Reporter Epitopes: A Novel Approach To Examine Transmembrane Topology of Integral Membrane Proteins Applied to the α1 Subunit of the Nicotinic Acetylcholine Receptor†

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ABSTRACT: The development of a novel immunological method called the "reporter epitope" technique to probe the transmembrane topology of integral membrane proteins is described. Using this method, synthetic oligonucleotides encoding epitopes (reporter epitopes) for well characterized monoclonal antibodies (reporter mAbs) were inserted at various locations within the human acetylcholine receptor (AChR) α1 subunit cDNA. The engineered subunits were then expressed along with *Torpedo* β1, γ, and δ subunits in *Xenopus* oocytes, and the transmembrane location of the site of insertion was determined by the binding of the ^{125}I-labeled reporter mAbs to whole oocytes. Control reporter epitope insertions at α347 exhibited the expected cytoplasmic location. Reporter epitopes inserted at α429 are located on the extracellular surface. Reporter epitopes that are 16–48 amino acids long do not disrupt assembly or function of hybrid AChRs when inserted near the carboxy terminus (at α429) or in the large cytoplasmic domain (at α347). However, because two reporter epitopes inserted at α157 obliterated subunit assembly and a third reporter epitope when tolerated at this position was inaccessible from the extracellular surface and only marginally accessible after detergent solubilization of the AChRs, a definitive transmembrane location for this region was not possible. Nonetheless, the use of this approach has been successfully demonstrated, and it may be generally applicable to the study of other integral membrane proteins.

Integral membrane proteins play a critical role in a number of cellular functions such as active transport (e.g., sugar and amino acid transporters), transmembrane signaling (e.g., G-protein coupled receptors), and ion transport (e.g., voltage or ligand-gated ion channels). However, they are also hard to crystallize, as evidenced by the paucity of crystallographic information available for structure–function analysis of proteins in this class. A first step toward gaining insight into the functioning of these proteins is to study the transmembrane topology of the protein subunits. A variety of elegant approaches have emerged in the past few years that allow one to study membrane protein topology, each with particular virtues and limitations [reviewed in Jennings, (1989)].

In this paper, we describe the development of a novel approach to examine the topology of any cloned integral membrane protein *in vivo*, by an immunological technique we term the "reporter epitope" technique. In this technique, a DNA sequence coding for a well-characterized mAb[1] epitope (reporter epitope) is inserted at different positions within a subunit cDNA, with the expectation that if the insertion site is near the surface of the functional protein, then the reporter epitope may be accessible to binding by the "reporter mAb". Further, the expectation is that if the surface site is away from active sites, the protein may continue to function normally, despite the additional loop of peptide on its surface. The engineered cDNA is then expressed in *Xenopus* oocytes along with other native subunits necessary for the assembly of a complex, and the expressed protein is tested to determine if the reporter epitope prevented function. Finally, the binding of the reporter mAb to the reporter epitope is used to determine whether the epitope appears on the extracellular or cytoplasmic surface of the oocyte expressing the functional protein.

Using this method, we have examined the transmembrane topology of the AChR α1 subunit, first, because it is an archetype for all other subunits of the AChR and second, because its subunit topology is better studied than that of any other subunit of the ligand-gated ion channel superfamily. The reporter epitopes (for mAb 236 and mAb 142) used in this study were mapped on *Torpedo* AChR α1 subunits to a precision of as few as five to eight amino acids (Das & Lindstrom, 1991). Because the reporter mAbs and their corresponding reporter epitope probes used were also species-specific for the α1 subunit of the *Torpedo* AChR, this technique was applied to the human α1 subunit rather than the *Torpedo* α1 subunit. Futhermore, because the human α1 subunits expressed in *Xenopus* oocytes assemble even more efficiently with *Torpedo* β1, γ, and δ subunits than do *Torpedo* α1 subunits (Conroy et al., 1990), there was no need to coexpress human β1, γ, and δ subunits with human α1 subunits containing reporter epitopes. Two different reporter epitopes were used to investigate whether either would prove less likely to disrupt the AChR or more likely to be recognized by its reporter mAb.

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[1] Abbreviations: αBgt, α-bungarotoxin; ACh, acetylcholine; AChR, acetylcholine receptor; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RIA, radioimmunoassays.

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Table I: Summary of Fusion Sequences at the Various Sites of Insertion of the Reporter Epitopes in the Human α1 Subunit[a]

site of insertion	native sequence at site of insertion	fusion sequence after insertion of reporter epitope 142	fusion sequence after insertion of reporter epitope 236
α157	SVVA*INPE	SVVA (QVTGEVIFQTPLIKNP)$_{n=1,2}$ AINPE	SVVA (VSISPESDRPDLSTF)$_{n=1}$ AINPE
α162	INPE*SDQP	INPE (EVIFQTPL)$_{n=1}$ SDQP	
α347	EDID*ISDI	EDID (EVIFQTLP)$_{n=1}$ ISDI	EDID (VSISPESDRPDLSTF)$_{n=1,2}$ ISDI
α347	EDID*ISDI	EDID (QVTGEVIFQTPLIKNP)$_{n=1}$ AINPE	
α355	SGLP*GPPP	SGLP (EVIFQTPL)$_{n=2}$ GPPP	SGLP (ESDRP)$_{n=2}$ GPPP
α429	FAGR*LIEL	FAGR (QVTGEVIFQTPLIKNP)$_{n=1,2}$ STH	FAGR (VSISPESDRPDLSTP)$_{n=1,2,3}$ STH
α429	FAGR*LIEL	FAGR (EVIFQTPL)$_{n=1}$ GSGYH	
α437	LIEL*NQQG	LIEL (EVIFQTPL)$_{n=1}$ GSGYH	

[a] Amino acids surrounding the site of insertion of the reporter epitope 142 and 236 and the resulting fusion sequence after insertion of the reporter epitopes are shown. Additional amino acids added on during the construction of the clones are underlined. The asterisks indicate the site of insertion, the amino acids shown in parentheses correspond to the reporter epitopes themselves, and $n = 1, 2$ refers to the number of copies of reporter epitopes inserted in tandem.

In developing this approach, we started by testing if a small reporter epitope (eight amino acids) would disrupt AChR assembly or function when inserted at least three different locations within the α1 subunit. We found that in all three cases neither assembly nor macroscopic function of the AChR was disrupted by insertion of this epitope. However, contrary to our expectation, the inserted reporter epitope was easily obscured by the surrounding native sequences. In a second generation of constructs, we were able to circumvent this problem to some degree by inserting multiple tandem copies of the same epitope. Finally, use of a third generation of constructs showed that insertion of multiple copies of longer epitopes containing the flanking amino acids that surrounded the minimal epitope in the *Torpedo* α1 subunit substantially increased both the epitope accessibility and perhaps the mAb binding affinity to a level that was useful for studying transmembrane topology of the AChR α1 subunit.

