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Design, synthesis, and characterization of a 39 amino acid peptide mimic of the main immunogenic region of the *Torpedo* acetylcholine receptor☆

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ABSTRACT

We have designed a 39 amino acid peptide mimic of the conformation-dependent main immunogenic region (MIR) of the *Torpedo* acetylcholine receptor (*T*AChR) that joins three discontinuous segments of the *Torpedo* α-subunit, α(1–12), α(65–79), and α(110 – 115) with two GS linkers:

1	2	3	4	5	6	7	8	9	10	11	12		65	66	67	68	69	70	71	72	73	74	75	76	77	78	79		110 – 115
S	E	H	E	T	R	L	V	A	N	L	L	**GGGS**	L	R	W	N	P	A	D	Y	G	G	I	K	K	I	R	**GS**	L D Y T G K

This 39MIR-mimic was expressed in *E. coli* as a fusion protein with an intein–chitin-binding domain (IChBD) to permit affinity collection on chitin beads. Six MIR-directed monoclonal antibodies (mAbs) bind to this complex and five agonist/antagonist site directed mAbs do not. The complex of MIR-directed mAb-132A with 39MIR has a K_d of $(2.11 \pm 0.11) \times 10^{-10}$ M, which is smaller than $(7.13 \pm 1.20) \times 10^{-10}$ M for the complex of mAb-132A with α(1–161) and about the same as 3.4×10^{-10} M for that of mAb-132A with *T*AChR. Additionally, the 39MIR-IChBD adsorbs all MIR-directed antibodies (Abs) from an experimental autoimmune myasthenia gravis (EAMG) rat serum. Hence, the 39MIR-mimic has the potential to inactivate or remove pathogenic *Torpedo* MIR-directed Abs from EAMG sera and to direct a magic bullet to the memory B-cells that produce those pathogenic Abs. The hope is to use this as a guide to produce a mimic of the human MIR on the way to an antigen specific therapeutic agent to treat MG.

1. Introduction

Human autoimmune myasthenia gravis (MG) develops from a B-cell directed T-cell regulated production of pathogenic antibodies (Abs) directed to protein components of neuromuscular junctions. In most cases the target of the Abs is the nicotinic acetylcholine receptor (AChR). The binding of anti-AChR Abs to the AChR initiates the immune system destruction of the post-synaptic membrane folded architecture and concomitant loss of receptor density (Lindstrom, 2000; Richman et al., 1993). Greater than 50% of the anti-AChR Abs in a myasthenic serum are blocked by a single monoclonal antibody (mAb) raised in rats to an electric organ AChR and directed to an epitope at the top of the AChR α subunit as part of a "main immunogenic region" (MIR) (Tzartos and Lindstrom, 1980; Tzartos et al., 1981, 1982; Beroukhim and Unwin, 1995).

In both *Torpedo* AChR (*T*AChR) and mammalian neuromuscular AChR, the two AChR α subunits each anchor an acetylcholine (ACh) binding domain and each possesses a MIR domain. The 2:1 stoichiometry of the MIR to AChR along with its peripheral location at the most extracellular reach of the nicotinic AChR α subunits certainly contribute to its prominence in eliciting an immune response. Another factor that weighs in on this susceptibility is the apparent lack of immune tolerance to this region compared, for example, to the β-hairpin loop, home to the cys 192–193 disulfide at the ACh binding site. This loop appears to be quite immune privileged in that mAbs that bind to the *Torpedo* loop do not

Abbreviations: ACh, acetylcholine; AChBP, acetylcholine binding protein; B-132A, biotinylated mAb 132A; cDNA, double stranded DNA complementary and identical sequences to mRNA; DAB, 3,3′ diaminobenzidine; EAMG, experimental autoimmune myasthenia gravis; EM, electron microscopy; GFP, green fluorescent protein; HRP, horseradish peroxidase; IChBD, intein chitin binding domain; IPTG, isopropylthiogalactopyranoside; mAb, monoclonal antibody; MCS, multiple cloning site; MG, myasthenia gravis; MIR, main immunogenic region; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; *T*AChR, *Torpedo* nicotinic acetylcholine receptor.

