α that are required for efficient assembly with δ subunit are not present in the homologous β subunit. Furthermore, we suggest that although glycosylation and a disulfide bridge between cysteines 128 and 142 are required for normal maturation of
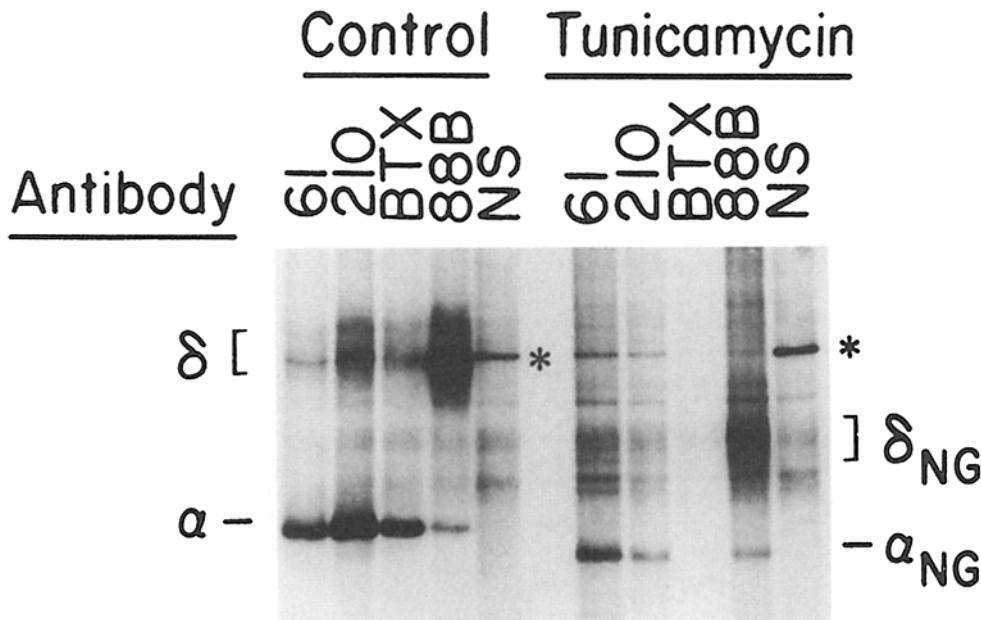
Control Tunicamycin

Antibody

Figure 3. Association of α and δ subunits expressed in tunicamycin-treated fibroblasts. Confluent 60-mm dishes of the αδ cell line were incubated in the absence (Control) or presence of 1.5 μg/ml Tunicamycin for 10 h. The cells were labeled with [³⁵S]methionine for 1 h, and then harvested and extracted as described in Materials and Methods. The α subunit was immunoprecipitated with the α-specific monoclonal antibodies mAb61 and mAb210. Toxin antitoxin was used to immunoprecipitate α subunit that had acquired high affinity BTX binding ($α_{TX}$). The δ subunit-specific monoclonal antibody mAb88B was used to immunoprecipitate δ subunit, and α subunit associated with the δ subunit. Precipitates were analyzed by SDS-PAGE and the fluorograms are shown. The lane NS is an immunoprecipitation where only the second antibody and S. aureus were added. Note the nonspecific band labeled with asterisks. The wild-type α and δ, and the nonglycosylated α and δ ($α_{NG}$ and $δ_{NG}$) subunits are labeled.

α subunit, they are not necessary for the assembly of α and δ subunits.

