Expression of Fusion Proteins of the Nicotinic Acetylcholine Receptor from Mammalian Muscle Identifies the Membrane-spanning Regions in the α and δ Subunits

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Abstract. We have investigated the topology of the α and δ subunits of the nicotinic acetylcholine receptor (AChR) from mammalian muscle synthesized in an in vitro translation system supplemented with dog pancreatic microsomes. Fusion proteins were expressed in which a carboxy-terminal fragment of bovine prolactin was attached downstream of each of the major putative transmembrane domains, M1-M4 and MA, in the AChR subunits. The orientation of the prolactin domain relative to the microsomal membrane was then determined for each protein by a proteolysis protection assay. Since the prolactin domain contains no information which either directs or prevents its translocation, its transmembrane orientation depends solely on sequences within the AChR subunit portion of the fusion protein. When subunit-prolactin fusion proteins with the prolactin domain fused after either M2 or M4 were tested, prolactin-immunoreactive peptides that were larger than the prolactin domain itself were recovered. No prolactin-immunoreactive peptides were recovered after proteolysis of fusion proteins containing prolactin fused after M1, M3, or MA. These results support a model of AChR subunit topology in which M1-M4, but not MA, are transmembrane domains and the carboxy terminus is extracellular.

THE nicotinic acetylcholine receptor (AChR),[1] which is found at the neuromuscular junction in vertebrate skeletal muscle and at the endplates of electric organs, is a pentameric complex composed of four glycoprotein subunits in an $\alpha_2\beta\gamma\delta$ stoichiometry (10). The subunits are arranged pseudosymmetrically around a central ion channel (40, 53). A comparison of the primary sequences of the subunits of the AChR reveals a high degree of homology both within and across species boundaries (52). The AChR is the best-studied member of a super-family of ligand-gated ion channels which includes the $GABA_A$, glycine, and neuronal nicotinic acetylcholine receptors (20, 43, 51). All of these channels are hetero-oligomers whose subunits are highly homologous, suggesting that they are derived from a common evolutionary precursor.

The subunits of the AChR and related channels also share a common domain structure. Hydropathic analysis of the primary sequence reveals five regions of high hydrophobicity comprised of an amino-terminal signal sequence (1, 2, 4) and four regions of sufficient length to form α-helical transmembrane domains (M1 through M4) (11, 15, 41). This domain pattern has led to a structural model of the AChR subunits in which the amino and carboxy termini are on the extracellular side of the membrane separated by four transmembrane domains (M1-M4) and a long cytoplasmic loop between M3 and M4 (see Fig. 1).

Although this model is commonly accepted, experiments on the topological arrangement of the AChR subunits have yielded conflicting results, leading to several alternative models of subunit structure. An amphipathic transmembrane domain between M3 and M4 (designated MA) was proposed on the basis of primary sequence analysis (17, 22), placing the carboxy terminus on the cytoplasmic side of the membrane. Epitope mapping experiments support models with a pair of transmembrane domains in the region before M1 (12, 44, 48), and other immunochemical and biochemical experiments support models with zero (48), one (16, 37), or two (17, 22, 56) transmembrane domains beyond M3. Thus, models with three to seven transmembrane domains can be constructed.

As an approach to this problem, we have investigated the transmembrane orientation of the AChR subunits synthesized in an in vitro translation system from rabbit reticulocytes supplemented with dog pancreatic microsomes. In earlier experiments, we investigated the topology of the amino terminal domain of the α subunit using glycosylation consensus sequences as reporter domains (8). In the present study, we fused a reporter domain derived from bovine prolactin to the α and δ subunits of the AChR after each of the proposed transmembrane domains beyond M1 (M1-M4 and MA) and expressed these constructs in the in vitro translation system. The accessibility of this reporter domain to

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; CIP, calf intestinal phosphatase; PK, proteinase K.

proteolytic enzymes was used to determine its orientation relative to the microsomal membrane. The results of these experiments, taken with our earlier findings, demonstrate that the amino and carboxy termini of the subunit polypeptides are sequestered within the vesicle and are separated by four transmembrane domains.

Materials and Methods

Subunit Clones and Molecular Biology Reagents

The clones for the α and δ subunit cDNAs of the mouse muscle AChR (26, 30) were obtained from Drs. J. P. Merlie (Washington University, St. Louis, MO) and N. Davidson (California Institute of Technology). The plasmids pSP BPI, which contains the cDNA for bovine prolactin, and pSP P^{tr}, a truncated form of pSP BPI which contains the cDNA for the carboxy terminal 142 amino acid residues of bovine prolactin, were generous gifts of Dr. V. R. Lingappa (University of California at San Francisco) and have been characterized elsewhere (35, 49). Several vectors (pGEM 1, pGEM 2, pSP72, pSP73) were obtained commercially (Promega Biotec, Madison, WI). The vector pSM was generously provided by M. Brodsky and Dr. D. Littman (University of California at San Francisco) (7). All enzymes were obtained commercially from either Promega Biotec, New England Biolabs (Beverly, MA), Boehringer Mannheim Biochemicals (Indianapolis, IN), or Bethesda Research Laboratories (Bethesda, MD). Sequencing of constructs was performed with the Sequenase kit (US Biochemicals, Cleveland, OH). Oligonucleotides used in the creation of new restriction sites in the α and δ subunit sequences were obtained from either the Biomolecular Resource Center at University of California at San Francisco or were synthesized by Dr. P. Garcia (University of California at San Francisco). Table I details the amino acid sequences at the subunit-prolactin fusion points.

pGEM PI. The plasmid pGEM Pl, which has several restriction sites upstream of the coding region for the carboxy terminal 142 amino acid residues of bovine prolactin, was constructed to facilitate the cloning of the prolactin fragment into the subunit coding regions. The vector pGEMl was digested with SmaI and calf intestinal phosphatase (CIP), and was ligated to the HincII/PvuII fragment from pSP BPI overnight at 16°C with T4 DNA ligase.