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cross-react with the rat loop, and attempts to raise mAbs in rats to this region of the rat receptor have been uniquely unsuccessful (Twaddle and Richman personal communication). An open question remains whether the MIR is accorded immune tolerance in most humans and is not immune privileged only in MG patients. This would argue that the essence of the disease perhaps concerns the proper education of the immune system. While most humans have correctly educated their immune systems so that the MIR is immune privileged, those unlucky individuals who develop the disease have had a failure to properly educate their immune systems. The alternative explanation for people without MG is that they have never encountered the trigger of the disease, which remains an enigma.

The MIR consists of a group of overlapping epitopes of mAbs raised in rats against the *Torpedo* and/or *Electrophorus* electric organ AChR (Conti-Tronconi et al., 1981; Papadouli et al., 1990; Tzartos et al., 1990). An initial clue about the MIR was provided by the ability of many of the initial mAbs produced in rats to mutually block the binding of one another to the eel/fish AChR (Tzartos and Lindstrom, 1980; Tzartos et al., 1981). The mutually blocking mAbs exhibit varying abilities to bind to the SDS denatured AChR α subunits, which gave rise to the notion that these mAbs bind to "conformational-dependent" epitopes available only in the native folded, assembled AChR. Likewise, the MIR must be "conformational-dependent." The stoichiometry of binding of this class of mAbs varied from one per receptor to two per receptor despite the ability of saturating concentrations of each to mutually inhibit the binding of the other (Fairclough et al., 1998a, 1998b; Morell et al., 2014). Some of the mAbs with overlapping epitopes cross react with the AChRs of many different species including humans (Gomez et al., 1981; Tzartos et al., 1982). Single MIR-directed mAbs injected into naïve rats induce generalized muscle fatigability in the short time span of 48 h rather than the two week induction period for experimental autoimmune myasthenia gravis (EAMG) generated by immunization of rats with *T*AChR(Gomez and Richman, 1985; Lindstrom et al., 1976; Tzartos et al., 1987). Based on electron micrographs of single chain Fv and/or Fab fragments of mAb 35 bound to 2-D crystalline arrays of *T*AChRs, the MIR is located on the top of the extracellular region of each of the two AChR α subunits (Fig. 1A) (Beroukhim and Unwin, 1995).

The sequence mapping of the MIR began with fusion protein technology fusing the cDNAs for small α subunit peptides to the cDNA of 52–85 amino acid fragments from the coding region of β-galactosidase in pUC8, which were then expressed in *E. coli* and checked for the ability of a MIR-directed mAb to bind to the construct. In this manner a core segment of the *mouse* MIR was identified to include α(61–80) (Barkas et al., 1988). In addition to the α(61–80) core, Morell (2006) found, using Western blots of the *Torpedo* α subunit N-terminal peptides fused to the green fluorescent protein, that α(1–12) and α(110–115) contribute to the ability of anti-MIR mAb 132A to bind to the peptide constructs in Western blots (Morell, 2006; Morell et al., 2014). Although, the presence of α(110–115) greatly improves mAb recognition, site-specific mutation of Y112 in α(110–115) did not significantly impair binding of mAb 132A, suggesting a supporting structural role for this segment (Morell et al., 2014). The present study also confirms the importance of α(110–115) in binding MIR directed mAbs. In another study, Luo et al. (2009), Luo and Lindstrom (2010) found that the homopentameric human α7 AChR and *Aplysia* AChBP, which do not bind anti-MIR mAb 35, become recognized by mAb 35 when human α1(2–14) and α1(60–81) are substituted for the corresponding stretches in those proteins, but not when just α1(66–76) is substituted (Luo and Lindstrom, 2010). These results emphasize the important contribution of the N-terminal α-helix to MIR as well as the conformational dependence of the MIR structure.

