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Nucleotide and deduced amino acid sequences of *Torpedo californica* acetylcholine receptor γ subunit

(cDNA clone/polyadenylylation consensus sequence/signal peptide/protein structure)

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ABSTRACT **The nucleotide sequence has been determined of a cDNA clone that codes for the 60,000-dalton γ subunit of *Torpedo californica* acetylcholine receptor. The length of the cDNA clone is 2,010 base pairs. The 5′ and 3′ untranslated regions have respective lengths of 31 and 461 base pairs. Data suggest that the putative polyadenylylation consensus sequence A-A-T-A-A-A may not be required for polyadenylylation of the mRNA corresponding to the cDNA clone described in this study. From the DNA sequence data, the amino acid sequence of the γ subunit was deduced. The subunit is composed of 489 amino acids giving a molecular mass of 56,600 daltons. The deduced amino acid sequence data also indicate the presence of a 17-amino acid extension or signal peptide on this subunit. From these data, structural predictions for the γ subunit are made such as potential membrane-spanning regions, possible asparagine-linked glycosylation sites, and the assignment of regions of the protein to the extracellular, internal, and cytoplasmic domains of the lipid bilayer.**

The nicotinic acetylcholine receptor (AcChoR) is a glycoprotein located in the postsynaptic membrane of the vertebrate neuromuscular junction. When the neurotransmitter acetylcholine binds to the receptor, a channel permeable to small cations opens. The resulting ion flow leads to depolarization and contraction of the muscle cell. AcChoR from the electric organ of *Torpedo californica* has been most extensively studied because it is a major component of the membrane in this tissue and can be purified in milligram amounts (for recent reviews, see refs. 1 and 2). The AcChoR from *T. californica* is an oligomeric complex with a molecular mass of ≈250,000 daltons. The complex is composed of four different polypeptide chains of approximately 40,000 (α), 50,000 (β), 60,000 (γ), and 65,000 (δ) daltons (3, 4) with a stoichiometry of 2:1:1:1, respectively (5, 6). The subunits have a number of common features: each subunit is glycosylated (4, 7), the subunits have similar amino acid compositions (8) and show 35–50% sequence homology in the NH_2-terminal regions (6), and each subunit spans the lipid bilayer (9). A number of experiments have demonstrated that a large part of the AcChoR extends into the synaptic cleft or extracellular side of the membrane (reviewed in ref. 10). The purified 250,000-dalton oligomer contains the binding sites for acetylcholine and the ligand-gated ion channel (11, 12). Acetylcholine analogs are known to bind to the α-polypeptide chains (3); as yet, no specific functions have been assigned to the other polypeptide chains.

This paper reports the complete nucleotide sequence of a cDNA clone (4D8) coding for the γ subunit of AcChoR from the electric organ of *T. californica*. The deduced amino acid sequence of the γ subunit and its putative signal peptide are also presented. Several structural predictions based on the amino acid sequence are discussed and incorporated into a model giving a possible partitioning of the γ-polypeptide chain into the extracellular, internal, and cytoplasmic domains of the lipid bilayer. The identification of clone 4D8 as coding for the γ subunit has been described elsewhere (13).

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MATERIALS AND METHODS

Cloning and Preparation of DNA for Sequence Determination. Preparation of a *T. californica* electric organ cDNA library, screening, and isolation of the γ-subunit clone (4D8) have been described (13). The cDNA library was made in a hybrid pBR322–simian virus 40 vector (14). Relevant features of this cloning procedure are that the mRNA is oriented in the vector and an oligo(dT) primer is used in the synthesis of the cDNA. The cDNA insert is flanked by an oligo(dG) tract at the 5′ end of the mRNA-sense strand and by an oligo(dA) tract at the 3′ end. Transformed HB101 cells were grown and amplified, and the plasmid was purified by standard techniques (15).

