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Mouse–*Torpedo* hybrid acetylcholine receptors: Functional homology does not equal sequence homology

(*Xenopus* oocytes/SP6 RNA polymerase/cDNA clones/ion channels/*in ovo* translation)

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ABSTRACT **The nicotinic acetylcholine (AcCho) receptor (AcChoR) is a multisubunit protein complex of stoichiometry $\alpha_2\beta\gamma\delta$. The several subunits show homology with each other within a given species; in addition, homology is found between analogous subunits between species. We have used the phage SP6 RNA polymerase transcription system to produce single-species RNA *in vitro* for various AcChoR subunits from cDNAs. Injection of an equimolar mixture of RNA for the α, β, γ, and δ subunits of *Torpedo californica* AcChoR into *Xenopus* oocytes results in the appearance of functional receptors in the oocyte membrane. No response to AcCho is detected when the β or γ subunit RNA is omitted, and a small response is seen when the δ subunit RNA is omitted. Replacement of *Torpedo* δ subunit RNA by the mouse BC3H-1 cell line AcChoR δ subunit RNA leads to the formation of functional receptors that show a 3–4-fold greater response to AcCho than does the full *Torpedo* complex. No response is seen when the mouse δ RNA replaces *Torpedo* γ RNA. By amino acid homology profile comparisons, the mouse δ subunit appears to be moderately but not highly similar to the *Torpedo* δ subunit; the apparent similarity to the *Torpedo* γ subunit is only slightly less. Therefore, the features of the primary sequence that determine the functional δ character of the mouse polypeptide are not revealed by simple homology comparisons.**

The nicotinic acetylcholine receptor (AcChoR) is involved in vertebrate neuromuscular transmission and the generation of electrical impulses by the electroplax of electric fish. Extensive electrophysiological, biochemical, structural, and molecular biological studies have made it the best-characterized membrane channel from excitable cells (for recent reviews, see refs. 1–5). The receptor complex consists of four subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. The binding of AcCho to each of the two α subunits results in the opening of a transmembrane channel that is permeable to small cations, resulting in the transmission of an impulse at the nerve–muscle synapse or in a high-voltage pulse from electric fish.

The recent cloning of cDNAs for the subunits of the *Torpedo* electric organ AcChoR (6–11) makes it possible to apply the powerful techniques of molecular biology to the study of the structure, evolution, biosynthesis, and mechanisms that underlie the operation of the complex. Our interest lies in the mechanism of ligand activation and ion permeation through the channel. Through the use of site-directed mutagenesis (12), we hope to identify the structural features and, thus, the mechanisms involved in the functioning of the receptor. Since the mutagenesis involves manipulations at the DNA level, a suitable expression system must be developed to study the properties of these "mutant" receptors.

Xenopus oocytes have proved to be an attractive system for the expression of proteins coded for by exogenous nucleic acids. Nuclear injection of DNA (13) or cytoplasmic injection of mRNA (14) results in the biosynthesis of functional products. Barnard and co-workers (15, 16) have shown that when *Torpedo* electric organ mRNA is injected into oocytes, functional *Torpedo* AcChoRs appear in the oocyte membrane. Mishina *et al.* (17) injected mRNA isolated from COS cells transfected with expression vectors containing cDNAs coding for each of the subunits of *Torpedo* AcChoR and obtained functional AcChoRs, an indication that the cDNAs contained all of the *Torpedo*-specific information required for assembly of functional receptors.

In this report, we describe another approach to the expression of *Torpedo* AcChoRs in *Xenopus* oocytes. We utilize the highly efficient phage SP6 RNA polymerase *in vitro* transcription system developed by Melton and colleagues (18, 19). This system allows the synthesis of microgram quantities of pure RNA from cDNA. When used with cDNAs for the individual subunits of the AcChoR, injection of the *in vitro* transcripts into oocytes gives rise to functional *Torpedo* AcChoRs in large quantities that can be studied readily by both biochemical and electrophysical techniques.