In this paper we successfully applied the reporter epitope approach to analyze two of the three regions targeted in the human AChR α1 subunit. Our success with this *in vivo* approach suggests its general applicability to the study of the topology of other integral membrane protein subunits, provided the reporter epitope used is tolerated at the site of insertion and the reporter mAb used shows no cross-reactivity with the native subunit under study or *Xenopus* oocyte proteins. In addition, we were able to assess perturbations in the macroscopic function of the AChR due to the insertion of reporter epitopes and thus were able to designate a transmembrane location for the site of insertion only after giving due consideration to the effect of the insertion of the reporter epitope on the function of the AChR. Voltage and ligand-gated channels are especially amenable to such functional tests to evaluate the structural integrity of the engineered protein and thus might be ideal candidates for this approach.

MATERIALS AND METHODS

cDNAs. The cDNAs for the *Torpedo* AChRs subunits α1, β1, γ, and δ were kindly provided by Dr. Toni Claudio (Yale University). The cDNA for the human AChR α1 subunit has been previously described (Schoepfer et al., 1988).

mAbs. mAb 142 was initially described by Tzartos et al. (1986) and mAb 236 by Criado et al. (1985). The minimum boundaries of epitopes on the *Torpedo* α1 subunit were measured by mAb binding to short overlapping synthetic α1 peptides and found to be α161–165 (ESDRP) for mAb 236 and α359–366 (EVIFQTPL) for mAb 142 (Das & Lindstrom, 1991). mAb 210 binds the main immunogenic region on the *Torpedo* α1 subunit and the human α1 subunit, an extracellular epitope that includes amino acids within the sequence α67–76 (Das & Lindstrom, 1989; Saedi et al., 1990).

Reporter Epitope Constructs. Two methods were employed to insert synthetic oligonucleotides coding for the amino acid sequences (reporter epitopes) recognized by mAb 142 and mAb 236 into the human muscle α1 subunit cDNA (TEα). The first method involved ligating double-stranded oligonucleotides into pre-existing restriction enzyme sites of TEα. Additional amino acid sequences not corresponding to the epitope sequence were often included to maintain the translational reading frame of the polypeptide. Using this method, reporter epitopes were inserted at position α157 after digestion of the cDNA clone TEα with *Msc*I (blunt end), at α347 after digestion with *Eco*RV (blunt end), and at α429 and α437 after digestion with *Sal*I followed by a fill-in reaction using the Klenow fragment of DNA polymerase I. All nonnative amino acid sequences including the reporter epitope sequences inserted at the various positions are shown in Table I. Insertion of reporter epitopes at position α157 resulted in the addition of an alanine residue at the end of the reporter epitope sequence. Insertion of the long reporter epitope for mAb 142 at position α429 resulted in the replacement of the last eight amino acids (LIELNQQG) of the subunit with the sequence STH and GSGYH with the short epitope for mAb 142. Insertion of the short epitope for mAb 142 at α437 resulted in the addition of the sequence GSGYH at the end of the inserted reporter epitope.

Multiple tandem insertions of the epitopes were achieved by screening for clones containing more than one insert. The DNA sequence at the site of insertion and the number of copies of the inserted epitope were determined by standard dideoxy sequencing. An alternative method was used to insert epitopes at position α162. In this method, as described by Kammann et al. (1989), three primers were used in two successive PCR reactions. In the first PCR reaction, a primer complementary to the sequences α262–268 and the second mutagenic primer complementary to the sequence at the site of insertion (α156–162 and 163–169), but containing the reporter epitope sequence in between, was used. The product of this reaction was used in a second PCR reaction with a third primer complementary to the vector sequences containing the SP6 promoter site. The amplified product containing the reporter epitope inserted between α162 and α163 was digested with *Hin*dIII (5′ end) and *Dra*III (3′ end) and used to replace the *Hin*dIII–*Dra*III (~700 bp) fragment of the parent TEα clone. The entire *Hin*dIII–*Dra*III fragment was sequenced to detect errors generated by PCR. A silent mutation at α155 converting GTT to GTC was observed. However, this change does not result in a change in the amino acid sequence at α155 (valine). Using a similar strategy but using only two primers, tandem reporter epitopes for mAb 142 and 236 were inserted at position α355. The mutagenic 5′ primer was a 102-mer for the tandem 142 epitope and a 86-mer for the tandem 236 epitope, which was complementary to the sequence at the site of insertion (α345–355 and α356–362) but contained the sequence encoding the tandem minimal reporter epitopes in

between. The 3′ primer was a 20-mer complementary to the vector sequence at the end of the inserted cDNA and spanned an *Eco*RI site. The final amplified product (~270 bp) was digested with *Eco*RV(5′ end) and *Eco*RI (3′ end) and used to replace the *Eco*RV–*Eco*RI fragment of the parent TEα clone. The entire insert was sequenced, and no PCR-generated errors were detected in either clone.

Expression in Xenopus Oocytes. RNA was prepared *in vitro* using the SP6 RNA polymerase as originally described by Melton et al. (1984) and more recently using the Megascript kit (Ambion, TX). Oocytes were prepared for microinjection as described by Colman (1984) and injected with ~15 ng of cRNA of each subunit. The oocytes were then incubated in saline solution [96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.6)] containing 5% heat-inactivated horse serum at 18 °C for 3–4 days before use.

Surface Expression of AChRs. The number of AChRs expressed on the surface of the oocytes after microinjection with cRNA from various constructs was measured by incubating oocytes in saline solution containing 10% horse serum and 10 nM [^{125}I]αBgt (~8–10 × 10^{17} cpm/mol) at room temperature for 2 h. After five successive 1-mL washes with saline solution, the amount of bound [^{125}I]αBgt was measured by γ counting individual oocytes. Nonspecific binding was determined by using noninjected oocytes in parallel assays.

Total Expression of AChRs. The total amounts of AChRs expressed per oocyte were measured by solid-phase radioimmunoassays (RIAs). Immulon 4 (Dynatech) microtiter wells were coated with 40 μg/mL of either mAb 210, mAb 142, or mAb 236 in 10 mM Na_2CO_3/$NaHCO_3$ buffer (pH 8.8) overnight. The wells were rinsed three times with 1 mL of the bicarbonate buffer and blocked with 3% BSA in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 2 h and then rinsed three times with 1 mL of the PBS–Tween 20 buffer. Oocyte lysates were prepared by homogenizing oocytes in lysis buffer [2% Triton X-100, 50 mM NaCl, 50 mM sodium phosphate (pH 7.5), 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, 5 mM iodoacetamide], incubating the homogenate at 4 °C for 30 min with shaking, and clearing the cellular debris by centrifugation in a microfuge for 10 min at 4 °C. The cleared lysates were then used for all assays. RIAs were performed in 100-μL reactions volumes in the presence of 5 nM [^{125}I]αBgt in the mAb-coated microtiter wells at 4 °C overnight. The wells were then washed five times with ~0.5 mL of PBS–Tween 20 buffer, and the amount of radioactivity bound was determined by γ counting.