Restriction Enzyme Digestions and End-Labeling. Restriction endonucleases *Acc* I, *Ava* II, *Bam*HI, *Bgl* II, *Cla* I, *Hae* III, *Hinf* I, and *Pvu* II from Bethesda Research Laboratories; *Eco*RI, *Hpa* II, *Nco* I, *Pvu* II, *Sal* I, and *Tth* III-I from New England BioLabs; and *Cla* I from Boehringer Mannheim were used essentially as recommended by the manufacturers. DNA 5′ ends were labeled with polynucleotide kinase and [γ-^{32}P]ATP (ICN; >7,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) (16). End-labeled restriction fragments were digested with a second endonuclease and separated on 6% (wt/vol) acrylamide (acrylamide/bisacrylamide, 30:0.8, wt/wt), 100 mM Tris borate, pH 8.3/2 mM Na_2EDTA preparative gels. The appropriate end-labeled fragments were located by ethidium bromide staining or autoradiography of the gel, and gel strips containing the fragments were cut from the gel. Each gel fragment was placed in a dialysis bag containing 300 μl of 10 mM Tris borate (pH 8.3), 0.2 mM Na_2EDTA, and 7.5 μg of carrier tRNA and was electroeluted 15–60 min at 30 V/cm (constant voltage) against 10 mM Tris borate, pH 8.3/0.2 mM Na_2EDTA to recover the end-labeled DNA fragment.

Nucleotide Sequence Determination. End-labeled fragments were cleaved by the base-specific chemical cleavage method of Maxam and Gilbert (17, 18) and electrophoresed by the thin-gel system of Sanger and Coulson (19).

RESULTS AND DISCUSSION

Nucleotide Sequence Analysis. The strategy used to determine the complete nucleotide sequence of clone 4D8 is shown

Abbreviations: AcChoR, acetylcholine receptor; bp, base pair(s).

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in Fig. 1. The sequence of both strands was determined in the coding region of the clone. Fig. 2 shows the DNA sequence (mRNA sense) from the 5′ end, beginning at the first base past the oligo(dG) tract (see *Materials and Methods* or ref. 14) and extending to the poly(A) tract. The full length of the cDNA clone is 2,010 base pairs (bp).

The DNA sequence contains an open reading frame of 1,548 bases beginning at nucleotide position 2. A comparison of the deduced amino acid sequence with the NH_2-terminal protein sequence data of each of the four *T. californica* subunits (6) positively identified this clone as coding for the γ subunit (13). The DNA sequence coding for the mature γ subunit begins at nucleotide position 83 (Fig. 2). Relationships between the nucleotide sequence and the deduced amino acid sequence noted in this section are discussed further in the next section.

The sequences flanking functional initiation codons in eukaryotic mRNAs are not random and most often are characterized by having a purine in position −3 or a G in position +4, or both (20). The sequences flanking the ATG at position 32, A-C-C-A-T-G-G, in clone 4D8 have these features. There are no other possible initiation codons in any of the three reading frames in the region 5′ to this sequence. The ATG at position 32 begins an open reading frame of 1,518 bases, which includes the sequence coding for the known NH_2-terminal portion of the γ subunit (6). Thus, it is concluded that initiation of translation of the mRNA coding for the γ subunit most likely begins at nucleotide position 32, whereas the coding region for the NH_2 terminus of the mature γ protein begins at nucleotide position 83.

The single open reading frame of 1,518 bases ends with two tandem termination codons, TAA and TAG, located at positions 1,550 and 1,553. Termination codons appear in all three reading frames in the remaining 461 bases 5′ to the poly(A) tract. Thus, the two tandem termination codons appear to represent the termination of translation of the γ-subunit mRNA, and the 461 bases after these codons constitute the 3′ untranslated region of clone 4D8.

