Determination of amino acids critical to the main immunogenic region of intact acetylcholine receptors by in vitro mutagenesis

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The main immunogenic region (MIR) of the acetylcholine receptor (AChR) is the target for the majority of high-affinity autoantibodies produced in myasthenia gravis patients. Some monoclonal antibodies (mAbs) to the MIR bind specifically, but with low affinity, to synthetic AChR α subunit peptides with the sequence α67–76. Studies of synthetic peptides suggest that amino acids α68 and α71 may be especially important to the antigenic structure of the MIR. We have studied the contribution of amino acids α68 and α71 to the antigenicity of the MIR on intact AChR by replacing α68 (N) and α71 (D) of *Torpedo* AChR α with α68 (D) and α71 (K) by site-directed mutagenesis, expressing the mutated transcripts in *Xenopus* oocytes along with wild-type *Torpedo* β, γ and δ subunits, and analyzing the expressed AChR for the binding of mAbs to the MIR. These mutations of the MIR greatly diminished binding of mAbs to the MIR. Thus, both α68 and α71 are critical to the antigenicity of the MIR in intact AChRs.

Acetylcholine receptor; Myasthenia gravis; Immunodominant region; Mutagenesis, in vitro; Subunit expression; *Xenopus* oocyte

1. INTRODUCTION

Nicotinic acetylcholine receptors (AChRs) from muscle and *Torpedo* electric organ are composed of 4 different subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ [1]. In the human neuromuscular disease myasthenia gravis (MG), autoantibodies are directed against AChRs and cause accelerated destruction and functional blockage of AChRs which causes failure of neuromuscular transmission and muscular weakness (reviewed in [2]). A similar autoimmune disease called experimental autoimmune MG (EAMG) can be induced in animals that are immunized with purified AChR [3]. More than 60% of the antibodies in sera of MG patients and rats with EAMG are directed at the main immunogenic region (MIR) on the AChR α subunit [4–6]. When injected into rats, monoclonal antibodies (mAbs) to the MIR induce an EAMG response, and when added to cultured muscle cells they cause loss of AChRs [7,8], further showing the importance of this region in the pathogenesis of MG.

Electron microscopy studies showed that the MIR is located on the extracellular surface of α subunits of the intact AChR [9]. Some mAbs to the MIR bind with low affinity but great specificity to synthetic peptides with the sequence α66–76 on *Torpedo* α subunits [10–16]. *Xenopus* muscle AChR is unique among muscle-type AChRs tested in not reacting with anti-MIR mAbs [17]. In *Xenopus* AChR α subunits [18] residues α68 (aspartic acid, D) and 71 (lysine, K) are quite different from the amino acids α68 (asparagine, N) and 71 (aspartic acid, D) in *Torpedo* AChR α subunits [19]. Synthetic peptides corresponding to *Xenopus* α66–76 did not bind mAbs to the MIR, whereas the corresponding *Torpedo* sequence did [14]. Asparagine α68 is critical to mAb binding to the peptides because the human sequence α68–76 binds MIR mAbs, but α69–76 does not [14]. Residues α68 and α71, among others, were also found to be important for binding of MIR mAbs when amino acid residues in synthetic peptides corresponding to human α67–76 were sequentially replaced by glycine and tested for antigenicity of the MIR [13].

The MIR epitope is highly conformation dependent [4,20]. mAbs to the MIR bind to synthetic peptides with much lower affinity than to native AChRs, and in some cases they do not bind at all to denatured subunits or synthetic peptides [4,14]. Synthetic peptides do not reflect the conformation seen in the native AChR. Therefore, it is uncertain to what degree the detailed binding characteristics of mAbs to synthetic peptides of MIR reflect the real binding characteristics of mAbs to native AChRs. To begin determining which amino acids are important to the antigenic structure of the MIR in vivo, in the present study we replaced α68 and α71 amino acid residues of *Torpedo* AChR α subunits with the corresponding residues of *Xenopus* α subunit by in vitro mutagenesis. Analysis of the mutated AChR subunit expressed in *Xenopus* oocytes along with *Torpedo* β, γ and δ subunits showed that changing either α68 or α71 or both amino acids of *Torpedo* α subunits to the corresponding *Xenopus* amino acids eliminates binding of mAbs to the MIR in the expressed AChRs. Because the mutation inhibited binding of

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even mAbs that depend strongly on the native conformation of the MIR and do not bind to synthetic peptides, these results show that the MIR is a compact region in the intact α subunit that depends strongly on residues α68 and α71 for its antigenicity.

