Assembly of the Nicotinic Acetylcholine Receptor

THE FIRST TRANSMEMBRANE DOMAINS OF TRUNCATED \( \alpha \) AND \( \delta \) SUBUNITS ARE REQUIRED FOR HETERODIMER FORMATION IN VIVO*

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To investigate the mechanism of assembly of the mouse muscle acetylcholine receptor, we have expressed truncated N-terminal fragments of the \( \alpha \) and \( \delta \) subunits in COS cells and have examined their ability to fold, to associate into heterodimers, and to form a ligand-binding site. Truncated fragments of the \( \alpha \) subunit that include all, part, or none of the first transmembrane domain (M1) folded to acquire \( \alpha \)-bungarotoxin binding activity. Neither the full-length \( \alpha \) subunit nor any of the fragments were expressed on the cell surface, although the shortest folded fragment lacking a transmembrane domain was secreted into the medium. When coexpressed with the \( \delta \) subunit, the \( \alpha \) subunit fragment possessing M1 formed a heterodimer containing a ligand-binding site, but shorter fragments, which lack transmembrane segments, did not associate with the \( \delta \) subunit. N-terminal \( \delta \) subunit fragments gave similar results. An N-terminal \( \delta \) subunit fragment that contains M1 associated with the \( \alpha \) subunit to form a heterodimer, while a fragment lacking M1 did not. These results show that a complete M1 domain is necessary for association of truncated N-terminal \( \alpha \) and \( \delta \) subunits into a heterodimer with high affinity ligand binding activity.

The nicotinic acetylcholine receptor (AChR)† of mammalian muscle and the electrical organ of Torpedo is the best characterized member of a family of ligand-gated ion channels that mediate rapid synaptic transmission in the nervous system (1). All members of the family are thought to have a common pentameric structure in which highly homologous subunits surround a central aqueous pore whose opening and closing is regulated by binding of the neurotransmitter ligand (2–4). The muscle receptor consists of four different subunits in the ratio \( \alpha _2\beta_2\gamma_\delta \) (5, 6). Each of the subunits is separately synthesized and translocated into the endoplasmic reticulum, where AChR assembly occurs (7–9). Before assembly, the \( \alpha \) subunit undergoes a folding reaction whose product can be recognized by its ability to bind \( \alpha \)-bungarotoxin (\( \alpha \)-BuTx) (10–12). The other subunits also presumably undergo folding reactions before assembly, although the intermediates have not been characterized. Association of the folded subunits to form the assembled receptor occurs by a defined pathway in which the first step is the formation of the heterodimers \( \alpha \delta \) and \( \alpha \gamma \) (13–16). The binding of the \( \alpha \) subunit to the \( \delta \) and \( \gamma \) subunits results in the formation of a ligand-binding site within each heterodimer. Each site is characterized by distinctive properties that correspond to those in the intact AChR (6, 17). Several observations suggest that the binding site for competitive antagonists occurs at or near the interface between \( \alpha \) and \( \delta \) subunits or \( \alpha \) and \( \gamma \) subunits, respectively (18–20).

In previous experiments, we have sought to define the domains of the AChR subunits that participate in the subunit interactions required for heterodimer formation. Chimeric subunits and dominant negative experiments were used to show that the N-terminal domains of the AChR mediate the specific interactions between the subunits and give them their identity in the assembly reaction (21–23). In addition, by comparing subunits of different species, we identified amino acid residues in the N-terminal domains that affect the efficiency of assembly (24, 25). Recent studies using chimeric \( \gamma \delta \) constructs (26) have identified a specific region in the N terminus of the \( \gamma \) subunit that apparently mediates contact with the \( \alpha \) subunit. In the present experiments, we have sought the minimal domains of the \( \alpha \) and \( \delta \) subunits that are necessary for folding and for specific subunit association in vivo. By expressing truncated N-terminal fragments of the \( \alpha \) and \( \delta \) subunits in COS cells, we found that, although the N-terminal extracellular domain of the \( \alpha \) subunit folds in vivo, heterodimer formation between truncated subunits requires the first transmembrane domains (M1) of both \( \alpha \) and \( \delta \) subunits. Our results suggest that the soluble N-terminal sequence plus a complete M1 segment in the \( \alpha \) and \( \delta \) subunits are the minimal structural units required to form a ligand-binding site in vivo whose pharmacological properties mimic those of the functional surface AChR pentamer. An incomplete M1 domain is adequate for the retention of proteins in the ER, but is not sufficient to promote assembly.