The consensus sequence (A-A-T-A-A-A) is thought to be the recognition sequence for the addition of poly(A) tracts to eukaryotic mRNA and is usually found within 11–30 bases of the poly(A) site (21). Three such consensus sequences can be found in the 3′ untranslated region of the γ-subunit clone; however, none of them lie within 30 bases of the poly(A) site but rather 156, 373, and 377 bases from the poly(A) site (positions 1,853, 1,636, and 1,632, respectively). Although the region from the consensus sequence (position 1,853) to the poly(A) addition site is A+T-rich (70% A+T vs. 57% overall), neither the consensus sequence A-A-T-A-A-A (21) nor the sequence A-T-T-A-A-A (22, 23) is located within 30 bases of the poly(A) site in this cDNA clone. The sequence A-A-T-A-A-A may not be required for polyadenylylation of this γ-subunit mRNA (from which clone 4D8 was derived), as has been observed by Setzer *et al.* (24) for at least three of four dihydrofolate reductase mRNAs. Setzer *et al.* (25) also have shown that there may be more than one mRNA that codes for dihydrofolate reductase and that these mRNAs may differ in the length of the 3′ untranslated region. This observation also may be true of mRNA coding for the γ subunit. One of the consensus sequences (position 1,853) in clone 4D8 is located 40 bases 5′ to a stretch of 10 adenosines (position 1,893). This stretch of 10 adenosines may represent the site of polyadenylylation in a second, shorter mRNA species that also codes for the γ subunit. Hybridization of radiolabeled clone 4D8 to size-fractionated electric organ polyadenylylated mRNA revealed a band 2,100 nucleotides in length and bands of smaller size (13). These smaller mRNA species may represent additional size classes of γ-subunit mRNA produced by using different sites of polyadenylylation.

Deduced Amino Acid Sequence. The probable initiation codon for translation of the γ-subunit clone 4D8 begins at nucleotide position 32, whereas the sequence coding for the NH_2 terminus of the mature protein begins at nucleotide position 83 or 18 amino acids COOH-terminal to the site of initiation. This 17-amino acid sequence (see Fig. 2) is typical of leader or signal sequences (26, 27) thought to be required for the translocation of secreted and membrane polypeptides across the membrane of the rough endoplasmic reticulum. Signal peptides are often cleaved while the protein is still nascent and usually are not present on processed, mature proteins. The 17-amino acid extension sequence of the γ subunit contains an internal stretch of hydrophobic amino acids and has hydrophilic amino acids on the COOH-terminal side of the hydrophobic stretch, and the amino acid located at the cleavage site (serine) has a short side chain. These features are all characteristic of signal sequences (28).

The region 5′ to base 32 of clone 4D8 is thought to be untranslated even though it does not contain a termination codon in phase with the reading frame coding for the signal and γ-subunit sequences. Because of the lack of a termination codon in this region and because it is not known if clone 4D8 is a full-length cDNA clone, the possibility remains that initiation of translation begins at an ATG located 5′ to the 5′ end of the cDNA clone described in this study. However, nucleotides 2–31 of clone 4D8 code for 10 amino acids with a random arrangement of hydrophobicities, making it unlikely that they are part of the leader sequence.

Nucleotides 83–244 code for the 54 NH_2-terminal amino acids of the γ subunit. This deduced sequence is identical in all but one position to the 54 NH_2-terminal residues of the 60,000-dalton subunit obtained from protein sequence assay (6). Protein sequence data list a methionine (which would be coded by ATG) as residue 46, whereas the amino acid sequence data deduced from clone 4D8 show a lysine (AAA) as residue 46. In this study, the sequences of both strands of clone 4D8 were determined, and they confirm the assignment of a lysine to this position. As discussed in an earlier study (13), an independent clone had the same DNA sequence as 4D8 had in this region. The reason for

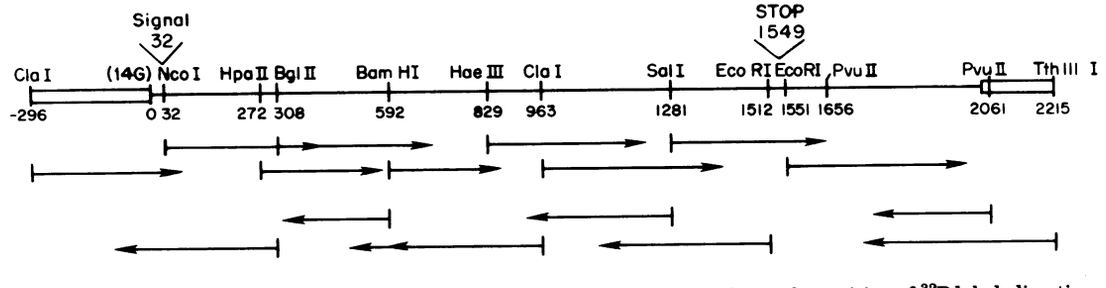

FIG. 1. Scheme for sequence determination of the γ-subunit cDNA clone 4D8. Arrows indicate the position of ^{32}P label, direction of assay, and length of fragment. Boxed regions indicate regions of cloning vector. Numbers correspond to nucleotide numbers shown in Fig. 2. Positions of the start of the putative signal peptide and termination of the coding region of the γ subunit are indicated.