α Subunit-Prolactin Fusion Proteins

αPM1. The construct pSM α_m was altered by oligonucleotide-directed mutagenesis (8, 19, 28) to create an SmaI site in the region between the first and second transmembrane domains. The resulting plasmid, pSM αA869, was digested with SmaI and CIP. pGEM Pl was digested with HincII and EcoRI, and the fragment was blunted with the Klenow fragment of DNA polymerase. This fragment was ligated with the SmaI-digested pSM αA869 to yield pSM αPM1. To create pSP73 αPM1, pSP73 was first digested with SphI and EcoRI, and treated with CIP. pSM αPM1 was digested with SphI and EcoRI, and the fragment was ligated into the digested pSP73.

αPM2. The construct pSM α_m was altered to create an HpaI site in the region between the second and third transmembrane domains. This construct, pSM αA967, was digested with EcoRI and XmnI to excise the modified α subunit coding region, and this fragment was ligated into pGEM2 cut with EcoRI and SmaI and treated with CIP. The construct pGEM2 αPM2 was created by digestion of pGEM2 αA967 with HpaI and CIP, into which the HincII/EcoRI/Klenow fragment from pGEM Pl was ligated (see above).

αPM3. A new Hpa I site was created in the region between the third transmembrane domain and the putative amphipathic helix in the construct pSM α_m. This construct, pSM αA1158, was digested with EcoRI/XmnI, and ligated into pGEM2 digested with SmaI/EcoRI/CIP. pGEM2 αPM3 was prepared by digestion of pGEM2 αA1158 with HpaI and CIP, into which the HincII/EcoRI/Klenow fragment from pGEM Pl was ligated.

αPMA. A new BamHI site was created in the region between the putative amphipathic helix and fourth transmembrane domain of pSM α_m. This construct, pSM αA1346, was digested with EcoRI/BamHI and the fragment was inserted into the plasmid vector pSP73 prepared by digestion with EcoRI/BamHI/CIP. To create pSP73 αPMA, pSP73 αA1346 was digested with BamHI/SphI/CIP, and a BamHI/SphI fragment from pGEM Pl was ligated into the prepared vector.

αPM4. The construct pSM α_m was altered to create an HpaI site in the carboxy-terminal tail of the α subunit beyond the fourth transmembrane domain. The modified α subunit coding region was excised from the resulting construct, pSM αA1453, by digestion with EcoRI and XmnI, and was ligated into pGEM2 that had been digested with SmaI/EcoRI/CIP. pGEM2 αA1453 was digested with BamHI/HpaI/CIP. pSP BPI was digested with PvuII and BamHI, and the fragment was ligated into the digested pGEM2 αA1453 to create pGEM2 αPM4.

δ Subunit-Prolactin Fusion Proteins

δPM2. pSP73 δ_m was prepared as a vector by complete digestion with NcoI, followed by Klenow treatment and digestion with PstI and CIP. A HincII/PstI fragment from pGEM Pl was ligated into this site to create pSP73 δPM2.

δPM3. pSP73 δ_m was digested with StuI and PstI and treated with CIP. A HincII/PstI fragment of pGEM Pl was ligated into the prepared pSP δ_m vector to create pSP δPM3.

δPMA. The construct pSM δ_m was modified to create an HpaI site in the region between the putative amphipathic helix and the fourth transmembrane domain. The altered δ subunit cDNA (δD1411), excised from this construct by digestion with EcoRI and XbaI, was ligated into pSP73 that had been digested with EcoRI/XbaI/CIP. pSP73 δD1411 was digested with HpaI and CIP, and a HincII/EcoRI/Klenow fragment from pGEM Pl was inserted into the prepared vector. This construct was designated pSP73 δPMA.

δPM4. The construct pSP73 δ_m was prepared by digestion with PstI, SphI, and CIP. A PstI/SphI fragment from pGEM Pl was ligated into the prepared vector. This construct was designated pSP73 δPM4.

In Vitro Transcription and Translation

Capped transcripts were synthesized from unlinearized plasmid DNA as described previously (8, 38) except that the digestion with RNAse-free DNAse at the end of the transcription was omitted. The synthesized transcript was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in RNAse-free water. Micrococcal nuclease–treated rabbit reticulocyte lysate and canine pancreatic microsomes were prepared as described (27, 54). The translations were performed as described (8). All translations included canine pancreatic microsomes, except those used in Fig. 3. At the end of the incubation the tubes were placed on ice, and a solution of $CaCl_2$ was added to a final concentration of 10 mM.

Proteolysis and Immunoprecipitation

Aliquots from the in vitro translation were prepared for digestion with proteinase K (PK; Sigma Chemical Co., St. Louis, MO). To 9 μl of the translation mixture, either 1 μl water, or 1 μl of 100 μg/ml PK (predigested for 30 min at 37°C), or 1 μl of 100 μg/ml PK plus 1.2 μl 10% Triton X-100 was added, and the digests were incubated on ice for 1 h. At the end of the digestion, PMSF was added quickly to the reaction mixture to a concentration of 2 mM, and the entire mixture was plunged into 100 μl of 1% SDS in 100 mM Tris-HCl (8.0) in a boiling water bath. After 10 min of boiling, a 50-μl aliquot of the sample was diluted with a solubilization buffer containing 1.25% Triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM Tris-HCl (7.4), 0.02% NaN_3 to make the Triton/SDS ratio 5:1 (1% Triton/0.2% SDS).

