SHORT REPORT

Selective cleavage of AChR cRNAs harbouring mutations underlying the slow channel myasthenic syndrome by hammerhead ribozymes

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ABSTRACT

Slow channel congenital myasthenic syndrome (SCCMS) is a dominant disorder caused by missense mutations in muscle acetylcholine receptors (AChR). Expression from mutant alleles causes prolonged AChR ion-channel activations. This 'gain of function' results in excitotoxic damage due to excess entry of calcium ions that manifests as an endplate myopathy. The biology of SCCMS provides a model system to investigate the potential of catalytic nucleic acids for therapy in dominantly inherited disorders involving single missense mutations. Hammerhead ribozymes can catalytically cleave RNA transcripts in a sequence-specific manner. We designed hammerhead ribozymes to target transcripts from four SCCMS mutations, αT254I, αS226F, αS269I and εL221F. Ribozymes were incubated with cRNA transcripts encoding wild type and mutant AChR subunits. The ribozymes efficiently cleaved the mutant allele cRNA transcripts but left the wild type cRNA intact. Cleavage efficiency was optimised for αS226F. We were able to demonstrate robust catalytic activity under simulated physiological conditions and at high Ca^{2+} concentrations, which is likely to be accumulated at the endplate region of the SCCMS patient muscles. These results demonstrate the potential for gene therapy applications of ribozymes to specifically down-regulate expression of mutant alleles in dominantly inherited disorders.

KEYWORDS: *Slow channel myasthenic syndrome, hammerhead ribozymes, AChR, gene therapy, allele-specific mRNA cleavage*

INTRODUCTION

Congenital myasthenic syndromes (CMS) are inherited disorders of neuromuscular transmission that have the common feature of fatigable muscle weakness (Engel et al, 2003). Many are due to mutations in the genes encoding the muscle acetylcholine receptor (AChR). One form of these disorders is the dominantly inherited slow channel congenital myasthenic syndrome (SCCMS), which is due to missense mutations that result in kinetic abnormalities of the AChR (Croxen et al, 1997; Croxen et al, 2002). The 'naturally occurring' mutations that underlie the SCCMS provide a series of cases for which it is possible to determine a direct causal relationship between molecular dysfunction of the ion channel and disease phenotype. They provide an excellent model system for investigating approaches for gene therapy in dominant neurological disorders.

Molecular genetic studies have revealed that SCCMS is caused by heterozygous missense mutations located in any one of the four genes that make up the adult form of the AChR. To date, 18 different SCCMS mutations in the AChR genes have been reported (Ohno and Engel, 2004). In general, AChR harbouring SCCMS mutations shows little difference in the receptor expression levels compared with the wild type AChR, as measured by surface ^{125}I-α-bungarotoxin (α-BuTx) binding, but do show changes in the kinetics of the ion channel activations. Ultrastructural analysis of the endplates shows myopathy in the affected muscles, with decay of the synaptic structure and the build up of calcium deposits (Engel et al, 1982; Gomez et al,

2002). SCCMS patients are refractory to anticholinesterase drugs, which are often used in other forms of CMS, but may show some response to quinidine sulphate (Harper et al, 1998), or fluoxitine (Harper et al, 2003).

An alternative therapy might be obtained by limiting expression of AChR pentamers containing mutant subunits, while maintaining expression of wild type AChRs. SCCMS might be a particularly good disorder for this approach since reduction of AChR expressions to below 30% of normal is required before the safety margin for neuromuscular transmission is affected (Engel et al, 2003), and thus haploinsufficiency will not be an issue, and even a modest reduction of the mutant channels is likely to have a beneficial effect on disease pathology. A possible therapeutic approach is to inhibit mutant expression at the transcription level using hammerhead ribozymes that, for certain sequences, can cleave the mutant but not the wild type RNA transcripts at the site of the mutation.