While this manuscript was in preparation, Mishina *et al.* (20) described an expression system essentially similar to that described here. They have used this system to study the effects of segment deletion or single amino acid changes introduced into the *Torpedo* AcChoR α subunit on receptor function. For our first study, we have chosen to test the effect of a much larger "mutation" in that we have asked whether a hybrid receptor containing the *Torpedo* α, β, and γ subunits and the mouse δ subunit is functional. This study necessarily required examination of the effects of one-by-one deletion of each individual *Torpedo* subunit on receptor assembly and function.

MATERIALS AND METHODS

Plasmids. Full-length *Torpedo* AcChoR cDNA clones were provided by D. Noonan of Scripps Research Institute (α subunit), T. Claudio of Yale University (β and δ subunits) and S. Heinemann of Salk Institute (γ subunit; ref. 10). The mouse BC3H-1 cell line AcChoR δ subunit cDNA clone was isolated in this laboratory (21). The cDNA inserts were excised from the vector and inserted into plasmid pSP62-PL (provided by D. Melton of Harvard University), which contains the phage SP6 RNA polymerase promoter followed by a polylinker. The resulting plasmids were maintained in *Escherichia coli* strain HB101.

***In Vitro* Transcription.** Plasmids containing the appropriate AcChoR subunit were linearized by digestion with either *Aat* II (*Torpedo* α,γ, and δ subunits), *Xmn* I (*Torpedo* β subunit), or *Sca* I (mouse δ subunit) (New England Biolabs) according to the supplier's recommendations. The digestion mixture

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Abbreviations: AcChoR, nicotinic acetylcholine receptor; AcCho, acetylcholine; GpppG, diguanosine triphosphate.

was extracted with phenol, and the DNA was precipitated with ethanol.

The transcription reaction contained 40 mM Tris (pH 7.5); 10 mM NaCl; 10 mM dithiothreitol; 6 mM $MgCl_2$; 4 mM spermidine; 0.5 mM each of ATP, CTP, and UTP; [α-^{32}P]CTP at 50 μCi/ml (Amersham; 1 Ci = 37 GBq). 0.1 mM GTP; 0.5 mM diguanosine triphosphate (GpppG; Pharmacia P-L Biochemicals), RNasin at 1000 units/ml (Promega Biotec, Madison, WI) SP6 RNA polymerase at 160 units/ml (Promega Biotec); and linearized DNA at 25 μg/ml. Total volume was 100 μl. Transcription was carried out for 70 min at 37°C. The reaction was stopped by the addition of RNase-free DNase (grade DPRF, Worthington) to a concentration of 20 μg/ml, and incubation was for 10 min at 37°C. The reaction mixture was extracted with phenol, unincorporated nucleotides were removed by the spun-column method of Penefsky (22) with 10 mM Na-phosphate (pH 7.0), and the RNA was recovered by precipitation by ethanol. A portion of the reaction product was treated with glyoxal and analyzed by gel electrophoresis (23).

Preparation of Oocytes and RNA Injection. Mature *Xenopus* females were obtained from Nasco (Ft. Atkinson, WI) and anesthetized by immersion in water containing 0.15% tricaine (3-aminobenzoic acid ethyl ester). An incision was made in the abdomen, and a portion of the ovary was removed and placed in ND-96 solution (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes/NaOH, pH 7.6). Follicle cells were removed by incubating the tissue in Ca^{2+}-free OR-2 solution (24) containing collagenase (type IA, Sigma) at 2 mg/ml for 30–45 min at room temperature. The oocytes were transferred to Ringer's solution (116 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/mM $MgCl_2$/5 mM Hepes/NaOH, pH 7.6), and the adhering follicular tissue was removed with forceps. Isolated stage V and VI oocytes were then transferred to ND-96 supplemented with 2.5 mM Na pyruvate.

