We have used mutagenesis to investigate the potential N-glycosylation sites in the δ subunit of the muscle nicotinic acetylcholine receptor (AChR). Of the three sites, Asn\(^{76}\), Asn\(^{143}\), and Asn\(^{169}\), only the first two were glycosylated when the δ subunit was expressed in COS cells. Because the heterologously expressed δ subunit was similar in its properties to that expressed in C2 muscle cells, the sites of glycosylation are likely to be the same in both cases. In COS cells, mutations of the δ subunit that prevented glycosylation at either of the sites did not change its metabolic stability nor its steady-state level. These results are in contrast to those found previously for the α subunit, in which glycosylation at a single site metabolically stabilized the polypeptide (Blount, P., and Merlie, J. P. (1990) J. Cell Biol. 111, 2613–2622). Mutations of the δ subunit that prevented glycosylation, however, decreased its ability to form an αδ heterodimer when the α and δ subunits were expressed together. When all four subunits of the AChR (α, β, δ, and ε) were coexpressed, mutation of the δ subunit to prevent glycosylation resulted in a reduced amount of fully assembled AChR and reduced surface AChR levels, consistent with the role of the heterodimer in the assembly reaction. These results suggest that glycosylation of the δ subunit at both Asn\(^{76}\) and Asn\(^{143}\) is needed for its efficient folding and/or its subsequent interaction with the α subunit.

The muscle nicotinic acetylcholine receptor (AChR),\(^1\) a heterooligomeric membrane receptor, is the best understood member of a family of closely related ligand-gated ion channels that include the neuronal acetylcholine, GABA\(_{\text{A}}\), glycine, and serotonin 5-HT\(_3\) receptors (1, 2). These receptors are clustered in the postsynaptic membrane, where they mediate rapid excitatory and inhibitory synaptic transmission. For each member of this family, the individual subunits are arranged in a pseudo-symmetric array around an aqueous channel. Binding of the ligand opens the channel, allowing ions to flow through, thus changing the potential of the membrane (3, 4).

The adult muscle nicotinic AChR is a pentamer of four subunits, α, β, δ, and ε, with the stoichiometry αβδε (reviewed in Refs. 3–5). The subunits, which are homologous, each comprise four transmembrane domains with a large extracellular N-terminal domain, a large intracellular loop between the third and fourth transmembrane domains, and a small extracellular C-terminal domain (Fig. 1A) (6–9). Each of the subunits is synthesized from a separate mRNA, and each undergoes translocation into the endoplasmic reticulum, where it undergoes post-translational processing, including signal sequence cleavage and glycosylation (10). Following synthesis, the α subunit undergoes a folding reaction that appears to involve disulfide bond formation and that confers upon the α subunit the ability to bind α-bungarotoxin (α-BuTx; Refs. 11–13). The other subunits presumably undergo folding reactions as well, although it has not been possible to measure these directly. The N-terminal domains of all four subunits possess a disulfide loop with a conserved glycosylation site contained within it; the ε subunit contains one, and the δ subunit contains two, other potential glycosylation sites as well. The folded subunits assemble in the endoplasmic reticulum (10, 14, 15), following a defined pathway in which the first step is the formation of αδ and αε heterodimers (16–19); these then associate with each other and with the β subunit to form the pentameric AChR. The fully assembled AChR is then transported to the Golgi, where some of its high mannose oligosaccharide chains are converted to complex oligosaccharides (20, 21), and where the α chain is myristoylated (22).

Although each of the subunits of the AChR contains consens glycosylation sites and each is glycosylated, little is known about the functional role of the oligosaccharide chains. Studies with other proteins indicate that sugar addition can affect various properties of a protein, including its three-dimensional conformation, solubility, stability, and ability to associate with other proteins (23, 24). The α subunit of the AChR has a single glycosylation site which undergoes sugar addition to form a simple (high mannone) oligosaccharide (21), and studies using glycosylation inhibitors and site-directed mutagenesis have shown that the abolition of oligosaccharide addition results in rapid degradation of the receptor subunit (25, 26). The β subunit has a single glycosylation site, and the δ subunit has three potential sites of glycosylation, all of which are in the N-terminal extracellular domain; the ε subunit, and its developmental homolog, γ, have two potential sites in this domain. Recent mutational studies of the highly conserved glycosylation site associated with the disulfide loop in all four subunits have shown that abolition of this site in the α, γ, and δ subunits, but not in the β subunit, eliminates surface expression of the assembled AChR (27).
We now report experiments investigating the function of each of the three putative glycosylation sites in the mouse muscle δ subunit using *in vitro* mutagenesis and expression in heterologous cells. We find that the mobility of the δ subunit made in COS cells and in muscle cells is similar, suggesting that the number of oligosaccharide chains is the same in the two cases. In COS cells, we find that only two of the three potential sites of the δ subunit are glycosylated and that muta-
tion of either of the two reduces the association of the δ subunit with α to form a heterodimer, thus affecting the intra-
cellular assembly of the AChR and its subsequent appearance on the cell surface. Mutation of the glycosylation sites did not affect the metabolic stability of the δ subunit. Thus, glycosyla-
tion at both sites is required for efficient folding of the subunit and/or for association with the α subunit to form a heterodimer.

