Membrane Tethering Enables an Extracellular Domain of the Acetylcholine Receptor α Subunit to Form a Heterodimeric Ligand-binding Site

Zuo-Zhong Wang,*‡ Stephen F. Hardy,* and Zach W. Hall*‡

*Department of Physiology, University of California School of Medicine, San Francisco, California 94143; and‡Laboratory of Cell Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20892

Abstract. The first step of assembly of the nicotinic acetylcholine receptor (AChR) of adult skeletal muscle is the specific association of the α subunit with either δ or ε subunits to form a heterodimer with a ligand-binding site. Previous experiments have suggested that heterodimer formation in the ER arises from interaction between the luminal, NH_2-terminal domains of the subunits. When expressed in COS cells with the δ subunit, however, the truncated NH_2-terminal domain of the α subunit folded correctly but did not form a heterodimer. Association with the δ subunit occurred only when the NH_2-terminal domain was retained in the ER and was tethered to the membrane by its own M1 transmembrane domain, by the transmembrane domain of another protein, or by a glycolipid link. In each case, the ligand-binding sites of the resulting heterodimers were indistinguishable from that formed when the full-length α subunit was used. Attachment to the membrane may promote interaction by concentrating or orienting the subunit; alternatively, a membrane-bound factor may facilitate subunit association.

THE nicotinic acetylcholine receptor (AChR)[1] in Torpedo electrical organ and mammalian skeletal muscle is the most widely studied member of a large family of ligand-gated ion channels (Betz, 1990; Unwin, 1993; Karlin and Akabas, 1995). The AChR is a heterooligomeric membrane protein formed by four different subunits in the stoichiometry $\alpha_2\beta\delta\epsilon$ (in adult mammalian skeletal muscle) or $\alpha_2\beta\delta\gamma$ (in fetal skeletal muscle and Torpedo electric organ) (Gu et al., 1990; Galzi et al., 1991; Karlin, 1991, 1993). The subunits of AChR are highly homologous and are arranged pseudosymetrically to surround a central aqueous pore through which ions flow (Karlin et al., 1983; Unwin, 1993). Each of the subunits is synthesized in the ER as a single polypeptide chain (Anderson and Blobel, 1981) that assumes a characteristic topology: a long, NH_2-terminal, extracellular domain, four transmembrane regions, and a short, COOH-terminal extracellular tail (Noda et al., 1982; Claudio, 1989; Chavez and Hall, 1991; Karlin, 1993). Like other oligomeric membrane proteins in the cell (Rose and Doms, 1988; Hurtley and Helenius, 1989), the peptide chains of AChR undergo folding reactions shortly after synthesis (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983). Properly folded subunits are assembled into a functional pentamer in the ER before being transported to the Golgi apparatus and from there to the cell surface (Smith et al., 1987; Gu et al., 1989; Blount et al., 1990; Green and Claudio, 1993).

Studies using heterologous expression systems with different combinations of subunits have delineated a distinctive scheme for AChR assembly, in which the first step is the formation of the heterodimers αδ and αε (or αγ) (Blount et al., 1990; Gu et al., 1991; Saedi et al., 1991). The interactions between subunits are highly specific, as heterodimer formation between α and β subunits does not occur even though they contact each other as neighbors in the fully assembled oligomer. The folded α subunit binds α-bungarotoxin (α-BuTx) in the absence of other subunits but only binds small, cholinergic ligands with high affinity after its association with δ or ε (γ) subunits (Blount and Merlie, 1988, 1989). The ligand-binding sites of the two heterodimers have distinct pharmacological features that correspond to the two nonequivalent sites found in the intact AChR (Karlin, 1991; Sine and Claudio, 1991; Fu and Sine, 1994). As the two α subunits in each receptor pentamer have identical primary polypeptide sequences, the nonequivalent binding of cholinergic ligands to identical α subunits is thought to be caused by structural influences of different neighboring subunits (Blount and Merlie, 1989; Sine, 1993). Indeed, photoaffinity labeling experiments have shown that δ and γ subunits contribute domains for

Address all correspondence to Dr. Zach W. Hall, National Institute of Neurological Disorders and Stroke, NIH, Building 31, Room 8A52, 31 Center Drive, Bethesda, MD 20892-2540. Tel.: (301) 496-9746. Fax: (301) 496-0296.

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; *d*TC, *d*-tubocurarine; GPI, glycosylphosphatidylinositol; HPAP, human placental alkaline phosphatase; M1, transmembrane domain 1; PI-PLC, phosphatidylinositol-specific phospholipase C; VSV, vesicular stomatitis virus.

ligand binding (Langenbuch-Cachat et al., 1988; Pederson and Cohen, 1990; Karlin and Akabas, 1995). The β subunit itself does not participate directly in the formation of a ligand-binding site, although it is essential for assembly of the final AChR pentamer (Blount et al., 1990; Gu et al., 1991; Saedi et al., 1991).

The association of subunits to form a heteromultimer with correct stoichiometry and subunit position requires domains within the sequence of each subunit that confer identity on it and allow it to recognize its neighbors. By studying the assembly of chimeric subunits and the dominant negative effects of truncated AChR subunit fragments, we and others have suggested that heterodimer formation occurs primarily through interactions between the extracellular NH_2-terminal domains of the AChR subunits (Yu and Hall, 1991, 1994; Hall, 1992; Verrall and Hall, 1992; Sumikawa and Nishizaki, 1994; Kreienkamp et al., 1995). We show here that although the soluble NH_2-terminal domain of the α subunit folds correctly, it does not associate with the δ subunit unless it is attached to the membrane, either by its own M1 transmembrane domain, by the transmembrane domain of another protein, or by a glycolipid link. Our data establish that the NH_2-terminal domain of the α subunit is an independent functional entity that possesses all of the structural information needed for folding, specific subunit association, and formation of a ligand-binding site. Efficient subunit association, however, requires retention of the subunit domain in the ER and association with the membrane.

Materials and Methods

Expression Vectors and Antibodies

Full-length cDNAs coding for the α, β, and δ subunits of mouse muscle nicotinic AChR were kindly provided by Drs. J.P. Merlie (Washington University, St. Louis, MO) and N. Davidson (California Institute of Technology, Pasadena, CA) (α, Isenberg et al., 1986; β, Buonanno et al., 1986; δ, Lapolla et al., 1984). The full-length cDNA for the mouse ε subunit was previously isolated in our own laboratory (Gu et al., 1990). Each of the cDNAs was subcloned into the multiple cloning sites of a SV-40–based expression vector, pSM (Brodsky et al., 1990).

mAb210 (Guillick and Lindstrom, 1983; Ratnam et al., 1986), a rat monoclonal antibody directed against the NH_2-terminus of α subunit, and mAb124 (Tzartos et al., 1981), a rat monoclonal antibody recognizing the β subunit, were generous gifts of Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). Mouse monoclonal antibody mAb88B (Froehner et al., 1983), which recognizes the cytoplasmic loop of the δ subunit, was kindly provided by Dr. Stanley Froehner (University of North Carolina, Chapel Hill, NC).

cDNA Constructs of AChR α Subunit

Intact, truncated, and chimeric proteins were derived from full-length cDNAs coding for the α subunit of the mouse muscle nicotinic AChR. The constructs α211 and αM1 were made by amplification of the corresponding sequences of the full-length α subunit cDNA with PCR. We engineered α211-CD8 and α211-VSVG by fusing the transmembrane and cytoplasmic domains of the human T-lymphocyte CD8 (Littman et al., 1985) and the vesicular stomatitis virus (VSV) G protein (Rose and Iverson, 1979) to the carboxyl side of amino acid residue 211 of the AChR α subunit, respectively (see Fig. 1). The α211-GPI was made by fusing the glycosylphosphatidylinositol (GPI) anchor signals from human placental alkaline phosphatase (HPAP) downstream of residue 211 of the α subunit (Lin et al., 1990). Each of the cDNA constructs was cloned into expression vector pSM and its sequence confirmed by dideoxynucleotide DNA sequencing (Sequenase kit; United States Biochem Corp., Cleveland, OH). Amino acids were numbered according to their position in the mature protein (signal peptide cleaved) sequence of α subunit.