Prolactin antiserum (US Biochemicals) was diluted 1:10 with 50 mM Tris-HCl (7.4), 0.02% NaN_3. 2 μl of this antiserum was added to 250 μl of the Triton-solubilized proteolysis mixture and incubated for 2 h on ice. 15 μl of a 1:1 slurry of prewashed protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was added, and incubated for 1–2 h with agitation at 4°C. At the end of the incubation, the beads were pelleted and washed twice with 500 μl 1% Triton X-100, 100 mM NaCl, 10 mM EDTA, 10 mM EGTA, 100 mM Tris-HCl, pH 8.0. PAGE gel loading buffer with 5% β-mercaptoethanol was added, the sample was heated to 100°C for 3 min and was subjected to SDS-PAGE (29). The gels were stained, fluorographed, and exposed to Hyperfilm MP (Amersham Corp., Arlington Heights, IL).

Three different antibodies were used to characterize the α subunit-prolactin constructs: mAb 210 (a rat mAb which has been mapped to α68–76 [14]), mAb 61 (a rat mAb which has been mapped to α371–386 [48]), and the prolactin antiserum described above. mAbs 210 and 61 were the generous gifts of Dr. J. Lindstrom (University of Pennsylvania). Two antibodies were used to characterize the δ subunit-prolactin constructs: mAb 88B (a mouse mAb that has been mapped to the cytoplasmic loop between M3 and M4 [18]) and the prolactin antiserum. mAb 88B was the generous gift of Dr. S. C. Froehner (Dartmouth University). Fig. 2 shows the location of the mAb epitopes on the primary sequences of the α and δ subunits. Aliquots (5 μl) were taken from the in vitro translation reactions

*Table I. Sequences at the junction points in the AChR subunit-prolactin fusion proteins**

Construct	Sequence
αPM1‡	...[234]Y L P T D S D S R G S P C H T S S L...
αPM2	...[266]S T S S A V D S R G S P C H T S S L...
αPM3‡	...[329]M K R P S R V D S R G S P C H T S S L...
αPMA	...[391]E S N N A A E G S P C H T S S L...
αPM4‡	...[426]F A G R L I E F C H T S S L...
δPM2‡	...[278]L P A T S M D S R G S P C H T S S L...
δPM3	...[392]R L T T A R R D S R G S P C H T S S L...
δPMA	...[443]Q V A R T V D S R G S P C H T S S L...
δPM4‡	...[465]A W I F L Q V D S R G S P C H T S S L...

* Amino acid sequences at the fusion points of the subunit-prolactin constructs, given in single-letter code. Amino acids derived from the AChR subunit coding sequences are in PLAIN text, amino acids derived from prolactin coding sequences are **BOLD**, and amino acids derived from vector sequences are UNDERLINED. Number to the right of the sequence indicates the position along the mature subunit sequence of the first amino acid residue given.
‡ Constructs sequenced for confirmation. All others predicted from construction protocol as well as immunoprecipitation results (Figs. 3 and 4).

and diluted to 50 μl with 2 mM PMSF in PBS. This sample was added to 200 μl of solubilization buffer and immunoprecipitated as described above, except that either rabbit-anti-rat-Sepharose (for mAb 210 and mAb 61) or mAb 88B-Sepharose was used instead of protein A-Sepharose.

Results

We investigated the transmembrane topology of the α and δ subunits of the AChR in a rabbit reticulocyte lysate in vitro translation system supplemented with dog pancreas microsomes. Fusion proteins were constructed in which a fragment of prolactin used as an antigenic marker (reporter domain) was placed after each of the postulated membrane-spanning domains of the AChR subunits. After synthesis of these constructs in vitro, the position of the marker with respect to the microsomal membrane was determined by proteolysis of the intact microsomes. The rationale of these experiments is that if the prolactin marker is translocated into the lumen of the microsome (topologically equivalent to the extracellular space) it will be protected from proteolysis, whereas if the marker remains outside the vesicle (i.e., the cytoplasmic surface) it will be susceptible. Once the location of the prolactin marker domain is determined for each construct in the series, we could then determine whether each of the postulated domains spans the membrane or not.

Construction of the Fusion Proteins

A schematic diagram of the fusion proteins that we constructed is given in Fig. 2. The prolactin fragment (M_r = 18) was introduced after each of the segments M1–M4 and MA of the α subunit, and after M2–M4 and MA of the δ subunit. The specificities of the antibodies that we used and the structures of the polypeptides encoded by our constructs were verified by immunoprecipitation after in vitro translation. The prolactin fragment, expressed alone, was recognized by the antiserum to prolactin, but not by antibodies to the α or δ subunits (Fig. 3 *A*); conversely, the prolactin antiserum recognized neither the intact α subunit (Fig. 3 *B*, lane *c*) nor δ subunit (Fig. 4 *A*, lane *d*).

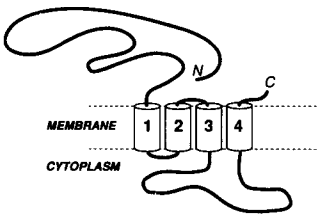

Figure 1. Diagram of the predicted transmembrane topology of the subunits of the AChR, based on the hydrophobicity of the primary sequence (11, 15, 41).