The αδ and αγ complexes form high affinity binding sites for small agonists and competitive antagonists that do not bind the unassembled α subunit (Blount and Merlie, 1989). In addition, a recent affinity labeling study suggests that the binding site for these ligands may be at the interface of αγ and αδ subunits (Pedersen and Cohen, 1990b). However, the structural requirements for the formation of this binding site are not known. Therefore, to determine if the 192 and 193 cysteines or the proline at position 136 were requirements for the formation of an agonist binding site, we tested the ability of a high concentration (1 mM) of carbamylcholine, a small agonist, to inhibit ¹²⁵I-BTX binding in membranes prepared from clones expressing mutant α and wild-type δ subunits. These studies demonstrated that in fibroblasts coexpressing the 192 + 193 CC→SS or the 136P→A mutant with δ subunit greater than 50% of the normal ¹²⁵I-BTX binding could be inhibited by carbamylcholine, a value similar to clones co-expressing the wild-type α and γ or α and δ subunits (Blount and Merlie, 1989); many of the ¹²⁵I-BTX binding sites that were not inhibited by carbamylcholine are presumably unassembled α subunits that bind ¹²⁵I-BTX but not carbamylcholine (Blount and Merlie, 1988, 1989). Therefore, the cysteines at positions 192 and 193 and the proline at position 136 in the AChR α subunit are not critical for formation of a carbamylcholine binding site.

Assembly of Nonglycosylated α and δ Subunits in Tunicamycin-treated Fibroblasts

Experiments using the N-linked glycosylation inhibitor, tunicamycin, suggested that glycosylation of subunits was important for normal maturation and assembly of mouse AChR subunits in BC3H-1 cells (Merlie et al., 1982). However, as seen in Fig. 2, a mutation that inhibits glycosyla-

tion in the α subunit did not prevent assembly of α with δ subunit in stably transfected fibroblasts. To determine if tunicamycin was preventing assembly of AChR subunits by a mechanism independent of inhibition of α subunit glycosylation, we incubated a wild-type αδ fibroblast cell line (Blount and Merlie, 1989, 1990) in the presence or absence of tunicamycin, pulse labeled the cells for 1 h with [³⁵S]methionine, and immunoprecipitated with subunit specific antibodies. As seen in the fluorogram presented in Fig. 3, tunicamycin treatment of αδ cells increased the mobility of α and δ subunits on SDS-PAGE and substantially decreased the amount of α subunit precipitated by toxin antitoxin, but did not prevent the assembly of α subunit with δ. These data are consistent with the characterization of the α subunit glycosylation mutation presented in Fig. 2, but are in apparent contradiction with the data obtained in BC3H-1 cells (Merlie et al., 1982).

Assembly and Degradation of Normal and Nonglycosylated AChR Subunits in Untreated and Tunicamycin-treated BC3H-1 Cells

The half-life for degradation of unassembled AChR subunits in BC3H-1 cells has been determined to be only 15-30 min (Merlie and Lindstrom, 1983). Therefore, it seemed possible that nonglycosylated subunits expressed in tunicamycin-treated BC3H-1 cells did assemble, but that assembled products were degraded as rapidly as unassembled subunits. In an attempt to detect subunits that assembled but degraded rapidly, we repeated the study of tunicamycin treated BC3H-1 cells using very short [³⁵S]methionine pulse-labeling and chase times. In this experiment, coimmunoprecipitation of α with either β or δ subunit specific antibodies (not available for the previous study) was used as an assay for assembly. BC3H-1 cells were pulse labeled with [³⁵S]methionine for