Hammerhead ribozymes are the shortest discovered RNA molecules that have the ability to cleave RNA molecules in an enzymatic fashion. They were first discovered in plant viroids cleaving RNA *in-cis* (Forster and Symons, 1987) and then modified to cleave *in-trans* (Uhlenbeck, 1987), in principle allowing the targeting of any RNA molecule. They consist of a catalytic motif of 35 consensus nucleotides (helix II) flanked by two arms of a complementary sequence (helices I and III) that binds to the target RNA molecules on a Watson-Crick basis. In the presence of Mg^{2+}, the catalytic core is activated and RNA cleaved at specific sites. The catalytic core catalyses a transesterification, cutting the 3', 5'-phosphodiester bond after the trinucleotide consensus motif in the target molecule 5′-NUH-3′, where N = any nucleotide and H = C, U or A (Figure 1A). The specificity of the ribozyme thus originates from the engineered antisense arms, helices III and I. Ribozymes have been used as therapeutic tools targeting various pathogenic genes, including mouse caspase-7 involved in apoptosis-related disorders (Zhang et al, 2004), the untranslated 5' region (U5) of HIV-1 (Hotchkiss et al, 2004), and in attenuation of pancreas cancer telomerase in cell lines (Hayashidani et al, 2005). Their applications have been also extended to target central nervous system-related diseases (Wood et al, 2003), and they have the potential for allele-specific silencing (Figure 1B) in dominant neurological disorders. Here we investigate the ability of hammerhead ribozymes to selectively cleave mutant transcripts encoding four different mutations that we identified in patients with the dominant neuromuscular junction disorder, slow channel myasthenic syndrome.

MATERIALS AND METHODS

Preparation of cRNA target substrates

cDNAs encoding the human AChR α and ε subunits were subcloned into pcDNA3.1hygro (Invitrogen). Missense mutations that underlie slow channel congenital myasthenic syndromes were introduced by the Sculptor™ *in vitro* mutagenesis system (Amersham Biosciences). These were: αT254I, αS226F, αS269I and εL221F. Plasmids harbouring the mutant cDNA were checked by DNA sequencing. ^{32}P- labelled cRNA substrates containing the full coding sequence of mutant and wild type subunits were synthesised using the MEGAscript™ T7 *in vitro* transcription kit (Ambion). Short cDNAs (451 bp, 459 bp and 482 bp) for both mutant and the wild type α-subunit sequences and for the ε-subunit sequence (528 bp) were subcloned into the plasmid vector pGEM-4Z. The clones were confirmed by sequencing. cRNA of the α wild-type and the sequence harbouring the mutation were generated by T7 *in vitro* transcription.

Design of hammerhead ribozymes

Sequence analysis of the mutations revealed that αS226F, in which C is mutated to T, creates a ribozyme cleavage site UUC. A symmetric-armed ribozyme (length of helix III + length of helix I) (12+12) was designed for the αS226F mutation. In order to confirm the catalytic activity of the ribozyme, an inactive ribozyme was also designed in which the G at the 3' of the catalytic core nucleotides was mutated to C. The ribozymes were created by synthesis as standard oligonucleotides of both sense and antisense strands. Two restriction endonuclease sites, *Eco*RI at the 5' and *Hind*III at the 3' ends were included at the end of the oligonucleotides in order to orientate of the ribozyme sequence. The oligonucleotides were annealed in a high salt buffer and cloned into pGEM-4Z vector (Promega). Plasmid constructs were subjected to DNA sequencing to confirm the ribozyme sequence. The ribozymes were generated by *in vitro* transcription using the T7 promoter, according to the manufacture's manual (Ambion).

In vitro cleavage reactions

cRNA substrates were incubated with the ribozymes in 50 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, at 37°C for 4 hr, or under simulated physiological conditions (2 mM $MgCl_2$, 150 mM KCl, 50 mM Tris-HCl, pH 7.5, 37°C) (Santoro and Joyce, 1997). The reaction products were size fractionated either on 6% (w/v) acrylamide/TBE gels or on 3% (w/v) agarose/TBE gels. Gels were dried and exposed overnight to a phosphor storage screen (Fuji). The cleavage efficiency of labelled transcripts was quantified by measuring the band intensity of the cleaved products (P1 and P2) and the remaining substrate (S). The percentage cleavage (%C) was calculated using the equation %C = (P1+P2/ P1+P2+S) x 100 where P1 and P2 are the 5' and 3'products, S is the substrate, modified from (Uhlenbeck, 1987). The data presented are the means of at least three separate experiments unless otherwise stated.