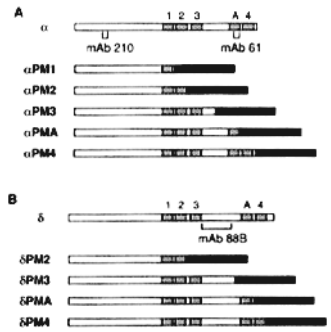

Figure 2. Diagram of the AChR subunit-prolactin fusion proteins. (*A*) α subunit-prolactin fusions; (*B*) δ subunit-prolactin fusions. Chimera names are given at the left. For each construct, the AChR subunit sequences are denoted by the open box (with the putative transmembrane domains M1-M4 and MA given as *stippled boxes*), and the prolactin sequences denoted by the black box. Details of the amino acid sequences at the junction points are given in Table I. The locations of the epitopes for mAbs mAb 210, mAb 61, and mAb 88B are indicated.

The fusion proteins were then tested. All of the α subunit-prolactin constructs were immunoprecipitated with the antiserum to prolactin and with an antibody (mAb 210) that recognizes an epitope in the amino terminal domain of the α subunit (Figs. 2 *A* and 3 *B*). Only those with the prolactin fragment inserted after MA (αPMA and αPM4) were immunoprecipitated with mAb 61, an antibody that recognizes an epitope in the segment between M3 and M4 (Fig. 2 *A*; Fig. 3 *B*, lanes *n* and *q*). Similarly, all of the δ subunit-prolactin constructs were immunoprecipitated with the antiserum to prolactin, but only δPM3, δPMA, and δPM4 were immunoprecipitated with mAb 88B (Fig. 4 *B*, lanes *c*, *e*, and *g*). The mAb 88B epitope is known to be located in the loop between M3 and M4 (Fig. 2 *B*) (18); since the prolactin domain in δPM3 was inserted ~85 amino acids after the end of M3, the epitope must lie within this 85 amino acid stretch. These results show that the α subunit- and δ subunit-prolactin fusion proteins were synthesized correctly, with a prolactin-immunoreactive domain located downstream from each of the transmembrane domains in the mouse α and δ subunits.

Protease Digestion of the α Subunit-Prolactin Fusion Proteins

We then investigated whether the prolactin domain in each of the subunit chimeras was translocated into the microsomal lumen or remained outside the vesicle. The polypeptides were expressed in vitro and the microsomes containing the newly synthesized polypeptides were subjected to proteolytic digestion with proteinase K (PK) and immunoprecipitated with the prolactin antiserum. When the five α subunit-prolactin constructs were tested, prolactin-immunoreactive peptides were recovered from αPM2 and αPM4 (Fig. 5 *A*, lanes *e* and *n*), but not from αPM1, αPM3, or αPMA (Fig. 5 *A*, lanes *b*, *h*, and *k*). When Triton X-100 was added before the digestion, no prolactin-immunoreactive peptides were recovered in any case (Fig. 5 *A*). Thus, the prolactin domains