Transfection of COS Cells

An adenovirus-mediated, DEAE-dextran transfection procedure was adopted to introduce expression vectors into COS cells (Forsayeth and Garcia, 1994). For a 10-cm dish of cells, 5.0 ml of Dulbecco's modified Eagle's medium (DMEM H-21), 1.0 ml of Adgpt lysate (an E1 defective adenovirus), 0.24 mg of DEAE-dextran, and an appropriate amount of plasmid DNA were mixed and added to cultured COS cells at ~60% confluence. For Western blot, immunoprecipitation, and pulse-chase labeling experiments in which only one or two subunits were expressed, 4 μg of each plasmid was used. For analysis of cell surface AChR expression, cDNAs for α, β, δ, and ε subunits were 2.64, 1.32, 0.52, and 1 μg, respectively, per 10-cm dish (Gu et al., 1991). After incubation in a CO_2 incubator at 37°C for 2 h, the transfection mixture was removed, and the cells were rinsed with PBS before being returned to the incubator in 10 ml of growth medium (10% FBS in DMEM H-21 supplemented with 2 mM glutamine, 100 U/ml of penicillin, and 100 U/ml of streptomycin).

Metabolic Labeling

COS cells were labeled metabolically 48 h after transfection. Briefly, after a 30-min preincubation at 37°C in cysteine- and methionine-free DMEM H-21, COS cells were labeled for 30 min at 37°C with 200 μCi/ml Tran^{35}S-label (1,159 Ci/mmol, ICN Biomedicals, Costa Mesa, CA) in cysteine- and methionine-free DMEM H-21. Cells were washed with DMEM H-21 supplemented with 2 mg/ml each of cysteine and methionine and then chased for 6 h with DMEM H-21 containing 10% FCS. At the end of the chase period, the media were collected, and the labeled cells scraped off the dish with ice-cold PBS, pelleted by centrifugation, and solubilized in an 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 PMSF, 20 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 U of aprotinin (Chavez et al., 1992; Gu et al., 1991). Cell lysates were pelleted to remove insoluble materials. The chasing media and the cleared cell extracts were each divided into two equal parts. Individual aliquots were assayed for total α subunit protein by immunoprecipitation with mAb210 and for folded α subunit by precipita-

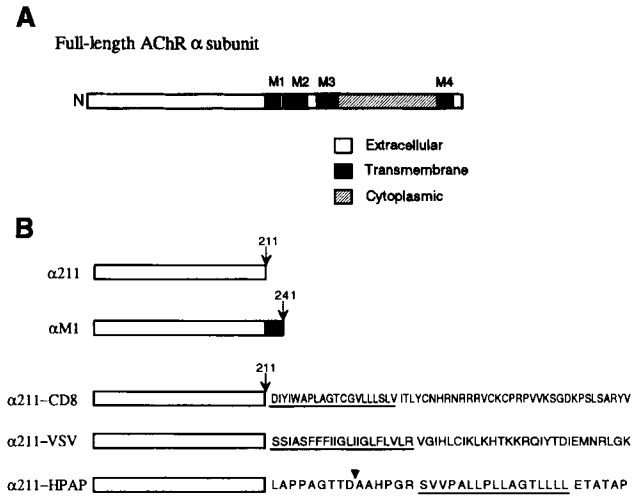

Figure 1. Diagram of the intact, truncated, and chimeric constructs of AChR subunit proteins. (*A*) Protein encoded by full-length mouse AChR α subunit cDNA. (*B*) Truncated proteins expressed by α211 and αM1 cDNAs, and fusion proteins encoded by a set of chimeric α subunit cDNAs. The arrows indicate the last amino acids encoded by AChR α subunit. Hydrophobic transmembrane domains of the chimeric constructs are underlined. For fusion protein α211-HPAP, the arrowhead indicates the site where peptide cleavage and addition of GPI anchor are predicted to occur within cells. Sequences are shown in one-letter amino acid notation.

tion with α-BuTx coupled to Sepharose 4B. The precipitations were performed for 2 h at 4°C with agitation. A rabbit anti-rat secondary antibody and protein G–Sepharose were used to precipitate the mAb210-containing samples. The Sepharose pellets were washed three times with 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl and 1% Triton X-100. The samples were heated for 5 min at 100°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 film (BioMax; Kodak, Rochester, NY). The relative optical density of the protein bands on the film was semiquantified using the software NIH Image (National Institutes of Health, Bethesda, MD) following scanning of the properly exposed films into a computer. Throughout this process, all samples were handled identically.

Surface Toxin Binding Assay

24 h after transfection, COS cells were trypsinized and replated into 12-well tissue culture dishes. After an additional 24 h in culture, surface expression of α-BuTx binding sites was determined by incubating intact cells in each well for 90 min at 37°C with 5 nM [^{125}I]α-BuTx (220 Ci/mmol; Amersham Corp., Arlington Heights, IL) in DMEM H-21. Unbound α-BuTx was then removed by washing the cells with PBS. The amount of bound toxin was determined by measuring the radioactivity with a gamma counter after solubilizing the cells in 0.1 N NaOH.

Immunoprecipitation of Toxin-binding Sites with Subunit-specific Antibodies

Immunoprecipitation of expressed proteins with subunit-specific antibodies was performed as described previously (Gu et al., 1989; Verrall and Hall, 1992). 48 h after the transfection, COS cells were scraped off the dish (10 cm), pelleted by centrifugation, and lysed in 0.4 ml of the Triton X-100 extraction buffer. One tenth of the lysates (0.04 ml) were incubated with 4 nM [^{125}I]α-BuTx and 80 nM of each of the primary antibodies in a total volume of 0.5 ml for 1 h on ice. 2 μl of rabbit anti-rat (for mAb210 and mAb124) or rabbit anti-mouse (for mAb88b) secondary antisera (Cappel Research Products, Durham, NC) were then added to each sample. After an additional 1-h incubation on ice, 50 μl of Standardized Pansorbin cells (10% suspension of fixed *Staphylococcus aureus* cells; Calbiochem-Novabiochem Corp., La Jolla, CA) was added, and the samples were continuously mixed in a rotatory mixer for 30 min at 4°C. The Pansorbin cells were then washed with the extraction buffer containing 0.5 M NaCl (without protease inhibitors), pelleted by centrifugation, and counted for radioactivity in a gamma counter. Control immunoprecipitation experiments were carried out using sham transfected cells.

Formation of a ligand-binding site by αδ heterodimers was determined by measuring the inhibition of [^{125}I]α-BuTx binding by *d*-tubocurarine (*d*TC) (Blount and Merlie, 1989). Immunoprecipitation described above was carried out with the following modifications: Primary antibodies (mAb88B) were first incubated with cell extracts for 1 h on ice. Secondary antibodies and *d*TC were added to the samples and incubated for 30 min. Finally, [^{125}I]α-BuTx (1 nM) was added and incubation was continued for 30 min. [^{125}I]α-BuTx bound to heterodimer was precipitated using the Standardized Pansorbin cells.

Velocity Sedimentation on Sucrose Gradient

48 h after the transfection, COS cells grown in a 10-cm dish were incubated on ice with 10 nM [^{125}I]α-BuTx in 10 mM phosphate, pH 7.4, 10 mM EDTA, 0.5% BSA, and 0.1% saponin for 2 h (Blount et al., 1990; Verrall and Hall, 1992). The labeled cells were then washed three times in the same buffer without [^{125}I]α-BuTx, scraped off the plate with a rubber policeman, pelleted by brief centrifugation, and lysed in 0.4 ml extraction buffer. Cell lysates (0.04 ml) were loaded onto a 5–20% continuous sucrose gradient. Bovine alkaline phosphatase (6.3 S) and bovine hemoglobin (4.3 S) were included in the cell lysates as markers. Gradients were centrifuged at 65,000 rpm in a rotor (model VTi65; Beckman Instrs., Fullerton, CA) at 4°C for 80 min and fractionated into 105 μl aliquots (Saedi et al., 1991, Verrall and Hall, 1992). Fractions were then counted in a gamma counter.