RNA was dissolved in distilled water at 0.2 mg/ml, and 30–50 nl were injected into the cytoplasm by using a device similar to that described by Contreras *et al.* (25). The oocytes were incubated at room temperature for 48–72 hr in 70% L-15 medium (26) supplemented with 10 μg of penicillin and streptomycin per ml and 0.5 mM theophylline.

Electrophysiology. Individual oocytes were transferred to the recording chamber and studied under voltage-clamp conditions by using a standard two-microelectrode voltage clamp (model 8500, Dagan Instruments). The electrodes were filled with 3 M KCl/100 mM potassium EGTA, pH 7.0, and had resistances of 0.5–3 MΩ. The chamber was continuously perfused with 96 mM NaCl/2 mM KCl/5 mM $MgCl_2$/0.3 mM $CaCl_2$/0.3 μM atropine/5 mM Hepes/NaOH, pH 7.6. Holding potential in all experiments was −60 mV.

RESULTS AND DISCUSSION

Transcription of Subunit-Specific RNAs. *In vitro* transcription using plasmids containing the highly efficient SP6 promoter and SP6 RNA polymerase produces large quantities of single-species RNA. Fig. 1 shows the products of transcription reactions using linearized plasmids containing cDNA of the α, β, γ, and δ subunits of the *Torpedo* AcChoR and the δ subunit of the mouse AcChoR. Each reaction produces a single RNA species that carries the coding sequence for the appropriate subunit. Under the conditions described, 2–5 μg of RNA are produced per μg of DNA, which corresponds to 10–30 transcripts per DNA template.

The 5′ cap structure found in eukaryote mRNAs is an absolute requirement for mRNA stability in the cytoplasm (27). In their initial studies, Green *et al.* (28) capped their *in vitro* SP6 transcripts by subsequent treatment of the RNA

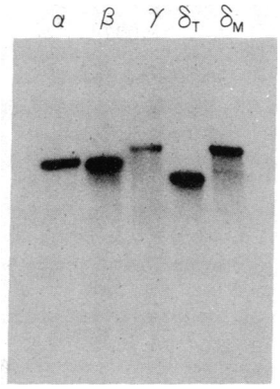

FIG. 1. *In vitro* synthesis of AcChoR subunit RNA by SP6 RNA polymerase. Plasmids containing the α, β, and γ AcChoR subunits of *Torpedo* and the δ AcChoR subunits of *Torpedo* and mouse, designated δ_T and δ_M, respectively, were linearized and transcribed as described. An aliquot from each reaction was treated with glyoxal and electrophoresed through 1% agarose. The transcripts are 2.5(α), 2.3(β), 2.65(γ), 2.1(δ_T), and 2.6(δ_M) kilobases long.

with vaccinia virus guanyltransferase. We obtained capped transcripts by inclusion of the cap analog diguanosine triphosphate (GpppG) in the transcription reaction. Contreras *et al.* (29) discovered that the cap analogs GpppA and GpppG can be incorporated at the 5′ end of RNAs produced *in vitro* with *E. coli* RNA polymerase. The capped transcripts are not rapidly degraded after injection into *Xenopus* oocytes and are efficiently translated. Konarska *et al.* (30) found that SP6 RNA polymerase also can incorporate the cap analog GpppG at the 5′ end of the transcripts. Whereas the cap analogs reduce the efficiency of the *E. coli* polymerase transcription reaction, we found that inclusion of GpppG stimulates transcription by SP6 RNA polymerase by 20–50%. Under the conditions described >95% of the transcripts are capped at the 5′ end (data not shown). We have not found it necessary to prepare transcripts with a 7-methylguanosine cap structure, which is not surprising because *Xenopus* oocytes contain a cytoplasmic methyltransferase (27). In addition, it is not necessary to include a polyadenylate tract at the 3′ end for translation of the RNA in oocytes, in agreement with Krieg and Melton (19).