**EXPERIMENTAL PROCEDURES**

\( \text{cDNAs and Expression Vectors—Full-length cDNAs coding for the } \alpha, \beta, \text{ and } \delta \text{ subunits of the mouse muscle AChR were kindly provided by Drs. J. P. Merlie and N. Davidson (27–29). The full-length cDNA for the mouse } \epsilon \text{ subunit was previously isolated in our own laboratory (24). Each of the } \text{cDNAs was subcloned into the multiple cloning sites of a SV40-based expression vector, pSM (30).}\)

\( \text{Constructs of Truncated AChR Subunits—All amino acids were numbered according to their position in the mature protein sequence of the given subunit. The CDNA constructs } \alpha 207 \text{ and } \alpha M1, \text{ which encode } \alpha \text{ subunit N-terminal fragments terminated immediately after amino acid 207 and after the first putative transmembrane domain, M1 (ami-}\)
no acid 241), respectively, were obtained from Drs. P. Blount and J. P. Mercurio at the University School of Veterinary Medicine. A 216-bp construct of subunit cDNA into the EcoRI site in pSM and a filled-in SalI site. a216 expresses an α subunit fragment containing the N-terminal extracellular domain (amino acid 210) plus the first 6 amino acids of M1 followed by serine and threonine residues generated in the cloning. The construct a211, encoding the entire N-terminal extracellular portion of the subunit (up to amino acid 211), was made by amplification of the corresponding sequences in the full-length subunit cDNA using polymerase chain reactions. The constructs 224, which encodes the entire N-terminal extracellular portion of the subunit (up to amino acid 224), and 3M1, which encodes the entire N-terminal extracellular portion and the first transmembrane domain of the subunit (up to amino acid 256), were made by amplification of their corresponding sequences in the full-length subunit cDNA with the polymerase chain reaction, respectively. To create truncated fragment 3M4, full-length δ subunit cDNA was terminated by a novel stop codon installed at the position immediately after the fourth transmembrane domain (amino acid 470) using site-directed mutagenesis (Transform Mutagenesis kit, CLON-TECH, Palo Alto, CA). Synthetic oligonucleotide primers for the polymerase chain reaction and mutagenesis were obtained from a commercial source (Integrated DNA Technology, Coralville, IA). Epitope-tagged 224 and 3M1 were also produced as fusion proteins with the FLAG peptide (224-FLAG) and the hemagglutinin sequence (2M1-HA) by polymerase chain reaction, respectively (see Fig. 1B) (31, 32). Each of the cDNA constructs was cloned into expression vector pSM, and its sequence was confirmed by dideoxynucleotide DNA sequencing (Sequenase kit, U. S. Biochemical Corp.).

Antibodies—mAb 210, a rat monoclonal antibody directed against the N terminus of the subunit (33, 34), was a generous gift of Dr. Jon Lindstrom (University of Pennsylvania). Mouse mAb 88B, which recognizes the cytoplasmic loop of the subunit (35), was kindly provided by Dr. Stanley C. Froehner (University of North Carolina, Chapel Hill, NC). The polyclonal antisera SFH. 224 (36) was raised by immunizing a rabbit with a purified, denatured N-terminal fragment of the mouse and the cleared detergent extract were evenly divided, and individual aliquots were assayed for expressed antigen at 37°C in gel loading buffer containing 5% β-mercaptoethanol and electrophoresed through 12.5% SDS-polyacrylamide gels. The gels were dried, fluorographed, and exposed to Kodak BioMax film. The relative optical density of the protein bands on the film was semiquantitated using the software NIH Image following scanning of the properly exposed films into a computer. Throughout this process, all samples were handled identically.

Surface and Intracellular Toxin Binding Assay—Twenty-four hours after transfection, COS cells were trypsinized and distributed into 12-well (2.5 cm) tissue culture plates. After an additional 24 h in culture, the transfected cell samples were harvested as described above and lysed in extraction buffer. The amount of bound toxin was determined by solubilizing the cells in 0.1 N NaOH and measuring the radioactivity with a γ-counter.

To measure the expression of species that did not retain the cell bound toxin binding activity2 were performed on COS cells permeabilized with saponin (13, 17). 125I-α-BuTx (4 nM) was applied in 10 mM HEPES buffer (pH 7.4), 0.1% saponin (Sigma), and 0.5% bovine serum albumin, and the cells were incubated on ice for 90 min. The cells were then washed with the same buffer without α-BuTx, solubilized in 0.1 N NaOH, and counted in a γ-counter. Non-specific binding was measured by the addition of a 100-fold excess of non-labeled α-BuTx to the mixture or by sham transfection. Each assay was performed in triplicate.}

Immunoprecipitation of Heterodimers—Immunoprecipitation of heterodimers with subunit-specific antibodies was performed as described previously (23). Forty-eight hours after transfection, COS cells were scraped off the dish and pelleted by centrifugation. The medium was collected, and the cells were solubilized in 0.4 ml of extraction buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM sodium tetrathionate, 1 mM N-ethylmaleimide, 1 mM benzamidine, 0.4 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 units of aprotinin. Cell lysate (400 μg protein) was incubated with 0.04 μl of antibody (anti-αAChR, anti-δ AChR, or 210 AChR) for 30 min at 4 °C. The Pansorbin cells were then washed with extraction buffer (without protease inhibitors), pelleted by centrifugation, and counted for radioactivity in a γ-counter. Control immunoprecipitation experiments were carried out using sham-transfected cells. The ligand-binding site of α and δ heterodimers was determined by measuring the inhibition of 125I-α-BuTx binding by d-tubocurarine (dTC) (17). Immunoprecipitation assays were carried out with the following modifications. Primary antibodies (mAb 88B, anti-FLAG mAb, and anti-HA mAb) were first incubated with cell extracts for 1 h on ice. Secondary antibodies and dTC were added to the samples 30 min prior to addition/ incubation with 125I-α-BuTx (30-min incubation) and with Pansorbin cells (30-min incubation).