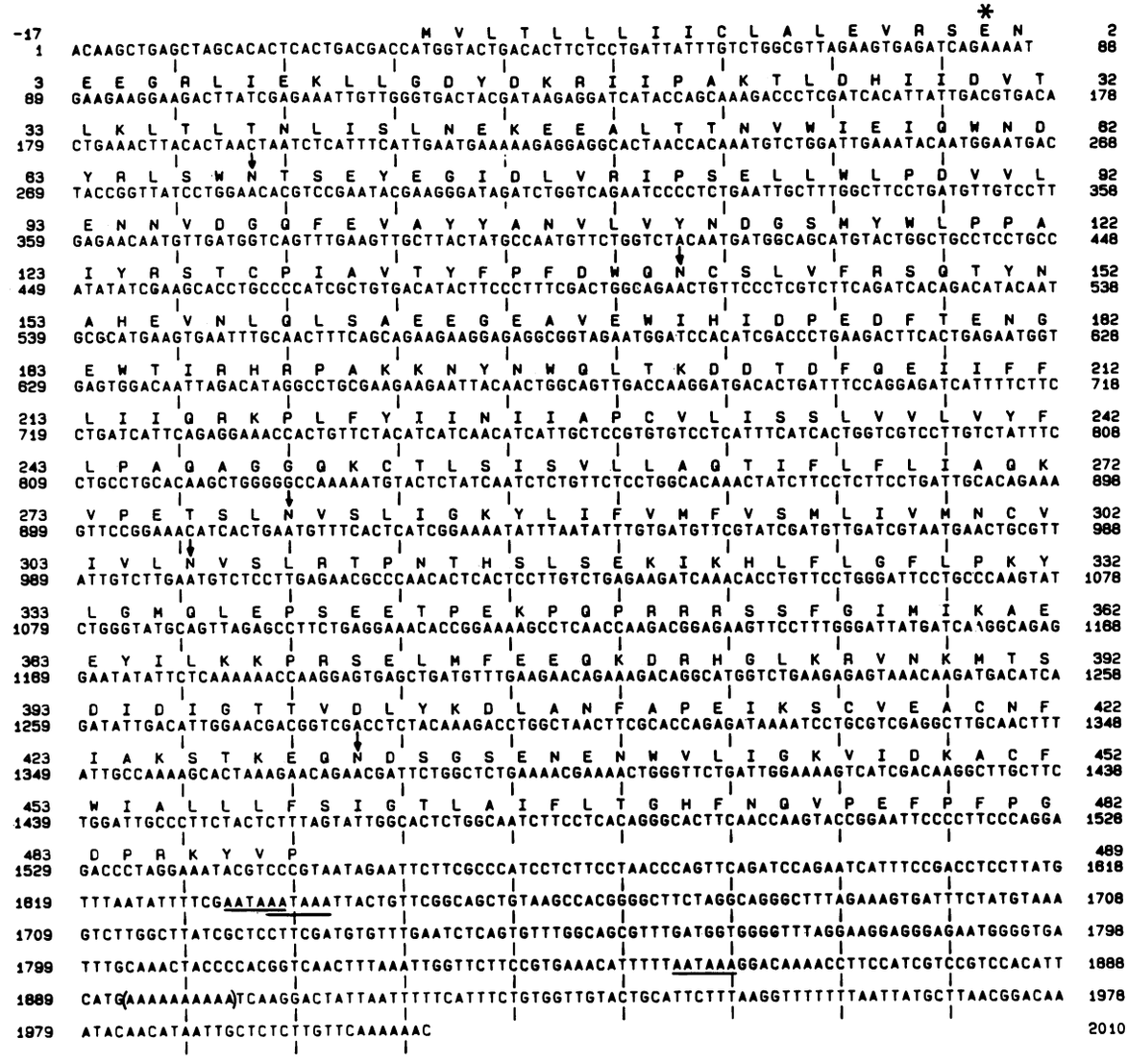

FIG. 2. Nucleotide sequence of a cDNA clone coding for the γ subunit and deduced amino acid sequence of the encoded protein. The cDNA sequence is numbered from the 5′ to the 3′ end as described in the text. The amino acid sequence is numbered from the NH_2-terminal end of the putative leader sequence (−17). *, NH_2 terminus of the mature subunit; ↓, potential asparagine-linked glycosylation sites. A possible alternate polyadenylylation site of 10 adenosines is in parentheses. The locations of the sequence A-A-T-A-A-A found in the 3′ untranslated region are underscored. Base composition for the 2,010 nucleotides is 21% G, 29% A, 28% T, and 22% C. The amino acid composition of the translated γ subunit (not including the signal sequence) is: $A_{23}R_{17}N_{28}D_{23}C_8E_{39}Q_{17}G_{20}H_8I_{44}L_{56}K_{29}M_8F_{26}P_{26}S_{29}T_{26}W_{11}Y_{18}V_{33}$. These 489 amino acids have a molecular mass of 56,600 daltons.

the discrepancy between the protein sequence data and the DNA sequence data is not known.

The DNA sequence from position 83 (NH_2 terminus of the mature protein) to position 1,549 (first termination codon) codes for a mature protein of 489 amino acids with a molecular mass of 56,600 daltons (amino acid composition is shown in the legend to Fig. 2). This figure, which does not account for the presence of carbohydrate, is consistent with the size of the mature, glycosylated subunit, which runs in $NaDodSO_4$/polyacrylamide gel electrophoresis with an apparent molecular mass of 60,000 daltons.