RESULTS

Target sites

Mutations that underlie SCCMS were scanned to identify those which create hammerhead ribozyme cleavage sites and thus could be used as targets for allele-specific down-regulation of the mutant RNA transcripts. We targeted four different mutations: εL221F (Hatton et al, 2003), αT254I, αS269I (Croxen et al, 1997) and αS226F (Abdelgany et al, 2003). εL221F results from a C→T transition at nucleotide position 661 in the human muscle AChR ε-subunit gene and creates a hammerhead ribozyme cleavage site UUC. αT254I results from mutation α761C→T creating the cleavage site AUU. αS269I results

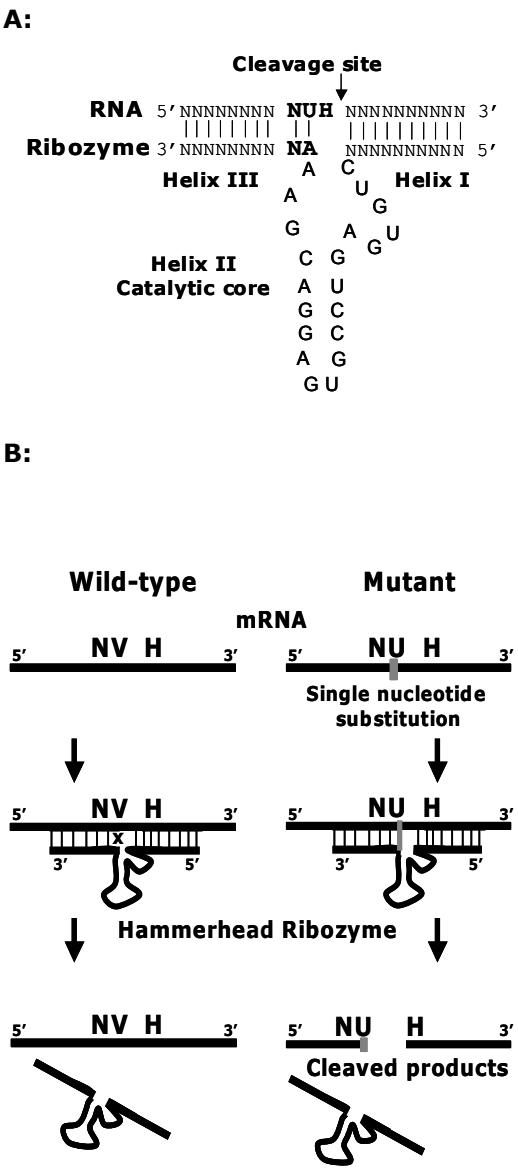

Figure 1. (A) Diagram of hammerhead ribozyme bound to substrate RNA. The substrate is bound to the enzyme portion of the ribozyme by the Watson-Crick base pairing of helices III and I. In the presence of divalent cations, the catalytic domain can then cleave the substrate 3′ of the trinucleotide cleavage sequence (arrow). The hammerhead motif can be separated into a 22-nucleotide conserved domain (Helix II) responsible for the catalytic function, and variable regions that dictate substrate specificity. Hence, modification of the substrate recognition domains enabled development of a versatile "*trans*-acting" ribozyme capable of intermolecular sequence-specific cleavage (Fedor and Uhlenbeck, 1990). **(B)** Selective cleavage of mutant RNA transcripts using hammerhead ribozymes. Schematic diagram illustrating how hammerhead ribozymes can be designed to discriminate between mutant and wild type transcripts. If a mutation results in the formation of a 5′-NUH-3′ sequence triplet , where N = any nucleotide, V = A, G or C, and H = C, U or A , a hammerhead ribozyme can be designed to specifically target and cleave the mutant sequence.

from the nucleotide substitution α806G→T, creating the cleavage site AUU, and αS226F is generated by nucleotide substitution α677C→T, resulting in the creation of the cleavage site UUC.