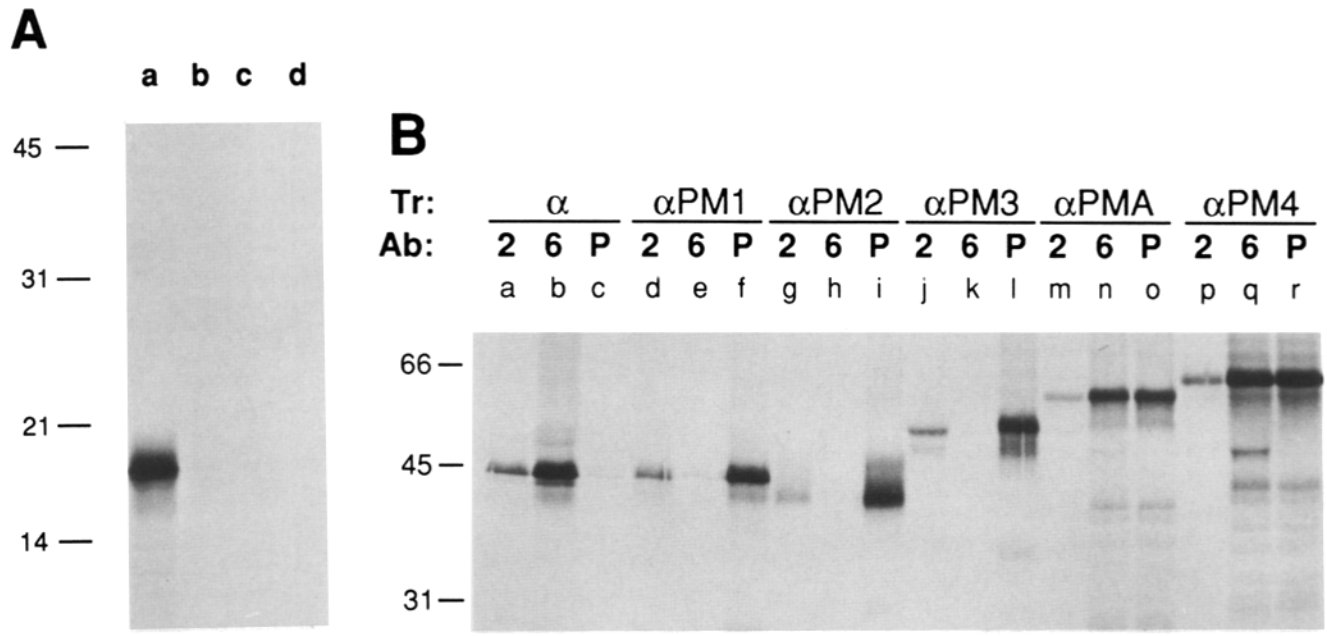

Figure 3. Characterization of prolactin fragment and α subunit-prolactin fusion proteins. (*A*) Immunoprecipitation of truncated prolactin fragment. A fragment of bovine prolactin encoding the carboxy-terminal 142 amino acids was expressed in vitro, solubilized in a Triton X-100 buffer, and immunoprecipitated with either the prolactin antiserum (lane *a*), α subunit–specific mAbs 210 (lane *b*) and 61 (lane *c*), or δ subunit-specific mAb 88B (lane *d*). (*B*) Immunoprecipitation of α subunit-prolactin fusion proteins. The indicated transcripts (*Tr*) were expressed in vitro, solubilized, and immunoprecipitated with either mAb 210 (2), mAb 61 (6), or the prolactin antiserum (*P*).

in αPM2 and αPM4 were protected and must lie within the lumen of the microsomes, whereas the prolactin domains in αPM1, αPM3, and αPMA are exposed on the cytoplasmic surface of the microsomes. These results indicate that M1, M2, M3, and M4 traverse the membrane, but that MA does not.

Comparison of the size of the fragments recovered from proteolysis of the α subunit-prolactin constructs with those resulting from digestion of the native α subunit (Fig. 5 *B*) are consistent with this conclusion. Synthesis of the α subunit in vitro gave rise to two bands, representing the glycosylated and unglycosylated forms of the α subunit (8). Immunoprecipitation with mAb 210 after proteolysis of microsomes containing the α subunit yielded two additional

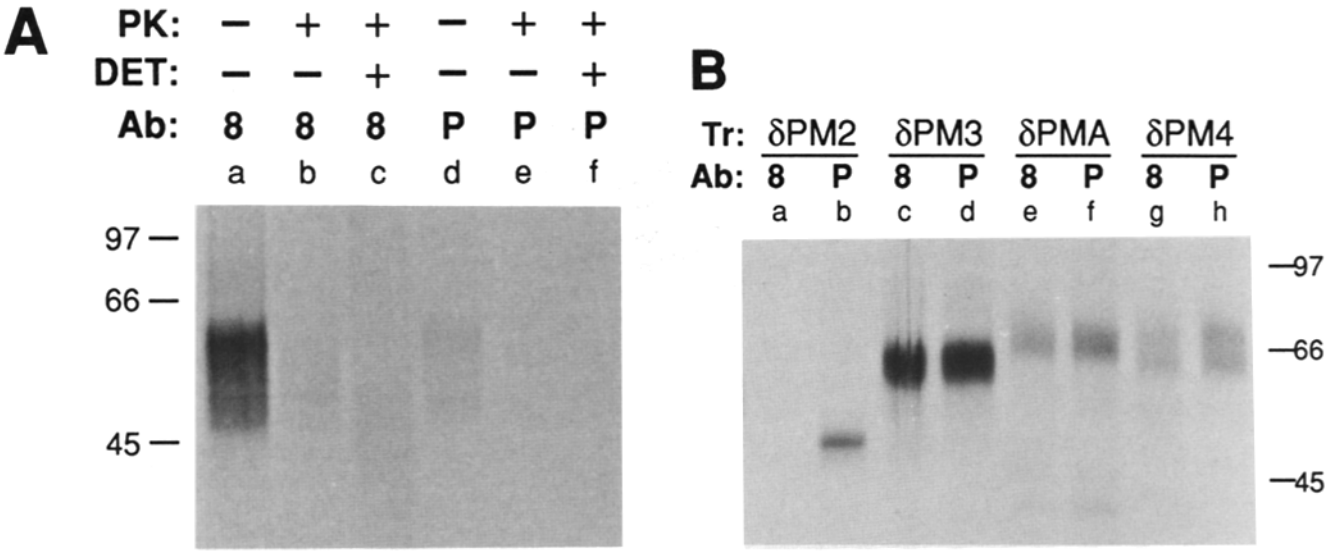

Figure 4. Characterization of δ subunit and δ subunit-prolactin fusion proteins. (*A*) Proteolysis and immunoprecipitation of normal δ subunit. The normal δ subunit was expressed in the presence of microsomes. The translation mixture was either left untreated or digested with proteinase K (*PK*) in the absence or presence of Triton X-100 (*DET*) as described in Materials and Methods. The solubilized mixture was immunoprecipitated with either mAb 88B (lanes *a–c*), or the prolactin antiserum (lanes *d–f*). (*B*) Immunoprecipitation of δ subunit-prolactin fusion proteins. The indicated transcripts (*Tr*) were expressed in vitro in the presence of microsomes, solubilized, and immunoprecipitated with either mAb 88B (8) or the prolactin antiserum (*P*).