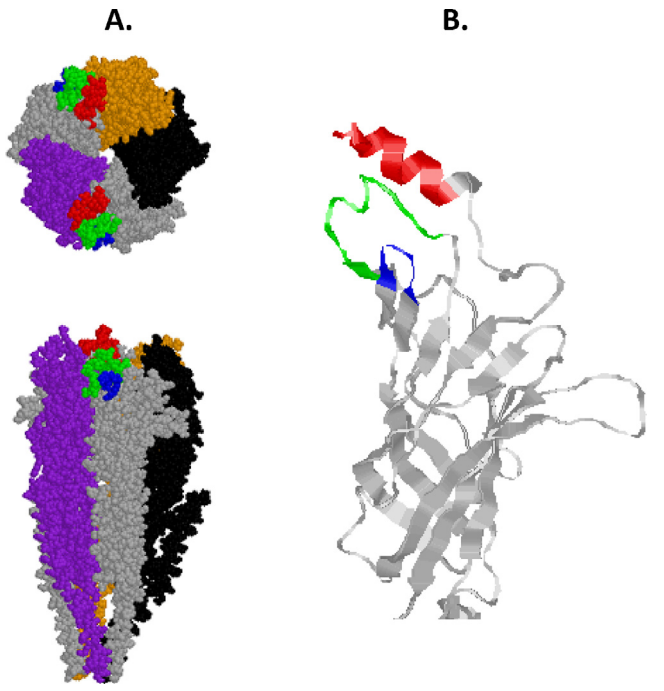

Fig. 1. *Torpedo* AChR structural model (Unwin, 2005). A. Top and side views of the *Torpedo* AChR in space filling format with subunits (grey-α, black-δ, orange-β, and purple-γ) and the MIR components [red, α(1–12); green, α(65–79); blue, α(110–115)] color coded. B. Side view of α-subunit extracellular domain with 2° structural elements displayed in cartoon format. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In Unwin's 4 Å resolution EM structure of the *T*AChR (Unwin, 2005) (Fig. 1), the red α(1–12) is part of the N-terminal α-helix, and it sits nestled next to the green α(65–79), the core segment of the MIR. In the *T*AChR structure, the segment, α(65–79), consists of a short helix, α(69–74), that lies between two coil/β-strand legs, α(65–68) and α(75–79). The α(65–79) segment is draped over the blue α(110–115), a β-turn (Fig. 1B) (Unwin, 2005). Thus α(1–12) and α(65–79) provide the AChR side chains recognized by mAb 132A while α(110–115) appears to serve as a structural anchor stabilizing the α(1–12)/α(65–79) complex (Morell, 2006).

Having mapped these components of the *T*AChR MIR, and recognizing the pathogenic potential of Abs-directed to this site in EAMG and human MG sera, we report in this paper the design, synthesis, and initial characterization of a 39 amino acid peptide that mimics the MIR and consists of the three non-contiguous segments of the *T*AChR: α(1–12), α(65–79), and α(110–115) joined by G/S linker stretches and fused to the amino terminus of an intein–chitin binding domain (IChBD). This MIR peptide mimic (39MIR-IChBD) selectively binds MIR-directed mAbs and such Abs in rat EAMG serum. Additionally, we find that mAb 132A, a MIR-directed mAb, has the same or smaller K_d for this peptide mimic than for either the assembled *T*AChR or the α(1–161) peptide. The prospect for using the immobilized human MIR mimic therapeutically to clear a major fraction of pathogenic Abs in an MG patient serum is considerably improved. In addition such constructs may be effectively used to clear an MG serum of just the anti-MIR component and the treated serum tested for pathogenicity, allowing us to address the question of whether the anti-MIR component is the major pathogenic component of MG serum. It is also evident that MIR mimics freed of the IChBD may neutralize anti-MIR Abs directly, and if conjugated to a magic bullet may eliminate the memory B-cells possessing the blueprint for production of these pathogenic Abs.