Binding of [^{125}I]mAb to AChRs to Oocytes Treated with Saponin. Oocytes expressing AChRs were incubated for 2 h at room temperature with or without 0.5% saponin, in saline solution containing 20 nM [^{125}I]mAbs, with gentle agitation. The oocytes were successively washed five times with 1 mL of saline solution and then incubated with saline solution containing 0.5% saponin for 1 h. Then, the oocytes were rinsed five times successively with 1 ml of the saline solution containing 0.5% saponin, following which the amount of [^{125}I]mAb bound was determined by γ counting. Nonspecific binding was monitored using noninjected oocytes.

Binding of [^{125}I]mAb to Surface AChRs. Binding of [^{125}I]mAb 210, [^{125}I]mAb 236, or [^{125}I]mAb 142 to the hybrid AChRs expressed in oocytes was determined by incubating oocytes with gentle agitation in saline solution containing 10% heat-inactivated horse serum and 20 nM of either [^{125}I]mAb 210, [^{125}I]mAb 142, or [^{125}I]mAb 236 for 2 h at room temperature. The mAbs were labeled with ^{125}I to specific activities of ~2–6 × 10^{18} cpm/mol, as described by Lindstrom et al. (1981). The oocytes were then washed five times successively with ~1 mL of saline solution, and the amount of radioactivity bound was determined by γ counting. Nonspecific binding was determined by using noninjected oocytes in parallel assays.

Electrophysiology. Electrophysiological recordings from oocytes injected with the various combinations of cRNA were made in a recording chamber continuously perfused with saline solution [96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.6), 0.3 μM atropine] at a flow rate of 10 mL/min. Responses were measured using a standard two-microelectrode voltage clamp (oocyte clamp OC-725, Warner Instrument Corp.). Electrodes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ for the voltage electrode and 0.2–0.4 MΩ for the current electrode.

RESULTS

To establish the feasibility of the reporter epitope technique, we engineered the minimum number of amino acids (EVIFQTPL) necessary for binding by the reporter mAb 142 (Das & Lindstrom, 1991) within the human AChR α1 subunit and coexpressed it with *Torpedo* β1, γ, and δ subunits in *Xenopus* oocytes. We measured the levels of expression of the hybrid AChRs formed on the surface of each oocyte by determining the amount of [^{125}I]αBgt bound to the surface AChRs. The efficacy of binding of the reporter mAb to the engineered epitope was then determined by measuring the amounts of Triton X-100-solubilized [^{125}I]αBgt-labeled AChRs bound by the reporter mAb in solid-phase RIA relative to that bound by mAb 210 (a mAb to an extracellular epitope including amino acids within the sequence α67–76). Finally, the binding of the ^{125}I-labeled reporter mAb to intact oocytes expressing these AChRs was measured to determine if the engineered epitope was located extracellularly with respect to the oocyte outer membrane.

Insertion of Short Reporter Epitopes at α162, α347, α429, and α437. In the first construct tested, the eight amino acid sequence (EVIFQTPL) corresponding to the minimum length of the synthetic peptide recognized by mAb 142 was inserted between M3 and M4 at position α347. This region is thought to be cytoplasmic because we had previously used electron microscopy to map mAbs to the cytoplasmic surface of muscle endplates and electric organ which recognized epitopes within α341–347, α349–365, α360–379, and α371–386 (Ratnam et al., 1986; Sargent et al., 1984) . In addition, this region is not well conserved between species and contains most of the epitopes for mAbs which recognize both native and denatured AChR, suggesting that the conformations of these sequences are not tightly constrained in the native AChR (Das & Lindstrom, 1991) and that the AChR was most likely to tolerate such an insertion without grossly perturbing its function. Co-injection of cRNA from this clone along with *Torpedo* β1, γ, and δ subunit cRNAs showed that ~9 fmol of AChRs was expressed on the surface of each oocyte (Table II). mAbs to epitopes in the large cytoplasmic domain often do not immune precipitate as many AChRs as do mAbs to the highly conformation-dependent main immunogenic region on the (more rigid) extracellular surface, perhaps because on the more amorphous surface of the large cytoplasmic domain the epitopes are accessible in only a subset of the AChRs. Because the Triton X-100-solubilized hybrid AChRs were bound by mAb 142 with a relative efficacy of only ~3% as compared to ~37% obtained for the binding of mAb 142 to the native epitope in the putative large cytoplasmic domain of the expressed *Torpedo* AChR (Figure 1 and Table II),

Table II: Summary of Reporter Epitope Constructs Tested[a]

reporter mAb	length of epitope	reporter epitope sequence used	copies of epitope inserted	position of insertion	efficiency of reporter mAb binding (% of mAb 210 binding)	surface expression (fmol of AChR/oocyte)	location of epitope	ACh sensitivity (μA/fmol of AChR)
mAb 142		native AChR with human α1	none	none	0	20	none	0.80
		native AChR with *Torpedo* α1	none	α359–366	37	8	cytoplasmic	1.38
	short	EVIF QTPL	1	α162	0	8	?	not tested
	short	EVIF QTPL	1	α347	3	9	?	not tested
	short	EVIF QTPL	1	α429	0	8	?	not tested
	short	EVIF QTPL	1	α437	1	8	?	not tested
	short	EVIF QTPL	2	α355	16	8	not tested	not tested
	long	QVTG EVIF QTPL IKNP	1	α157	0	0	?	0
	long	QVTG EVIF QTPL IKNP	2	α157	0	0	?	0
	long	QVTG EVIF QTPL IKNP	1	α347	33	17	cytoplasmic	0.81
	long	QVTG EVIF QTPL IKNP	1	α429	11	17	?	0.91
	long	QVTG EVIF QTPL IKNP	2	α429	25	16	extracellular	0.58
mAb 236		native AChR with human α1	none	α161–165	0	20	?	0.80
		native AChR with *Torpedo* α1	none	α161–165	0	8	?	1.38
	short	ESDRP	2	α347	29	6	not tested	not tested
	long	VSISP ESDRP DLST F	1	α157	14	7	?	0.80
	long	VSISP ESDRP DLST F	1	α347	26	18	cytoplasmic	0.71
	long	VSISP ESDRP DLST F	2	α347	36	17	cytoplasmic	0.99
	long	VSISP ESDRP DLST F	1	α429	0.7	17	?	0.39
	long	VSISP ESDRP DLST F	2	α429	52	16	extracellular	0.45
	long	VSISP ESDRP DLST F	3	α429	60	21	extracellular	0.45

[a] A summary of all the results obtained used the reporter epitope constructs is shown. Mean values obtained for solid-phase RIAs were done in duplicate. Mean values for three to five oocytes were obtained from measurements of either surface binding of [^{125}I]αBgt, surface binding of [^{125}I]mAbs, or normalized currents evoked by 100 μm ACh, made on individual oocytes.

these results suggested that insertion of the epitope sequence constrained its conformation so that it was recognized with a lower affinity by mAb 142 or it was accessible only in 3% of the AChRs. These results also indicated that the human α1 subunit could tolerate an eight amino acid insertion at position α347 and still associate efficiently with *Torpedo* β1, γ, and δ subunits.