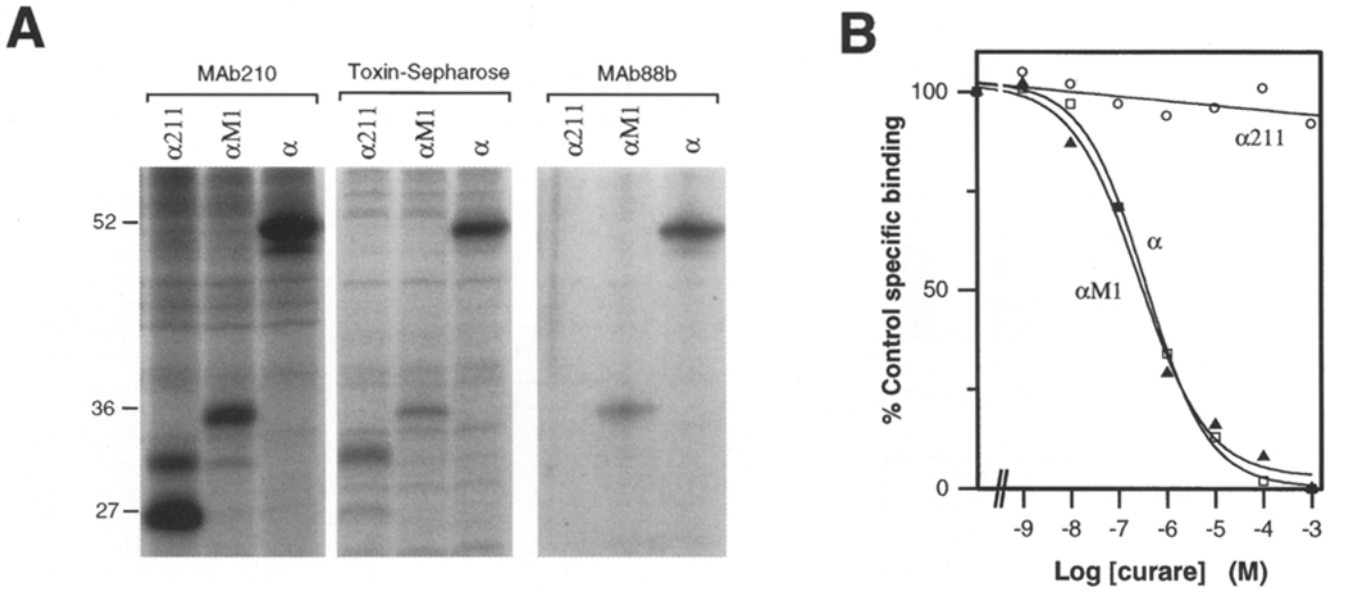

Figure 2. Expression, folding, and assembly of the truncated NH_2-terminal fragments and the full-length α subunit. (*A*) Fluorography showing the expression, folding, and assembly of α211, αM1, and the full-length α subunit. COS cells transfected with each of the cDNAs encoding α211, αM1, or the full-length α subunit in combination with δ were labeled overnight with [^{35}S]methionine. Cell lysates were divided into three equal parts. Total, correctly folded, and assembled α subunits were isolated by immunoprecipitation with antibody mAb210, by precipitation with α-BuTx–Sepharose, and by immunoprecipitation with antibody mAb88B, respectively. The precipitates were analyzed by 12.5% reducing SDS–acrylamide gels and fluorography. The lower bands in lane *1*, *2*, and *3* correspond to the nonglycosylated peptide. The positions of molecular mass markers (expressed in kD) are indicated on the left. (*B*) α211 failed to form a high affinity ligand-binding site when coexpressed with the full-length δ subunit. Formation of a ligand-binding site was determined by measuring *d*TC inhibition of α-BuTx binding. Triton X-100 lysates of the transfected COS cells were incubated for 90 min with [^{125}I]α-BuTx (1 nM) in the presence of *d*TC. Toxin bound to heterodimers was immunoprecipitated using mAb88b. Nonspecific binding was determined by addition of excess unlabeled α-bungarotoxin (1 μM). Control specific binding was defined by addition of no ligand other than [^{125}I]α-BuTx (total minus nonspecific binding).

Immunocytochemical Staining

Immunofluorescence staining was performed as described before (Ralston and Hall, 1989). Briefly, transfected COS cells cultured in chamber slides were fixed for 10 min in 2% paraformaldehyde in PBS, pH 7.4, and rinsed in cold PBS. After a preincubation in PBS containing 10% bovine serum and 0.05% saponin at 4°C for 20 min to permeabilize the membrane, cells were incubated at 4°C for 1 h with rhodamine-conjugated α-BuTx (for staining of α211 and α211-GPI), mAb88B (for δ subunit), and a polyclonal antibody to ER membrane proteins (for ER marker; Louvard et al., 1982). After a brief rinse in cold PBS, cells were incubated with rhodamine-conjugated goat anti-mouse (for δ subunit) or FITC-conjugated sheep anti-rabbit (for ER marker) secondary antibodies. The slides were examined under a fluorescence microscope (Nikon Inc., Melville, NY) equipped with the appropriate filters.

Western Immunoblotting

Lysates of the transfected COS cells were denatured by heating at 95°C for 5 min in SDS gel-loading buffer containing β-mecaptoethanol, electrophoresed on a 12.5% SDS–polyacrylamide gel (Laemmli, 1970), and then transferred to nitrocellulose membranes. After preincubation in PBS/0.3% Tween 20 containing 3% BSA to block nonspecific binding, the membranes were incubated with primary antibodies in the same blocking buffer and washed with PBS/Tween 20. Antibodies bound to the membrane were detected with horseradish peroxidase–conjugated secondary antibodies and enhanced chemoluminescent reagents (ECL; Amersham Corp.).

Results

The NH_2-terminal Domain of the α Subunit Folds but Does Not Associate with the δ Subunit

Before the α subunit can be assembled into the pentameric receptor, it must undergo a folding reaction in which it acquires the ability to bind α-BuTx (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983). To determine if a signal peptide-cleaved fragment of the AChR α subunit truncated at the NH_2-terminal boundary of the first transmembrane domain (α211; Fig. 1) can fold and associate with the δ subunit, we expressed the fragment in COS cells that were metabolically labeled with [^{35}S]methionine. Radioactive α peptide was precipitated from a cell lysate, either by an antibody to the NH_2-terminal domain (mAb 210, Guillick and Lindstrom, 1983; Ratnam et al., 1986) or by α-BuTx-Sepharose. Thus, the α211 peptide, like the full-length α subunit or a truncated α fragment containing the first transmembrane domain (αM1), can fold to the toxin-binding conformation (Fig. 2 *A*; Wang et al., 1996). When α211 is coexpressed in metabolically labeled COS cells with the δ subunit, however, no association between the two subunits was detected as measured by immunoprecipitation with an antibody to the δ subunit, mAb88B (Froehner et al., 1983). In contrast, both αM1 and the full-length α subunit were coimmunoprecipitated by the δ subunit antibody, indicating that in each case a complex with the δ subunit had been formed (Fig. 2 *A*). Sucrose gradient sedimentation experiments also failed to demonstrate association between folded α211 and the δ subunit (data not shown). As a final test, the antagonistic cholinergic ligand, *d*TC, failed to inhibit toxin binding in lysates of cells cotransfected with cDNAs for α211 and the δ subunit. In contrast, the association of both αM1 and the α with δ subunit results in the formation of a high affinity ligand binding site (Fig. 2 *B*; Blount and Merlie, 1989; Verrall and