We conclude that in contrast to the α subunit, in which glyco-
sylation serves to stabilize the protein (26), glycosylation of the δ subunit promotes efficient folding and assembly.

### EXPERIMENTAL PROCEDURES

**cDNA and Vector**—The cDNAs encoding the full-length murine α and β subunits were kindly given to us by Dr. Paul Gardner (University of Texas, San Antonio) and Dr. Norman Davidson (CalTech, Pasadena). The δ subunit (31) was given to us by Dr. Paul Gardner (University of Texas, San Antonio). Each of the cDNAs was subcloned into the multiple cloning site of the SV-40-based pSM vector (32).

**Generation of Mutants**—Site-directed mutagenesis was used to dis-
rupt the three consensus glycosylation sites (N\(\alpha\)GnN\(\beta\)) of the δ subunit corresponding to the asparagine residues at positions 76, 143, and 169 (Fig. 1B). The serine or the threonine residues were converted to alanine using the thermal cycling-based QuikChange site-directed mutagenesis kit (Stratagene). Briefly, two complementary oligonucleotides (125 ng each) containing the desired mutation were added to a solution of 50 ng of template δ subunit DNA, 10 mM dNTP, and Pfu DNA polymerase buffer (final volume). Pfu DNA polymerase (1 μl) was then added to start the extension process in a thermal cycler. Extensions involved one cycle at 95 °C for 30 s to denature the DNA, followed by 15 cycles of 30 s at 95 °C, followed by 1 min at 55 °C and then 10 min at 68 °C. The extension product was incubated with DpnI to digest the methylated parental plasmid and used to transform DH5-α cells, which were then plated on ampicillin plates. Individual colonies were picked, and the cDNAs were sequenced using dideoxy nucleotide sequencing (Sequene-
nase kit, U.S. Biochemical Corp.). The mutagenic oligonucleotides used in the transfection mixture was identical except that the quantities were adjusted for surface area. C2C12 (C2) cells were maintained as described previously (35).

Briefly, myoblasts were seeded at low density in DMEM supplemented with 4.5 g/liter fucose, 20% fetal bovine serum, 0.5% chick embryo extract, 2 mM glutamine, and 100 units/ml each of penicillin and strepto-
mycin. After about 96 h of culture, the cells were induced to form myotubes by changing the medium to differentiating medium, which comprised DMEM, 5% horse serum, and 2 mM glutamine. Fusion of the myoblasts into myotubes occurred within 1 day. Contracting myotubes were observed a day later, at which time the C2 cultures were used for experiments.

**Surface Binding Studies**—Twenty-four hours after cotransfection with all four subunits, the cells were replated into 12- well cluster dishes, and an additional 24 h later, surface binding studies were conducted. Briefly, the cells were washed once with PBS and then incubated for 90 min in a reaction mixture consisting of DMEM contain-
ing \(^{125}\)I-a-BuTx (4 nM). The reaction mixture was then aspirated, and the cells were washed three times with PBS. The cells in each well were dissolved in 1 ml of 0.2 N NaOH and counted in a γ-counter.