2. MATERIALS AND METHODS

2.1. *General methods and supplies*

The characterization of the mAbs has previously been described [4,6,10,21]. Radioiodinations were done by the chloramine-T method as described by Lindstrom et al. [22]. The *Torpedo* AChR subunits cloned under the control of the SP6 promoter in SP64T vector [23] (SP64T α, β, γ, δ) were a generous gift from Dr Toni Claudio (Yale University). All chemicals were obtained from Sigma (St. Louis, MO), and frogs were obtained from NASCO (Fort Atkinson, WI).

2.2. *In vitro mutagenesis*

An in vitro mutagenesis technique, slightly modified from that of Kammann et al. [24], using the polymerase chain reaction (PCR) was used to generate the mutants using the SP64T α clone as a template (as shown in Fig. 1). Two successive PCR steps were used (Fig. 1). In the first step the sequence between the 'mutagenic primer' (mut) and the 'SP6 primer' (SP6) was amplified. The mutagenic primer consisted of the mismatched nucleotide(s) at the MIR flanked by 10 nucleotides complementary to the *Torpedo* α sequence. The SP6 primer consisted of the sequence complementary to the SP6 promoter which is located 130 bp 5′ of the AChR α coding sequence on SP64T α. In the second PCR, the amplified fragment was used as a primer in combination with primer 3 (P3). P3 consisted of 20 bases complementary to the *Torpedo* α sequence located between 373 and 393 bases 3′ to the MIR. Thus, the product of the second PCR was the amplified sequence between the SP6 primer and P3, and it contained the desired mutation(s). This amplified product was digested with *Hin*dIII and *Dra*III restriction enzymes, which cut the SP64T α clone on opposite sides of the MIR, with *Hin*dIII on the 5′ side and *Dra*III on the 3′ side. The digested fragment was then used to replace the unmutated *Hin*dIII/*Dra*III fragment of SP64T α sequence. The nucleotide sequence between the *Hin*dIII and *Dra*III restriction sites of the mutant clones was determined using the dideoxy method to confirm the desired base change and also to make sure that no anomalies in the sequence had been generated by PCR.

2.3. *Expression of AChR in oocytes*

An in vitro transcription system using the SP6 RNA polymerase [25] was used to generate mRNA transcripts from *Torpedo* AChR subunit clones (SP64T α, β, γ, and δ). Oocytes were prepared for microinjection as described by Colman [26], injected with ~1.6 ng RNA of each subunit, and incubated at 19°C for 2–3 days for AChR expression. The number of AChRs capable of binding [^{125}I]α-bungarotoxin (αBgt) on the surface of the oocyte was measured by incubating the oocytes individually with 200 μl of 2 nM [^{125}I]αBgt in Barth's solution [26] for 2 h at 19°C, washing the oocytes 4 times in 3 ml of Barth's solution, and measuring the radioactivity by γ counting. Oocyte lysates were prepared by homogenizing the oocytes in lysis buffer (50 mM Tris, 100 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 1.5% Triton X-100, 0.05% SDS, pH 7.5), incubating the homogenate at 4°C for 30 min, and clearing debris by centrifugation in a microfuge at 4°C for 30 min. Radioimmunoassay (RIA) was used to test for mAb binding. Oocyte lysates (containing 10–20 fmol of αBgt binding sites) were incubated at 4°C overnight with 2 nM [^{125}I]αBgt, 0.5 μM of test mAb, and 5 μl normal rat serum in 100 μl final volume of lysis buffer. Following a 2 h incubation at 4°C with 100 μl goat anti-rat IgG, the sample was diluted with 1 ml of phosphate-buffered saline containing 0.5% Triton X-100 (PBS-Triton), centrifuged, the pellet washed with 1 ml of PBS-Triton, and the radioactivity in the pellet measured by γ counting. For sucrose gradient analysis, 100 μl of the oocyte lysate (from 10 oocytes) was sedimented on a 5 ml 5–20% sucrose gradient at 65k rpm in a VTi 65.2 rotor for 70 min at 4°C, and fractions (140 μl) were collected on microtiter plates precoated with mAb 142. Following an overnight incubation at 4°C with 2 nM [^{125}I]αBgt in lysis buffer, the microwells were washed 4 times with 200 μl of PBS-Triton and radioactivity measured by γ counting. Precoating of microtiter plates was accomplished by applying 50 μl of 10 mM sodium carbonate buffer, pH 8.8, containing 40 μg/ml mAb 142 to each well, incubating the plates at 4°C overnight, and quenching with 200 μl of PBS containing 0.05% Tween-20 and 2% BSA for 2 h at room temperature