Structural Predictions. A method has been devised by Kyte and Doolittle (29) that evaluates the hydrophobicity of a polypeptide along its sequence. Each amino acid is assigned a hydrophobicity value that reflects the measured hydrophobic properties of the amino acid side chain. The computer program (SOAP) determines the average hydrophobicity of a fixed sequence length as it moves along the sequence from the NH_2 to the COOH terminus. The deduced amino acid sequence of the γ subunit (including the leader sequence) was analyzed by using the SOAP program with a sequence length of seven amino acids. The profile is shown in Fig. 3. The hydrophobicity index in Fig. 3 is identical to the hydropathy index of Kyte and Doolittle (29). Hydrophobic regions of the protein lie above the midpoint line [the average hydrophobicity of the amino acid compositions found in most of the proteins of known sequence in the "Atlas of Protein Sequence and Structure" and in NEWAT (29)], while hydrophilic regions of the protein lie below the midpoint line.

Analysis of the γ-subunit amino acid sequence reveals four stretches of hydrophobic residues (Fig. 3) with respective lengths of 24, 19, 19, and 19 residues and average hydrophobicity values of 2.5, 2.2, 2.9, and 2.4 (Table 1). A region in a membrane protein has a high probability of being a membrane-spanning region if it is at least 19 residues long with an average hydrophobicity >1.6 (29). The four regions marked 1–4 in Fig. 3 each meet these two criteria and, thus, are all likely to be regions where the γ subunit of the AcChoR spans the lipid bilayer. However, it cannot be ruled out that other regions of the protein with a lower hydrophobicity index value also span the membrane. At present there is no criteria for identifying such re-