**Metabolic Labeling and Pulse-Chase Studies**—COS cells (60-mm dishes) transfected with δ plasmids were washed once with 2 ml of cysteine- and methionine-free DMEM medium (serum-free) and then incubated for 30 min in 2 ml of identical medium, which was replaced with 2 ml of a labeling mixture consisting of ~200 μCi of Pro-mix\(^{35}\)S (a mixture of \(^{35}\)S-labeled cysteine and methionine) in serum-
free medium (30 min). The labeling mixture was incubated and the cells were quickly washed once with serum-free DMEM supplemented with 2 mg/ml each of cysteine and methionine (start of the countdown for the chase), prior to the addition of regular DMEM growth medium and replacement in the incubator. All of the above operations were at 37 °C. To terminate the reaction at each time point, the cells were washed with ice-cold PBS and harvested in 140 μl of cold extraction buffer comprising Tris-HCl (50 mM, pH 7.4), NaCl (50 mM), EDTA (1 mM), EGTA (1 mM), Triton X-100 (1%), sodium tetrathionate (1 mM), N-ethylmaleimide (1 mM), benzamidine (1 mM), phenylmethylsulfonyl fluoride (0.4 mM), leupeptin (20 μg/ml), pepstatin (10 μg/ml), and aproti-
in (10 units). The lysate was then spun at high speed (14,000 rpm) in an Eppendorf microcentrifuge for 10 min at 4 °C, and the pellet debris was discarded. The δ subunit-specific antibody mAb 88B (36) was added to 40 μl of the clear extract to a final concentration of 80 nm. The volume was then adjusted to 0.5 ml with cold extraction buffer, and the solution was tumbled for 90 min at 4 °C. Protein G-Sepharose (40 μl of slurry) was then added to each of the samples, which were then tumbled at 4 °C for an additional 90 min. Following this, the samples were centrifuged at 14,000 rpm, and the pellets were washed three times with ice-cold extraction buffer. The immunoprecipitate was solubilized by adding 65 μl of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer to the beads and boiling for 6 min. The supernatants were run on a 12.5% SDS-polyacrylamide gel, enhanced in 1% salsylactic acid, and subjected to autoradiography. The band intensities were quantitated using NIH Image software (National Institutes of Health, Bethesda, MD) and subjected to quantitative analysis.

**a-BuTx-Bead Affinity Purification and Immunoblotting**—

\(\alpha\)-BuTx-Bead affinity purification and immunoblotting procedures were performed by an adenovirus-mediated DEAE-dextran procedure as described previously (33, 34). Briefly, cells (grown in 60-mm dishes) were washed with phosphate-buffered saline (PBS) and then treated with a solution of 2.5 ml of serum-free DMEM and 0.5 ml of a replication deficient adenoviral extract containing 240 μg of DEAE-
dextran (the appropriate plasmid). Where all four subunits were cotransfected, 1.0 μl of each plasmid was used in the transfection mixture; where two plasmids were to be coex-
pressed, 2 μg of each was used. When cells were grown in larger dishes, the transfection mixture was identical except that the quantities were

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**Glycosylation of the δ Subunit of the AChR**

Cell Culture and Transfections—COS cells were maintained in a cell culture incubator at 37 °C and 5% CO\(_2\) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml each of penicillin and streptomycin. Transfections were performed by an adenovirus-mediated DEAE-dextran procedure as described previously (33, 34). Briefly, cells (grown in 60-mm dishes) were washed with phosphate-buffered saline (PBS) and then treated with a solution of 2.5 ml of serum-free DMEM and 0.5 ml of a replication deficient adenoviral extract containing 240 μg of DEAE-dextran and the appropriate plasmid(s). Where all four subunits were cotransfected, 1.0 μl of each plasmid was used in the transfection mixture; where two plasmids were to be coex-
pressed, 2 μg of each was used. When cells were grown in larger dishes, the transfection mixture was identical except that the quantities were
COS cells (150-mm dishes) transfected with all four subunits or untransfected C2 cells were harvested in 1 ml of extraction buffer. AChR was isolated by incubating the extract with \( \alpha \)-BuTx-conjugated Sepharose beads (35, 37), following which the beads were pelleted and then boiled in SDS-PAGE sample loading buffer for 6 min. Glycosidase digestions of the samples were carried out as described above. The samples were applied to a 12.5% SDS-PAGE gel and transferred to a nitrocellulose filter, and the blot was probed for the \( \delta \) subunit with the 88B antibody as described previously (35, 37).