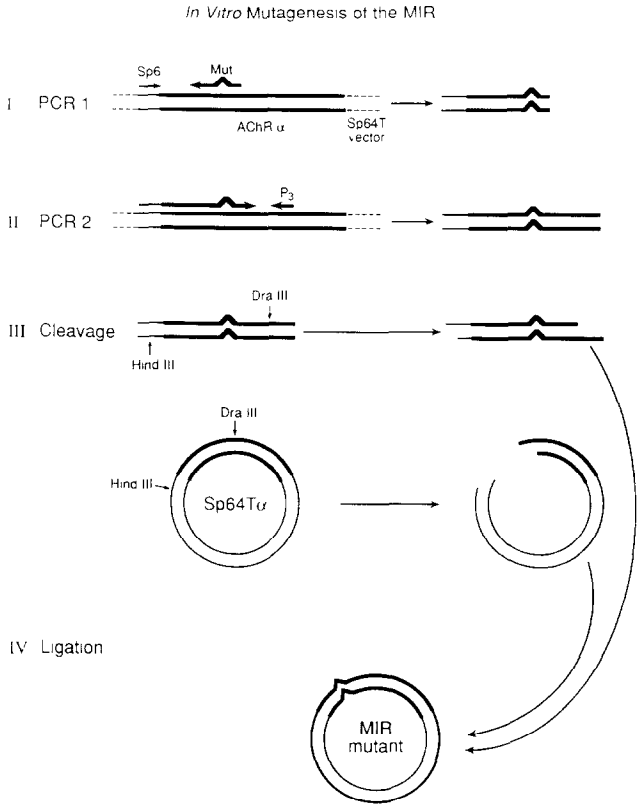

Fig. 1. In vitro mutagenesis of MIR.

3. RESULTS

3.1. *In vitro mutagenesis and the expression of MIR mutants*

Amino acids α68 (N,AAT) and α71 (D,GAT) of the *Torpedo* AChR α subunit were replaced individually (α68 or α71 mutants) or in tandem (α68 + 71 mutant) with the corresponding residues α68 (D,GAT) and α71 (K,AAG), of *Xenopus* α_1b subunit [27] using a PCR mutagenesis technique. mRNA transcripts were prepared from the mutant subunits in vitro and injected into *Xenopus* oocytes in combination with transcripts of *Torpedo* wild-type β, γ, and δ AChR subunits. To assess the expression of the α subunit mutants, oocyte lysates were prepared and were subjected to RIA in the presence of [^{125}I]αBgt and mAb 142. mAb 142 binds to the cytoplasmic region of the *Torpedo* α subunit away from the MIR [28]. Results showed that about 9 fmol/oocyte of AChR were present in the oocytes

that were injected with the α68, α71 or α68+71 mutants, in combination with β, γ, and δ subunits, as compared to ~13 fmol/oocyte in oocytes that were injected with wild-type *Torpedo* subunits. The mutant AChRs were then analyzed for their insertion into the plasma membrane by testing for [^{125}I]αBgt binding to the oocyte surface. Results showed that about 1.3 fmol of αBgt binding sites were present on the surface of each oocyte that was injected with α68, α71 or α68+71 mutants, in combination with the wild-type *Torpedo* β, γ, and δ subunits, as compared to ~2 fmol of [^{125}I]αBgt binding sites on the oocytes that were injected with the wild-type AChR α subunit. These results showed that mutation in α68 and/or α71 did not prevent the assembly of the mutant α subunits with native β, γ, and δ subunits, or prevent the insertion of the mutant AChRs in the surface membrane.

To assess the proper association of the mutant α subunits with the *Torpedo* β, γ and δ subunits, lysates from oocytes injected with the α68+71, β, γ, δ combinations and those injected with wild-type α, β, γ, δ combinations were subjected to sucrose gradient sedimentation and the fractions were tested for [^{125}I]αBgt binding (Fig. 2). Results showed that both the lysates from oocytes expressing the mutant AChR and the lysates expressing the wild-type AChR had the same gradient profile. Two toxin-binding peaks were present in each sample, a peak at position for AChR monomer (~9 S) and a peak at position for synthetic intermediates probably corresponding to combinations of α with γ and/or δ subunits (M. Saedi, W.G. Conroy and J. Lindstrom, unpublished).