Targeting a short cRNA substrate harbouring SCCMS mutations

A symmetric ribozyme with eight nucleotide helix III and helix I binding arms (8+8) was designed against the εL221F mutation, L221FRz. In order to confirm the catalytic activity of the ribozyme, an inactive ribozyme was also designed in which the G at the 3' end of the catalytic core nucleotides was mutated to C. The ribozyme cleaved the AChR ε-subunit cRNA transcript harbouring mutation εL221F, producing two products with the expected sizes of 5' product (137 nt) and 3' (391 nt). The wild type transcripts were not cleaved confirming the ability of the ribozyme to discriminate between the mutant and wild type transcripts. In addition, the inactive ribozymes did not cleave the mutant substrate (Figure 2A). Similar experiments were performed to assess cleavage for cRNA transcripts of the AChR α subunit harbouring mutations αT254I, αS269I and αS226F (Figure 2B-D). In each case ribozymes with symmetric arms were synthesised. αT254IRz cleaved the mutant transcripts producing two products with the expected sizes of 5' product (137 nt) and 3' (314 nt); αS269IRz cleaved the mutant transcripts producing two products with the expected sizes of 5' product (195 nt) and 3' (264 nt); and αS226FRz cleaved the mutant transcripts although only the 3' product of 406 nt was visualised. In each case the wild type transcripts were not cleaved, confirming the selectivity of the ribozymes, and the respective inactive ribozymes did not cleave the cRNA transcripts.

Optimisation of reaction conditions

Having established that separate cRNAs containing the four different naturally occurring mutation sites could be cleaved by our hammerhead ribozymes, we next examined the ability of the αS226F ribozyme to cleave the substrate cRNA at physiological ionic concentrations that might be present at the neuromuscular junction subsynaptic cytosol. We performed cleavage of target cRNA using the simulated physiological conditions, described by Santoro and Joyce (1997) of 2 mM $MgCl_2$, 150 mM KCl, 50 mM Tris-HCl pH 7.5 and 37°C. The cRNA target was efficiently cleaved (Figure 3A). Moreover, since the endplate myopathy in SCCMS patients is thought to be due to excess calcium entry through the mutant AChR, which accumulates in the muscle fibres, we examined the cleavage activity under a relatively high concentration of Ca^{2+} (3 mM). The high Ca^{2+} concentration made no obvious difference to cleavage activity.

To investigate the reaction conditions of αS226FRz (8+8), we varied the amount of ribozyme required for cleavage of the cRNA substrate (Figure 3C). 15 pmol of cRNA substrate was incubated with varying amounts of the ribozyme, and a clear cleaved band was obtained with as little as 10 pmol of the ribozyme. Increasing the ribozyme concentration did not show a dramatic increase in cleavage products generated from the 15 pmol of cRNA substrate.