To further investigate the feasibility of the approach, the same epitope was inserted at two controversial areas of the AChR α1 subunit: the regions between α152 and α167 and at the carboxy terminus. To examine these areas, the reporter epitope was inserted at positions α162, α429, and α437. As indicated in Table II, all three constructs when injected in oocytes showed ~8 fmol of AChRs capable of specifically binding [^{125}I]αBgt on the surface of each oocyte. However, in all three cases, no detectable binding of mAb 142 was observed, neither to Triton X-100-solubilized hybrid AChRs containing the reporter epitope at positions α162 and α429 in solid-phase RIA nor to surface AChRs. Only in the case of insertion at α437 could we detect marginal binding of mAb 142 to Triton X-100-solubilized AChR (~1%). Thus, insertions of eight amino acid reporter epitopes at positions α162, α347, α429, and α437 were well tolerated by the AChRs, but the altered conformation or accessibility of these small reporter epitope sequences greatly reduced the ability of the mAb to bind them.

Insertion of Tandem Short Reporter Epitopes at α355. In the next generation of constructs, we tested whether the efficiency of binding of the reporter mAbs would be augmented if we inserted tandem reporter epitopes within the putative large cytoplasmic domain. The rationale for making these constructs was that tandem epitope sequences with two mAb binding sites would be longer and thus at least one of the two sites might not be occluded by the native sequences surrounding the site of insertion. Two constructs were generated containing the tandem epitope sequences EVIFQTPLEVIFQTPL for mAb 142 and ESDRPESDRP for reporter mAb 236 (minimum epitope sequence ESDRP; Das & Lindstrom, 1991). mAb 236 was raised against the synthetic peptide *Torpedo* α152–167, and although this sequence is a major epitope in denatured α1 subunits, the epitope is not accessible in the native *Torpedo* AChR. mAb 236 binds to *Torpedo* AChRs only after treatment with chaotropic agents such as 3 M KSCN or denaturation with SDS, as on western blots. In addition, a near-native epitope sequence for mAb 236 (VAINPESDNPDLSNF) is also present in the human α1 subunit sequence at position α156–170 and, as in the *Torpedo* AChR, it is not accessible in the native human AChR. Thus, even though a near-native epitope for mAb 236 is normally present in the human α1 subunit, it was not expected to interfere with the use of this sequence as a reporter epitope. As seen from Table II, insertion of up to 16 amino acids at position α355 within the cytoplasmic loop was well tolerated with little change in the efficiency of assembly of the hybrid AChRs and the relative efficacy of binding by mAb 142 (16%). Thus, it appeared that, in addition to accessibility, the other factor limiting the affinity of the mAb 142 for its epitope in the putative large cytoplasmic domain was the sequence of the minimal epitope itself, as mAb 142 has a relative binding efficacy for its native epitope in the *Torpedo* AChR that is about 2-fold higher under identical assay conditions (~37%). We also confirmed that the five amino acid sequence recognized by mAb 236 *in vitro* could still be recognized *in vivo* and the binding occurred to the tandem epitope sequence with an efficacy of ~29%. Thus, at this point we had generated two different reporter epitopes for use in this technique.

Insertion of Long Reporter Epitopes. In the third generation of constructs we tried to improve the antigenicity of the reporter epitopes by including the amino acid sequences which normally surround the minimum epitope in the *Torpedo* α1 subunit, and to increase accessibility, we inserted multiple copies of the longer reporter epitopes in tandem at various positions in the human α1 sequence. In all the rest of the new constructs we used as reporter epitopes either a 16 amino acid sequence (QVTGEVIFQTPLIKNP), corresponding to the minimal epitope for mAb 142 and the surrounding native *Torpedo* α1 sequence, or the 15 amino acid sequence (VSISPESDRPDLSTF) corresponding to the minimal epitope for mAb 236

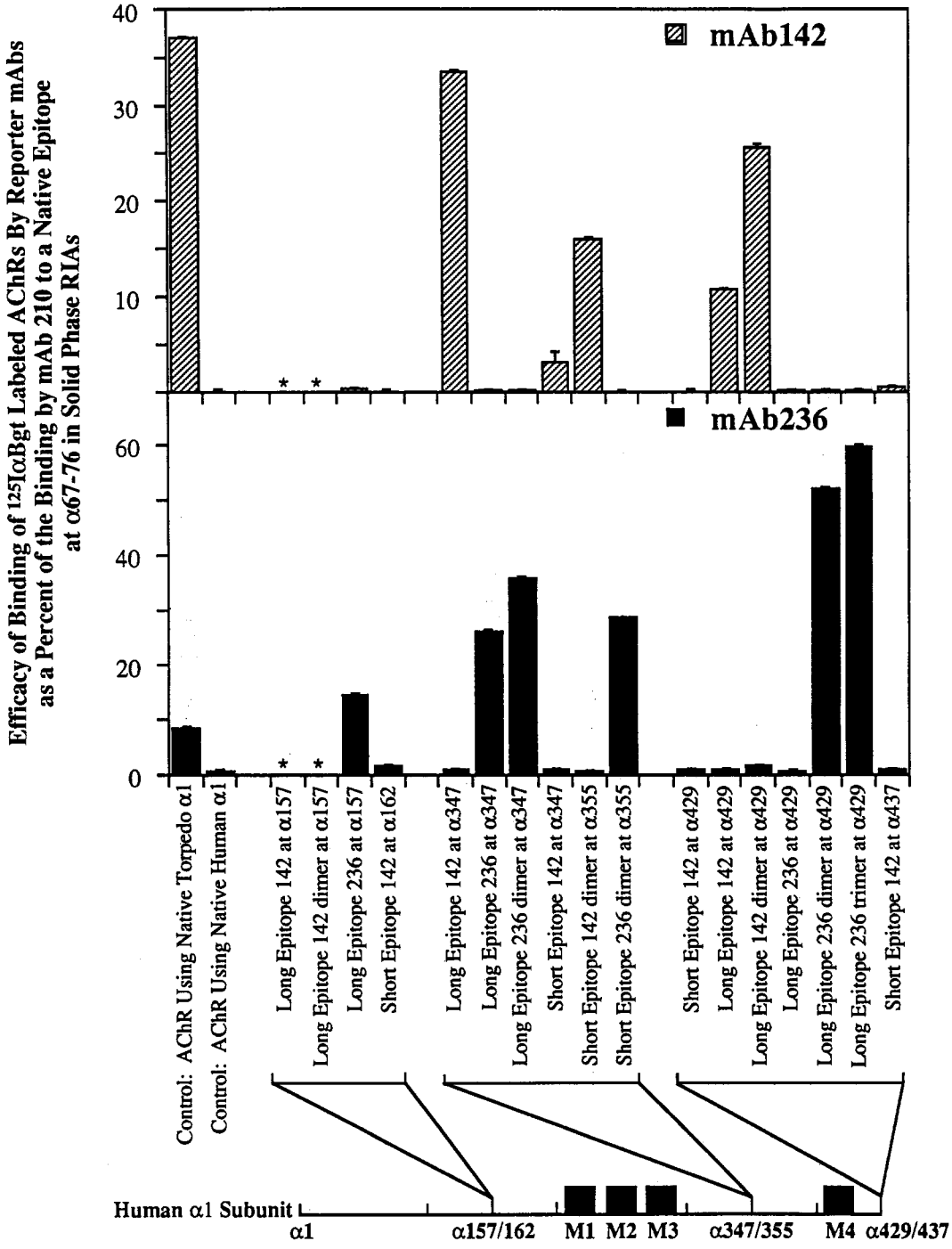

FIGURE 1: Detection of reporter epitopes in detergent-solubilized AChRs. The efficacies of binding to Triton X-100-solubilized [^{125}I]αBgt-labeled AChRs in solid-phase RIAs by reporter mAbs, as a percentage of the binding by a control mAb 210, whose native epitope is located on the extracellular surface of the α1 subunit at α67–76, are shown. The asterisks indicate α1 constructs for which no assembly of AChRs was detected. The values shown were obtained from solid-phase RIAs done in duplicate on mAb-coated microtiter wells. Nonspecific binding was determined using BSA-coated wells.