**RESULTS**

Expression of Mutated \( \delta \) Subunits in COS Cells—We made mutations in each of the three potential \( N \)-glycosylation sites (Asn\(^{76} \), Asn\(^{143} \), and Asn\(^{169} \)) in the mouse muscle \( \delta \) subunit and tested the mutated constructs (called G1, G2, and G3, respectively; see Fig. 1B and “Experimental Procedures”) by expression in heterologous cells. We examined the \( \delta \) subunit produced in transfected COS cells in two ways: first, by immunoblotting with the monoclonal antibody 88B, which is specific for the \( \delta \) and \( e \) subunits (Fig. 2), and second, by immunoprecipitating extracts of metabolically labeled cells with the same antibody (Fig. 3A). After immunoprecipitation, both wild-type and mutant \( \delta \) subunits consistently appeared as two major bands separated by 7–10 kDa (Fig. 3A); after immunoblotting, the subunit appeared as a broad and diffuse band whose resolution into two or more individual bands was only variably seen (Fig. 2). The apparent molecular mass and breadth of the band were consistent with those previously seen for the \( \delta \) subunit expressed either normally in C2 muscle cells (15) or following transfection in COS cells (31). Because the glycosylation-incompetent G1G2G3 mutant also ran as a doublet (Figs. 2 and 3A), the multiple bands do not result from differential glycosylation as previously suggested (21, 38) but are likely to result from partial proteolytic degradation.
Despite the uncertainty about the identity and significance of the sub-bands, the results of the mutation experiments were very clear, and were the same, whether the proteins were detected by immunoblotting or immunoprecipitation. When the mobilities of G1 and G2 mutant proteins were compared with that of the wild-type protein, the mutants showed increased mobilities in both cases (Figs. 2 and 3A). In contrast, the mobility of the G3 mutant protein was less than that of either G1 or G2 and appeared to be identical to that of the wild-type protein. These results suggested that in the wild-type δ subunit, sites Asn 78 and Asn 143 are glycosylated in COS cells but not at site Asn 169.

Finally, the lack of glycosylation at Asn 169 was confirmed by an experiment in which the G1G2 mutant protein was treated with either of the glycosidases, Endo H or PNGase. These treatments had no effect on the mobility of the G1G2 subunit (Fig. 3B); under similar conditions, the mobilities of the wild-type and the G1G3 and G2G3 subunits were shifted (Fig. 3, C–E). The upper band of the G1 protein was shifted to a smaller extent than that of the G2 protein, suggesting that Asn 169 is more extensively glycosylated than Asn 78 (Fig. 3A). Taken together, the results of all of these experiments provide strong evidence for glycosylation of the δ subunit in COS cells at sites Asn 78 and Asn 143 but not at site Asn 169.

To further examine the pattern of glycosylation in the two cell types, the δ subunits were subjected to glycosidase treatment, either with Endo H, selective for high mannose oligosaccharides, or with the nonspecific PNGase. The banding patterns of the δ subunit from COS cells and from C2 cells were essentially the same after PNGase treatment (Fig. 4C, lanes 1 and 3). The mobilities of the major δ bands from COS cells were similar following either Endo H or PNGase treatment (Fig. 4, B, lane 1, and C, lane 1), indicating that a majority of the δ subunit expressed in COS cells undergoes simple sugar addition. A small fraction of COS-expressed δ subunit was, however, resistant to Endo H (Fig. 4B, lane 1, uppermost band), indicating the presence of complex oligosaccharide chains. Identical treatment of the native δ subunit from C2 cells showed that a larger fraction of the protein, in this case, was resistant to Endo H (Fig. 4B, lane 3, upper bands), suggesting that sugar processing was more complete in C2 cells. A considerable fraction of C2-expressed δ, however, was also sensitive to treatment with Endo H (Fig. 4B, lane 3, lower band) and showed a pattern of mobility identical to that of COS-expressed δ after treatment with PNGase (Fig. 4B, lane 1, lower bands), suggesting that the oligosaccharide chains in this fraction were simple ones. Taken together, these results suggest that the δ subunit expressed in both COS and C2 cells contains simple and complex oligosaccharide chains but that in COS cells most of the oligosaccharide chains are simple ones. In C2 cells, processing is more complete, and complex chains comprise a significant fraction of the total.

**Effect of the Mutations on Cell Surface AChR Expression**—We then sought to examine the role of glycosylation at the two sites in δ subunit folding and AChR assembly. We began by examining the effect of mutating each of the sites on surface expression of the fully assembled AChR. As only the completely assembled pentameric receptor is transported to the cell surface, this is an indirect measure of the efficiency of AChR assembly. When we cotransfected all four subunits into COS cells and measured surface 125I-α-BuTx binding, we observed that the G1 and G2 mutations each resulted in a greater than 60% decrease in binding, whereas the G3 mutation had no effect (Fig. 5). When both functional glycosylation sites were removed (G1G2) there was a further loss of surface AChR expression, whereas the double mutants G1G3 and G2G3 gave results that were similar to those seen with G1 and G2, respectively. We conclude that glycosylation at both Asn 78 and Asn 143 of the δ subunit is required for complete surface expression of the AChR. The results of the experiments with the G3 mutation are consistent with the earlier conclusion that Asn 169 is not glycosylated in COS cells.