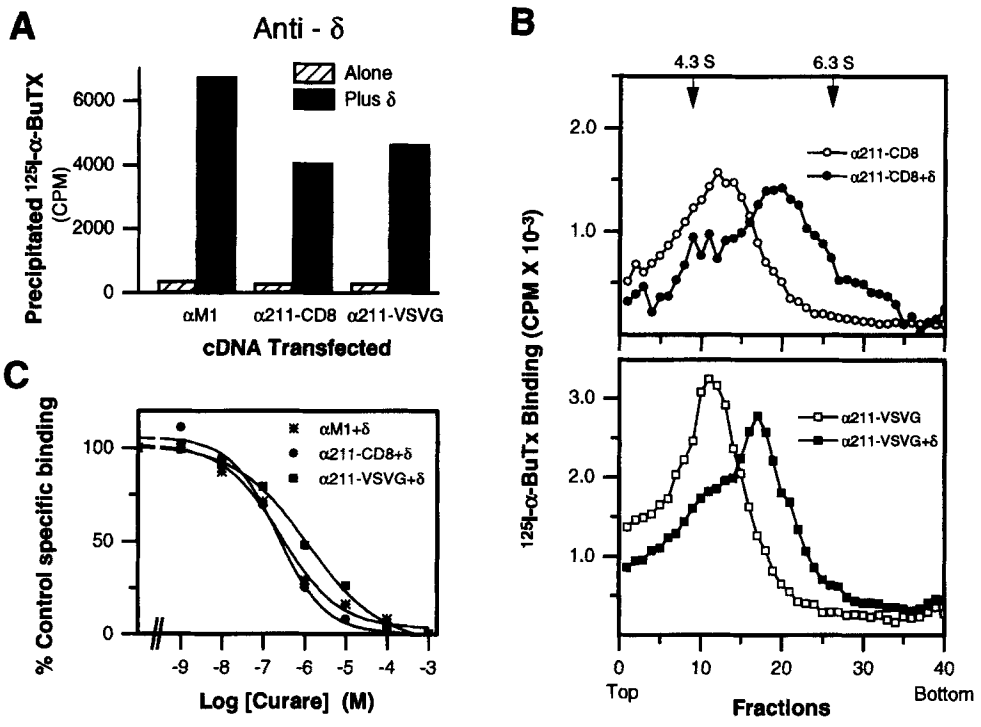

Figure 3. The α NH_2-terminal fragments fused to nonspecific transmembrane domains assemble with δ to form a heterodimer with a high affinity ligand-binding site. (*A*) COS cells grown in a 10-cm dish were transfected with each of the chimeric α subunit cDNAs alone (*cross-hatched bars*) or with the full-length δ subunit (*black bars*). One tenth of the cell extracts from each dish were incubated with [^{125}I]α-BuTx (10 nM), and assembled heterodimers were immunoprecipitated using mAb88b. (*B*) Sucrose gradient sedimentation was performed to identify heterodimers formed by chimeric α constructs and the δ subunit. COS cells grown in a 10-cm dish were transfected with cDNAs encoding, in the upper panel, α211-CD8 (*open circles*) or α211-CD8 plus δ (*filled circles*); in the lower panel, α211-VSVG (*open squares*) or α211-VSVG plus δ (*solid squares*). Cells were permeabilized with 0.1% saponin and incubated with 10 nM of [^{125}I]α-bungarotoxin for 90 min at 4°C, and washed with PBS. One tenth of Triton X-100 extracts in a culture dish were applied to a 5–20% sucrose gradient as described. (*C*) Chimeric α NH_2-terminal domains form a high affinity ligand-binding site with the δ subunit. COS cells were transfected with cDNAs encoding αM1 plus δ (*asterisks*), α211-CD8 plus δ (*filled circles*), and α211-VSVG plus δ (*solid squares*). Cell lysates were processed for analysis of *d*TC inhibition of [^{125}I]α-BuTx binding as described in Fig. 2 *B*.

Hall, 1992). Thus, the soluble, folded NH_2-terminal domain of the α subunit does not assemble with the δ subunit.

Nonspecific Peptide Sequences Can Replace M1 to Support Heterodimer Assembly

One explanation for the inability of α211 to associate with the δ subunit is that information required for subunit association is contained within the M1 region. To test this possibility, constructs were made encoding chimeric proteins in which α211 was fused to the transmembrane region of either the VSV glycoprotein (VSV G protein) or the lymphocyte surface antigen CD8 (Rose and Iverson, 1979; Littman et al., 1985; Fig. 1 *B*). Each of these chimeric proteins was then expressed in COS cells along with the δ subunit. In each case the fusion protein folded to a toxin-binding form that associated with the δ subunit as shown by immunoprecipitation experiments (Fig. 3 *A*). Sucrose gradient experiments in both cases showed a major peak at approximately 5.3 S, indicating the formation of a heterodimer (Fig. 3 *B*). Moreover, the heterodimers bound *d*TC with a high affinity, as shown by inhibition of toxin binding (Fig. 3 *C*) that was indistinguishable from that of the $\alpha_{M1}\delta$ heterodimer (Fig. 2 *D*). We conclude from these experiments that the M1 region of the α subunit is not specifically required for heterodimer formation with the δ subunit and that transmembrane domains from other proteins are equally effective.

Retention of α211 in the ER Does Not Result in Association with the δ Subunit

Assay of [^{125}I]α-BuTx binding activity on the surface of transfected COS cells showed that each of the fusion proteins, like αM1, is almost completely retained intracellularly (Fig. 4, *A* and *B*). In contrast, a substantial amount of α211, which lacks a transmembrane domain, is secreted into the medium (Fig. 4 *B*; Wang et al., 1996). A possible role of the attached transmembrane region may thus be to retain α211 in the endoplasmic reticulum, allowing association to occur. We tested this idea by treating cells expressing the α211 peptide and the δ subunit with brefeldin A, which prevents transport of secretory and membrane-bound proteins from the ER to the Golgi apparatus (Klausner et al., 1992). Even after brefeldin A treatment, however, no evidence was found for the association of α211 with the δ subunit (Fig. 5 *A*). To verify that α211 was retained after treatment with brefeldin A and that it was present in the same cellular compartment as the δ subunit, transfected cells treated with brefeldin A were examined by immunofluorescence. The distribution of the α211 polypeptide in brefeldin A–treated cells was exactly the same as that of an authentic ER marker (Ralston and Hall, 1989); moreover, the δ subunit was also demonstrated to be in the ER (Fig. 5 *B*). We conclude that both α211 and δ are in the ER but that α211 is unable to associate with the δ subunit because it is not tethered to the membrane.

Membrane Tethering via Glycolipid, Coupled with ER Retention, Enables α211 to Assemble

To see if the NH_2-terminal domain can be attached in other ways that would allow it to associate with the δ subunit, we expressed α211 as a fusion protein attached to a segment (the COOH-terminal 29 amino acids) of HPAP that serves as a signal sequence for the attachment of a GPI residue, linking the polypeptide to the cell membrane (Fig. 1 *B*; Lin et al., 1990). Cells transfected with cDNA for the fusion protein expressed toxin-binding activity on their surface in a form that could be released into the medium by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC; Fig. 6 *A*, lane 5), indicating that a GPI anchor had been formed (Lin et al., 1990; Kemble et al., 1994). When coexpressed with the δ subunit, however, α211-GPI did not associate with the δ subunit, either on the cell surface or in the ER. Thus, the truncated α