Alkaline Buffer Extraction—Transfected COS cells were homogenized in ice-cold Tris buffer (50 mM, pH 7.4) with protease inhibitors in glass grinders. The homogenates were centrifuged (12,000 rpm) at 4 °C for 5 min to remove the nuclei. Supernatants were collected and incubated with 1 M sucrose (in Tris buffer (pH 7.4)) or 1 M Na2CO3 (pH 11) on ice for 15 min. The samples were then separated by ultracentrifugation at 50,000 rpm in a Beckman Ti-70 rotor for 1 h at 4 °C. The pellets were dissolved directly in reducing SDS-PAGE sample buffer. Proteins in the supernatant fraction were precipitated with trichloroacetic acid. They were then applied to a 10% reducing SDS-polyacrylamide gel and immunoblotted with mAb 210 (38, 41).

Velocity Sedimentation—Transfected COS cells were harvested as described above and lysed in extraction buffer. Cell lysates were layered on top of a continuous 5–20% sucrose gradient with bovine alkaline phosphatase (6.3 S) and, in some tubes, bovine hemoglobin (4.3 S) included as markers. Gradients were centrifuged at 65,000 rpm in a Beckman VTi-65 rotor for 90 min at 4 °C (23) and then collected from the bottom of the tubes in fractions of 120 μl. Each of the
FIG. 1. Schematic representation of the intact and truncated α and δ subunit proteins. A, proteins encoded by mouse full-length AChR α or δ subunit cDNA; B, proteins encoded by a set of truncated α and δ subunit cDNAs containing deletions in the carboxyl-terminal portions of the coding regions. The arrows indicate the last amino acid encoded by each of the truncated AChR subunits. The sequences of residues following the arrows show amino acids added during cloning (α216) or for epitope tags (α224-FLAG and δM1-HA).

fractions was analyzed by immunoprecipitation with subunit-specific antibodies as described above.

Immunoblot Analysis—Proteins in COS cell extracts or culture media were denatured in reducing gel loading buffer, electrophoresed on a 12.5% SDS-polyacrylamide gel (39), and transferred to nitrocellulose membranes. Nonspecific binding to the membranes was blocked by incubation in blocking buffer (10% calf serum and 3% bovine serum albumin in PBS and 0.3% Tween 20). The membranes were then incubated with the primary antibodies in the same blocking buffer, washed with PBS/Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibodies. After extensive washing in PBS and 0.3% Tween 20, the bound antibodies were detected with enhanced chemiluminescent reagents (ECL, Amersham Corp.) by exposure to Kodak BioMax film.

RESULTS

Folding of the α Subunit Fragments and Their Disposition in the Cell—To determine the minimal domain of the α subunit required for folding, we made plasmid constructs that were truncated at progressively longer distances from the N terminus (Fig. 1A). α207 and α211 were terminated just before M1, the first transmembrane domain, which extends from positions 212 to 241; α216 was terminated within M1; and αM1 was terminated at amino acid 241, thus including the entire first transmembrane domain. When each of the plasmids was introduced into COS cells by adenovirus-mediated transfection (36), polypeptides of the appropriate length were expressed as demonstrated by immunoblotting (Fig. 2). In each case, two major species were observed. The higher mobility protein had an apparent molecular mass that corresponds to the polypeptide after cleavage of the 20-amino acid signal sequence from the precursor. The protein band with lower mobility corresponded to the signal sequence-cleaved, glycosylated peptide because it was not made or was dramatically reduced when N-linked glycosylation was prevented by treating the cells with tunicamycin (Fig. 2) (38, 40). The proportion of the total peptide that was glycosylated in each case increased with chain length of the fragment. Only a small fraction of α207 or α211, the two shortest forms, was glycosylated, whereas virtually all of polypeptides αM1 and α, the two longest forms, was glycosylated. α216, a fragment with intermediate chain length, expressed approximately equal amounts of glycosylated and non-glycosylated species.