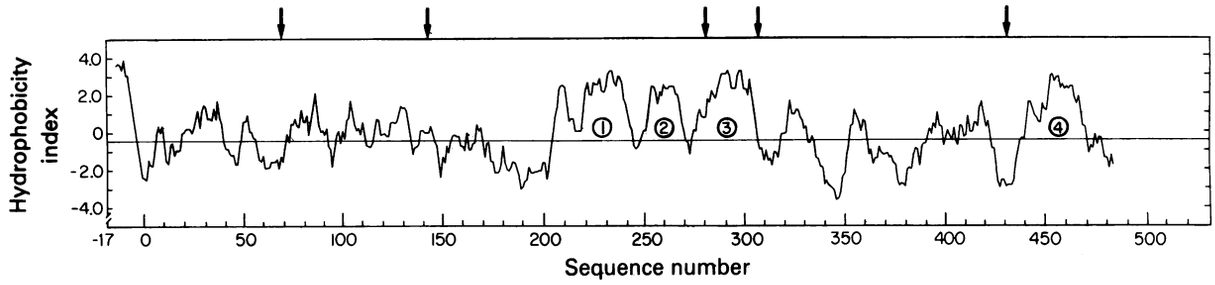

FIG. 3. SOAP profile (29) of the deduced γ subunit and its putative signal peptide. Hydrophobic regions of the protein lie above the midpoint hydrophobicity index line and hydrophilic regions lie below the midpoint line. Numbers correspond to amino acid sequence numbers in Fig. 2. Putative membrane-spanning regions are marked 1–4. Arrows indicate potential asparagine-linked glycosylation sites.

gions, if they exist, so we have considered only the four regions marked in Fig. 3. If one uses a different set of amino acid hydrophobicity values (30) from those used in this study (29), a profile similar to the one presented in Fig. 3 is obtained (data not shown). If the homology between subunits seen in the NH_2-terminal regions (6) is found throughout the protein, then it may be a general feature that all of the subunits span the membrane more than once, as is predicted for the γ subunit. In support of this conclusion, x-ray diffraction studies have suggested that the AcChoR (composed of five subunits) spans the membrane at least 12 times and possibly more (R. M. Stroud, personal communication).

The translocation of an integral membrane protein across the membrane of the rough endoplasmic reticulum is interrupted such that the protein becomes lodged in the membrane. It has been proposed that the information for this interruption is contained in the primary sequence of the protein, referred to as a "stop-transfer" sequence (31). The stop-transfer sequence may be represented by the hydrophobic membrane-spanning segment or by clusters of charged residues that flank the hydrophobic stretch, or by both. An analysis of possible stop-transfer sequences in the γ subunit is given in Table 1. The putative membrane-spanning segments of the protein and the charged amino acids within five residues of these segments are listed. Comparable numbers of charged residues are found flanking transmembrane sequences of other proteins (32–35). Although these amino acids appear to flank (rather than to be a part of) the membrane-spanning segments, the precise assignment of amino acids to the membrane-spanning regions is particularly difficult with this protein because some or all of these regions may form an ion channel in the membrane and may not be used merely to anchor the protein in the membrane. Charged or polar amino acid residues within or near the membrane-spanning segments could be used for the translocation of ions, as has been suggested for bacteriorhodopsin (32, 33), a protein that pumps protons across a hydrophobic membrane environment. In addition, with more than one segment spanning the membrane, intra- and interchain salt bridges or hydrogen bonds could be formed between polar amino acids that would mask partially their hydrophilicity in the hydrophobic environment of the membrane (36).

Table 1. Analysis of putative membrane-spanning segments 1–4

Segment characteristics				Nearby charged residues*	
No.†	Sequence position‡	AA, no.	Mean H index§	NH_2-terminal	COOH-terminal
1	220–243	24	2.45	K(2),R(3)	none
2	252–270	19	2.21	K(1)	K(2),E(5)
3	287–305	19	2.91	K(2)	R(5)
4	450–468	19	2.42	K(1),D(2)	H(3)

AA, amino acid.
* Charged residues (or His) within five residues of the hydrophobic sequence. The numbers are the positions from the ends of the hydrophobic sequence. D, Asp; E, Glu; H, His; K, Lys; R, Arg.
† Membrane-spanning segment number as shown in Fig. 3.
‡ Amino acid sequence positions defining membrane-spanning segments. Numbers correspond to amino acid number in Fig. 2.
§ Mean hydrophobicity (H) index value.