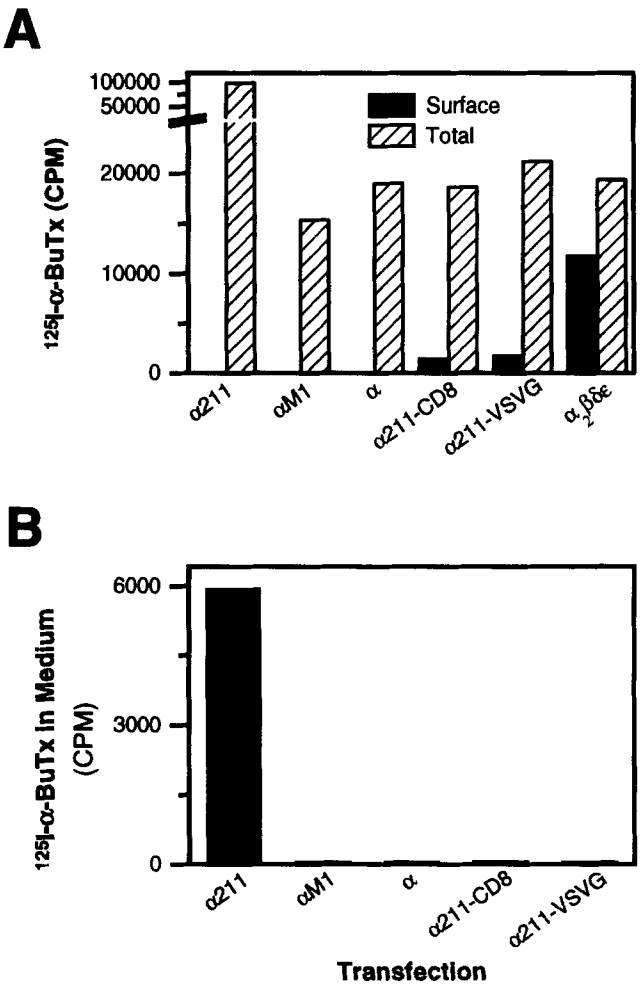

Figure 4. α-BuTx binding activity of the truncated and chimeric α subunit proteins on the cell surface and in the culture medium. (*A*) [^{125}I]α-BuTx binding sites expressed on the plasma membrane of the transfected COS cells (*dark bars*), and total toxin binding in cell lysates immunoprecipitated with mAb210 (*cross-hatched bars*). The data represent [^{125}I]α-BuTx bound per well (2.5-cm diam) of transfected cells. (*B*) Secretion of the expressed α subunit proteins was detected by measuring [^{125}I]α-BuTx binding activity in the culture media of COS cells transfected with each of the cDNAs as indicated. 1 ml of the culture media from each well of the transfected cells was used for immunoprecipitation assay with mAb210, and the results were expressed as [^{125}I]α-BuTx bound.

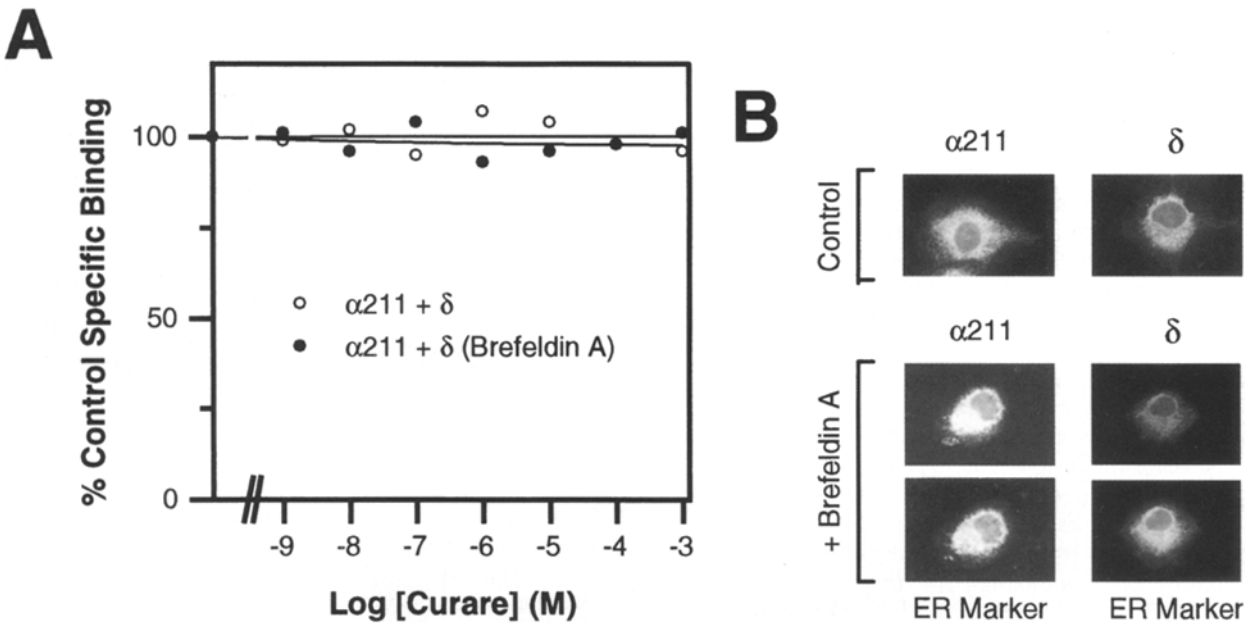

Figure 5. Brefeldin A fails to facilitate α211 assembly with the δ subunit, though it retains the peptide in the ER. (*A*) COS cells transfected with α211 plus δ cDNAs were incubated in control growth medium (*open circles*) or in growth medium containing brefeldin A (5 μg/ml, *filled circles*) at 37°C for 6 h. They were then lysed with extraction buffer containing Triton X-100. Inhibition of [^{125}I]α-BuTx binding by *d*TC was determined as described in Fig. 2 *B*. (*B*) Brefeldin A causes the retention of α211 in the ER. Cells transfected with cDNAs encoding α211 and δ were treated with brefeldin A as in *A*. Immunofluorescence staining was done using rhodamine-conjugated α-BuTx (for α211), mAb88B (for δ), and a polyclonal antibody to ER membrane (for ER marker). In the brefeldin A–treated group, the bottom row stained with anti-ER antibody shows the same cells as the top row.