The expressed fragments were then tested in two ways for their ability to bind α-BuTx. In the first, the transfected cells were permeabilized with saponin and incubated with 125I-α-BuTx (Fig. 3A). In the second, detergent extracts of the transfected cells were incubated with 125I-α-BuTx and immunoprecipitated with mAb 210 (34), a rat monoclonal antibody directed against an epitope between amino acid residues 46 and 127 of the α subunit (Fig. 3B). In both assays, all forms of the truncated subunits except α207 showed toxin binding activity. This activity decreased with increasing chain length of the α fragments. Thus, α216 and α211, which bear either a short portion of the transmembrane region or none at all, appear to fold more efficiently than αM1 and α. An exception was observed with α207, which exhibited no high affinity binding to α-BuTx. To identify whether glycosylated or non-glycosylated species folded to assume the toxin binding conformation, we precipitated detergent extracts of [35S]methionine-labeled cells with α-BuTx coupled to Sepharose beads. Analysis by SDS-PAGE showed that in each case only the glycosylated species was precipitated by toxin beads (Fig. 3C), consistent with the results of other experiments showing that glycosylation is necessary for efficient folding of the full-length α subunit to the toxin-binding form (37, 40, 52). In each case, the folded α subunit fragments constituted only a minor fraction (≤5%) of the total population of the glycosylated species immunoprecipitated by mAb 210.

To determine if the folded subunit fragments were expressed on the cell surface, the intact transfected cells were incubated with 125I-α-BuTx. No surface binding of toxin was detected for any of the constructs (Fig. 4A). Previous studies also have shown that unassembled full-length AChR subunits are retained in the ER and that surface expression of the AChR in transfected cells requires the assembly of all four subunits into a pentamer (13–15). To ascertain whether any of the truncated α fragments can substitute for the full-length α subunit in the assembly of a pentameric receptor, COS cells were cotransfected with cDNAs for each of the α fragments along with...

FIG. 2. Immunoblot analysis of the truncated and full-length forms of the α subunit. COS cells were transfected with each of the cDNAs encoding the expressed proteins as indicated. Detergent extracts were loaded onto a 12.5% reducing SDS-polyacrylamide gel. Proteins on the gel were then transferred to a nitrocellulose membrane and probed with an α subunit-specific antibody (mAb 210). The glycosylation inhibitor tunicamycin (1.5 μg/ml) was added to growth medium of the indicated samples 10 h before the cells were lysed with extraction buffer. The positions of molecular mass markers (expressed in kilodaltons) are indicated on the left.
full-length β, δ, and ε subunits. In all cases tested, surface toxin binding was not measurable, indicating that none of these α fragments is able to form a hetero-oligomeric AChR that is competent for surface transport and expression (Fig. 4A).

Assay of the cell culture medium, however, showed that substantial amounts of α211, the fragment lacking a M1 domain, were secreted into the medium. In contrast, α216, truncated fragments with part or all of the M1 domain (α216 and αM1), and the full-length α subunit were not secreted (Fig. 4B). The profile for secretion of α211 was determined with a pulse-chase experiment in which transfected COS cells were metabolically labeled for 10 min with a mixture of [35S]methionine and [35S]cysteine. Following a 30-min chase, α211 was found in the medium by immunoprecipitation with mAb 210 (Fig. 5A). Densitometric analysis of the intensity of protein bands in the fluorography shows that 23% of all of the folded α subunit in the cells was secreted into the medium after chasing for 8 h (Fig. 5, compare C and D). The secreted molecule corresponds to the glycosylated form of α211 found in detergent cell extracts (Fig. 5, A and B; also see Fig. 2). A comparison of the amount of α211 in the medium detected by precipitation with mAb 210 (Fig. 5A) and with α-BuTx-Sepharose (Fig. 5C) showed that most or all of the secreted subunit was folded. The truncated fragments retained in the cell are presumably contained within the endoplasmic reticulum (8, 9). To determine whether they are integral membrane proteins, we extracted crude membrane preparations from transfected COS cells with Na2CO3 (1 M, pH 11) (38, 41). In sucrose buffer, α216, αM1, and the full-length α subunit were found mostly in the membrane. When treated with Na2CO3 solution, only α216 was extracted into the supernatant fraction, indicating that this fragment is not anchored to the membrane by a hydrophobic segment (Fig. 6). α211, which lacks M1, was soluble both in sucrose and alkaline buffers. Thus, only αM1, the fragment with an intact transmembrane domain, appears to be an integral membrane protein.

**Association of the α Subunit Fragments with the δ Subunit**—We have previously found that αM1 forms a heterodimer when expressed with the δ subunit (23, 25). To determine if other folded α fragments lacking all or part of the M1 domain can associate with the δ subunit, COS cells were transfected with each of the cDNAs encoding the α subunit proteins alone (hatched bars) or in combination with the β, δ, and ε subunits (closed bars). The data show surface 125I-α-BuTx bound per well (2.5 cm in diameter) of COS cells and represent the results from four or more separate experiments. B, secretion of α211 into culture medium. COS cells transfected with cDNAs encoding the proteins as indicated were metabolically labeled overnight with a mixture of [35S]methionine and [35S]cysteine. The culture medium was collected. Secreted proteins were immunoprecipitated with mAb 210 and analyzed by SDS-PAGE and fluorography.

