SHORT REPORT

Selective DNazyme-mediated cleavage of AChR mutant transcripts by targeting the mutation site or through mismatches in the binding arm

Amr Abdelgany¹, John Ealing¹, Matthew Wood² and David Beeson¹*

¹Neurosciences Group, Weatherall Institute of Molecular Medicine, University of Oxford, The John Radcliffe Hospital, Oxford OX3 9DS, UK. ²Department of Human Anatomy and Genetics, South Parks Road, Oxford, OX1 3QX, UK

*Correspondence to: David Beeson, Email: dbeeson@hammer.imm.ox.ac.uk, Tel: +44 1865 222311, Fax: +44 1865 222402

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ABSTRACT

Many dominantly inherited disorders are caused by missense amino acid substitutions resulting from a single nucleotide exchange in the encoding gene. For these disorders, where proteins expressed from the mutant alleles are often pathogenic and present throughout life, gene silencing, through intervention at the mRNA level, holds promise as a therapeutic approach. We have used mutations that underlie the slow channel congenital myasthenic syndrome (SCCMS) as a model system to study allele-specific gene silencing of RNA transcripts by DNazymes. We tested the ability of DNazymes to give allele-specific cleavage for i) mutations that create cleavage sites, and ii) mutations located close to a DNazyme cleavage site that create a potential mismatch in the binding arms. For both we demonstrate selective cleavage of mutant transcripts under simulated physiological conditions. For DNazymes with binding arm mismatches the degree of selectivity for mutant over wild type may be enhanced by optimising the mismatch position as well as the binding arm length. The optimal sites for mismatches are 1.1 and 1.2 in arm I, and 16.2 in arm II. Asymmetric binding arm DNazymes with a shorter arm I are more discriminative. Our results show it should be possible to apply DNazyme-mediated cleavage of mutant alleles even when the mutant does not itself create a putative cleavage site. This therapeutic approach may be well suited to dominantly inherited disorders such as SCCMS, where loss of some wild type transcripts is unlikely to have pathogenic consequences.

KEYWORDS: DNazyme, allele-specific silencing, binding arm mismatch, slow channel myasthenic syndrome, gene therapy

INTRODUCTION

To devise successful therapy for dominant genetic disorders is a challenging goal. These disorders are often both progressive and incurable. An optimal therapy requires the elimination of the pathogenic mutant protein without detrimental effects on the protein generated from the corresponding wild type allele. One approach is to preferentially target the mutant transcripts using the antisense catalytic nucleic acids, hammerhead ribozymes (Fedor and Uhlenbeck, 1990) or their DNA analogues, DNazymes (Santoro and Joyce, 1997).

DNazymes are short oligonucleotides that have the capacity to cleave RNA molecules in an enzymatic fashion. They consist of a central catalytic motif flanked by two arms (arm I and II) of complementary sequence that bind to the target RNA molecules on a Watson-Crick basis (see Figure 1A). The catalytic activity is metal ion-dependent and breaks the phosphodiester bond between purine and pyrimidine nucleotides (Santoro and Joyce, 1998). Like hammerhead ribozymes (reviewed in Wood et al, 2003; Bagheri and Kashani-Sabet, 2004) DNazymes have been used to suppress pathogenic genes in a growing number of disease models (reviewed in (Khachigian, 2002; Achenbach et al, 2004)). Most recently, LMP1 mRNA in Epstein-Barr virus-mediated carcinogenesis was successfully targeted in vivo (Lu et al, 2005). However, the potential of DNazymes as a therapy for dominant genetic disorders has not yet been explored.
To test the ability of DNAzymes to cleave mutant cRNA sequences we used mutations underlining the slow channel congenital myasthenic syndrome (SCCMS). Thus far, 18 different SCCMS mutations have been described (Ohno and Engel, 2004). It is thought that prolonged activation of the receptors, due to the mutations, leads to excess calcium entry resulting in an “endplate myopathy” and muscle weakness (Engel et al, 1982; Gomez et al, 2002). Thus SCCMS is an example of a dominant excitotoxic disorder caused by “gain of function” mutations. At the neuromuscular junction, the safety margin for neuromuscular transmission is only compromised if AChR levels fall below around 30% of normal (Engel et al, 2003) and thus for gene silencing strategies problems arising from haploinsufficiency should not be a problem.