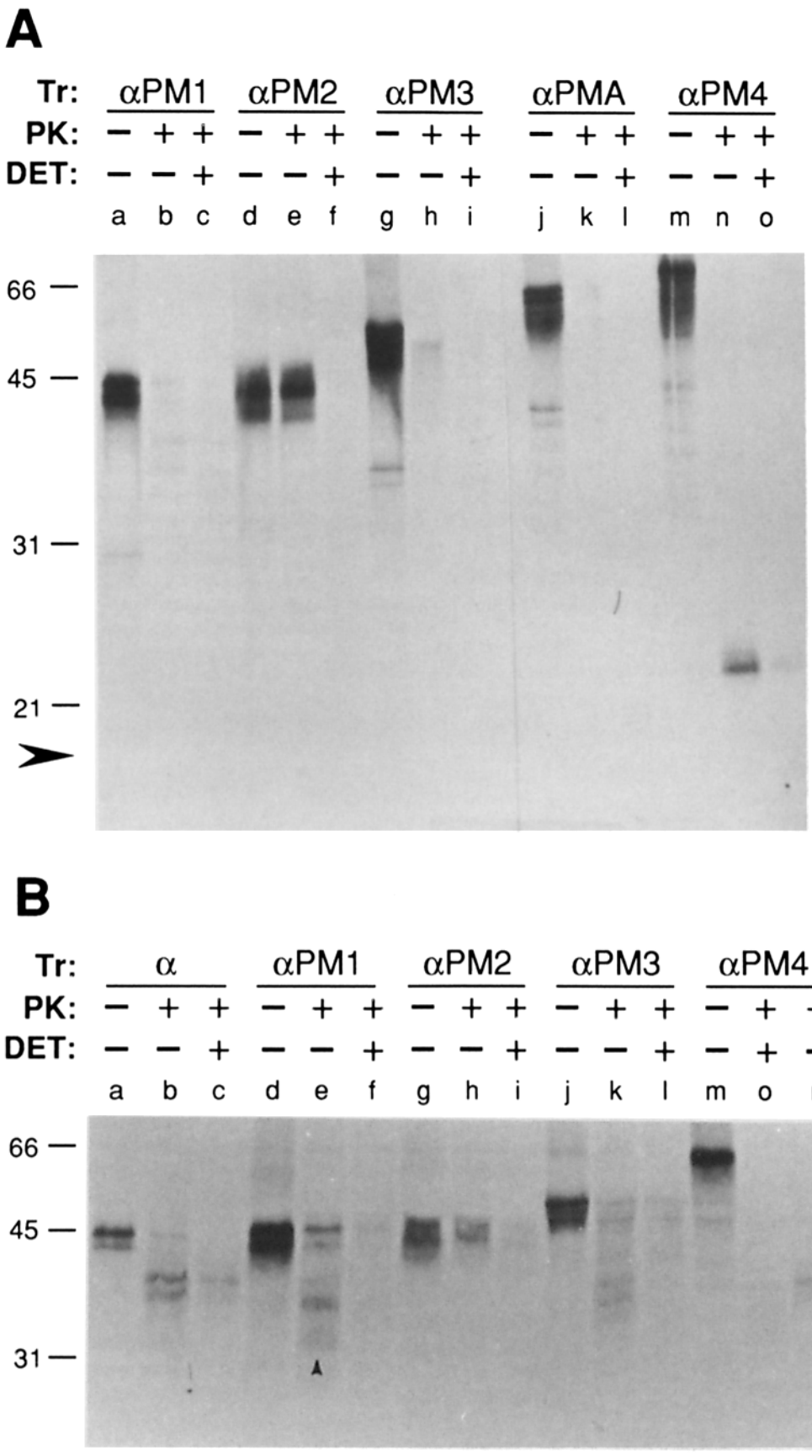

Figure 5. Proteolysis and immunoprecipitation of α subunit-prolactin fusion proteins. The indicated transcripts (*Tr*) were expressed in vitro in the presence of microsomes. The translation mixtures were either left untreated or digested with proteinase K (*PK*) in the absence or presence of Triton X-100 (*DET*) as described in Materials and Methods. (*A*) Immunoprecipitation with the prolactin antiserum. Large arrowhead at left indicates the migration of the prolactin fragment. (*B*) Immunoprecipitation with mAb 210. Note reversal of lanes *n* and *o*. Small upwards arrowhead in lane *e* indicates migration of smallest mAb 210-immunoreactive fragment derived from αPM1.