Expression of *Torpedo* AcChoRs in *Xenopus* Oocytes. When the *Torpedo* subunit-specific RNAs are mixed in equimolar proportions and injected into *Xenopus* oocytes, functional AcChoRs are synthesized and inserted into the oocyte plasma membrane. Fig. 2*A* shows the response of a voltage-clamped oocyte previously injected with *in vitro* synthesized RNA to bath application of various concentrations of AcCho. Atropine (300 nM) was included in the perfusion solution to block the endogenous muscarinic AcCho receptors (31), and the Ca^{2+} concentration was kept low (0.3 mM) to obtain dose–response data without the complications introduced by desensitization (32). When a physiological Ca^{2+} concentration (1.8 mM) is used, the receptors do desensitize (data not shown). Membrane current increases as AcCho activates the receptor; the time course of this increase is limited by perfusion mixing rather than by the molecular events of channel gating. The response is nonlinear; doubling the AcCho concentration from 330 nM to 660 nM results in a 5-fold increase in current. No response was seen in uninjected oocytes. Furthermore, *d*-tubocurarine (5 μM), a competitive inhibitor of the nicotinic receptor, blocked the response (data not shown). Fig. 2*B* shows the results of many measurements of this type plotted on double-logarithmic coordinates. Data from each experiment are normalized for comparison to a response of 100 for 1 μM AcCho. The data are fit well by a relation with a Hill coefficient, n, of 2.0 ± 0.1.

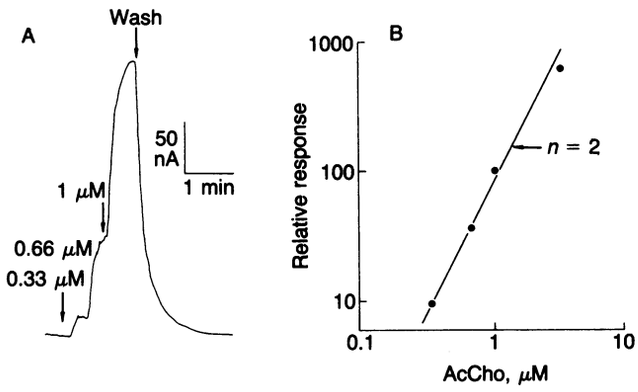

FIG. 2. *In vitro* transcribed RNA directs the synthesis of AcChoRs in *Xenopus* oocytes. (*A*) An oocyte injected with 10 ng of RNA 48 hr previously was voltage-clamped, and membrane current was monitored in response to bath application of 0.33, 0.66, and 1.0 μM AcCho. Note the nonlinear dependence of the response on AcCho concentration. (*B*) Pooled data from measurements on four oocytes. Data from measurements like those in *A* were normalized to a response of 100 for 1 μM AcCho. Each data point represents the mean ± SEM of four to six determinations; the error bars are smaller than the points. The solid curve is drawn according to the relation:

$$\text{response} = 100 \cdot \left(\frac{[\text{AcCho}]}{1\ \mu M}\right)^2.$$

The slight deviation from $n=2.0$ for 3.3 μM AcCho may be due to the effect of the small amount of desensitization seen at this concentration. The same quadratic dependence of response on ligand concentration has been observed both in electrophysiological dose–response curves using nerve-muscle and nerve-electroplaque preparations (33, 34) and in ligand-activated flux measurements on isolated membrane vesicles (35). This basic finding is consistent with the hypothesis that two AcCho molecules must bind to the receptor complex to effect a response.

These data indicate that the *in vitro* transcribed RNAs are capable of directing the synthesis of *Torpedo* AcChoRs in *Xenopus* oocytes. The pharmacological porperties (activated by AcCho, blocked by curare, unaffected by atropine), the desensitization, and the functional stoichiometry ($n=2$) are all hallmarks of normal receptors. In particular, the requirement for two agonist molecules is thought to manifest an interaction between the two liganded α subunits within an individual receptor molecule. Thus biosynthesis of the protein complex in oocytes appears to have all of the features of the *in vivo* process in *Torpedo* electroplax and muscle cells.