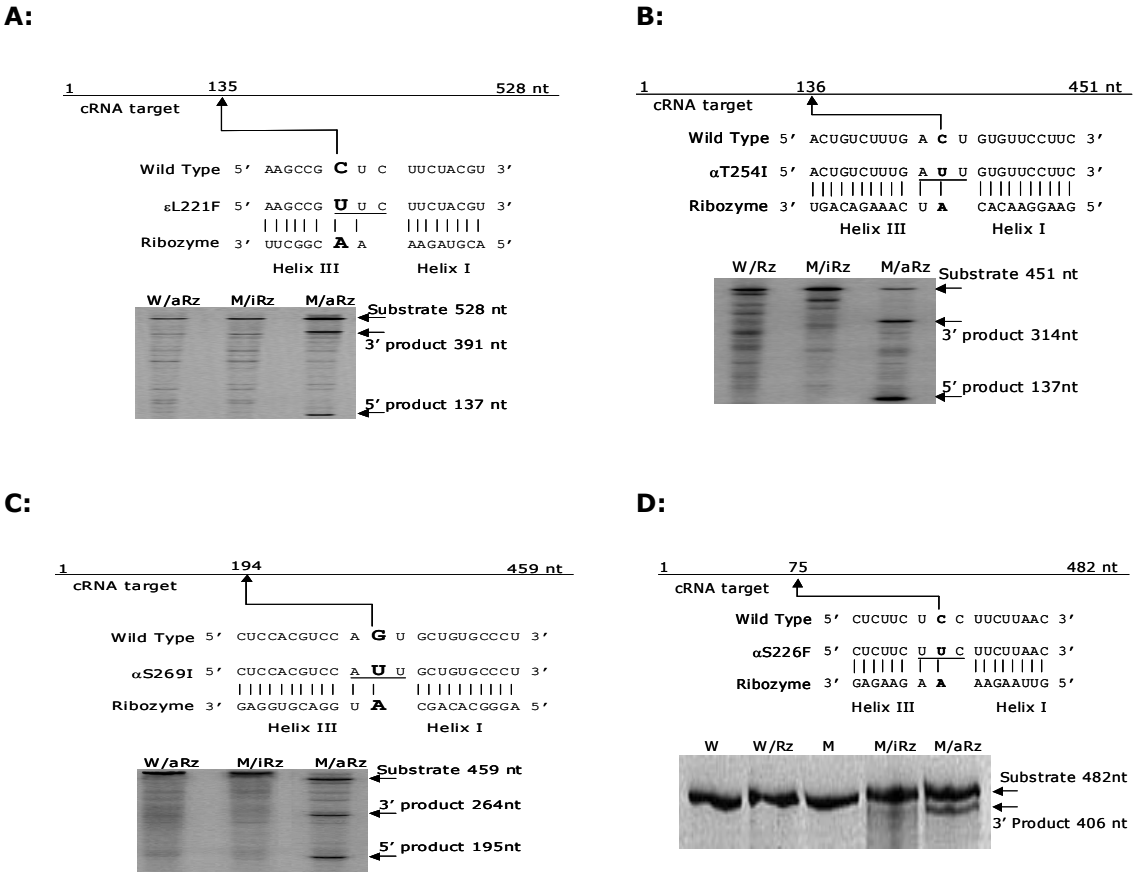

Figure 2. Selective cleavage of cRNA transcripts harbouring mutations that underlie the slow channel myasthenic syndrome. ^{32}P-labelled cRNA transcripts were generated by *in vitro* transcription, and following the ribozyme reaction, size-fractionated on a 6% (w/v) PAGE and subjected to autoradiography. **(A)** Selective cleavage of εL221F cRNA. Lane W/aRz, wild type ε-AChR cRNA incubated with active ribozyme; lane M/iRz, L221F mutant ε-AChR cRNA incubated with inactive ribozyme; lane M/aRz, L221F mutant ε-AChR cRNA incubated with active ribozyme. **(B)** Selective cleavage of αT254I cRNA. Lane W/aRz, wild-type AChR α-subunit cRNA incubated with active ribozyme; lane M/iRz, T254I mutant cRNA incubated with inactive ribozyme; lane M/aRz, T254I mutant cRNA incubated with active ribozyme. **(C)** Selective cleavage of αS269I cRNA. Lane W/aRz, wild type AChR α-subunit cRNA incubated with active ribozyme; lane M/iRz, mutant cRNA incubated with inactive ribozyme; lane M/aRz, mutant cRNA incubated with active ribozyme. **(D)** Selective cleavage of αS226F cRNA. Lane W, wild-type cRNA; lane W/aRz, wild-type α-subunit cRNA incubated with active ribozyme; lane M, mutant cRNA; lane M/iRz, mutant cRNA incubated with inactive ribozyme; lane M/aRz, mutant cRNA incubated active ribozyme.