and the surrounding native *Torpedo* α1 sequence.

Single and multiple copies of the longer reporter epitopes for both mAb 142 and mAb 236 were inserted in the human α1 subunit at positions α157, α347, and α429. Two different reporter epitopes were used to address the possibility that one of the reporter epitopes was not topogenically neutral or was not well tolerated at some of the sites of insertion. As before, we used the amount of [^{125}I]αBgt bound to surface AChRs as a measure of the levels of expression of the various constructs. In addition, their electrophysiological response to application of 100 μM ACh was measured and the total current evoked normalized to the amount of surface AChRs (as determined by [^{125}I]αBgt binding) per oocyte. Finally, we tested if the engineered epitope had an extracellular location by determining the binding of [^{125}I]mAb 142 or [^{125}I]mAb 236 to surface AChRs in intact oocytes.

Insertion of Reporter Epitopes at α157. Three constructs of the human α1 subunit with insertions at α157 containing either one copy of the longer reporter epitope for mAb 236 or one or two copies of the longer reporter epitope for mAb 142 were coexpressed with *Torpedo* β1, γ, and δ subunits. Of these, constructs containing the reporter epitopes for mAb 142 failed to assemble into hybrid AChRs, as no [^{125}I]αBgt-labeled AChRs were detected in solid phase RIAs using microwells coated with mAb 210 (shown with asterisks in Figure 1) or in cell surface binding assays (shown with asterisks in Figure 2). Similar results were obtained with injections of up to 100ng of these construct cRNAs per oocyte. However,

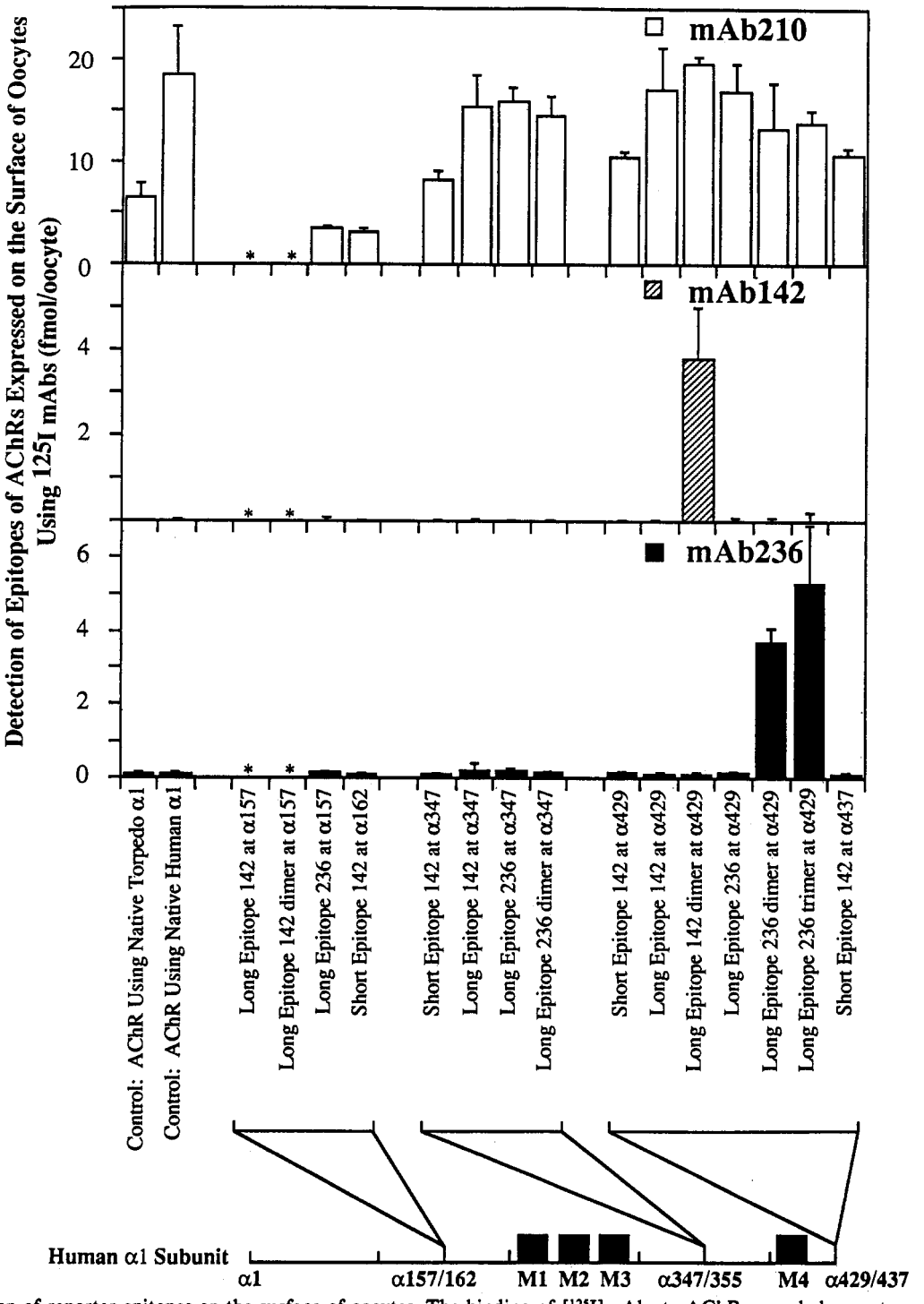

FIGURE 3: Detection of reporter epitopes on the surface of oocytes. The binding of [^{125}I]mAbs to AChRs on whole oocytes expressing the various hybrid AChRs is shown. The asterisks indicate α1 constructs for which no assembly of AChRs was detected. Values shown are the mean of results obtained from three to five oocytes. Nonspecific binding was determined using noninjected oocytes.