Subunit Deletion Experiments. The amino acid sequences of the four *Torpedo* subunits show homology among each other (8). In addition to this intrareceptor homology, each subunit also exhibits homology to the corresponding subunit from other species (21, 36–40). We have taken advantage of the flexibility of our expression system to determine if this sequence homology is also a functional homology. Fig. 3 and Table 1 show the results obtained from oocytes injected with various mixtures of *in vitro* transcribed RNAs. No current is elicited by bath application of 1.5 μM AcCho when either the *Torpedo* β or γ subunit RNA is omitted from the injection mixture. When the *Torpedo* δ subunit RNA is omitted, the elicited current is small (3% of control) but nonetheless present. This result differs somewhat from that of Mishina *et al.* (17), who detected a response in only 3 of 105 oocytes injected with an αβγ mixture prepared by synthesis in COS cells. We ascribe this difference to a higher sensitivity in our measurements, rather than any real difference in expression.

Formation of Hybrid Receptors. LaPolla *et al.* (21) have described the isolation of a cDNA clone for the δ subunit of the mouse BC3H-1 cell line AcChoR. It is probable that this

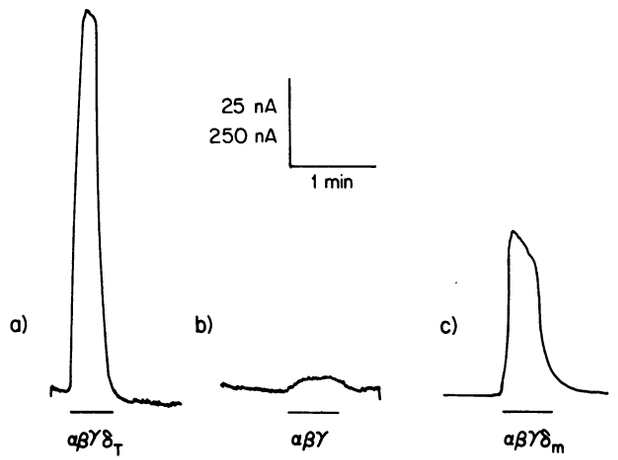

FIG. 3. Responses of oocytes injected with various combinations of AcChoR subunit RNAs. Oocytes were injected with the indicated mixture of AcChoR subunit RNAs, incubated, and voltage-clamped as described. The current elicited by bath application of 1.5 μM AcCho (solid bar below current traces) was determined. The current scale is 25 nA for traces a and b and 250 nA for trace c.

is the cDNA for the gene expressed in the skeletal muscles of the mouse. These authors reported that the amino acid sequence of the mouse δ subunit (δ_M) shows 59% overall amino acid homology to the *Torpedo* δ subunit (δ_T). The clone also shows 50% homology to the *Torpedo* γ subunit. Using a somewhat refined sequence alignment, we find that δ_M shows 59% homology to δ_T and 48% homology to the *Torpedo* γ subunit. We find that when γ RNA is replaced by δ_M RNA, no response is seen (Table 1). On the other hand, when δ_T RNA is replaced by δ_M RNA, the elicited current is 3- to 4-fold greater than the control (all *Torpedo* subunits; Fig. 3 and Table 1). The difference in the response of the hybrid receptor compared to the control is not due to a greater number of receptors on the surface of the oocyte, as we do not detect a significant difference in the number of surface α-bungarotoxin biding sites for the two populations (data not shown). The enhanced response is due to a change in the intrinsic properties of the receptor complex. Possibilities include a 4-fold increase in the single-channel conductance, a 2-fold decrease in the AcCho concentration for half-maximal activation, or an increased channel mean open time.