3.2. *Binding of MIR-specific mAbs to mutant AChRs*

Having established that mutations in *Torpedo* α subunit at α68, α71, or α68+71 do not greatly affect the association of the α subunit with β, γ and δ subunits or the insertion of the mutant AChRs into the surface membrane of *Xenopus* oocytes, we tested the effect of these mutations on the binding of MIR-specific mAbs. Lysates of the oocytes expressing these mutant AChRs were subjected to RIA in the presence of [^{125}I]αBgt, using various mAbs. mAb 142, which binds to the cytoplasmic region of the α subunit at α359–366 ([28], M. Das and J. Lindstrom, unpublished) which is distant from the MIR, was used to quantitate the number of AChRs present in each lysate. Also, to eliminate any species-specific interactions that might exist between mAb and MIR mutants, the test mAbs were chosen from mAbs raised against different species. As seen in Fig. 3, mutations in α68, α71 or α68+71 eliminated the binding of MIR-specific mAbs raised against AChRs from human (mAb 198), rodent (mAb 210), *Torpedo* (mAb 6), and *Electrophorus* (mAbs 22 and 47). mAb 35 is frequently used as the archetypic MIR mAb [5,7]. mAb 35 does not bind to synthetic peptides [12,14]. In muscle cells, α subunits undergo a confor-

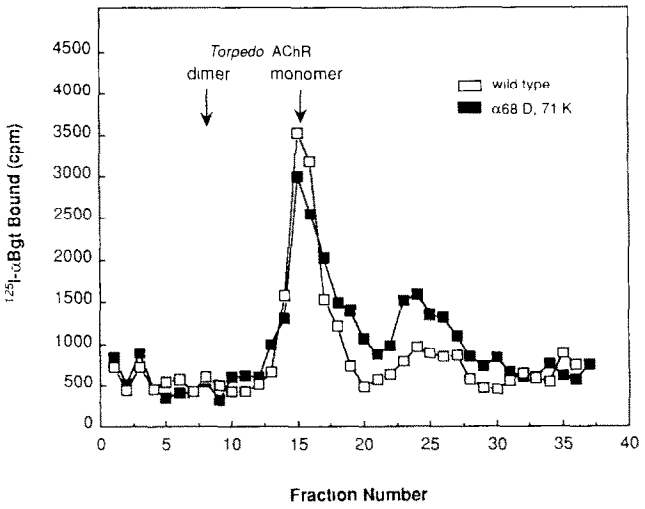

Fig. 2. Sucrose-gradient sedimentation analysis of mutant AChR. Lysates of oocytes injected with transcripts of the *Torpedo* AChR β, γ, and δ subunits in combination with the transcripts of the wild-type (□) or α68+71 mutant form (■) of *Torpedo* AChR α subunit were sedimented on 5–20% sucrose gradients and the fractions were tested for [^{125}I]αBgt binding as described in section 2. Each figure is the average of duplicate gradients. Fractions are numbered from the bottom of gradients. The positions of the monomer and the dimer forms of *Torpedo* AChR were determined from sucrose-gradient analysis of lysates of *Torpedo* electric organ.

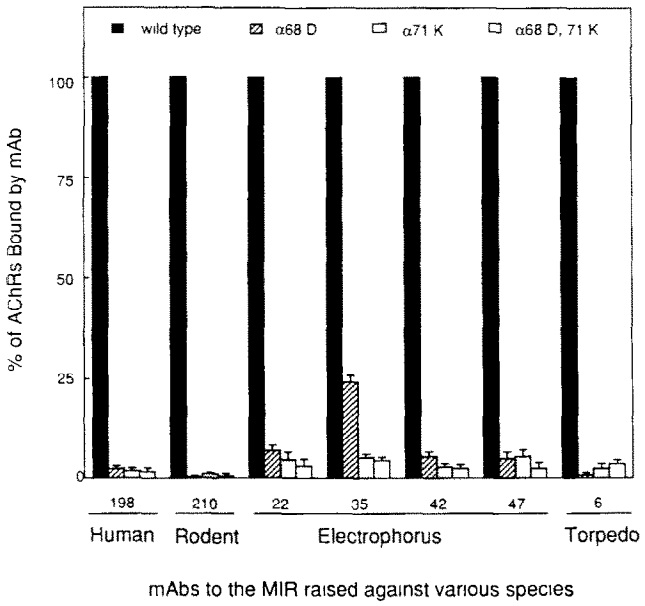

Fig. 3. Binding of anti-MIR mAbs to mutant AChRs. Binding of the indicated mAbs to the AChRs expressed in oocytes injected with wild-type or MIR mutants of *Torpedo* AChR α subunits in combination with the *Torpedo* β, γ, and δ subunits was measured by RIA. For each sample, results were first calculated as binding of test mAbs relative to the binding of mAb 142, and were then expressed as mean of triplicates ± SE relative to the binding of test mAbs to the wild-type sample (100%). The background, lysates of noninjected oocytes subjected to RIA, was subtracted from each sample before calculations.

mational maturation after synthesis and before assembly with other subunits, during which they acquire the ability to bind mAb 35 with high affinity ([29], W.G. Conroy and J. Lindstrom, unpublished). Mutation of α68 to the *Xenopus* residue reduced binding of mAb 35, and the corresponding mutations at α71 or α68+71 eliminated binding of mAb 35.