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### FIG. 3. Folding of the intact and truncated forms of the α subunit. A, 125I-α-BuTx binding activity in saponin (0.1%-)-permeabilized COS cells. B, immunoprecipitation of 125I-α-BuTx binding activity in detergent extracts of cells using an α subunit-specific monoclonal antibody (mAb 210). C, fluorography of the total and folded α subunit proteins in cell lysates following overnight [35S]methionine metabolic labeling of transfected COS cells. COS cells grown in a 10-cm dish were transfected with each of the cDNAs as indicated and lysed with 0.4 ml of Triton X-100-containing buffer. Equal aliquots (0.04 ml) of the cleared cell extracts were used for immunoprecipitation with mAb 210 and for precipitation with α-BuTx coupled to Sepharose, respectively. Lanes 1–3, total α subunit proteins immunoprecipitated using mAb 210; lanes 4–6, folded α proteins precipitated using α-BuTx-Sepharose.

### FIG. 4. Disposition of the truncated fragments of the AChR α subunit. A, surface 125I-α-BuTx binding activity in COS cells transfected with each of the cDNAs encoding the α subunit proteins alone (hatched bars) or in combination with the β, δ, and ε subunits (closed bars). The data show surface 125I-α-BuTx bound per well (2.5 cm in diameter) of COS cells and represent the results from four or more separate experiments. B, secretion of α211 into culture medium. COS cells transfected with cDNAs encoding the proteins as indicated were metabolically labeled overnight with a mixture of [35S]methionine and [35S]cysteine. The culture medium was collected. Secreted proteins were immunoprecipitated with mAb 210 and analyzed by SDS-PAGE and fluorography.
with each of the α construct cDNAs along with the full-length δ subunit cDNA. Association of the toxin-binding α fragments with the δ subunit was examined in two ways. First, the ability of the δ subunit to increase toxin binding was examined in saponin-permeabilized cells. Previous studies have shown that the association of α with δ stabilizes the folded α subunit and thus increases the amount of folded α in the ER (13, 23). When coexpressed with the δ subunit, both αM1 and full-length α showed higher toxin binding activity in saponin-permeabilized cells, suggesting that heterodimer formation might have occurred. No change was seen when α211 or α216 was coexpressed with the δ subunit (Fig. 7A).

We then tested directly for association of the δ subunit with the truncated forms of the α subunit by immunoprecipitation of toxin binding activity in lysates of transfected cells. Lysates were incubated with 125I-α-BuTx and precipitated with a monoclonal antibody to the δ subunit (mAb 88B). In this assay, both the full-length α subunit and αM1 were associated with the δ subunit, but neither α211 nor α216 showed evidence of association (Fig. 7B). A comparison of the radioactive toxin immunoprecipitated by mAb 88B, which represents the heterodimer, and that by mAb 210, which recognizes all of the α subunit, showed that ~70% of the total α subunit was associated with the δ subunit.

Unassembled α subunit binds α-BuTx with high affinity, but has very low affinity for small competitive ligands. αδ heterodimers, in contrast, bind small ligands such as dTC and carbamylcholine with high affinity (17). Thus, the competitive antagonist dTC does not inhibit α-BuTx binding to the isolated α subunit expressed in transfected cells, but does block toxin binding to the αδ heterodimer. To determine if the complexes formed between the α fragments and the δ subunit generated a ligand-binding site, we permeabilized transfected cells with saponin and incubated them with 125I-α-BuTx in the presence and absence of dTC. For the shorter forms, α211 and α216, dTC had no effect on toxin binding. For αM1 and the intact α subunit, however, 10^{-5} M dTC inhibited toxin binding by ~50% (Fig. 8A). Because the saponin assay does not distinguish toxin-binding sites associated with heterodimers from those of unassembled α fragments, the extent of dTC inhibition underestimates the inhibition of toxin binding specific to heterodimers. To examine specifically the sites associated with the heterodimers, we used immunoprecipitation with an anti-δ subunit monoclonal antibody. Detergent extracts of transfected...
cells were incubated with $^{125}$I-$\alpha$-BuTx in the presence and absence of dTC, and mAb 88B was then used to precipitate toxin bound to the heterodimer. A more substantial inhibition by the antagonist was seen with this assay. When different concentrations of dTC were tested, both α1 and the intact α subunit showed 50% inhibition at $-3 \times 10^{-7}$ M (Fig. 8B).

Expression of Truncated δ Subunit Fragments—To determine the minimum domain of the δ subunit essential for the formation of the αδ heterodimer, we created plasmid vectors containing cDNAs encoding truncated fragments of the mouse muscle AChR δ subunit and analyzed their protein products in a manner similar to that for the truncated α subunits. Construct δ224 encodes the extracellular N-terminal sequence of the δ subunit, but the peptide is terminated just before the first transmembrane domain; δM1 encodes the same sequence plus the first transmembrane domain; and δM4 encodes a δ subunit fragment truncated after the fourth transmembrane domain (Fig. 1B). Because antibodies that recognize the nondenatured N terminus of the δ subunit are not available, we attached the epitope tags FLAG and a sequence from the influenza virus hemagglutinin protein to the C terminus of fragments δ224 and δM1, respectively (Fig. 1B).