In order to construct a model of the γ subunit, one needs to know the orientation of the protein in the lipid bilayer. We have assigned the NH_2 terminus to the extracellular side of the membrane based on the following data. The γ subunit has a signal peptide, which is removed during maturation of the protein. Signal peptidase has been localized to the cisternal face of the rough endoplasmic reticulum (37); in agreement with this finding, most proteins whose signal peptide is cleaved have been shown to have their NH_2 terminus on the extracellular side of the membrane (35). Evidence presented for the α subunit (38) and the δ subunit (39, 40) of the AcChoR (whose homologies with the γ subunit have already been noted) suggests that the NH_2 termini of these two subunits reside on the extracellular surface of the membrane. By assuming that the NH_2 terminus of the γ subunit is located on the extracellular surface of the membrane and the assignment of membrane-spanning regions is correct, then it is possible to propose a model for the partitioning of the γ-subunit polypeptide chain into the extracellular, internal, and cytoplasmic domains of the lipid bilayer (see the model in Fig. 4). From this model, one can calculate the distribution of the polypeptide mass (not including the mass contribution from carbohydrate) among these three domains. The model predicts that 52% (29,300 daltons) of the protein lies on the extracellular side of the membrane, 31% (17,600 daltons) lies on the cytoplasmic surface, and 17% (9,700 daltons) is buried within the lipid bilayer. These results are consistent with those obtained from x-ray diffraction (41) and from immunoelectron microscopic (42) and neutron scattering (43) studies of the AcChoR, all of which indicate that the bulk of the protein resides on the extracellular side of the membrane. Further sup-

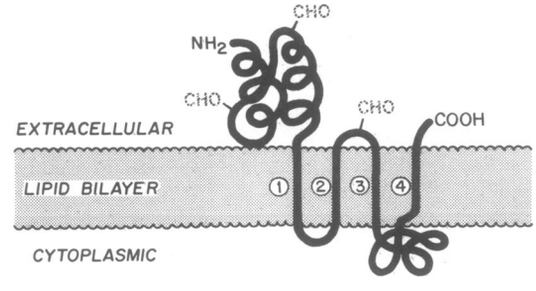

FIG. 4. Model depicting possible partitioning of the γ-subunit polypeptide chain in the lipid bilayer. Potential asparagine-linked glycosylation sites are marked with hatched CHO. The numbers 1–4 correspond to putative membrane-spanning regions 1–4 shown in Fig. 3.

port comes from results of trypsinization experiments, which indicate that ≈15,000 daltons of the γ subunit are exposed to the cytoplasmic surface of the lipid bilayer (44). The model (Fig. 4) also predicts that a 2,200-dalton segment containing the COOH terminus resides on the extracellular side of the membrane. If the model is correct, then both the NH_2 and COOH termini would be located on the extracellular side of the membrane; this configuration has not been found in the integral membrane proteins examined to date (35).

All four subunits of the AcChoR are glycosylated, but the locations of the sites have not been determined (4, 7, 8). Asparagine-linked glycosylation sites can be recognized by the sequence Asn-X-Ser/Thr. Although all asparagine-linked glycosylation sites have been shown to have this sequence, not all such sequences are glycosylated (reviewed in ref. 45). Examination of the deduced amino acid sequence in Fig. 2 reveals five potential glycosylation sites at amino acid residues 68, 141, 279, 306, and 431. These potential glycosylation sites are indicated with arrows in Fig. 3. By assuming that the model presented in Fig. 4 is correct, then two of the potential glycosylation sites (positions 323 and 448) would not be available for carbohydrate attachment because they would be on the cytoplasmic surface of the membrane (46). The prediction that the γ subunit has three asparagine-linked glycosylation sites is consistent with suggestive evidence (see figure 3A in ref. 44) that indicates three glycosylation sites in the γ subunit.

Analysis of the amino acid sequence of the γ subunit of AcChoR has led to a model for the partitioning of the polypeptide chain into the extracellular, internal, and cytoplasmic domains of the lipid bilayer. The model presented for the disposition of the γ subunit in the membrane is consistent with available biochemical data; however, further experimentation is needed in order to test the specific predictions of the model. The predictions can be tested by localizing the NH_2 and COOH termini, determining the number and positions of the glycosylation sites, and establishing the membrane-spanning regions of the protein. It should be remembered that the AcChoR is an oligomeric complex that consists of four different polypeptide chains, and possible interactions among the subunits have not been considered in the model presented for the γ subunit. Once the validity of making structural predictions based on amino acid sequence data is established and sequence data for the α, β, and δ subunits become available, then a detailed model of the AcChoR complex can be made. Such models should prove useful in determining relationships among structure, function, and regulation of the AcChoR.

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