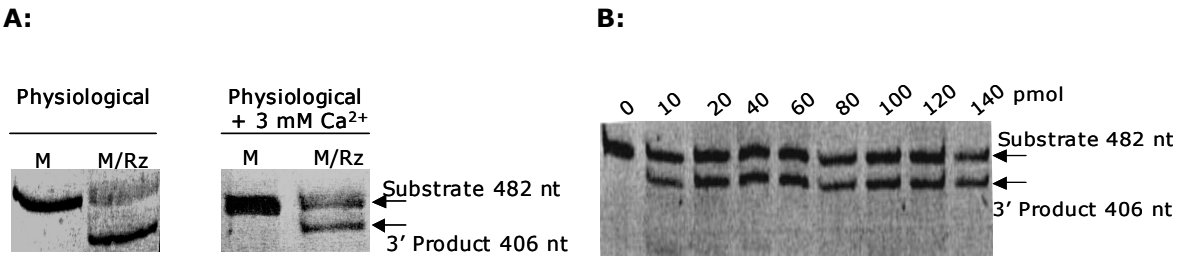

Figure 3. cRNA transcripts harbouring the αS226F mutation were incubated with the αS226F ribozyme for 4 hours at 37°C, size-fractionated on a 3% (w/v) agarose/TBE gel containing ethidium bromide, and visualised under uv light. **(A)** Cleavage of the αS226F mutant cRNA under simulated physiological conditions and at 3 mM Ca^{2+}. **(B)** Effect of varying the ribozyme concentration. cRNA substrate incubated with 0, 10, 20, 40, 60, 80, 100, 120, 140 pmol, respectively, under simulated physiological conditions.

Cleavage of cRNA transcripts containing the full coding region

We next examined the ability of the hammerhead ribozyme targeted at the αS226F mutation to cleave longer cRNA transcripts (1552 nucleotides) that contain the full coding sequence of the AChR α subunit. Efficient cleavage of the mutant but not the wild type sequence resulted in two cleaved products with the expected sizes (Figure 4A). Finally, we compared two ribozymes with different length binding arms (8 + 8 and 12 + 12). cRNA harbouring the αS226F was incubated with each ribozyme under simulated physiological conditions. Cleavage after four hours was similar for both ribozymes (Figure 4B). In order to measure the difference between the two ribozymes more precisely, we calculated the % of cleavage by quantifying the ^{32}P signals using a phosphoimager (see methods) and, although using this method the (12+12) ribozyme cleaved 48% of the substrate as opposed to 42% of the substrate for the (8+8) ribozyme, the difference was not statistically significant.

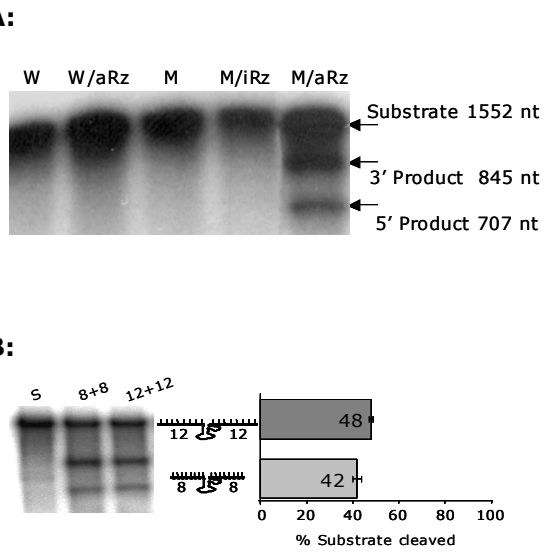

Figure 4. Cleavage of cRNA transcripts containing the full AChR α-subunit coding sequence. ^{32}P-labelled cRNA was generated by *in vitro* transcription, incubated with respective ribozymes under simulated physiological conditions for 4 hours, size-fractionated on a 6% (w/v) PAGE and visualised by autoradiography or using a phosphoimager. **(A)** Cleavage reactions of wild type or mutant αS226F cRNA containing the full coding sequence. Lane W, wild type cRNA; lane W/aRz, wild type cRNA incubated with ribozyme; lane M, αS226F mutant cRNA; lane M/iRz, αS226F mutant cRNA incubated with the inactive ribozyme; lane M/aRz, αS226F mutant cRNA incubated with active ribozyme. Arrows indicate the cleaved products. **(B)** Cleavage of the αS226F mutant cRNA transcript by active ribozymes with different length binding arms. Lane S, cRNA transcript; lane 8+8, mutant cRNA incubated with a two 8-nucleotide arm ribozyme; lane 12+12, mutant cRNA incubated with a two 12-nucleotide arm ribozyme. The cleavage efficiency was calculated using the results from phosphorimaging (see Materials and Methods).