4. DISCUSSION

In the autoimmune disease MG, more than 60% of the autoantibodies are directed against the MIR of the AChR [4,5]. Therefore, detailed investigation of antigen-antibody interactions at this region is critical for the understanding of the pathogenesis of MG. Several laboratories, including ours, have used synthetic or bacterially expressed peptides to monitor the critical amino acid residues involved in the binding of MIR-specific mAbs to this region [10–16,30]. These in vitro studies, however, cannot accurately reflect the interactions that exist between the antigens and antibodies in the native molecule, thereby necessitating the investigations of mAbs with the MIR on intact AChRs.

To accomplish this we chose to mutagenize the *Torpedo* α subunit and study the expression of the mutant α subunits in *Xenopus* oocytes. Results presented here show that converting α68 (N) or α71 (D) amino acids to their respective residues in *Xenopus*, α68 (D) and α71 (K), inhibited the binding of the MIR-specific mAbs while not greatly affecting the expression of AChRs containing the mutant α subunit. Sucrose gradient analysis showed that the same size αBgt-binding molecules are synthesized in oocytes injected with α68+71, β, γ, δ combinations as oocytes injected with the wild-type subunits, indicating that the association of the α subunit with β, γ, and δ subunits is not greatly affected by these mutations. This indicates that normal expression, αBgt binding, and subunit assembly of *Torpedo* AChR are not absolutely dependent on the native conformation of the MIR.

Binding studies using MIR-specific mAbs showed that α68 and α71 were critical for binding of all mAbs to the MIR tested. This correlates well with the finding that binding of these mAbs to synthetic peptides containing the α67–76 sequence is inhibited when α68 or α71 is substituted by glycine [13]. Results also showed that mutations in either α68 or α71 inhibited the binding of anti-*Electrophorus* mAbs 22 and 47. This is particularly interesting since glycine substitution for α68 or α71 in human α67–76 synthetic peptide did not have any effect on the binding of mAbs 22 or 47 to the oligopeptide. We cannot explain this apparent discrepancy, but it suggests that binding of mAbs 22 and 47 to native AChR α is dependent on the conformation of the MIR and that interactions of mAbs with synthetic peptides do not always reflect in detail their interactions with native AChRs.

The importance of MIR conformation in mAb binding is evident from mAb 35 binding studies. mAb 35 binding is highly conformation dependent and it does not bind to synthetic peptides [12,14,31]. Our results show that converting α68 (N) to α68 (D) reduces the binding of this antibody to AChR and that converting α71 (D) to α71 (K) eliminates it. This suggests that mAb 35 binding is less dependent on the individual amino acid residues within the MIR, but more dependent on the overall conformation of this region. Thus, a more drastic change in the charge of the amino acid at α71, substituting K (basic) for D (acidic), affects the conformation of the MIR, and thereby mAb 35 binding, more drastically than a more subtle change in the charge of the amino acid at α68, substituting D (acidic) for N (neutral). This finding also suggests that in cases of other anti-MIR mAbs, the binding depends more on the nature of the amino acid at α68 than on the overall conformation of the MIR.

Studies of the crystal structures of complexes of antibodies with lysozyme reveal that many (~18) amino acids of the antigen may be in contact with the antibody binding site [32], but that many of these interactions are permissive and that alteration of a single, critical amino acid can greatly alter the affinity of the antibody for the protein [33]. Our results are consistent with these observations. Our results also suggest that the MIR is not a series of closely spaced epitopes that allow competitive binding of mAbs because each occludes a large area of AChR (perhaps 35 Å in diameter [4,33]), but rather that the MIR is a single, compact epitope [31] in which amino acids α68 and α71 are critically important to the interaction of this structure with many mAbs which may bind to the MIR in slightly different ways.

Further studies using similar mutagenesis and expression of human AChR α subunits [34,35] may permit better definition of the specificities of autoantibodies to AChRs from MG patients.

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