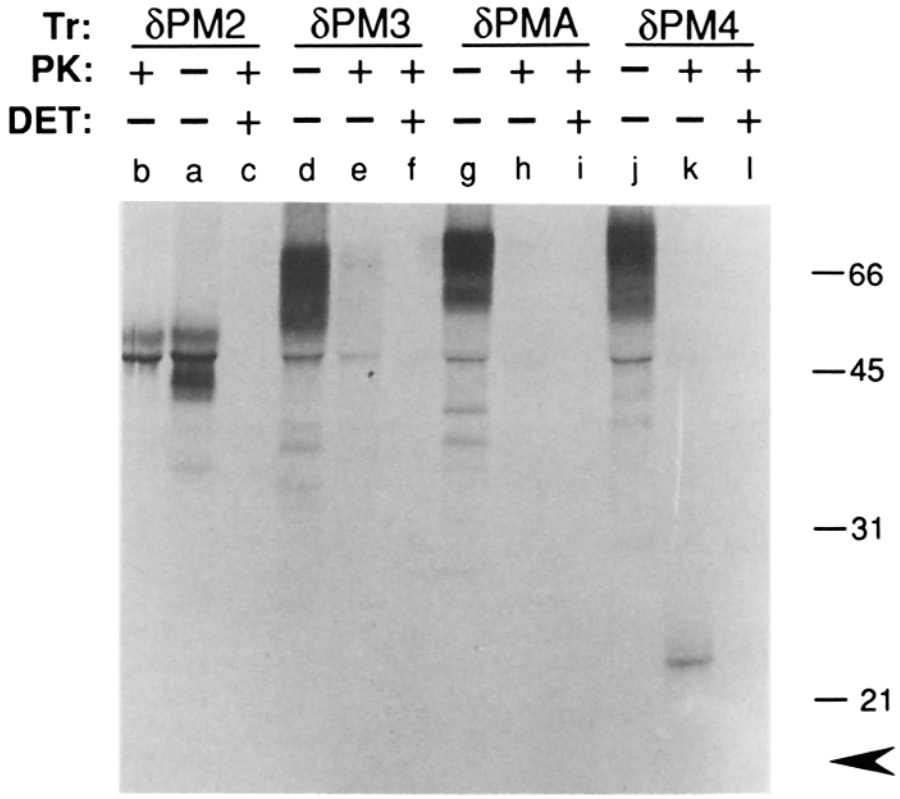

Figure 6. Proteolysis and immunoprecipitation of δ subunit-prolactin fusion proteins. The indicated transcripts (*Tr*) were expressed in vitro and treated as described in Fig. 5. The solubilized mixture was immunoprecipitated with the prolactin antiserum. Note reversal of lanes *a* and *b*. Large arrowhead at right indicates the migration of the prolactin fragment.

bands of 38 and 36 kD (Fig. 5 *B*, lane *b*). Protected fragments of the same size were obtained when microsomes containing either αPM3 or αPM4 were immunoprecipitated with mAb 210 after PK digestion (Fig. 5 *B*, lanes *k* and *n*). These results suggest that the cytoplasmic segment between M3 and M4 is the principal site of PK attack of the α subunit, and that little proteolysis occurs between M1 and M2. The segment between M1 and M2 may not be susceptible because of its short length, which is predicted to be <10 amino acids long. The results obtained after mAb 210 immunoprecipitation of the proteolytic products of PK digestion of αPM1 and αPM2 demonstrate that the short loop between M1 and M2 is not accessible to PK. The size of αPM2 was unaffected by PK digestion, whereas digestion of αPM1 yielded a smaller fragment (~32 kD) which is recognized by mAb 210 (Fig. 5 *B*, lane *e*) and which is similar in size to the primary translation product of a truncated α subunit cDNA that terminates just after M1 at αG240 (8). Thus the prolactin fragment is removed in αPM1, but not αPM2, and the segment between M1 and M2 is not cleaved.

This analysis of the proteolytic products immunoprecipitated by mAb 210 allows rationalization of the sizes of the protected prolactin-immunoreactive fragments seen with αPM2 and αPM4 (Fig. 5 *A*, lanes *e* and *n*). Proteolysis of αPM2 yielded a full-length prolactin-immunoreactive peptide, indicating that the polypeptide chain of αPM2 was not accessible to PK attack. For αPM4, in contrast, a single prolactin-immunoreactive fragment of ~22 kD was obtained (Fig. 5 *A*, lane *n*). This peptide is presumably slightly larger than the prolactin peptide (Fig. 3 *A*) because of the additional amino acids that comprise M4.

Protease Digestion of the δ Subunit-Prolactin Fusion Proteins

The pattern of protection seen with the δ subunit-prolactin fusion proteins after PK digestion and immunoprecipitation with the prolactin antiserum was similar to that of the α subunit-prolactin fusion proteins (Fig. 6). The entire δPM2 polypeptide was immunoprecipitated with the prolactin antiserum after digestion with PK (Fig. 6, lane *b*), whereas a small fragment of δPM4 slightly larger than the prolactin peptide (23 kD) was recovered (Fig. 6, lane *k*). No fragments of the normal δ subunit were recovered after digestion with PK, whether immunoprecipitated by mAb 88B or the prolactin antiserum (Fig. 4 *A*). As with αPM4, the prolactin-immunoreactive fragment recovered after PK digestion of δPM4 was slightly larger than the prolactin polypeptide (Fig. 6, lane *k*). No prolactin-immunoreactive fragments were recovered after the proteolysis of either δPM3 or δPMA (Fig. 6, lanes *e* and *h*). Therefore, for analogous α- or δ-subunit-prolactin chimeras, the location of the prolactin domain is identical. For either subunit, when placed after M1, M3, or MA, the prolactin domain remained accessible to proteolysis, indicating that the regions that follow these domains are oriented outside of the microsome (cytoplasmic in vivo). When placed after either M2 or M4, however, the prolactin domain was inaccessible to proteolysis, indicating that the carboxy terminal ends of M2 and M4 are oriented within the microsome (extracytoplasmic in vivo).

Discussion

We have made a systematic investigation of the transmem-

brane topology of the subunits of the mammalian muscle AChR in an in vitro translation system by inserting a prolactin reporter domain after each of the major proposed transmembrane domains. Our experiments demonstrate that the carboxy termini of the newly translated, unassembled α and δ subunits of the AChR reside in the lumen of the ER, indicating that the subunit polypeptides span the membrane an even number of times. Furthermore, our results are consistent with a topological model in which the AChR subunits span the membrane at least four times and in which M1–M4 traverse the membrane but MA does not. Thus prolactin domains placed to the carboxy-terminal side of M1, M3, and MA are susceptible to proteolysis, whereas those placed after M2 and M4 are protected.