Whatever similarity exists between δ_T and δ_M that is not present in the *Torpedo* γ subunit is not obvious from comparison of the amino acid sequences. Fig. 4 presents homology profiles for δ_M vs. δ_T, δ_M vs. γ, and δ_T vs. γ subunits. There are no obvious differences between the profiles to suggest a region that defines the mouse subunit as a δ rather than a γ. It is possible that the regions of sequence nonhomology contain structural features that determine the

Table 1. Electrophysiological responses of oocytes after injection of various AcChoR mRNAs

Mixture	Response, nA ± SEM
$\alpha\beta\gamma\delta_T$	235.6 ± 25.5
$\alpha\gamma\delta$	0
$\alpha\beta\gamma$	6.5 ± 1.1
$\alpha\beta\delta_T$	0
$\alpha\beta\gamma\delta_M$	792.0 ± 153.9
$\alpha\beta\delta_T\delta_M$	0

Oocytes were injected with various combinations of AcChoR subunit-specific RNAs as indicated, incubated, and voltage-clamped as described. The current elicited by bath application of 1.5 μM AcCho was determined. Each value represents the mean ± SEM of 10–20 determinations.

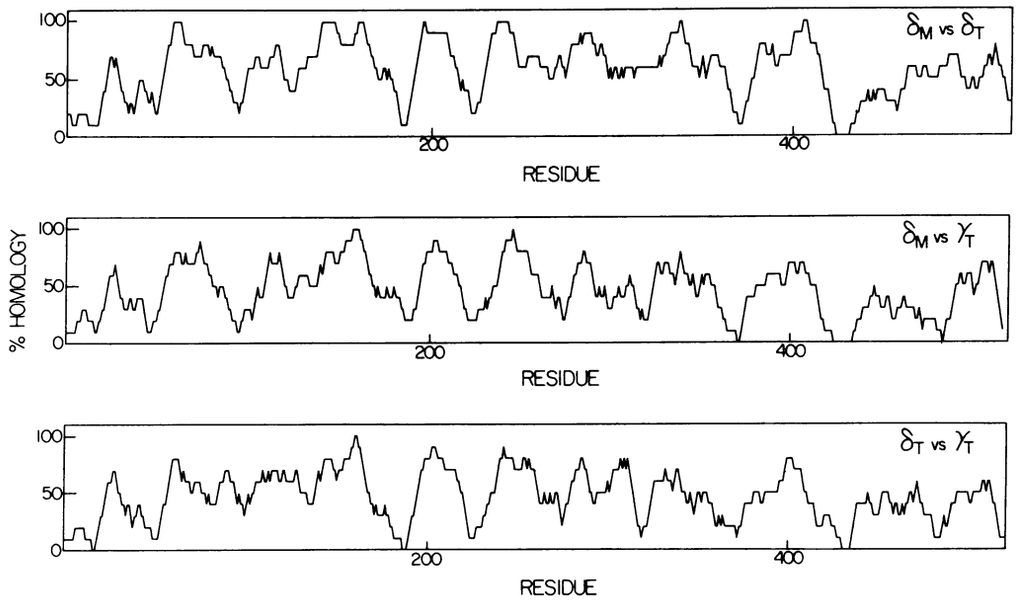

FIG. 4. Homology profile comparisons. Percent homology values for strings of 10 amino acids were calculated by using refinements of the sequence alignments described in ref. 21. In this analysis, the gaps inserted in the sequences for alignment purposes were scored as regions of 0% homology.