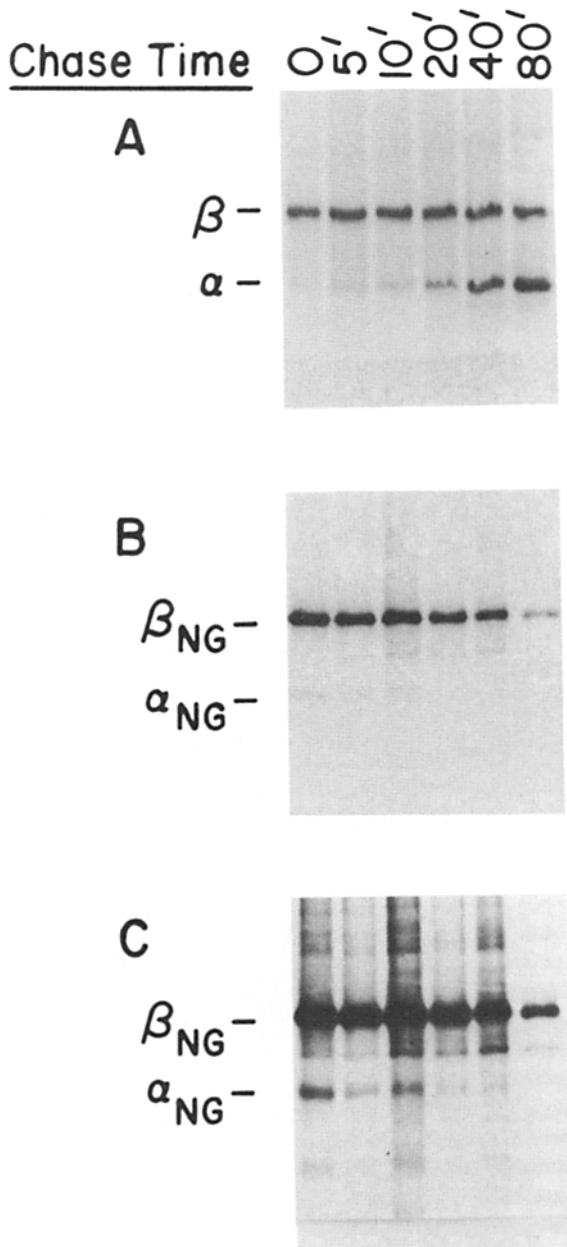the first 80 min as assayed by the coimmunoprecipitation of α subunit by a β subunit-specific antibody. After a 5-min pulse only a small amount of α is assembled with β subunit and the amount of assembly increases with increasing chase times. In contrast, in tunicamycin-treated BC3H-1 cells, no increase of assembled nonglycosylated α subunit was observed, and the nonglycosylated β subunit was quickly degraded with a $t_{1/2}$ of ~43 min (Fig. 4 B). However, in long fluorographic exposures, a small amount of assembly was detected in tunicamycin-treated cells (Fig. 4 C). Nonglycosylated but assembled α subunit was degraded with a $t_{1/2}$ of ~16 min, essentially the same as the unassembled, nonglycosylated α subunit (Table II). Although we obtained similar results in this experiment using coimmunoprecipitation of α with δ subunit-specific antibody, the efficiency of the β subunit-specific antibody, mAb148 (Gullick and Lindstrom, 1983), and the resistance of the β subunit to proteolysis made the coimmunoprecipitation of α with the β subunit

Figure 4. Kinetics of assembly and/or degradation of subunits in normal and tunicamycin-treated BC3H-1 cells. Confluent 10-cm dishes of the BC3H-1 cell line were incubated in the absence (A) or presence of 1.5 μg/ml tunicamycin (B and C) for 10 h. The cells were labeled with [³⁵S]methionine for 5 min and chased with conditioned media supplemented with 1 mM methionine for the times (in minutes) indicated (*Chase Time*). The cells were then harvested and extracted as described in Materials and Methods. The β subunit was immunoprecipitated with the β-specific monoclonal antibody mAb148. The precipitates were analyzed by SDS-PAGE, and the fluorograms are shown. A is a 6-d fluorographic exposure; B and C are from the identical gel, 5- and 27-d fluorographic exposures, respectively. The β, the nonglycosylated β (β_NG), and the coimmunoprecipitating α and nonglycosylated α subunit (α_NG) are labeled.

only 5 min; some cells were then harvested while others were chased with conditioned media for increasing times before harvesting. Fig. 4 A shows a fluorogram from untreated BC3H-1 cells showing the normal course of assembly over

Table II. Half-Life for Degradation of Unassembled and Assembled Subunits

Subunit	Half-life for degradation	
In BC3H-1 Cells*		
Unassembled		
α subunit	15–30 min	
Surface AChR	8 h	
Nonglycosylated		
unassembled		
α subunit	13 min	
Nonglycosylated		
α assembled with		
β subunit	16 min	

Subunit	Unassembled	Assembled with δ
In transfected fibroblasts		
Wild-type δ subunit	2 h	Not applicable
Wild-type α subunit	2 h	> 13 h
192 + 193 CC→SS		
α subunit	ND	> 13 h
136P→G α subunit	ND	> 13 h
143S→G α subunit	17 min	22 min
128C→S+ 142C→S		
α subunit	1.2 h	1.0 h
142C→A α subunit	2.0 h	1.8 h
Δ128-142 glycosylated		
α subunit	1.5 h	1.7 h