Proteins of the appropriate length were produced in COS cells transfected with each of the plasmids. Like the truncated fragments of the α subunit, two or more major bands were present for each of the expressed δ constructs in Western blots of the reducing SDS-polyacrylamide gel (Fig. 9, A and B). The upper bands, which were virtually absent when cells were grown in the presence of tunicamycin, correspond to the signal sequence-cleaved products modified by N-linked glycosylation. The identity of the low mobility species was further confirmed by their association with concanavalin A-Sepharose beads (data not shown), a lectin specific for mannose and glucose (42).
infect with tation techniques sequentially. Lysates of COS cells transfected with one of the FLAG epitope (for like the truncated M1 domain, was also secreted by the cell. Immunoblotting of detergent extracts from control and tunicamycin-treated COS cells was performed using a polyclonal antibody against the N terminus of the α subunit (A) and using mAb 88B, which recognizes the cytoplasmic loop of the δ subunit (B). Tunicamycin (1.5 μg/ml) was added to growth medium 10 h before the cells were harvested for the immunoblot assay. Shown in C is an immunoblot of the secreted δ224 fragment in the culture medium of the transfected COS cells. The positions of molecular mass markers (expressed in kilodaltons) are indicated on the left.

**TABLE I**

| cDNA cotransfected | αM1 | α |
|--------------------|-----|---|
| Alone              | 4812 ± 171 | 5731 ± 195 |
| δ224              | 4671 ± 147 | 4629 ± 219 |
| δM1              | 4594 ± 213 | 5207 ± 602 |
| δM4              | 5743 ± 243 | 6790 ± 228 |
| δ              | 12,925 ± 536° | 19,905 ± 471° |

*p < 0.01 versus alone.

**FIG. 9. Immunoblot analysis of the truncated δ fragments and the full-length δ subunit in cell lysates and culture medium.** Immunoblotting of detergent extracts from control and tunicamycin-treated COS cells was performed using a polyclonal antibody against the N terminus of the δ subunit (A) and using mAb 88B, which recognizes the cytoplasmic loop of the δ subunit (B). Tunicamycin (1.5 μg/ml) was added to growth medium 10 h before the cells were harvested for the immunoblot assay. Shown in C is an immunoblot of the secreted δ224 fragment in the culture medium of the transfected COS cells. The positions of molecular mass markers (expressed in kilodaltons) are indicated on the left.

We further assayed for the formation of a ligand-binding site in heterodimers composed of αM1 and one of the truncated δ fragments. Detergent extracts of cells transfected with the cDNAs encoding αM1 and the truncated δ fragments were incubated with 125I-α-BuTx in the presence of dTC. Monoclonal antibodies against specific epitopes in δ fragments were used to precipitate the toxin binding activity associated with heterodimers. For δ224-FLAG, dTC had no effect on toxin binding. For δM1-HA and δM4, however, this competitive antagonist displaced toxin binding by >60% at 10−5 M (Table II). When different concentrations of dTC were tested, we found that the αM1-δM1 complex showed a similar dTC inhibition curve as the αM1-δ heterodimer, with an IC50 of ~5 × 10−7 M (Fig. 10B).

**DISCUSSION**

To determine the contribution of various protein domains of the muscle AChR subunits to receptor assembly, we have expressed truncated N-terminal fragments of the α and δ subunits in COS cells and have examined their ability to fold, to
COS cells were transfected with cDNAs encoding αM or the full-length α subunit in combination with each of the δ subunit fragments as indicated. $^{125}\text{I-α-BuTx}$ binding activity in cell lysates was immunoprecipitated using epitope tag-specific antibodies (for δ224-FLAG and δM1-HA) or δ subunit-specific mAb 88B (for δM4 and δ). The data represent the results from four or more experiments.

| cDNA cotransfected | Control | α | Plus $10^{-5}$ μdTC | α |
|---------------------|---------|---|---------------------|---|
| δ224-FLAG           | 92 ± 21 | 71 ± 19 | 109 ± 24 | 89 ± 44 |
| δM1-HA              | 1727 ± 50 | 2489 ± 73 | 390 ± 74$^*$ | 492 ± 54$^*$ |
| δM4                 | 1983 ± 121 | 2101 ± 109 | 357 ± 55$^*$ | 509 ± 83$^*$ |
| δ                   | 5991 ± 198 | 7831 ± 249 | 1109 ± 115$^*$ | 1533 ± 169$^*$ |

$^*$p < 0.01 versus control.