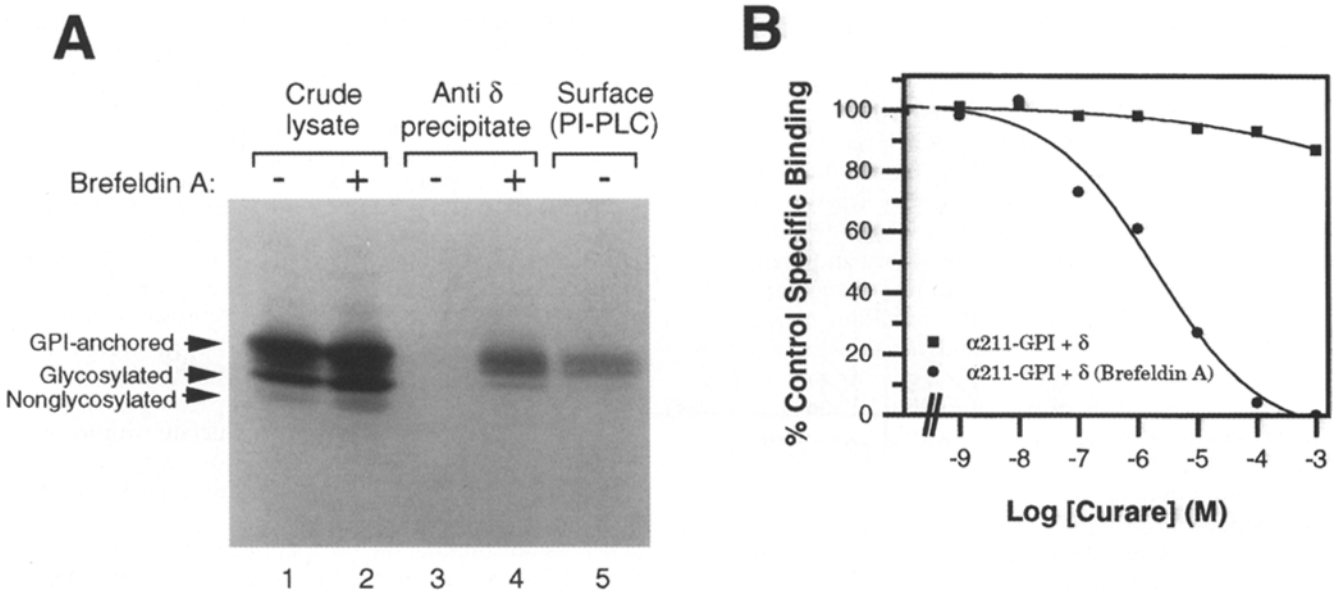

Figure 6. When retained in the ER, the GPI-tethered α211 assembles with the δ subunit to form a high affinity ligand-binding site. (*A*) An immunoblot with mAb210 shows specific association of the α211-GPI with δ after brefeldin A treatment. Cells coexpressing α211-GPI and the δ subunit were extracted with β-D-octylglucoside. Immunoblotting was performed using crude cell lysates (lanes *1* and *2*) or using mAb88b immunoprecipitates of the crude cell lysates (lanes *3* and *4*). In lanes *2* and *4*, cells were treated with brefeldin A (5 μg/ml) at 37°C for 6 h. Lane *5* shows the surface GPI-anchored α211 released into culture medium following cleavage by PI-PLC. (*B*) Formation of a high affinity ligand-binding site. COS cells transfected with α211-HPAP plus δ were incubated in control growth medium (*squares*) or in growth medium containing brefeldin A (5 μg/ml; *filled circles*) at 37°C for 6 h. They were then lysed with extraction buffer containing 60 mM β-D-octylglucoside. Inhibition of [^{125}I]α-BuTx binding by *d*TC was determined as described in Fig. 2 *B*.

polypeptide could not be coimmunoprecipitated with an anti-δ antibody (Fig. 6 *A*), and inhibition of toxin binding by *d*TC was not observed (Fig. 6 *B*).

A possible explanation for the failure of folded α211-GPI to form a heterodimer is that its effective concentration in the ER is too low for assembly. A large portion (80–90%) of α211-GPI is expressed on the cell surface (Fig. 7) to which it is rapidly transported within 10 min of synthesis (data not shown). To test this idea, we treated cells expressing α211-GPI and the δ subunit with brefeldin A. In the presence of the drug, folding of α211-GPI was not affected (Fig. 7 *A*), and the peptide was completely retained in the ER (Fig. 7). Moreover, the α211-GPI that was retained in the ER associated with the δ subunit as shown by coimmunoprecipitation with δ-specific antibody mAb88B (Fig. 6 *A*, lane *4*). In contrast, when α211-GPI was coexpressed with the β subunit in the presence of brefeldin A, a monoclonal antibody (mAb124; Tzartos et al., 1981) recognizing the β subunit precipitated no toxin binding activity (data not shown). Thus, the interaction between the α211-GPI and the δ subunit is specific, as the fragment does not associate with the β subunit. The association between α211-GPI and the δ subunit resulted in the formation of a high-affinity binding site for *d*TC that is indistinguishable from that of the full-length αδ heterodimer (Fig. 6 *B*). We conclude that α211 contains all of the sequence information necessary for folding, for specific subunit association, and for formation of a high-affinity ligand-binding site. Efficient interaction with the δ subunit, however, requires that α211 be retained in the ER and be tethered to the membrane.

Discussion

Previous experiments on AChR assembly in our own and other laboratories have implicated the luminal, NH_2-terminal domain as the part of the AChR subunits that is responsible for their specific association into heterodimers with ligand-binding sites (Yu and Hall, 1991, 1994; Verrall and Hall, 1992; Sumikawa and Nishizaki, 1994; Kreienkamp et al., 1995). Here we show that although the NH_2-terminal domain of the α subunit expressed in COS cells can fold autonomously into a conformation that allows it to bind α-BuTx, it does not associate with the δ subunit unless two conditions are met: It must be retained in the ER, and it must be attached to the membrane.

Like the subunits of other oligomeric membrane proteins (Hurtley and Helenius, 1989; Doms et al., 1993), unassociated subunits and assembly intermediates of the AChR are normally retained in the ER. When expressed alone, none or only trace amounts of the AChR subunits are seen on the surface (Smith et al., 1987; Gu et al., 1989;

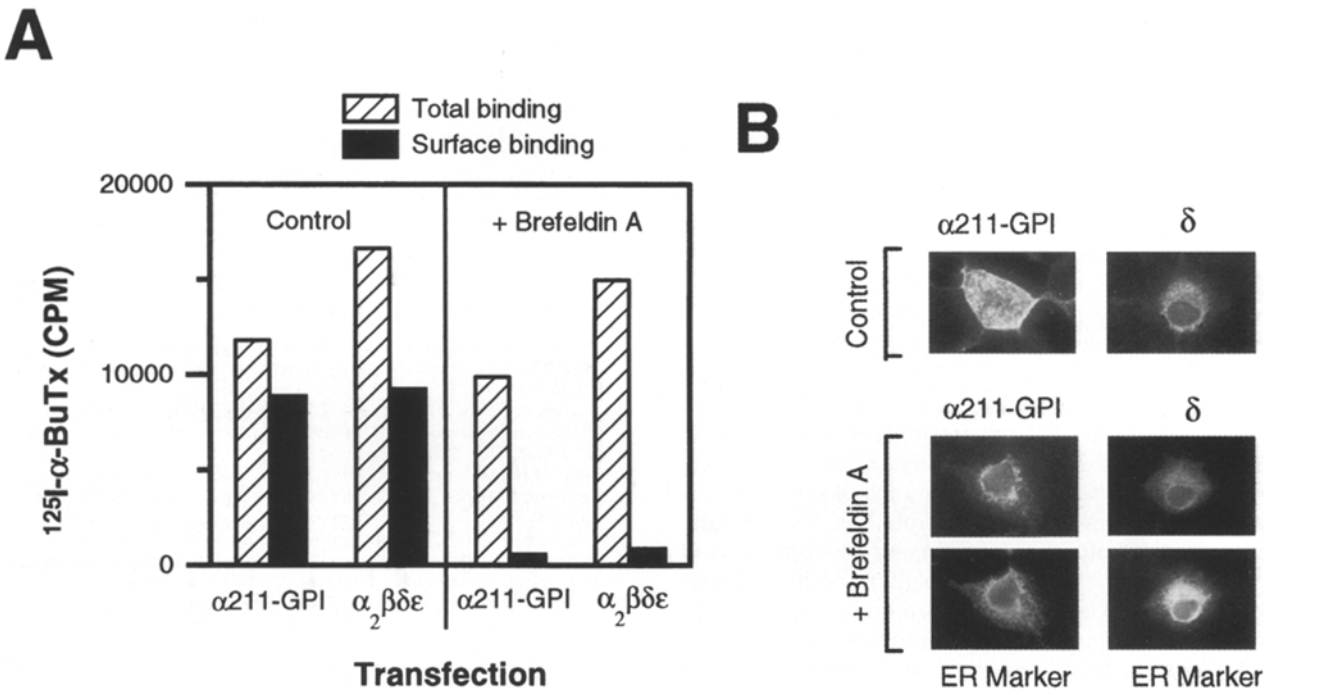

Figure 7. (*A*) Brefeldin A blocked the surface transport of α211-GPI and AChR pentamer ($\alpha_2\beta\delta\epsilon$), but had little effect on α subunit folding. COS cells grown in a 6-cm dish were transfected with cDNAs for α211-GPI, or for the α, β, δ and ϵ subunits, and replated to a 12-well culture dish. Intact cells in each well (2.5-cm diam) were preincubated with unlabeled α-BuTx (1 μM, 37°C for 1 h) to block preexisting surface receptor. After washing with PBS to remove unbound toxins, cells were incubated with control medium or medium containing brefeldin A (5 μg/ml) for 6 h, and processed for surface [^{125}I]α-BuTx binding. An equal number of cells in separate wells were treated in the same manner and lysed with detergent. Total α-BuTx binding activity in the lysates was determined by immunoprecipitation with mAb210. (*B*) Retention of the GPI-tethered α211 in the ER by brefeldin A. Cells transfected with cDNAs as indicated were treated with brefeldin A as in *A*. Immunofluorescence staining was done using rhodamine-conjugated α-BuTx (for α211-GPI), mAb88B (for δ), and a polyclonal antibody to ER membrane (for ER marker). The brefeldin A–treated cells were double labeled with antibodies against the indicated subunits (*top row*) and with the ER marker antibody (*bottom row*).