**Effect of the Mutations on α6 Heterodimer Formation**—The reduced expression of the surface AChR could result either from reduced assembly of the AChR or from incomplete transport to the surface of assembled AChR containing an incompletely glycosylated δ subunit. To distinguish these possibilities, we cotransfected COS cells with the wild-type or mutated δ subunits, along with α, β, and ε subunits. Extracts from the transfected cells were then incubated with 125I-α-BuTx and immunoprecipitated with mAb 88B. As under these conditions, most of the δ associated with the α subunit is part of the fully assembled receptor, the immunoprecipitated toxin represents total assembled AChR, both surface and intracellular (39). We found that use of either the G1 or G2 mutated δ subunit appeared to be slightly faster (Fig. 4A, lanes 1 and 3). These results suggest that in muscle cells, as in COS cells, the δ subunit is glycosylated at only two sites but that the pattern of glycosylation in COS cells is slightly different from that seen in C2 cells.
resulted in an over 80% decrease of immunoprecipitated α-BuTx; a further reduction occurred when the double mutant G1G2 was used (Fig. 6A). Because the effects of the mutants on the total amount of AChR were similar to their effects on surface AChR, the decreased expression at the cell surface is unlikely to result from defective transport of the assembled AChR and thus must result from reduced assembly of the AChR. As expected from the results described earlier, mutation of the G3 site caused no change in the amount of immunoprecipitated 125I-α-BuTx.

Because one of the first steps in AChR assembly is the specific association of the α and δ subunits, we examined the effect of the G1 and G2 mutations on αδ heterodimer formation. In this case, COS cells were cotransfected with only the α and δ subunits, and cell extracts were incubated with 125I-α-BuTx and immunoprecipitated with the 88B antibody as above. Here, the amount of coprecipitated 125I-α-BuTx reflects the extent of αδ heterodimer formation. The effect of the mutations on αδ dimer formation precisely mirrored their effect on the expression of the pentameric receptor at the cell surface (Fig. 6B). The G1 and G2 mutants each showed a diminished ability (13 and 17% of control) to dimerize with the α subunit. When both the G1 and G2 sites were disrupted together (G1G2), the loss of dimerization was even more marked than that seen with the single mutants (9% of control). Finally, the αδ dimerization abilities of the G2G3 and G1G3 mutants were compromised to the same extents as that of the G2 and G1 mutants, respectively, again consistent with the observation that G3 is not glycosylated in COS cells. Thus, the effect of δ subunit mutations on AChR surface expression can be entirely accounted for by decreased formation of the αδ heterodimer. These results suggest that glycosylation at Asn76 and Asn143 is essential for the efficient dimerization of the δ subunit with the α subunit and thus explains their effect on the subsequent expression of the pentamer at the surface.

Stability of the Mutants in Vivo—The reduced αδ heterodimer formation shown by the glycosylation-deficient mutants of δ could result either from a reduced steady-state amount of the mutated δ proteins or from a loss in its proper folding and subsequent assembly with α. Both immunoblotting (Fig. 2) and immunoprecipitation experiments (Fig. 3) indicate that the steady-state levels of the mutated δ subunits are roughly comparable to those of the wild-type protein. Nevertheless, mutagenic disruption of the single glycosylation site in the mouse muscle α subunit results in a dramatic decrease in the metabolic stability of the protein (26), leading us to examine this point directly. If the loss of a glycosylation site in δ had a similar effect, this could explain both the reduced incorporation of the mutated subunits into the receptor and its subsequent surface expression. In order to test this possibility, we used pulse-chase experiments to determine the turnover rates of the wild-type protein and the three mutants G1, G2, and G3. Fig. 7 shows the results obtained with wild-type and mutant δ
subunits in COS cells. The estimated turnover times from the various experiments are indicated in Table I. The results indicate that lack of glycosylation at Asn76 and Asn143 does not appreciably affect the intracellular stability of the δ subunit in COS cells.

**DISCUSSION**

The principal findings reported here are, first, that the δ subunit of the AChR, when expressed in COS cells, is glycosylated at two of its three potential N-glycosylation sites and, second, that mutation at either of these sites to prevent glycosylation affects the ability of the subunit to form a specific heterodimer with the α subunit but does not change the stability of the protein. Thus, glycosylation at both sites is required for efficient assembly and expression of the AChR. Because the δ subunit expressed in COS cells is similar in its properties to that seen in muscle cells, these conclusions are likely also to apply to the δ subunit normally made in muscle cells.