human α1 subunits containing one copy of the longer reporter epitope for mAb 236 coassembled with the *Torpedo* subunits to form functional AChRs, albeit at lower levels (Table II). mAb 236 bound the Triton X-100-solubilized hybrid AChRs with a relative efficacy of ~14%, but no detectable binding of [^{125}I]mAb 236 to surface AChRs was observed (Figure 2). The hybrid AChR responded functionally to applications of 100 μM ACh with normalized currents of ~0.8 μA/fmol, which was the same as the value for hybrid AChRs containing native human α1 subunits (Table II). Thus, it appears that perturbation of subunit assembly was not caused by insertion of the reporter epitopes at α157 per se but instead by the particular sequence of the reporter epitope for mAb 142. In addition, because only one of the three reporter epitopes was tolerated at this position, and because the binding of the reporter mAb 236 to these Triton X-100-solubilized AChRs was marginal, we were not able to confidently determine the transmembrane location of the site of insertion. We used saponin to permeabilize oocyte membranes expressing these hybrid AChR to see if the epitope was intracellular; however, no specific binding of [^{125}I]mAb was observed (data not shown). These results are consistent with the fact that even in Triton X-100-solubilized AChRs this epitope is only poorly accessible to mAb 236. Binding of [^{125}I]mAb 142 to oocytes expressing *Torpedo* AChRs (~1 fmol/oocyte) was observed only after permeabilization with 0.5% saponin. However, caution must be used in interpretation of positive results after saponification of oocytes because, unlike synaptic vesicles,

[125I]mAbs traversing the oocyte outer membrane could possibly have multiple intracellular targets including assembled subunits in intracellular membranes of the endoplasmic reticulum and the Golgi bodies, partially assembled subunits, and unassembled subunits in immature conformations, binding to any of which would complicate the results.

Insertion of Reporter Epitopes at α347. Three constructs of the human α1 subunit with insertions at α347 containing either one copy of the longer reporter epitope for mAb 142 or one or two copies of the longer reporter epitope for mAb 236 were coexpressed with *Torpedo* β1, γ, and δ subunits in *Xenopus* oocytes. All three constructs efficiently assembled into hybrid AChRs yielding ~17–18 fmol/oocyte (Table II). In addition, mAb 142 bound the hybrid AChRs containing one copy of its reporter epitope with a relative efficacy of ~33%, while mAb 236 bound AChRs containing one copy or two copies of its long reporter epitope with relative efficacies of 26% and 36%, respectively (Figure 2). The binding efficiencies of mAb 236 to its inserted epitopes in solubilized AChRs were not sufficient in themselves to conclude that the epitopes were cytoplasmic. However, on the basis of the fact that one copy of the reporter epitope for mAb 142 at this site of insertion is bound by mAb 142 with an efficiency (~33%) comparable to that of the native epitope in *Torpedo* AChR (~37%) only after solubilization, we were able to conclude that the inserted epitope is cytoplasmic in location, in keeping with the results of other investigators discussed earlier. All three hybrid AChRs containing the reporter epitopes at α347 responded functionally to applications of 100 μM ACh and showed little change in the normalized current evoked (~0.7–0.9 μA/fmol) from those containing native human α1 subunits (Table II). Both [125I]mAb 142 and [125I]mAb 236 showed no specific binding to any of the surface AChRs, in keeping with the expected cytoplasmic location of the site of insertion of these reporter epitopes.

Insertion of Reporter Epitopes at α429. A total of five constructs of the human α1 subunit with insertions at α 429 containing one or two copies of the reporter epitope for mAb 142 or one, two, or three copies of the reporter epitope for mAb 236 were coexpressed with *Torpedo* β1, γ, and δ subunits in *Xenopus* oocytes and tested in similar assays. mAb 142 bound the Triton X-100-solubilized AChRs containing a single copy of its epitope with a low relative efficacy of ~0.7%, while mAb 236 failed to bind AChRs containing a single copy of its epitope (Figure 1) . mAb 142 and mAb 236 bound AChRs containing two copies of their reporter epitopes with relative efficacies of ~25% and ~52%, respectively (Figure 2). AChRs containing three copies of the reporter epitope for mAb 236 were bound by mAb 236 with a relative efficacy of ~60%. Thus, it appeared that, as with the shorter minimal epitopes, the longer epitopes were not accessible to the mAbs, and addition of multiple copies appeared to increase the accessibility by the first copy serving as a spacer arm. All of the AChRs containing the reporter epitopes at α429 appeared to be functional, and all but one of the AChRs responded to application of 100 μM ACh with normalized currents of ~0.4–0.5 μA/fmol, which is about half of the ACh sensitivity observed in AChRs containing the native human α1 subunit (Table II). The hybrid AChR containing one copy of the long reporter epitope 142 showed a near-native normalized current of 0.91 μA/fmol. Both [125I]mAb 142 and [125I]mAb 236 showed no specific binding to surface AChRs containing single reporter epitopes at position α429 on intact oocytes. However, binding of the iodinated mAbs to the surface AChRs containing two copies of their respective epitopes was very evident with an average of ~4 fmol of AChRs detected per oocyte using either [125I]mAb 142 or [125I]mAb 236. Binding of [125I]mAb 236 to surface AChRs containing three copies of its reporter epitope was only marginally better, detecting ~5 fmol of AChR per oocyte (Figure 2). Thus, as with the Triton X-100-solubilized AChRs, the failure of the mAbs to bind the AChRs containing the single reporter epitopes at position α429 appeared to be due to their inaccessibility, probably as a result of their close proximity to the lipid bilayer. Thus, we conclude that the C terminus of the human α1 subunit is located on the extracellular side of the membrane.

DISCUSSION

One of the approaches used to delineate the transmembrane topology of specific sequences of integral membrane proteins has been to map the binding site of mAbs raised against the corresponding synthetic peptides. Unfortunately, this approach has proven to be both costly and time-consuming due to the effort involved in making antibodies and because the mAbs thus produced often did not bind those sequences in their native conformation. Another interesting approach to study subunit topology of single subunit membrane bound bacterial proteins has been to engineer the genes for enzymes such as alkaline phosphatase (Boyd et al., 1987; Manoil & Beckwith, 1986), β-galactosidase (Froshauer et al., 1988), or β-lactamase (Forst et al., 1987) at various locations within the protein and use the sideness of the enzymatic activity to infer orientation of the insertion site. In these studies, however, no attempt was made to retain the function of the chimeric protein, as the inserted gene was quite large. In multisubunit integral membrane proteins, such large insertions are likely to disrupt subunit assembly.

In this paper we describe a reporter epitope technique that allows one to determine the transmembrane topology of an integral membrane protein whose cDNA has been cloned. This technique uses well-characterized high-affinity mAbs as reporter mAbs and their corresponding epitopes as reporter epitopes, to probe the topology at the site of insertion, thus eliminating the need to raise new antibodies. Ideally the sites of insertion would not be in sequences suspected of forming parts of active sites or transmembrane domains but instead in hydrophilic regions that are likely to be on the protein surface. The experiments use low concentrations of reporter mAbs (~10–20 nM) to help ensure specific binding. Other artifacts observed in vesicle preparations, due to a small fraction of leaky or wrong-sided vesicles, are also eliminated as one can determine the intactness of the outer membrane by simple microscopic examination of individual oocytes. In addition, one can also use the membrane resting potential measured electrophysiologically to gauge the "leakiness" of the oocyte after a binding assay. We find that the signal from the binding of [125I]mAbs to their reporter epitopes expressed in a single oocyte is often 10–100-fold higher than the nonspecific binding to noninjected oocytes, and this unequivocally allows one to designate a transmembrane location for the expressed epitope when located on the extracellular surface. Engineered epitopes that are intracellular should be easily localized using electron microscopy in conjunction with colloidal gold-conjugated mAbs to differentially label intracellular and extracellular epitopes of AChR on the surface membrane.