Blount et al., 1990; Maimone and Merlie, 1993). Since assembly occurs in the ER, retention presumably promotes efficient interaction of the subunits with each other and possibly with assembly cofactors in the ER. In our experiments, αM1 and each of the chimeric proteins α211-CD8 and α211-VSVG were retained in the ER, where they efficiently formed heterodimers with the δ subunit. In contrast, α211, which lacks a transmembrane domain and is secreted into the medium, and α211-GPI, which is rapidly transported to the surface, do not associate with the δ subunit. These results suggest that the signal for retention of the α subunit resides in its transmembrane domains. The interactions that are responsible for retention are likely to be complex. Thus, the transmembrane domain of CD8 in its normal context does not result in ER retention (Littman et al., 1985; Ralston and Hall, 1989), but does so when attached to α211. Studies of the α chain of the T cell receptor have identified a 23-amino acid sequence in the transmembrane domain that is both necessary and sufficient for retention in the ER (Bonifacino et al., 1990).

The importance of retention in the ER for heterodimer formation is seen most clearly for α211-GPI. When expressed under normal conditions, α211-GPI was transported to the cell surface within 10 min, and no association with the δ subunit was observed. After brefeldin A treatment, however, which prevents transport of proteins out of the ER, α211-GPI readily associated with the δ subunit to form a heterodimer with a ligand-binding site. Since the δ subunit is confined to the ER, the steady-state concentration of α211-GPI in the ER under normal conditions is presumably too low for efficient heterodimer formation to occur. To see if heterodimer formation could occur if both subunits were on the surface, we made a GPI construct of the δ NH_2-terminal domain (δ224-GPI) and coexpressed it with α211-GPI. Although δ224-GPI was transported to the surface along with α211-GPI, no heterodimer formation was detected (Wang, Z.-Z., and Z.W. Hall, unpublished experiments). We were also unable to obtain heterodimer formation, however, when α211-GPI and δ224-GPI were coexpressed in the presence of brefeldin A. Thus, we cannot rule out the possibility that δ224-GPI is not folded correctly.

The NH_2-terminal domain of the α subunit must not only be present in the ER for assembly to occur, it must also be attached to the membrane. Since α211 can be effectively anchored to the membrane by each of several transmembrane sequences or by a glycolipid link, the information for specific subunit interaction and for formation of the ligand-binding site must arise solely from the NH_2-terminal domain. Attachment to the membrane appears to be necessary to facilitate the assembly process. This may be a general feature of membrane protein assembly as homooligomeric viral membrane proteins are known to assemble less efficiently when they are expressed as anchor-free fragments (Singh et al., 1990; Doms et al., 1993).

Tethering a folded polypeptide to the membrane confines its diffusional movement to a two-dimensional surface and orients it for interaction with other subunits. Such an effect could explain the effect that we observe. Alternatively, interaction with a membrane-bound cofactor may be required for efficient subunit association. For assembly of the AChR, calnexin could be one such candidate; interaction with other proteins may also be important. Calnexin, a membrane protein residing in the ER, has been shown to associate with the α subunit of the AChR and is thought to play a role in the folding and assembly of oligomeric proteins (Gelman et al., 1995). Although the interaction of calnexin with viral membrane proteins appears to require oligosaccharide trimming (Hammond and Helenius, 1995), inhibitors of the trimming reaction have no effect on heterodimer formation in an in vitro translation system (Shtrom and Hall, 1996). Further investigation will be required to determine the importance of membrane-bound factors. The present experiments give impetus to a search for assembly cofactors by establishing that the NH_2-terminal domain of the α subunit, in the absence of other parts of the protein, is competent to fold, to assemble with the δ subunit, and to form an authentic ligand-binding site, but only does so when tethered to the ER membrane.

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References

Anderson, M.J., and G. Blobel. 1981. *In vitro* synthesis, glycosylation, and membrane insertion of the four subunits of torpedo acetylcholine receptor. *Proc. Natl. Acad. Sci. USA.* 78:5598–5602.

Betz, H. 1990. Ligand-gated ion channels in the brain: the amino acid receptor superfamily. *Neuron.* 5:383–392.

Blount, P., M.S. McHardy, and J.P. Merlie. 1990. Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell Biol.* 111:2601–2611.

Blount, P., and J.P. Merlie. 1988. Native folding of an acetylcholine receptor α subunit expressed in the absence of other subunits. *J. Biol. Chem.* 263:1072–1080.

Blount, P., and J.P. Merlie. 1989. Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. *Neuron.* 3: 349–357.

Bonifacino, J.S., C.K. Suzuki, and R.D.Klausner. 1990. A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. *Science (Wash. DC).* 247:79–82.

Brodsky, M.H., M. Warton, R.M. Myers, and D.R. Littman. 1990. Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. *J. Immunol.* 144:3078–3086.

Buonanno, A., J. Mudd, V. Shah, and J.P. Merlie. 1986. A universal oligonucleotide probe for acetylcholine receptor genes: selection and sequencing of cDNA clones for the mouse β subunit. *J. Biol. Chem.* 261:16451–16458.

Chavez, R.A., and Z.W. Hall. 1991. The transmembrane topology of the amino terminus of the α subunit of the nicotinic acetylcholine receptor. *J. Biol. Chem.* 266:15532–15538.

Chavez, R.A., J. Maloof, D. Beeson, J. Newsom-Davis, and Z.W. Hall. 1992. Subunit folding and αδ heterodimer formation in the assembly of the nicotinic acetylcholine receptor. *J. Biol. Chem.* 267:23028–23034.

Claudio, T. 1989. Molecular genetics of acetylcholine receptor-channels. *In* Frontiers in Molecular Biology: Molecular Neurobiology. D.M. Glover and B.D. Hames, editors. IRL Press, Oxford. 63–142.

Doms, R.W., R.A. Lamb, J.K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology.* 193:545–562.

Forsayeth, J.R., and P.D. Garcia. 1994. Adenovirus-mediated transfection of cultured cells. *Biotechniques.* 17:354–358.

Froehner, S.C., K. Douville, S. Klink, and W.J. Culp. 1983. Monoclonal antibodies to cytoplasmic domains of the acetylcholine receptor. *J. Biol. Chem.* 258:7112–7120.