The conclusion that only two sites, Asn76 and Asn143, are glycosylated whereas the third, Asn169, is not based on the behavior of δ subunit mutants with single and combined mutations at each of these sites. With respect to electrophoretic mobility after either immunoblotting or immunoprecipitation, or to surface AChR expression, or to ability to form an αδ heterodimer, we consistently found that mutations preventing glycosylation at either Asn76 or Asn143 affected the properties of the protein and that mutation preventing glycosylation at Asn169 did not. Moreover, unlike the wild-type protein and all mutant proteins with an intact site at either Asn76 or Asn143, the doubly mutated polypeptide lacking Asn76 and Asn143 sites did not show an altered electrophoretic mobility after treatment with glycosidases. All of these results are consistent with a pattern of glycosylation at Asn76 and Asn143 but not at Asn169. Because of the similarity in electrophoretic mobilities, the δ subunit in muscle cells is likely to be also glycosylated at only two sites, presumably the same ones. Thus, the difference in molecular mass between the glycosylated and non-glycosylated forms of the δ subunit is about the same (8–10 kDa) in COS cells, in muscle cells, and in an *in vitro* translation system (10). This is also consistent with results indicating that N-linked glycosylation adds 4–7 kDa to the δ subunit of the *Torpedo* AChR (21).

All of the δ proteins appeared on SDS gels either as a broad diffuse band or as a doublet, as previously reported for the δ subunit in AChRs of adult muscle cells (20), cultured myotubes (37, 38), or transfected heterologous cells (31). The breadth and/or multiplicity of the bands has been thought to be due to variable glycosylation (21, 38), but this explanation cannot account for the fact that the unglycosylated peptide also appears to be heterogeneous. Thus, the heterogeneity is not a function of differential glycosylation but most likely arises because of proteolytic degradation of the δ subunit.

In normal adult muscle (20) and in C2 muscle cells (Ref. 15 and this study), the δ subunit of the fully assembled AChR has both simple and complex oligosaccharide side chains. Treatment of the δ subunit of the AChR expressed by C2 muscle cells with PNGase produces a larger shift in mobility than does treatment with Endo H (Fig. 4). When expressed in COS cells along with other subunits, most of the δ subunit in the AChR shows the same mobility shift after Endo H treatment as after PNGase treatment, suggesting that most of the protein has simple, high mannose oligosaccharides at the two sites. A small fraction of the δ subunit (Fig. 4), however, appears to be completely resistant to Endo H but susceptible to PNGase, suggesting that that fraction has only complex sugars. These observations suggest that the conversion of simple to complex oligosaccharide chains, which occurs in the Golgi complex (15, 38), is less efficient for the δ subunit expressed in COS cells than in C2 cells and possibly shows an altered pattern. This interpretation must be a tentative one, however, as the heterogeneity of the bands in both C2 and COS cells complicates the interpretation.

Surprisingly, we found that changes in glycosylation did not affect degradation of the δ subunit. Experiments on the α subunit have shown that after blocking glycosylation either pharmacologically (25) or by mutation (26), the subunit becomes metabolically destabilized, with its half-life decreasing from 120 to 17 min (26). The glycosylation-deficient α subunit fails to acquire α-BuTx binding ability, and this misfolding is thought to cause its rapid degradation. Under this circumstance, the fraction of α subunit that is not degraded associates normally with other subunits to form an intact receptor but fails to reach the cell surface (26, 27). In contrast to the α subunit, the metabolic half-life of the δ subunit is unaffected by deficient glycosylation at either of its glycosylation sites. Thus, our results suggest that the glycosylation state of the δ subunit does not affect its intracellular degradation rate and so, presumably, the steady-state expression levels of the polypeptide.

Although the degradation rate is unchanged, mutation of either glycosylation site in the δ subunit affects its ability to associate with the α subunit, thus reducing expression of the fully assembled, pentameric AChR. These findings are similar with previous results from our laboratory (17–19) and others (38, 40, 41), showing that conditions that decrease αδ het-
Glycosylation of the δ Subunit of the AChR

erodimer formation also decrease the expression of the fully assembled AChR. Taken together, these results are consistent with the idea that formation of the specific αδ heterodimer is the first step in assembly of the AChR. They are also in agreement with recent findings of Sugiyama et al. (42) who, through mutations engineered in αδ, showed that capacity to form αδ and αγ dimers intracellularly determines the amount of surface pentameric AChR that is expressed. Thus, the results of our laboratory and those of others do not support the more complex model of assembly based on experiments carried out at lower temperatures (43).