All half-lives for degradation were calculated from densitometric scans of appropriate fluorographic exposures from pulse-chase labeling experiments. The half-life for degradation of unassembled wild-type α subunit in transfected fibroblasts was previously published (Blount and Merlie, 1988) and has been confirmed in three independent clones. The half-life for degradation of wild-type δ subunit was obtained from a cell line producing only the δ subunit. Mutations were made by site-directed mutagenesis as described in Materials and Methods. Amino acid changes resulting from these mutations are designated by position, wild-type amino acid→mutant amino acid. The half-life for degradation of unassembled α was calculated from densitometric scans of fluorograms of α subunit pulse-chase labeled and immunoprecipitated with mAb61; ND, not determined. The half-life for degradation of the unassembled 128C→S + 142C→S α subunit was not significantly different between a clone producing both the mutated α and δ subunit and a clone producing only this mutated α subunit. The half-lives for degradation of wild-type and mutated α subunits assembled with δ were calculated from densitometric scans of fluorographs with α coimmunoprecipitated with the δ-specific antibody mAb88B.
* The half-lives for degradation of unassembled α subunit (Merlie and Lindstrom, 1983) and surface AChR (Patrick et al., 1977) in BC3H-1 cells were previously published. The half-lives for degradation of the nonglycosylated α subunit in tunicamycin-treated BC3H-1 cells were calculated from densitometric scans of appropriate fluorographic exposures from the experiment shown in Fig. 4.
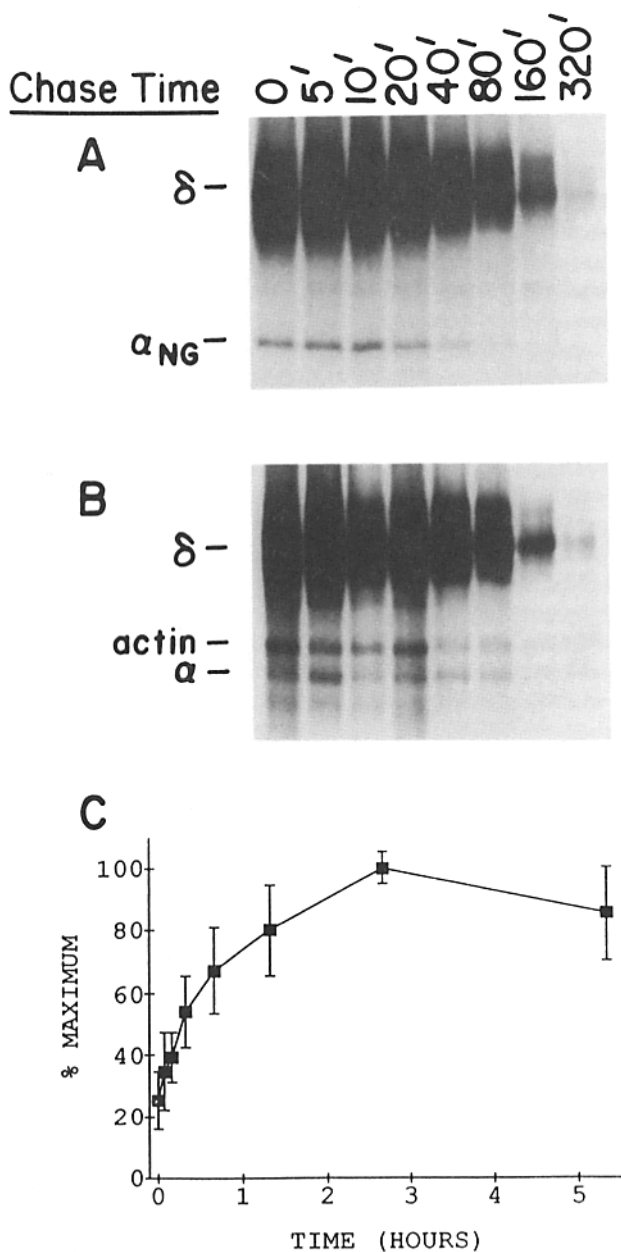
Chase Time