Fu, D.-X., and S.M. Sine. 1994. Competitive antagonists bridge α-γ subunit interface of the acetylcholine receptor through quaternary ammonium aro-

matic interactions. *J. Biol. Chem.* 269:26152–16157.
Galzi, J.F., F. Revah, A. Bessis, and J.P. Changeux. 1991. Functional architecture of the nicotinic acetylcholine receptor: from electric organ to brain. *Annu. Rev. Pharmacol.* 31:37–72.
Gelman, M.S., W. Chang, D.Y. Thomas, J.J. Bergeron, and J.M. Prives. 1995. Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of nicotinic acetylcholine receptors. *J. Biol. Chem.* 270:15085–15092.
Green, W.N., and T. Claudio. 1993. Acetylcholine receptor assembly: subunit folding and oligomerization occur sequentially. *Cell.* 74:57–70.
Gu, Y., E. Ralston, C. Murhy-Erdosh, R. Black, and Z.W. Hall. 1989. Acetylcholine receptor in a C2 muscle cell variant is retained in the endoplasmic reticulum. *J. Cell Biol.* 109:729–738.
Gu, Y., A. Franco, P.D. Gardner, J.B. Lansman, and Z.W. Hall. 1990. Properties of embryonic and adult muscle acetylcholine receptors transiently expressed in COS cells. *Neuron.* 5:147–157.
Gu, Y., J.R. Forsayeth, S. Verrall, X.-M. Yu, and Z.W. Hall. 1991. Assembly of the mammalian acetylcholine receptor in transfected COS cells. *J. Cell Biol.* 114:799–807.
Guillick, W., and J. Lindstrom. 1983. Mapping the binding of monoclonal antibodies to the acetylcholine receptor form Torpedo californica. *Biochemistry.* 22:3312–3320.
Hall, Z.W. 1992. Recognition domains in assembly of oligomeric membrane proteins. *Trends Cell Biol.* 2:66–68.
Hammond, C., and A. Helenius. 1995. Quality control in the secretory pathway. *Curr. Opin. Cell Biol.* 7:523–529.
Hurtley, S.M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277–307.
Isenberg, K.E., J. Mudd, V. Shah, and J.P. Merlie. 1986. Nucleotide sequence of the mouse muscle acetylcholine receptor α subunit. *Nucleic Acids Res.* 14: 5111.
Karlin, A. 1991. Explorations of the nicotinic acetylcholine receptor. *Harvey Lect.* 85:71–107.
Karlin, A. 1993. Structure of nicotinic acetylcholine receptors. *Curr. Opin. Neurobiol.* 3:299–309.
Karlin, A., and M.H. Akabas. 1995. Toward a structural basis for the function of nicotinic acetylcholine receptor and their cousins. *Neuron.* 15:1231–1244.
Karlin, A., E. Holtzman, N. Yodh, P. Lobel, J. Wall, and J. Hainfeld. 1983. The arrangement of the subunits of the acetylcholine receptor of Torpedo californica. *J. Biol. Chem.* 258:6678–6681.
Kemble, G.W., T. Danieli, and J.M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell.* 76:383–391.
Klausner, R.D., J.G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071–1080.
Kreienkamp, H.-J., R.K. Maeda, S.M. Sine, and P. Taylor. 1995. Intersubunit contacts governing assembly of the mammalian nicotinic acetylcholine receptor. *Neuron.* 14:635–644.
Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
Langenbuch-Cachat, J., C. Bon, C. Mulle, M. Goeldner, C. Hirth, and J.-P. Changeux. 1988. Photoaffinity labeling of the acetylcholine binding sites on the nicotinic receptor by an aryldiazonium derivative. *Biochemistry.* 27: 2337–2345.
Lapolla, R.J., K.M. Mayne, and N. Davidson. 1984. Isolation and characterization of a cDNA clone for the complete protein coding region of the δ subunit of the mouse acetylcholine receptor. *Proc. Natl. Acad. Sci. USA.* 81:7970–7974.
Lin, A., B. Devaux, A. Green, C. Sagerstrom, J. Elliott, and M. Davis. 1990. Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science (Wash. DC).* 249:677–679.
Littman, D.R., Y. Thomas, P.J. Maddon, L. Chess, and R. Axel. 1985. The isolation and sequence of the gene encoding T8: a molecule defining functional classes of T lymphocytes. *Cell.* 40:237–246.
Louvard, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi complex and the rough endoplasmic reticulum. *J. Cell Biol.* 92:92–107.
Maimone, M.M., and J.P. Merlie. 1993. Interaction of the 43 kd postsynaptic protein with all subunits of the muscle nicotinic acetylcholine receptor. *Neuron.* 11:53–66.
Merlie, J.P., and J. Lindstrom. 1983. Assembly in vivo of mouse muscle acetylcholine receptor: identification of an α subunit species that may be an assembly intermediate. *Cell.* 34:747–757.
Merlie, J.P., and R. Sebbane. 1981. Acetylcholine receptor subunits transit a precursor pool before acquiring α-bungarotoxin binding activity. *J. Biol. Chem.* 256:3605–3608.
Noda, M., H. Takahashi, T. Tanabe, M. Toyosato, Y. Furutani, T. Hirose, M. Asai, S. Inayama, T. Miyata, and S. Numa. 1982. Primary structure of alpha-subunit precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. *Nature (Lond.).* 299:793–797.
Pederson, S.E., and J.B. Cohen. 1990. *d*-tubocurarine binding sites are located at αγ and αδ subunit interfaces of the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA.* 87:2785–2789.
Ralston, E., and Z.W. Hall. 1989. Intracellular and surface distribution of a membrane protein (CD8) derived from a single nucleus in multinucleated myotubes. *J. Cell Biol.* 109:2345–2352.
Ratnam, M., P.B. Sargent, V. Sarin, J.L. Fox, D.L. Nguyen, J. River, M. Criado, and J. Lindstrom. 1986. Location of antigenic determinants on primary sequences of subunits of nicotinic acetylcholine receptor by peptide mapping. *Biochemistry.* 25:2621–2632.
Rose, J.K., and R.W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257–288.
Rose, J.K., and L. Iverson. 1979. Nucleotide sequences from the 3'-ends of vesicular stomatitis virus mRNAs'as determined from cloned DNA. *J. Virol.* 32:404–411.
Saedi, M.S., W.G. Conroy, and J. Lindstrom. 1991. Assembly of Torpedo acetylcholine receptors in Xenopus oocytes. *J. Cell Biol.* 112:1007–1015.
Shtrom, S.S., and Z.W. Hall. 1996. Formation of a ligand-binding site for the acetylcholine receptor in vitro. *J. Biol. Chem.* 271:25506–25514.
Sine, S.M. 1993. Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. *Proc. Natl. Acad. Sci. USA.* 90:9436–9440.
Sine, S.M., and T. Claudio. 1991. Gamma- and delta-subunits regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. *J. Biol. Chem.* 266:19369–19377.
Singh, I., R.W. Doms, K.R. Wagner, and A. Helenius. 1990. Intracellular transport of soluble and membrane bound glycoproteins: folding, assembly and secretion of anchor-free influenza hemagglutinin. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:631–639.
Smith, M.M., J. Lindstrom, and J.P. Merlie. 1987. Formation of the α-bungarotoxin binding site and assembly of the acetylcholine receptor subunits occur in the endoplasmic reticulum. *J. Biol. Chem.* 262:4367–4376.
Sumikawa, K., and T. Nishizaki. 1994. The amino acid residues 1–128 in the α subunit of the nicotinic acetylcholine receptor contain assembly signals. *Mol. Brain Res.* 25:257–264.
Tzartos, S.J., D.E. Rand, B.L. Einarson, and J. Lindstrom. 1981. Mapping of surface structures of Electrophorus acetylcholine receptor using monoclonal antibodies. *J. Biol. Chem.* 256:8635–8645.
Unwin, N. 1993. Neurotransmitter action: opening of ligand-gated ion channels. *Cell 72/Neuron* (Suppl.):31–41.
Verrall, S., and Z.W. Hall. 1992. The N-terminal domains of acetylcholine receptor subunits contain recognition signals for the initial steps of receptor assembly. *Cell.* 68:23–31.
Wang, Z.-Z., S.F. Hardy, and Z.W. Hall. 1996. Assembly of the nicotinic acetylcholine receptor: the first transmembrane domains of truncated α and δ subunits are required for heterodimer formation in vivo. *J. Biol. Chem.* In press.
Yu, X.-M., and Z.W.Hall. 1991. Extracellular domains mediate subunit interactions of the muscle acetylcholine receptor. *Nature (Lond.).* 352:64–67.
Yu, X.-M., and Z.W. Hall. 1994. Amino- and Carboxyl-terminal domains specify the identity of the δ subunit in assembly of the mouse nicotinic acetylcholine receptor. *Mol. Pharmacol.* 46:964–969.