In designing the site of epitope insertion, we took advantage of pre-existing restriction enzyme sites within the AChR α1 subunit cDNA to ligate in oligonucleotides encoding the reporter epitopes, but with a little more effort unique restriction sites can be created at the desired site of insertion. Ideally such a site would correspond to a restriction enzyme recognition

sequence that would create a blunt end at the end of a codon such that by simple blunt-end ligation of double-stranded oligonucleotides corresponding to one copy of the reporter epitope (thus eliminating the need to synthesize both long and different oligonucleotides), one can get both single and multiple tandem insertions of the epitope by varying the ratio of the plasmid vector to oligonucleotide insert during the ligation. We have had only limited success using the PCR-based method described by Kammann et al. (1989) to engineer the epitopes into the human α1 cDNA, because the long primers needed to encode the entire epitope (whose sequence is also highly restricted to the site of insertion), formed either primer–dimers, or hairpin structures, yielding no final product.

The muscle-type AChRs examined in this study consist of four homologous subunits that are assembled in a circular array, probably in the order $\alpha 1\gamma\alpha 1\beta 1\delta$, to form an ACh-gated cationic channel [reviewed in Karlin (1991); Changeux, 1990]. Hydropathic analysis of the deduced amino acid sequence of the subunits predicts four putative hydrophobic, α-helical, transmembrane domains termed M1–M4 (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983). On the basis of this analysis, the most widely accepted model has the amino terminus on the extracellular side of the membrane, followed by M1–M3, a large cytoplasmic domain, M4, and a short extracellular carboxy-terminal sequence. However, experimental verification of this model has proven to be controversial.

The amino-terminal sequence of α1 subunits up to M1 (α1–α210) is thought to be extracellular because it contains the main immunogenic region at α68–71, the N-glycosylation site at α141, and the ACh and neurotoxin binding sites near α192, 193 [reviewed in Karlin (1991); Changeux, 1990]. However, a mAb raised against the synthetic peptide α152–167, when used in solid-phase assays, appears to bind to AChRs in *Torpedo* vesicles only after permeabilization of the membrane (Criado et al., 1985). It is apparent now that, unlike the reporter epitope method described here, such solid-phase assays may yield false results if a minority of the vesicles are disrupted or a small minority of the AChRs are denatured in a way that permits binding of the mAb. Immunoelectron microscopy experiments indicate that other mAbs specific for the region α156–179 also appear to bind to the cytoplasmic side of the membrane (Pederson et al., 1990). However, such experiments might also be subject to error due to nonspecific binding of mAbs when used at concentrations vastly higher than that at which their antigenic specificity was determined. Nonetheless, the results from both of these experiments favor a model in which two transmembrane domains exist between α141 and α192. More recently, however, support for the conventional model came from two separate sets of experiments. In the first, α1 subunits containing newly introduced N-glycosylation sites at α154 and α200, but not at the native site (α141), are glycosylated at the new sites in *in vitro* translation systems, as a result of their transmembrane translocation (Chavez & Hall, 1991). In the second, α1 subunit fusion proteins terminating with the carboxy-terminal fragment of prolactin (a presumably topogenically neutral marker) after M1, M2, M3, or M4, were synthesized in an *in vitro* translation system supplemented with pancreatic microsomes. The prolactin marker was then deemed extracellular if detection of the marker by western blots was eliminated by addition of proteases to the outside of the vesicles or intracellular if proteolysis of the marker occurred only after detergent solubilization of the vesicles (Chavez & Hall, 1992). These two methods examine subunits synthesized *in vitro* which are incapable of maturation or assembly with other subunits and thus would be blind to insertion of subunit domains into the membrane favored as a result of subunit-subunit interaction occurring during assembly. Furthermore, these methods also fail to address the effect of the modification of the subunits on the function of fully assembled AChRs. However, the results obtained are consistent with those predicted by hydropathic analysis of the subunit sequence.

Recent data bring into question the idea that all of the proposed transmembrane sequences M1–M4 are α helical. Electron microscopic analysis of tubular crystals of *Torpedo* AChRs at 9-Å resolution reveal only a single transmembrane α helix in each subunit rather than the four proposed by the conventional model (Unwin, 1993). In addition, Akabas et al. (1992) have employed a combination of mutagenesis and covalent modification experiments which suggest that amino acids in M2 which line the cation channel are probably in a β strand conformation rather than in an α helix.

There is good agreement between theory and immunological evidence that the domain between M3 and M4 is exposed to the cytoplasmic surface. In α1, M3 ends at α298 and the putative large cytoplasmic domain extends to the beginning of M4 at α408; within this sequence we have mapped five epitopes extending between α339 and α378 to mAbs which have been shown by electron microscopy of colloidal gold-labeled mAbs to bind to the cytoplasmic surface of electric organ membranes (Ratnam et al., 1986). Most other data are also consistent with the idea of a large cytoplasmic domain within this region [Young et al., 1985; Chavez et al., 1992; LaRochelle et al., 1985; Lei et al., 1992; but not Moore et al. (1989)].

There are conflicting reports on the location of the carboxy terminus. Antibody binding experiments of the type subject to false positives, as discussed earlier, initially suggested a cytoplasmic location for the carboxy terminus (Ratnam & Lindstrom, 1984; Young et al., 1985). However, more recent carefully controlled experiments testing accessibility of membrane-impermeable reducing agents to the penultimate cysteine residue of the carboxy terminus of the *Torpedo* AChR δ subunit indicate that it is located extracellularly (DiPaola et al., 1989; McCrea et al., 1987).

Using the reporter epitope approach, we have provided definitive topographic data for two regions of the α1 subunits and presumably for corresponding regions of homologous subunits. We have shown that the carboxy terminus of the AChR α1 subunit has an extracellular transmembrane location in keeping with the results obtained for the δ subunit by DiPaola et al. (1989) and McCrea et al. (1987). Similarly, we have shown that the location of the sequence around α347 in the large cytoplasmic domain between M3 and M4 is consistent with the expectations of the conventional model and the results of Ratnam et al. (1986) and Sargent et al. (1984), though these results are in conflict with those of Moore et al. (1989). A more controversial sequence around α160, however, remains refractory to analysis because the longer epitope for mAb 142 inserted at α157 completely obliterates subunit assembly, while the longer epitope for mAb 236, though tolerated, remains inaccessible to [^{125}I]mAb 236 on the surface of oocytes and poorly accessible even after solubilization with Triton X-100.