A

δ —

α NG —

B

δ —

actin —
α —

C

Figure 5. Kinetics of assembly and degradation of subunits in mutated and wild-type α/δ cell lines. Confluent 10-cm dishes of cell lines expressing mutated or wild-type α and δ subunits were labeled with [³⁵S]methionine for 5 min and chased with conditioned media supplemented with 1 mM methionine for the times indicated (*Chase Time* in minutes, and *TIME (HOURS)* in *C*). The cells were then harvested and extracted as described in Materials and Methods. The δ subunit was immunoprecipitated with the δ-specific monoclonal antibody mAb88B and the precipitates were analyzed by SDS-PAGE. Fluorograms are shown in *A* and *B*. *A* shows an experiment from a cell line coexpressing 143S→G α and δ subunit, and *B* is from a cell line coexpressing 128C→S + 142C→S α and δ subunit. The δ and the coimmunoprecipitating nonglycosylated α (α$_{NG}$) and mutated α subunit are labeled. *C* shows quantitative analysis, by densitometric scanning, of assembled α in a cell line coexpressing wild-type α and δ subunits. The error bars in *C* are SEM of three independent experiments; data for each experiment were normalized to the maximum assembled α subunit.

a more sensitive assay. In addition, data demonstrating that α and β subunits associate only inefficiently in transfected fibroblasts (Blount and Merlie, 1989) suggests that the association of these subunits may be a late event in assembly, and perhaps, a better assay for more complete and normal AChR assembly. Thus, the nonglycosylated α subunit expressed in tunicamycin-treated BC3H-1 cells has the potential to assemble with other subunits, but the assembled products are rapidly degraded.

Kinetics of Assembly and Degradation of Mutant and Wild-type αδ Subunit Complexes in Transfected Fibroblasts

As demonstrated in Fig. 4, tunicamycin inhibition of N-linked glycosylation in the BC3H-1 cell line did not prevent assembly of nonglycosylated subunits; however, assembled products were rapidly degraded. To determine if a similar process occurs in transfected fibroblasts coexpressing a mutant, nonglycosylated, α subunit with wild-type δ subunit, we labeled these cells with [³⁵S]methionine for 5 min and immunoprecipitated using a δ subunit specific antibody. As seen in Fig. 5 *A*, nonglycosylated α subunit coimmunoprecipitated with δ subunit was degraded with a $t_{1/2}$ of 22 min, a degradation rate similar to the unassembled nonglycosylated α subunit (Table II). Similarly, the degradation rates of α subunits with mutations at cysteines 128 and 142 or a deletion in this region did not decrease upon association with δ subunit (Fig. 5 *B* and Table II). In contrast, wild-type α and δ subunits assembled in fibroblasts in a time-dependent manner and, as previously described (Blount and Merlie, 1990), the resulting complex had a half-life of 13 h, 6–10-fold greater than either the unassembled α or δ subunits (Fig. 5 *C* and Table II). Note that a small amount of assembly of wild-type α with δ was observed at the earliest time point measured (Fig. 5 *C*, 5-min pulse with no chase). This early assembly may be the same as that observed at the earliest time points in Fig. 5, *A* and *B*; however, in contrast to cells expressing wild-type α, accumulation of assembled complexes from these mutant α subunits was not observed.

Mutations that do not prevent acquisition of high affinity BTX binding could have subtle effects on kinetics of α subunit maturation and assembly. However, experiments similar to those presented in Fig. 5 demonstrated that in fibroblast cell lines co-expressing wild-type δ with either the 136P→G or the 192 + 193 CC→SS α subunit, α subunit acquired high affinity BTX binding and assembled with δ subunit with kinetics and efficiency indistinguishable from wild-type α subunit (not shown). In addition, the αδ complex formed in these cell lines had a degradation rate similar to the wild type (Table II).