"deltaness" of the mouse subunit. Such structural/functional homology despite a lack of sequence homology is by no means uncommon. For example, the structure of the NAD-binding domain of NAD-dependent dehydrogenases is remarkably conserved despite a lack of sequence homology (41). In the absence of high-resolution structural data concerning the AcChoR, this possibility remains a viable hypothesis. However, another possibility does exist. There may be sequences conserved in the two δ subunits but not in the γ subunit that are not detected by the overall sequence analysis shown in Fig. 4. If subunit–subunit interactions are the forces that hold the complex together, then the points of contact could define the identity of each subunit. If one considers interactions between α helices, then these contact points should repeat every 3.6 residues (the periodicity of the helix). This periodicity may easily be missed in the plot shown in Fig. 4. We have examined the sequences of the four putative hydrophobic transmembrane domains of the subunits for such a periodicity. Fig. 5 shows helical net projections of these four domains. Residue positions in which δ_M, δ_T, and γ have identical amino acids are outlined with rectangles, and residue positions in which δ_M and δ_T but not γ have identical amino acids are outlined with ovals. Our hope was to find a stripe of sequence conservation between the two δ subunits that is not in the γ subunit. We have discounted such benign differences as valine to isoleucine, etc., and searched for rather drastic differences. Helix IV appears to be the best candidate for such a difference. A stripe of sequence conservation runs down the length of the helix for the δ subunits. The γ sequence differs in various positions with substitutions of tryptophan for phenylalanine, leucine for proline, and phenylalanine for methionine. These major

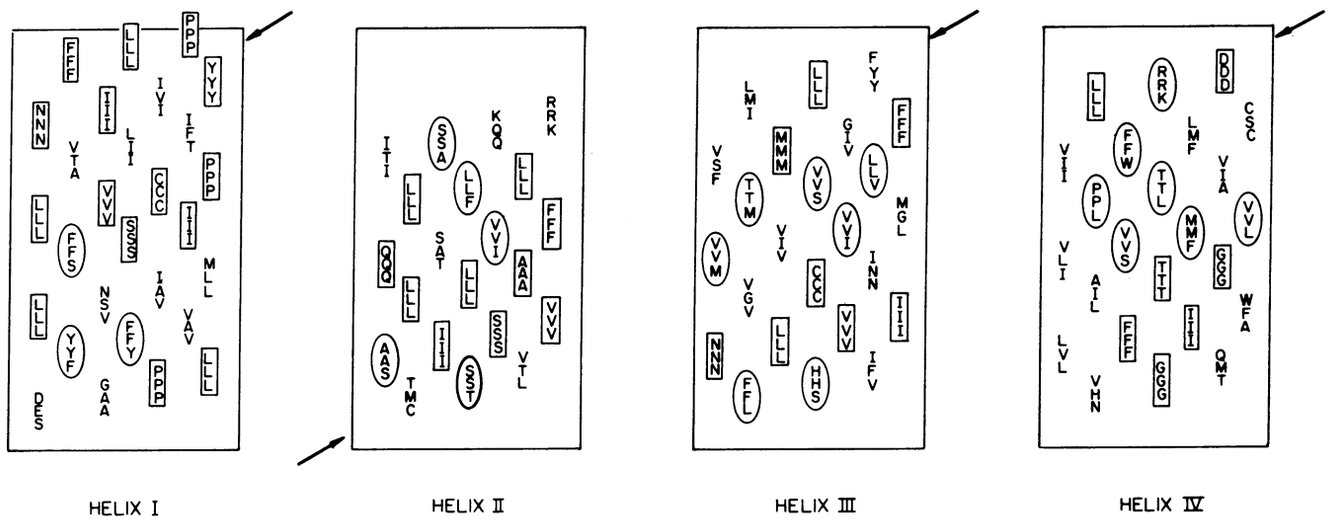

FIG. 5. Helical net representation of the four transmembrane hydrophobic regions. The residues are displayed as triplets in the order (top to bottom) δ_M, δ_T, and γ. Triplets in which all subunits have identical amino acids are outlined with a rectangle; triplets in which the two δ subunits but not the γ subunit have identical amino acids are outlined with an oval. The arrows indicate the amino terminus of each helix. Note the stripe of δ homology in helix IV.

side-chain changes could well prevent the neighbor–neighbor interactions that hold the δ subunit in its normal position.

This study illustrates the principle that the subtle ways in which conservation of function is encoded in the primary sequence of evolutionarily related proteins may not be revealed by a simplistic sequence homology comparison.

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