Allele-specific cleavage of mutant RNA transcripts by catalytic nucleic acids normally depends on the mutation itself creating a cleavage site (Figure 1B(ii)). However, single nucleotide mutations will not always result in the creation of a cleavage site that can be used to discriminate between wild type and mutant transcripts, apparently limiting this strategy. Here, we also adopt an alternative approach for targeting mutant alleles by identifying putative cleavage sites near the mutation, and then designing the DNAzyme binding arm to target the mutant sequence, creating a mismatch with the wild type (Figure 1B(ii)).

**MATERIALS AND METHODS**

**DNAzyme design and synthesis**

DNAzymes bearing the 10-23 catalytic motif were designed with different binding arm lengths and were synthesised as standard oligonucleotides (Invitrogen). To facilitate DNAzyme nomenclature, we refer to them, for example, as αS269IAI5(13+9), where αS269I is the target subtype. AI is arm II, 5 is the distance of the mismatch from the cleavage site, and (13+9) indicates the lengths of arm II and arm I respectively (see Figure 3A). DNAzymes that directly cleave the target are termed, for example, εL221F(6+12), where εL221F is the target substrate and (6+12) are the lengths of the binding arms (Figure 2).

**cRNA substrate preparation**

Missense mutations that underlie SCCMS were introduced by the Sculptor™ in vitro mutagenesis system (Amersham Biosciences). Plasmids harbouring the mutant cDNA were checked by DNA sequencing. 32P-labelled DNA substrates containing the full coding sequence of mutant and wild-type subunits were synthesised using the Megascript™ T7 in vitro transcription kit (Ambion Biosciences).

**In vitro cleavage**

Substrate cRNA and DNAzymes were incubated either in 10 mM MgCl2, 50 mM Tris.HCl, pH 7.5 or under simulated physiological conditions (Santoro and Joyce, 1997) (2 mM MgCl2, 150 mM KCl, 50 mM Tris.HCl, pH 7.5, 37°C) for 4 hr. The reactions were carried out under single turn over conditions (i.e. with excess enzymes) using a molar ratio for enzyme:substrate of 10:1. The reactions were loaded on to a 6% (w/v) denaturing polyacrylamide gel and run at 200V for 7 hr. Gels were dried and exposed overnight to a phosphor storage screen (Fuji) and scanned in a PhosphorImager (Fuji). The amount of cleavage products were expressed as a percentage of the total of cleaved and uncleaved material (see below).

**Calculations of % of cleavage and selectivity**

The percentage of cleavage was calculated as (P1+P2/P1+P2+S) x 100 where P1 and P2 are the 5' and 3' products, and S is the substrate as previously described by Werner and Uhlénbeck (1995). The catalytic activities of mismatched DNAzymes were calculated by normalising the percentage of cleavage to the matched ones as % activity = % cleavage mismatched DNAzyme / % cleavage matched DNAzyme x 100. The degree of selectivity was calculated as (100 – mismatched activity).

**RESULTS**

**Cleavage of cRNA transcripts at the site of mutation**

DNAzymes with the 10-23 catalytic core can cleave RNA specifically at RY junctions, where R = A or G, and Y = U or C (Santoro and Joyce, 1997). εL221F and αS269I are two missense mutations that reside in different AChR subunit genes (Croixen et al, 1997; Croxen et al, 2002). Each underlies a SCCMS and also creates putative DNAzyme cleavage sites. εL221F results from a C to T transition at nucleotide position 661, creating a GU cleavage site in the mRNA, and αS269I, which results from a G to T nucleotide substitution at position 806, creates an AU cleavage site. The DNAzyme εL221F(6+12) (see methods for DNAzyme nomenclature) was designed to target the εL221F mutation. In vitro cleavage showed εL221F(6+12) cleaved its cRNA target (Figure 2), whereas the wild-type, which lacks the cleavage site, was not cleaved, thus demonstrating the discriminatory nature of the DNAzyme. To confirm the catalytic activity, an inactive DNAzyme with the same arm lengths and but with two nucleotides (C → T at position 3 and A → G at position 12) mutated in the catalytic core was used as a control. No cleavage was detected after incubation with this inactive DNAzyme. Similar results were obtained for a second target, αS269I within the α-subunit gene transcript, with the DNAzyme αS269I(13+13) (data not shown).