A summary of our data on degradation rates of assembled and unassembled subunits is presented in Table II. Collectively, these data suggest that glycosylated and cysteine 128 and 142 disulfide bridged α subunits that have assembled into a heterologous αδ complex accumulate, while unassembled subunits and αδ complexes composed of α subunits that are not glycosylated or have not made a normal 128–142 disulfide bridge are degraded.

Discussion

Expression of functional multimeric transmembrane recep-

tors requires assembly of subunits into a correct hetero-subunit complex. However, the requirements for and regulation of subunit assembly are not well understood. Because of our current knowledge of the structure of muscle-type AChRs, we can use this molecule as a model for studying requirements for normal subunit association. Here we have used the assembly of AChR α and δ subunits in transfected fibroblasts (Blount et al., 1990) to analyze the effects of site-directed mutations of the α subunit in an attempt to identify structural elements required for normal maturation and assembly of subunits.

The interpretation of our results depends upon our using BTX binding as an assay for α subunit maturation. Merlie and Lindstrom (1983) previously demonstrated that AChR α subunit acquired high affinity BTX binding in a time-dependent manner. Subsequently, we demonstrated that in stably transfected fibroblasts, α subunit could acquire high affinity BTX binding in the absence of other subunits (Blount and Merlie, 1988). These data suggested a covalent modification was required for α subunit maturation as assayed by BTX binding. The data presented here implicate α subunit cysteines 128 and 142 in this maturation. However, interpretation of effects of mutation is not straightforward. The change of a single amino acid may cause loss of drug binding by interfering with folding (as we believe is the case for mutations of cysteine 128 and 142), or, alternatively, mutations may directly influence the drug binding site. Several studies have suggested that an α subunit domain to which BTX (Wilson et al., 1985; Neumann et al., 1986; Barakas et al., 1987; Wilson and Lentz, 1988; Radding et al., 1988) and competitive antagonists (Kao et al., 1984; Dennis, et al., 1988) bind is located at or near the 192 and 193 cysteines. However, a study in which mutated *Torpedo* AChR α subunits were co-expressed with wild-type β, γ, and δ in *Xenopus* oocytes suggested that cysteines 192 and 193 were not requirements for BTX binding (Mishina et al., 1985). The authors found that when cysteine at position 192 or 193 was mutated to serine, although no agonist gated ion channels were detected on the cell surface, a significant amount of carbamylcholine-inhibitable BTX binding sites reached the plasma membrane. These data suggested that the 192 and 193 cysteines were not required for expression of agonist or BTX binding sites, but were required for channel gating. In contrast, when cysteine at position 128 or 142 was mutated to serine, neither ligand-gated channels nor high affinity BTX binding sites were detected on the surface of the oocytes. Because only BTX binding sites that reached the oocyte plasma membrane were monitored, these results were inconclusive in determining if the cysteines at 128 and 142 were necessary for formation of α subunit with high affinity BTX binding sites, or, alternatively, if these amino acids were required for the assembly of α with other subunits or transport of assembled AChR to the cell surface. Consistent with the results of Mishina et al., 1985, we found that mutation of cysteines 192 and 193 to serines did not interfere with acquisition of high affinity BTX binding, assembly with δ subunit, or formation of a carbamylcholine binding site. In addition, our data indicate that cysteines at position 128 and 142 are required for expression of any detectable high affinity BTX binding sites. The observation that mutation of proline at position 136 does not inhibit α_{Tx} formation confirms that not all amino acids in this region are important for α subunit maturation; how-

ever, the deletion mutants suggest that the secondary structure formed by these amino acids is critical for a mature conformation. Analysis of double cysteine mutants (192 + 193 CC$\rightarrow$SS and 128C$\rightarrow$S + 142 C$\rightarrow$S) support the conclusion that our observations are due to lack of disulfide bridging, not formation of improper disulfide linkages or the presence of free sulfhydryls. Because mutation of either one or both of the cysteines at position 128 and 142 leads to absence of detectable BTX binding in our assay, and because these amino acids have not been implicated in agonist or BTX binding (as 192 and 193 cysteines have), our data strongly suggest that a disulfide bridge between cysteines at position 128 and 142 is required for acquisition of high affinity BTX binding and a mature conformation of AChR α subunit.