Loss of glycosylation at either Asn⁷⁶ or Asnⁱ⁴³ of the δ subunit caused a 3–4-fold reduction in the expression of both total cell surface receptor, and loss of glycosylation at both Asn⁷⁶ and Asnⁱ⁴³ resulted in a more than 11-fold reduction. Because the reductions were similar for total and cell surface AChR, the effect of the mutations cannot be explained by defective trafficking of assembled ACHR to the surface. Reductions of the same order of magnitude were seen when only the α and δ subunits were coexpressed in COS cells and the extent of αδ heterodimer was measured. Thus, because the primary effect of the mutations is to reduce formation of the heterodimer, our results indicate that either glycosylation of the δ subunit is required for its correct folding, or that the sugars of δ are directly involved in its dimerization with α, or both.

What is the role of the oligosaccharide chains in subunit folding and heterodimer formation? Unfortunately, there are no means for following the folding reaction of the δ subunit, and direct measurement of the αδ heterodimer association rate is not possible, since specific association between the subunits has not been achieved except within the endoplasmic reticulum (15, 38). However, our results, taken with those of others (41), suggest that folding may, in fact, be compromised, at least in the Asnⁱ⁴³ glycosylation mutant of δ. In the intact ACHR, the δ subunit is flanked on either side by an α and a β subunit (3, 38, 41, 44). Kreienkamp et al. (41) have found evidence that suggests that Lys⁴¹⁴ and Lys³⁵⁰ of δ are positioned at the α-δ subunit interface, and away from the δ-α interface. Similarly, Asn¹⁴³ of δ is expected to be located close to the δ-β interface and away from the δ-α interface. If the sugars of δ-Asn¹⁴³ are locally involved in δ-β subunit association, then this would predict that altered glycosylation at δ-Asn¹⁴³ would have little effect on its ability to form the early α-δ intermediate. Our observation that abolishing glycosylation at δ-143 abolishes its capacity to associate with α suggests that the mutation has more than simply a local effect and that it probably causes the tertiary structure of the mutant δ subunit to be altered significantly.

In support of this interpretation, studies of the homologous α subunit indicate that mutation of the single highly conserved N-glycosylation site causes its rapid degradation and suggests that the protein has misfolded. Thermodynamic analysis of a peptide comprising residues 127–144 of the α subunit of the Torpedo AChR, which includes the conserved glycosylation site and a critical cystine disulfide, indicates that glycosylation alters both the equilibrium for disulfide bond formation in favor of the disulfide and also affects cis/trans isomerization of a vicinal proline (45). As this sequence is highly conserved among all four subunits, it is likely that the δ subunit is similarly dependent on glycosylation for its efficient folding. Further, in many proteins, multiple sites of N-glycosylation act cooperatively to aid the folding process (46). Consistent with this, we found that loss of glycosylation at both Asn⁷⁶ and Asn¹⁴³ in the δ subunit caused a 13-fold decrease in αδ heterodimer formation compared with a 6–8-fold reduction when the sites were disrupted individually. Interestingly, if the δ subunit is misfolded under these circumstances as we suggest, the rate of subunit degradation is unaffected. Thus, the degrada-


tion rate of the δ subunit, unlike that of α, is unaffected by loss of glycosylation. One plausible explanation of our results is that the glycosylation-deficient δ subunits do misfold but that the misfolded polypeptides do not undergo enhanced degrada-


tion (Fig. 7, Table I). An alternative explanation is that the δ subunit folds correctly even in the absence of glycosylation but that the oligosaccharide chains are directly involved in subunit association.

Although the δ subunit expressed in COS cells may have minor alterations in oligosaccharide processing compared with that found in muscle cells, the principal conclusions of our experiments in COS cells are likely to be applicable to muscle cells as well. The only difference between the δ subunit expressed in the two cell types appears to be less efficient conversion of one of the oligosaccharide chains from simple to complex in COS cells. As both subunit folding and complete αβδε ACHR pentamer formation occur in the endoplasmic reticulum, before the processing of the oligosaccharide chain to the complex type occurs (14, 15), these processes should be unaffected by the later processing of the sugar chains of the intact ACHR in the Golgi complex. Thus, in muscle, as in heterologous cells, glycosylation of the δ subunit is likely to promote efficient folding and subsequent assembly into the intact AChR.