**Selective cleavage of mutant transcripts through mismatches in the binding arms**

To target mutations that do not introduce cleavage sites we identified putative cleavage sites near the mutation and designed DNAzymes with arms that perfectly match the mutant sequence but have a mismatch in the binding arm for the wild type counterpart (Figure 1B(ii)). Thus, both wild type and mutant cRNA have the same DNAzyme cleavage site, but differ through the binding arm mismatch with the wild type sequence. Selectivity is obtained through the effects of the mismatch on cleavage activity. In these experiments we measured the efficacy of the DNAzyme in terms of selectivity rather than cleavage efficiency.

SCCMS mutation αS269I has two additional GU cleavage sites in close proximity either side of the AU mutation cleavage site. We examined the binding arm mismatch approach by targeting these two GU cleavage sites. Two asymmetric DNAzymes were designed. αS269IAI5(9+13)
position 1.5. The second cleavage site was targeted by the nomenclature). Under simulated physiological conditions, DNAzyme αV156MAI3(10+10), was designed to target the cleavage site three nucleotides upstream of the mutation site, creating a mismatch with the wild type cRNA in arm I at position 1.3. Similarly, DNAzyme αV156MAI6(10+10) created a mismatch in arm II at 16.6. αV156MAI6(10+10) cleaved 25% of the mismatched wild type, and 75% of the matched mutant substrate. αV156MAI3(10+10) showed an even greater difference in activity, cleaving only 11% of the wild type but 61% of the mutant (Figure 3B).

Optimization of the DNAzyme design to enhance the mismatch-based selectivity

i. The effect of the position of the mismatch

Targeting the cleavage sites either side of αS269I and αV156M revealed differing effects of the mismatch position along arm I and arm II in reducing cleavage activity and hence the degree of selectivity. For αS269I, DNAzymes with the same arm length (9+13), but different mismatch positions, 1.5 in arm I for αS269IAI5(9+13) and 16.5 in arm II for αS269IIAII5(9+13), showed dramatic changes in the degree of selectivity (39% and 4% respectively) (Figure 4A). Similarly, targeting the two cleavage sites either side of the αV156M mutation with the same arm length (9+13) but different positions for the mismatches, 1.3 for αV156MAI3(9+13) and 16.6 for αV156MAI6(9+13), also showed different degrees of selectivity (74% and 55% respectively) (Figure 4A).

Figure 1. (A) The structure of the 10-23 DNAzyme. The top strand is an RNA substrate with an RY cleavage site, where R represents A or G and Y represents U or C. Arms I and II are antisense binding arms. (B) Schematic diagram showing the steps involved in targeted cleavage of mutant transcripts by DNAzymes. (i) Allele-specific cleavage of mutations that create a DNAzyme cleavage site. (ii) Allele-specific cleavage using mismatches in the binding arms.

targets the cleavage site five nucleotides upstream of the mutation creating a mismatch with wild type in arm I at position 1.5. The second cleavage site was targeted by the αS269IAI5(13+9) DNAzyme creating the mismatch with the wild type in arm II at position 16.5 (see Figure 1A for nomenclature). Under simulated physiological conditions, αS269IAI5(9+13) DNAzyme cleaved 43% of the mismatched target (wild type) compared with 72% of its matched one (mutant), giving a degree of selectivity of 39%. The αS269IAI5(13+9) DNAzyme, which cleaved 57% of the mutant transcript, showed a dramatic reduction for the wild type target, cleaving only 3%, which was at least 20-fold less than its matched mutant counterpart giving 95% selectivity (Figure 3A). A second SCCMS mutation in the AChR ε subunit was targeted, αV156M results from a G to A nucleotide substitution at nucleotide position 466 (Croxen et al, 1997), it does not create a cleavage site but has two putative cleavage sites in close proximity. Consequently, DNAzyme αV156MAI3(10+10), was designed to target the cleavage site three nucleotides upstream of the mutation site, creating a mismatch with the wild type cRNA in arm I at position 1.3. Similarly, DNAzyme αV156MAI6(10+10) created a mismatch in arm II at 16.6. αV156MAI6(10+10) cleaved 25% of the mismatched wild type, and 75% of the matched mutant substrate. αV156MAI3(10+10) showed an even greater difference in activity, cleaving only 11% of the wild type but 61% of the mutant (Figure 3B).