The maturation of the α subunit is a slow event; maximum levels of α_{Tx} are not achieved until several minutes after α subunit synthesis. Possible explanations for this delay include (a) a slow isomerization of the proline at position 136, or, (b) slow isomerization of disulfide bonds among the 4 cysteines at positions 128, 142, 192, and 193 (Fig. 1 A) until the correct disulfide configuration is achieved. Several previous studies have suggested that either proline or disulfide bond isomerization can be rate limiting for normal protein folding (for review see Fischer and Schmid, 1990). If isomerization of proline 136 or disulfide bond isomerization is rate limiting, mutation of proline 136 or a double mutation of cysteines at position 192 and 193 should significantly increase the rate of acquisition of high affinity BTX binding. Our data, however, indicate that α subunit mutated at 136P$\rightarrow$G, and the double mutant α 192 + 193 CC$\rightarrow$SS, acquire high affinity BTX binding with kinetics and efficiency indistinguishable from wild-type α subunit. Hence, neither isomerization of the proline at position 136 nor incorrect intramolecular disulfide bridging can account for the time-dependent nature of α subunit maturation.

Merlie et al. (1982) demonstrated that tunicamycin treatment of BC3H-1 cells decreased α_{Tx} formation and AChR assembly, but not α subunit synthesis. When it was discovered that α subunit could acquire high affinity BTX binding subsequent to glycosylation but before assembly with other subunits (Merlie and Lindstrom, 1983), a sequential model for processing and assembly of AChR subunits was proposed. In this model, α subunit is translated and cotranslationally glycosylated, acquires high affinity BTX binding in a time-dependent manner, then is assembled with other subunits to form the AChR. The time-dependent nature of formation of α_{Tx} seemed sufficient to suggest this as rate limiting for assembly. However, because experimental intervention in the normal process of α subunit maturation was not possible, the evidence that α_{Tx} was required for subunit assembly relied entirely on the effect of tunicamycin on BC3H-1 cells and on kinetic studies. And indeed, this model was recently challenged when *Xenopus* oocytes injected with messenger RNA of *Torpedo* α, β, γ, and δ AChR subunits were treated with tunicamycin, and assembly of non-glycosylated subunits was observed (Sumikawa and Miledi, 1989). The results presented here for mouse AChR subunits expressed in fibroblasts confirm Sumikawa and Miledi's observations. In addition, we demonstrate that although immature α subunit can assemble, the assembled products are short lived when compared to the correctly assembled products. Because *Xenopus* oocytes have a relatively slow rate of

AChR expression (Kobayashi and Aoshima, 1986), and, most likely a slow rate of degradation of all unassembled and assembled subunit species, it seems probable that even relatively rapidly degraded subunits are more easily observed in the oocyte expression system than the BC3H-1 or transfected fibroblast cell lines. Thus, our data present a resolution to an apparent paradox of the effects of tunicamycin on assembly of AChR subunits. Moreover, our data suggest that correctly processed and assembled subunit complexes are recognized by cellular transport or degradation machinery in a manner different from incorrectly processed and assembled complexes or unassembled subunits, thereby leading to the selective accumulation of only correct assembly intermediates.

In conclusion, by studying individual and combinations of subunits expressed in fibroblasts, we have gained insight into subunit maturation and regulation of assembly of the AChR. Mutational analysis suggests that acquisition of high affinity BTX binding requires the formation of a disulfide bridge between cysteines 128 and 142 in α subunit. Because this processing step is time-dependent and relatively inefficient, it remains a possible site of regulation for AChR expression. The assembly of α_{Tx} with other subunits and surface expression of AChR appear to be specific and highly regulated. Previous work has suggested that discrete assembly intermediates are formed in and confined to the endoplasmic reticulum when α and γ or α and δ subunits are coexpressed in fibroblasts (Blount et al., 1990). Mutational analysis now suggests that subunits lacking N-linked glycosylation or a disulfide bridge between cysteines 128 and 142 can assemble, but these incorrect assembly intermediates are degraded, thus leading to accumulation, and ultimately, expression of only correctly processed AChR.

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