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REFERENCES

1. Betz, H. (1990) Neuron 5, 383–392
2. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
3. Karlin, A. (1993) Curr. Opin. Neurobiol. 3, 299–309
4. Unwin, N. (1995) Cell/Neuron 72/10, Suppl., 31–41
5. Hall, Z. W. (1992) Trends Cell Biol. 2, 66–68
6. Chavez, R. A., and Hall, Z. W. (1991) J. Biol. Chem. 266, 15532–15538
7. Chavez, R. A., and Hall, Z. W. (1992) J. Cell Biol. 116, 385–393
8. Devillers-Thierry, A., Giraudat, J., Bentaboulet, M., and Changeux, J. P. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2067–2071
9. Noda, M., Takahashi, H., Tanabe, T., Toyoshita, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., and Numa, S. (1982) Nature 299, 793–797
10. Anderson, D. J., and Blabel, G. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5998–5602
11. Merlie, J. P., and Sebbane, R. (1981) J. Biol. Chem. 256, 3605–3608
12. Merlie, J. P., and Lindstrom, J. (1983) Cell 34, 747–757
13. Blount, P., and Merlie, J. P. (1988) J. Biol. Chem. 263, 1972–1980
14. Smith, M. M., Lindstrom, J., and Merlie, J. P. (1987) J. Biol. Chem. 262, 4367–4376
15. Gu, Y, Ralston, E., Murphy-Erdosh, C., Black, R. A., and Hall, Z. W. (1989) J. Cell Biol. 109, 729–738
16. Yu, X.-M., and Hall, Z. W. (1991) Nature 352, 64–67
17. Gu, Y., Forsayeth, J. E., Verrelli, S., Yu, X.-M., and Hall, Z. W. (1991) J. Cell Biol. 114, 789–807
18. Verrelli, S., and Hall, Z. W. (1992) Cell 68, 23–31
19. Chavez, R. A., Malof, J., Breeze, D., Newsom-Davis, J., and Hall, Z. W. (1992) J. Biol. Chem. 267, 23028–23034
20. Gu, Y., and Hall, Z. W. (1988) J. Biol. Chem. 263, 12878–12885
21. Nomoto, H., Takahashi, N., Nagaki, Y., Endo, S., Arata, Y., and Hayashi, K. (1986) Eur. J. Biochem. 157, 233–242
22. Olson, E. N., Glaser, L., and Merlie, J. P. (1984) J. Biol. Chem. 259, 5364–5367
23. Dwek, R. A. (1995) Biochem. Soc. Trans. 23, 1–25
24. Dwek, R. A. (1995) Science 269, 1234–1235
25. Merlie, J. P., Sebbane, R., Tzartus, S., and Lindstrom, J. (1982) J. Biol. Chem. 257, 2694–2701
26. Blount, P., and Merlie, J. P. (1990) J. Cell Biol. 114, 2613–2622
27. Gehle, V. M., Walcott, E. C., Nishizaki, T., and Sumikawa, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3996–4001
28. Sugiyama, Robin Taylor, and Zuo-Zhong Wang (1997) Nature 383, 793–797
29. Anderson, D. J., and Blabel, G. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5998–5602
30. Lapolla, R. J., Mayne, K. M., and Davidson, N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7970–7974
31. Gu, Y., Franco, A., Gardner, P. D., Lansman, J. B., Forsayeth, J. R., and Hall, Z. W. (1990) Neuron 5, 147–157
32. Brodsky, M. H., Warton, M., Myers, R. M., and Littman, D. R. (1990) J. Immunol. 144, 3078–3086
33. Forsayeth, J. R., and Garcia, P. D. (1994) BioTechniques 17, 354–358
34. Wang, Z.-Z., Hardy, S. F., and Hall, Z. W. (1996) J. Biol. Chem. 271, 27575–27584
35. Fuhrer, C., and Hall, Z. W. (1996) J. Biol. Chem. 271, 32474–32481
36. Froehner, S. C., Douville, K., Klink, S., and Culp, W. J. (1983) J. Biol. Chem. 258, 7112–7120
37. Fuhrer, C., Sugiyama, J. E., Taylor, R. G., and Hall, Z. W. (1997) EMBO J. 16, 4951–4960
38. Blount, P., Smith, M. M., and Merlie, J. P. (1990) J. Cell Biol. 111, 2601–2611
39. Gu, Y., Camacho, P., Gardner, P., and Hall, Z. W. (1991) Neuron 6, 879–887
40. Saedi, M. S., Conroy, W. G., and Lindstrom, J. (1991) J. Cell Biol. 112, 1007–1015
41. Kreienkamp, H.-J., Maeda, R. K., Sine, S. M., and Taylor, P. (1995) Neuron 14, 635–644
42. Sugiyama, N., Boyd, A. E., and Taylor, P. (1996) J. Biol. Chem. 271, 26575–26581
43. Green, W. N., and Wanamaker, C. P. (1998) J. Neurosci. 18, 5555–5564
44. Sine, S., and Claudin, T. (1991) J. Biol. Chem. 266, 19369–19377
45. Rickert, K. W., and Imperiali, B. (1995) Chem. Biol. 2, 751–759
46. Helenius, A. (1994) Mol. Biol. Cell 5, 253–265

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