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To investigate further the effect of the mismatch position on cleavage efficiency, a set of DNAzymes were designed to target the n472AU site close to the αV156M mutation. Symmetrical (10+10) DNAzymes were designed with sequential mismatches in the binding arms for the mutant target, arm I (1.1, 1.2, 1.3, 1.4, 1.5) and arm II (16.1, 16.2, 16.3, 16.4, 16.5). The cleavage efficiency of each mismatched enzyme was normalised to the matching one (Figure 4B). For arm I, a mismatch at position 1.1 showed no cleavage. This is to be expected since it is a required position for cleavage. A mismatch at position 1.2 gave very low cleavage activity (15%). As the distance of the mismatch from the cleavage site increases, the enzymes become more tolerant. Position 1.3 mismatch showed 44% activity, which is 2-fold less than the matched substrate. Positions 1.4 and 1.5 reduced the cleavage by 2.3- and 2-fold respectively. By contrast, except for position 16.2, mismatches in arm II have less effect on catalytic activity. In general a mismatch in helix I had a far more pronounced effect than a mismatch in arm II, suggesting a vital role for this arm in establishing the DNAzyme catalytic activity.

**ii. The effect of binding arm length**

We then examined the effect of the binding arm length on the two mutant cRNA substrates, αS269I and αV156M. For the αV156M mutation, over 70% selectivity was obtained with three DNAzymes with different length binding arms, αV156MAI3(9+13), αV156MAI3(13+9) and αV156MAI3(10+10), when the mismatch is at position 1.3 in arm I (Figure 4Ci). However, when the mismatch lies further from the cleavage site, such as at position 1.5, the length of the binding arm appears to affect the degree of selectivity. DNAzyme αS269IAI3(13+13), which targets the GU site five nucleotides upstream of the αS269I mutation, showed only 14% selectivity. Shortening the arms to (10+10) dramatically increases the selectivity to 69% (Figure 4Civ). Although, no general rule can be applied to all cases (as exemplified by the results in figure 4Ciii) our data suggests that of the various possibilities for the mismatch location and binding arm length, the selectivity is likely to be greatest when the mismatch is close to the cleavage site and in a short binding arm (Figure 4Civ).