Most previous experiments on the AChR have used antibodies to determine the location of specific regions on particular subunits in the intact receptor (12, 13, 18, 31, 32, 33, 34, 44, 46, 47, 48, 50, 56). Such studies have been limited to regions for which antibodies are available and in some cases by the specificity of the antibodies used. Our approach differs from these experiments in two ways. First, we have identified transmembrane regions by determining the orientation of a neutral reporter domain whose susceptibility to proteolysis we can detect reliably using specific antibodies. Reporter domains have been used widely and successfully to study the topology of other polytopic membrane proteins (5, 6, 9, 21, 36, 57). A possible disadvantage of this use of a reporter domain is that its introduction could disrupt the normal topology of the subunits. To minimize this possibility, we chose for our experiments a cDNA fragment encoding a carboxy-terminal region of bovine prolactin of ~142 amino acids in length (predicted molecular mass 16,786 D). This fragment lacks the codons for the signal sequence found in full-length prolactin and has been shown to be topogenically neutral when used as a reporter in other experiments (45, 49, 55). Thus, its transmembrane orientation in our experiments presumably is determined solely by topogenic elements within the AChR subunit sequences. The addition of the prolactin domain is also unlikely to disturb the topology of the AChR subunit in the regions examined as experiments in other systems have shown that the removal of downstream topogenic domains does not affect the topology of upstream regions (6, 36) and that topogenic domains exert their function on nonnative domains in predictable ways (49, 55).

A second feature of our experiments is the use of an in vitro translation system to give information about transmembrane orientation. Although assembly of the intact receptor has not been achieved in vitro, subunits in an in vitro translation system supplemented with microsomes are faithfully synthesized, inserted into the membrane, glycosylated, and their signal sequences cleaved (1, 3, 8). In vitro experiments have the advantage that the translocation of domains in subunits can be investigated in constructs that may not undergo assembly and transport to the cell surface and may not form functional AChR. Transmembrane domains that arise from subsequent rearrangements during assembly, however, might not be detected (see below).

The amino termini of AChR subunits expressed in vitro are translocated into the lumen of microsomal vesicles (equivalent topologically to the extracellular space) as are the amino termini of the native AChR subunits (Fig. 5) (1, 3, 8). Although the experiments described here offer no information on the position and number of transmembrane regions on the amino-terminal side of M1, we have previously described experiments in vitro showing that an α subunit fragment terminating at position α207 is not an integral membrane protein and that novel glycosylation sites introduced at positions α154 and α200 are on the lumenal side of the membrane (8). These findings, which are consistent with previous data on the amino-terminal signal sequence, the glycosylation sites, and the location of the binding site for α-bungarotoxin in the intact AChR (10), suggest that the entire amino-terminal domain preceding M1 is translocated into the microsomal lumen.

Our experiments show that in the newly synthesized α subunit both M1 and M2 span the membrane and do so in opposite directions. The peptide containing the amino-terminal domain, M1, and a carboxy-terminal prolactin domain (αPM1) is, in contrast to the α207 fragment, an integral membrane protein and has the prolactin domain on the cytoplasmic side; when placed after M2 (in both αPM2 and δPM2), the prolactin domain is lumenal. By a similar argument, M3 and M4 are also transmembrane domains. The assignment of M1–M4 as transmembrane domains is consistent with experiments in oocytes showing that after deletion of any of the segments M1–M4 functional AChRs are not expressed (39).

Our observation that the carboxy termini of the α and δ subunits are located on the lumenal side of the membrane are supported by previous experiments demonstrating that the AChR oligomers of *Torpedo* are linked as dimers via an extracellular disulfide bond between cysteine residues near the carboxy terminus of neighboring δ subunits (16, 23, 37). Earlier reports based on immunocytochemical localization (47, 48, 56) placed the carboxy terminus on the cytoplasmic side of the membrane.

Our results clearly indicate that MA is cytoplasmic, consistent with earlier immunochemical studies (47, 48). Thus, prolactin domains placed on either side of MA are susceptible to proteolysis. Because MA is amphipathic, one might argue that MA does not adopt a transmembrane orientation until the AChR oligomer is assembled. To be compatible with a placement of the carboxy terminus on the extracellular side of the membrane, however, this hypothesis would require either that the polypeptide chain traverse the membrane twice between M3 and M4 or that M4, the most hydrophobic of all the putative transmembrane domains in the AChR subunits, become extracellular in the assembled receptor. Neither possibility seems likely.

An important question is whether the orientation assumed by the newly synthesized subunits is identical to that of the subunits in the mature AChR. Although a rearrangement of transmembrane domains is theoretically possible, the domain structure of the AChR subunits makes this unlikely. All of the transmembrane domains, even M2 which lines the aqueous pore (24, 25, 42), are highly hydrophobic, and are likely to be quite stable as transmembrane segments. We cannot rule out the possibility that assembly might cause other segments to insert into the membrane, giving rise to additional transmembrane regions, but with the possible exception of the M6 and M7 segments on the amino-terminal side of M1, postulated by Criado et al. (12) to span the membrane, no other segments of the subunit polypeptides are plausible candidates for such a role. We have argued previ-

ously that M6 and M7 are also unlikely to insert during assembly (8).

Because of the high degree of primary sequence homology between the AChR subunits and other members of the family of ligand-gated ion channels, our conclusions can be extended to these molecules as well. The characterization of specific conformational changes that accompany ligand binding and desensitization await a more detailed analysis involving the determination of the crystal structure of the receptor in a variety of functional states. However, our studies show that alternative strategies such as the use of reporter domains can be very useful in the elucidation of the basic topology of polytopic membrane proteins.

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