DISCUSSION

Recent studies of RNA interference and microRNAs demonstrate that RNA can play a dynamic role in the control of gene expression. These systems, potentially, could be adapted for therapeutic application in dominantly inherited genetic disorders where intervention at the level of mRNA could give allele-specific silencing of pathogenic mutants. RNA interference may be modified for allele-specific gene silencing by using short sequences (siRNA or shRNAi) that match the mutant but have a central mismatch with the wild type sequence (Davidson and Paulson, 2004). However, the ability to discriminate between mutant and wild type alleles may depend on the nature of the mismatch and sequence context. Proposed therapy involving RNAi uses naturally occurring gene silencing machinery, and some concerns have been raised about off target effects (Scacheri et al, 2004). Here, as an alternative approach, we have investigated the ability of hammerhead ribozymes to generate allele-specific cleavage of mutant cRNA transcripts. We show cleavage by hammerhead ribozymes of cRNA transcripts harbouring four different mutations that underlie slow channel myasthenic syndromes. In each case the mutant transcript is cleaved whilst the wild type transcript remains intact. For the ribozymes targeted at mutation αS226F we defined the conditions for efficient cleavage and demonstrate effective allele specificity against full-length cRNA transcripts under simulated physiological conditions.

Ribozyme cleavage efficiency can be influenced by a number of factors, such as metal ion concentrations, the accessibility of the target site and the binding affinity of ribozyme helices I and III to their target sequence. Magnesium ions are important both for the catalytic activation of hammerhead ribozymes (Hammann and Lilley, 2002) and for the pairing of the arms to the target sequence. In general, higher Mg^{2+} concentrations result in more efficient ribozyme cleavage (Shimayama et al., 1995), but the effects of Mg^{2+} concentration varies with different ribozymes (Trulzsch, 2003). Cellular levels of Mg^{2+} are low at around 0.25 – 2.0 mM (Grubbs, 2002), and thus if the ribozyme is to have potential therapeutic application it is important to demonstrate efficient cleavage at physiological Mg^{2+} concentrations. For the αS226FRz we were able to demonstrate cleavage occurring at as little as 1 mM Mg^{2+} (Figure 3A). Moreover it was equally efficient at the high Ca^{2+} concentrations (Figure 3B) that could occur at SCCMS patient endplates (Gomez et al, 2002).

Cleavage of short substrates does not necessarily indicate that a ribozyme will effectively cleave a full-length mRNA transcript, where complex secondary structure folding is likely to occur. We were able to demonstrate that incubation of a full-length cRNA transcript with the αS226FRz for up to 4 hr led to cleavage of the mutant transcript without affecting the wild type sequence (Figure 4A), and that similar cleavage efficiency was obtained using 10 pmol of ribozyme after 30 minutes, in presence of 1 mM Mg^{2+} and 3 mM Ca^{2+} (data not shown). Finally, we performed cleavage reactions with ribozymes of different binding arm length. Short arm lengths for helix I and helix III would be expected to generate a hammerhead ribozyme that would

rapidly recycle from one substrate molecule to the next, whereas longer arms might be expected to show more efficient cleavage, but not to recycle so effectively. With an incubation period of four hours there was no significant difference between hammerhead ribozymes with arm lengths of (8+8) or (12+12).

CONCLUSIONS

- Hammerhead ribozymes may efficiently discriminate between mutant and wild type cRNA transcripts that differ by only a single nucleotide substitution.
- Discrimination can be achieved in conditions that simulate physiological conditions, and the environment of high Ca^{2+} that might be present at the endplate of patients with slow channel myasthenic syndrome.
- Hammerhead ribozymes may have a role in therapeutic allele-specific gene silencing in situations where RNAi proves to be inappropriate or ineffective.

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STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

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