The criterion we established to designate a region as cytoplasmic or intracellular on the basis of reporter mAb binding to the engineered reporter epitope after solubilization of the AChRs with Triton X-100 was that a very large fraction of these AChRs should be bound by the reporter mAb. Since this criterion was satisfied with at least some of the constructs containing multiple reporter epitopes at α347, an unambiguous designation of the cytoplasmic location of this region could

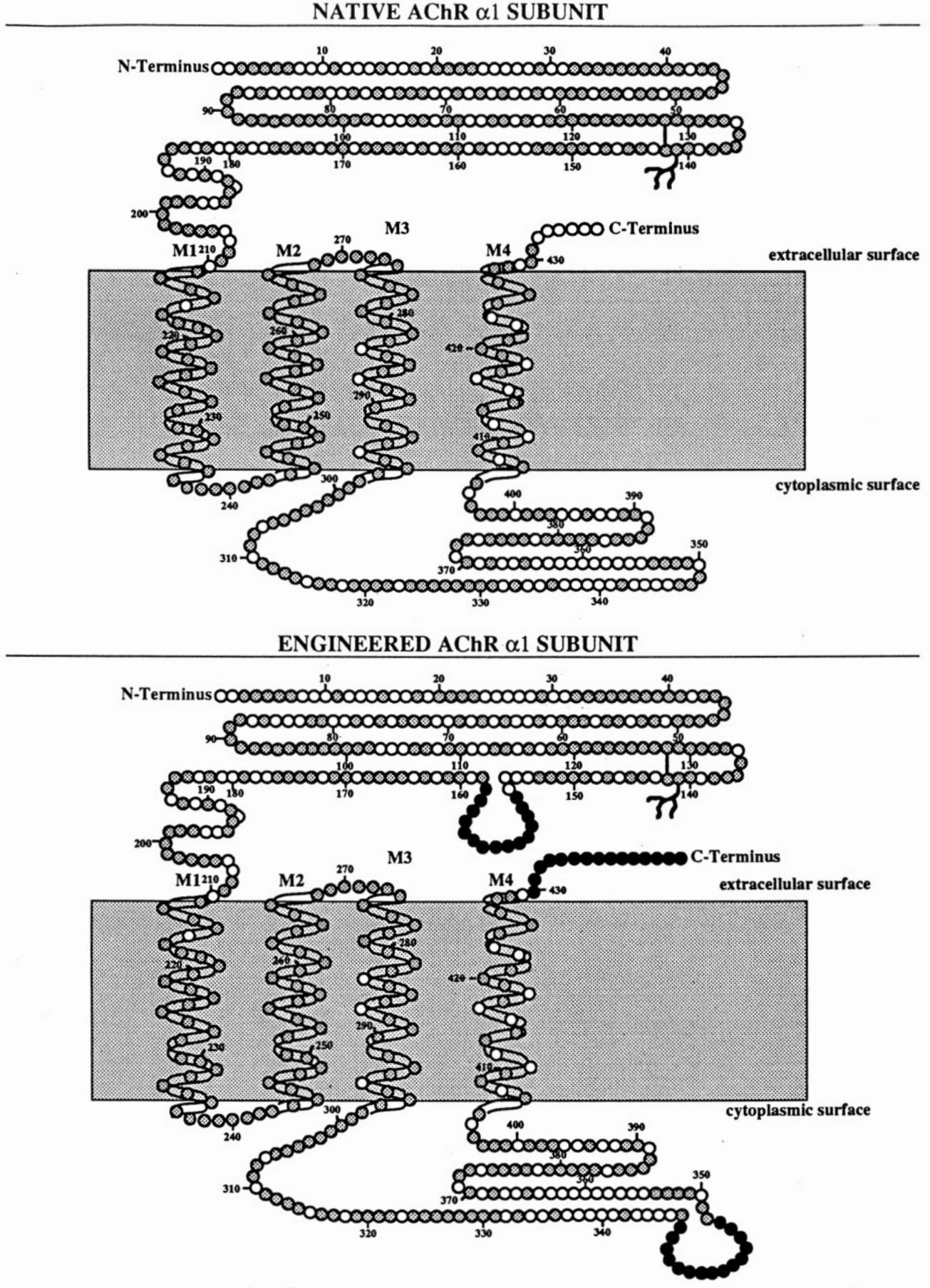

FIGURE 3: Transmembrane orientation of the α1 subunit polypeptide chain. The top panel shows a schematic of a generic α1 subunit transmembrane orientation. The white residues are variable between species (human, mouse, calf, *Xenopus*, and *Torpedo*), and the gray residues are invariant (Luther et al., 1989). The bottom panel shows the results obtained by the reporter epitope technique and is consistent with the conventional model, with the possible exception of α157, as discussed in the text. For illustration purposes, only insertions of one copy of the long reporter epitope 236 at positions α157, α347, and α429 are shown as black residues in the model.

be made. However, since the reporter epitope at α157 was detected in only one construct and that too in only a small fraction of the Triton X-100-solubilized AChRs, we have been cautious in interpreting this result to be indicative of the cytoplasmic location of this region. Because this region of the α1 subunit has proven to be sensitive to the insertion of epitopes, we are separately investigating the possibility that these reporter epitopes may be better tolerated in other homologous subunits wherein the amino acid sequence around this region is more hydrophilic and thus perhaps more tolerant to the insertion of a foreign sequence.

It is instructive to consider which sequences of the α1 subunit proved to be amenable to probing by the reporter epitope technique. Figure 3 (top panel) shows a schematic for a generic muscle α1 subunit. The white residues are variable between α1 subunits of different species and reflect regions expected to be more tolerant of variation and hence insertions of epitopes, while the gray residues reflect invariant residues between species thus expected to be less tolerant of insertions of epitopes (Luther et al., 1989). This appears to be the case; reporter epitopes were well tolerated at α347, α355, α429, and α437, in regions that were primarily variable, but not as well tolerated at α157 and α162, in a region that is primarily conserved. Also, epitope insertions at α157 and α162 were tolerated only when the surrounding sequence could occlude the epitope and thus allow efficient assembly and function. Thus, the sequence around α157–162 may be buried beneath the surface of the protein or strongly conformationally constrained. This appears to be one of the limitations of this technique: insertion of certain reporter epitope sequences at some sites could cause gross perturbation of expression, assembly, or insertion of the protein into the membrane. Figure 3 (bottom panel) shows a schematic diagram summarizing the results of our analysis of the transmembrane topology of human α1 subunit. The results appear to be consistent with the conventional model for the AChR subunits with the possible exception of the insertions at α157. Most of the hybrid AChRs used to study the AChR α1 subunit topology were functionally assayed by monitoring the currents evoked by application of ACh.

The reporter epitope technique should prove to be useful in the topological analysis of the subunits of the other members of the ligand-gated ion channel gene superfamily, such as the glutamate receptors, where the putative transmembrane domains assigned on the basis of hydropathic analysis of the amino acid sequence of the subunits conflict with functional analysis of variants derived as a result of RNA editing [see Gasic and Hollmann (1992) for a review].

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