**DISCUSSION**

DNAzymes may provide a tool for gene silencing in vivo, in particular if stabilised by phosphoramidate or locked nucleic acids (Takahashi et al, 2004; Vester et al, 2004). We show selective cleavage by DNAzymes of missense mutations that underlie a dominantly inherited disorder. If a mutation creates a putative cleavage site, DNAzymes can be designed to target specifically the mutant transcripts leaving the wild-type counterpart intact. cRNA transcripts containing the SCCMS mutations εL221F or αS269I were cleaved to target specifically the mutant transcripts leaving the mutant allele. We also investigated whether we could obtain selective cleavage of mutant transcripts through mismatches in binding arms I and II. Under simulated physiological conditions a single nucleotide mismatch in the binding arm reduced the catalytic activity of DNAzymes, thus generating the ability to discriminate...
Figure 4. (A) Effects of the mismatch position on DNAzyme cleavage activity. (a) The effects of the position of the binding arm mismatch in targeting the two cleavage sites located either side of αS269. The mismatches are in arm I and arm II, both 5 nucleotides from the mutation. The sequence of the binding arms are shown, and the position of the mismatch indicated in the diagrams. The cleaved products and the calculated degree of selectivity for mutant versus wild type cRNA transcripts are shown. (B) The effect of the position of the binding arm mismatch on the DNAzyme cleavage site at nucleotide position α472. 32P-labelled cRNA for wild type or mutant αV156M AChR α subunits were synthesised in vitro and incubated with the DNAzymes under simulated physiological conditions for 4 hr. Cleaved products were run on 6% (w/v) polyacrylamide gels and subjected to autoradiography. (i) Example autoradiograph showing in lane 1, αV156M cRNA; lanes 2-6, DNAzymes with mismatches in helix III; lane 7, DNAzyme matched to the mutant transcript; lanes 8-12, DNAzymes with mismatches in helix I. (ii) Cleavage activity normalised to the activity for the matched arms. Values represent mean ± SD of 5 experiments. (C) Effects of binding arm length on selectivity due to binding arm mismatches. 32P-labelled cRNA substrates containing the wild type sequence or SCCMS mutations were incubated with the indicated DNAzymes that contain mismatches in arm I or arm II and have binding arms of varying length. Diagrammatic representations of the DNAzymes (left) and calculated degree of selectivity (right) are shown. (i) αV156M cRNA transcripts incubated with DNAzymes targeting the cleavage site 3 nucleotides upstream of the mutation. (ii) αS269I cRNA cleaved by symmetric DNAzymes of differing arm length with the mismatch to the wild type helix I at position 15. (iii) αS269I cRNA cleaved at the upstream or downstream DNAzyme sites (A15 or A15) with variation of the DNAzyme binding arm length. (iv) DNAzymes targeted to α-subunit cRNAs containing three different cleavage sites, illustrating that greater selectivity is obtained with mismatches in the shorter binding arm.
between mutant and wild-type RNA transcripts. We found that mismatches in arm I reduce the catalytic activity more than mismatches in arm II (except for arm II position 16.2). The degree of selectivity may be enhanced by optimising the position of the mismatch as well as the binding arm length. Positions 1.2 and 16.2 were the most sensitive, correlating with results from mismatch studies on hammerhead ribozymes (Sun et al, 1995; Werner and Uhlenbeck, 1995), and the greatest discrimination was obtained by generating asymmetric DNAzymes with a shorter arm I.

The reduction in DNAzyme activity due to the mismatches in the arms varied according to the length and the mismatch position. Our results, which were obtained using long cRNA transcripts, correlated well with previous studies on the influence of arm length asymmetry and base substitutions performed on a short target substrate (Cairns et al, 2000a; Cairns et al, 2000b). We found that, in general, mismatches in arm I are more effective in reducing the enzyme activity and thus increasing the degree of selectivity, although this is not always the case. For example, αs269IAI5(13+9), with a mismatch at 16.5 in arm II, showed a very high degree of selectivity of 95%, compared with 45% for its counterpart αs269IAI5(13+9) with the mismatch in arm I at position 1.5. Factors such as target site and sequence composition of the arms may influence the catalytic activity of the DNAzyme (Santoro and Joyce, 1998; Cairns et al, 1999). In addition, the nature of the mismatch will influence the catalytic properties of DNAzymes; for instance, the wobble mismatch (G:U) is likely to cause least disruption to binding.

Having located the most sensitive positions for placing mismatches, we tested the effect of altering the arm length for both symmetric and asymmetric DNAzymes. The principle of mismatch-mediated selectivity is based on weakening the enzyme-substrate binding which leads to a reduction in the cleavage activity. Thus, as expected, mismatches in shorter arms give greater selectivity. However, we also noted the dominant role of arm I in governing catalytic activity, suggesting that shortening arm I as opposed to arm II may be used to enhance selectivity without compromising cleavage efficiency.

CONCLUSIONS

• DNAzymes provide a potential alternative to RNAi or ribozymes for allele-specific silencing.

• DNAzymes can target allele-specific RNA transcripts where a mutation introduces a cleavage site.

• Where a mutation does not introduce a cleavage site, DNAzymes may be designed to preferentially target the mutant allele through mismatches in the binding arms.

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STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

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