![Fig. 10. Formation of the heterodimer and a high affinity ligand-binding site by truncated δ fragments with αM.](image)

**Fig. 10.** Formation of the heterodimer and a high affinity ligand-binding site by truncated δ fragments with αM. A, analysis by sucrose gradient sedimentation and immunoprecipitation of the αM-δM1-HA complex. COS cells were transfected with cDNAs encoding αM1 alone (open circles), αM1 plus δ (open triangles), or αM1 plus δM1-HA (filled circles). Cell lysates were incubated with $^{125}\text{I-α-BuTx}$ and separated on 5–20% sucrose gradients. The fractions were immunoprecipitated with mAb 210 (for αM1 alone), mAb 88B (for αM1-δ), or monoclonal antibodies against the hemagglutinin epitope (for αM1-δM1-HA). Bound $^{125}\text{I-α-BuTx}$ in the immunoprecipitates was then quantitated with a γ-counter. B, dose-response curves of dTC inhibition of $^{125}\text{I-α-BuTx}$ binding to the heterodimer. COS cells were transfected with combinations of subunit cDNAs as indicated. $^{125}\text{I-α-BuTx}$ bound to heterodimers in cell lysates was immunoprecipitated using epitope-specific antibodies (for αM1-δ224-FLAG and αM1-δM1-HA) or δ subunit-specific mAb 88B (for αM1-δ). Nonspecific binding for these high affinity sites was determined by addition of 1 μM dTC to the sample (17). Control specific binding was defined by addition of no ligand other than $^{125}\text{I-α-BuTx}$ (total minus nonspecific binding). All data represent the average of two or more experiments.

The efficiency of glycosylation corresponds to the relative association of each of the fragments with the membrane. The truncated N-terminal domains of the α subunit showed a marked variation in glycosylation, with the longer peptides α and αM1 being readily glycosylated, whereas only a small fraction of the short fragments became glycosylated. Thus, α211 and presumably α207 are readily soluble; fragment α216 is membrane-associated, but can be released by treatment with alkaline buffers, and αM1 and α are integral membrane proteins. A possible explanation for this variation is that most of the enzymes responsible for nascent chain glycosylation are integral membrane proteins and may have better access to membrane-associated polypeptides. In agreement with observations for the full-length α subunit (40, 48, 52), we found glycosylation of all α forms to be necessary for folding. Nonetheless, the extent of glycosylation does not determine the efficiency of folding. Shorter forms such as α211 and α216, which were glycosylated to a lesser extent, folded more efficiently (Fig. 3). Whether the transmembrane regions actually constrain the folding of the luminal sequence of the protein or associate into heterodimers, and to form a ligand-binding site. Truncated fragments of the α subunit that included all, part, or none of the first transmembrane domain (M1) all folded to acquire α-BuTx binding activity. When coexpressed with the δ subunit, the fragment containing M1 formed a heterodimer with a ligand-binding site, but shorter fragments did not associate with the δ subunit. Examination of N-terminal δ subunit fragments showed a similar pattern. A δ subunit fragment containing M1 associated with αM1 to form a heterodimer, but a fragment lacking M1 did not. These results demonstrate that a complete M1 domain is necessary for truncated N-terminal peptides derived from the α and δ subunits to assemble into a heterodimer with high affinity ligand binding activity.

**Folding of Truncated α and δ Subunits in COS Cells—**As Merlie and Lindstrom (10) and Blount and Merlie (12) first demonstrated, the newly synthesized α subunit does not bind α-BuTx, but acquires the ability to do so over a period of 15–30 min after its synthesis and prior to its specific association with other subunits. Subsequent experiments in which the α subunit was expressed alone in transfected heterologous cells showed that other subunits are not required for the α subunit to fold to the toxin-binding form (12) and that a truncated subunit containing the N-terminal domain and M1 can achieve folding (23). The experiments reported here show that N-terminal fragments terminating at amino acid 216 or 211 can fold to assume high affinity α-BuTx binding when expressed in COS cells, but that a fragment terminating at amino acid 207 does not. Since the synthetic peptide derived from amino acid sequence 173–204 of the Torpedo α subunit can bind α-BuTx with relatively high affinity (44), it seems unlikely that residues 208–211 participate directly in interacting with the toxin molecule. Alternatively, the last 3 amino acids before M1 (208–210) may be critical for peptide folding to acquire a proper tertiary structure with an accessible high affinity toxin-binding site. The absence of α207 in the culture medium also supports this idea since misfolded or unfolded proteins are known to be generally prevented from entering the secretory pathway (45). Identifying the quality control mechanism responsible for the selective retention of misfolded α207 and for the efficient secretion of folded α211 remains an important problem of cell biology (46, 47).
whether the folded shorter forms have a longer half-life in the ER and thus accumulate to a greater extent remains to be determined.

Glycosylation of the δ subunit fragments, which have three glycosylation sites and both high-mannose and complex chains (9, 29), also appeared to occur most efficiently with the longer forms. Following synthesis, the δ subunits presumably undergo a folding reaction analogous to that of the α subunit, which makes them competent for assembly with other subunits. Because methods are not available for distinguishing folded and unfolded forms of the δ subunit, however, the effect of subunit truncation and glycosylation on folding of this subunit could not be determined.

Retention of the Truncated and Unassembled AChR Subunits in the Cell—Like other membrane proteins (45, 47), unfolded and unassembled AChR subunits or partially assembled complexes are poorly transported to the cell surface and remain in the ER (9, 13). The experiments shown here, in agreement with previous results (13, 22, 23), show that the folded full-length α subunit and α-M1 are not expressed on the surface in the absence of the other receptor subunits, but are retained intracellularly. Unassembled δ subunit and fragments with one or more transmembrane domains are likewise retained within the cell. In contrast, some truncated forms of the α and δ subunits without transmembrane domains are transported to the surface, where they are secreted into the medium. In the case of the α subunit, only folded forms are secreted. Thus, neither a207 nor non-glycosylated forms of α211 are seen in the medium, and virtually all of the α211 found in the medium is in the folded form. Like α211, δ224, which is truncated just before M1, is also secreted into the medium. Its secretion may imply that it is properly folded. These results suggest that only folded forms are recognized as competent for transport to the surface. We have previously shown that while some of the unfolded α subunit is rapidly degraded, a portion of this population is retained in the ER as a long-lived complex with BiP, a resident ER protein (49). Association with BiP or with other resident ER proteins could be responsible for retention of the unfolded, soluble α subunit peptides.

Although folded, a216, like α-M1 and full-length α, is not secreted. δ peptides containing the M1 domain are also not secreted. These results suggest that the M1 domain carries some signal that is responsible for retention in the ER. Studies of the α chain of the T cell receptor have identified a 23-amino acid sequence that is both necessary and sufficient for retention in the ER (50, 51), and based on its homology to this region, the M2 domain of the AChR subunits has been suggested as the targeting sequence for retention (37). Our results show that M2 is not required and that all or part of the M1 domain is sufficient for retention in the ER of both the α and δ subunits.

A Complete M1 Domain Is Required for the Formation of the αδ Heterodimer and a Ligand-binding Site—The specific association of homologous subunits to form hetero-oligomeric ligand-gated ion channels requires that the subunits each have domains within them that give them identity and that allow them to recognize each other. In earlier experiments, we used chimeric subunits to show that for the ε, γ, and δ subunits of the AChR, the identity of the subunits in the assembly of the AChR was determined by their extracellular N- and C-terminal domains (21, 22). In other experiments, we showed that the α-M1 subunit formed a specific heterodimer with the δ subunit, implying that the C-terminal domain was not necessary, and we showed that N-terminal fragments of the δ subunit lacking a transmembrane domain could exert a dominant negative effect on the formation of the αδ heterodimer and thus on assembly of the AChR (23). On the basis of these results, we postulated that all of the information necessary for the subunit recognition and interaction to form heterodimers was contained within the luminal N-terminal portion of the AChR subunits (16). More recent experiments by Kreienkamp et al. (26) have shown that a chimeric subunit, in which residues 21–131 of the γ subunit were substituted into the β subunit, can associate specifically with the α subunit to form a ligand-binding site. Taken together, these experiments provide strong evidence that subunit association is mediated exclusively through the luminal N-terminal domains of the subunits and that the transmembrane domains are not required for folding or subunit association.

We have further explored the requirements for subunit association by determining if an N-terminal fragment lacking a transmembrane domain can undergo specific heterodimer formation. Our results show by several criteria that the N-terminal domains of the α and δ subunits cannot associate with the appropriate partner when the M1 domain is missing. Although we have only circumstantial evidence that the δ fragment is folded properly (i.e. it is secreted), both α211 and α216 are folded into toxin binding conformations. In experiments reported elsewhere, we show, for the α subunit, that M1 contains no specific information for subunit association as α211 can form a heterodimer when attached to transmembrane domains from other proteins.

The failure of δ224 to associate with the α subunit leaves unexplained our previous dominant negative results showing that soluble fragments of the δ subunit can reduce the formation of the αδ heterodimer. The observed inhibition cannot have arisen by competition of the δ fragments with intact δ for the α subunit, an interpretation that is also consistent with our observation that the inhibition was not relieved by increased α subunit expression (23). In recent experiments, we have found that dominant negative effects can be exerted by α subunit fragments that do not fold (e.g. a207), suggesting that the fragments interfere in a more complex way with subunit folding and/or association.

One effect of αδ subunit association is to metabolically stabilize the two subunits (13, 14, 25). αδ heterodimers have a turnover time of >13 h, compared with 2 h for unassembled α or δ subunits. The increased amount of the toxin-binding form of the α subunit resulting from coexpression with the δ subunit reflects this stabilization (Fig. 7A). Our experiments show that the stabilizing effect of the δ subunit depends on its C-terminal tail. Thus, both δ-M1 and δ-M4 associate with the α subunit as shown by immunoprecipitation experiments and by the formation of a ligand-binding site (Fig. 10B and Table II), but association with these fragments does not increase the amount of toxin binding activity in the same way that association with full-length δ does (Table I). Deletion of the tail also reduces the amount of δ subunit when expressed alone (Fig. 9), suggesting that the C-terminal sequence plays a critical role in determining stability, perhaps by obscuring a sequence that targets the protein for rapid degradation.

One aim of our experiments on the assembly of the subunits of the AChR is to obtain a soluble heterodimer complex containing a ligand-binding site that will allow structural and functional studies of protein-ligand interactions. Although the experiments described here show that formation of such a soluble heterodimer within cells is unexpectedly complex, they provide a foundation for further studies on subunit assembly and suggest that assembly may involve factors other than the folded subunits themselves.

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