Allele-specific silencing of a pathogenic mutant acetylcholine receptor subunit by RNA interference

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Slow channel congenital myasthenic syndrome (SCCMS) is a disorder of the neuromuscular synapse caused by dominantly inherited missense mutations in genes that encode the muscle acetylcholine receptor (AChR) subunits. Here we investigate the potential of post-transcriptional gene silencing using RNA interference (RNAi) for the selective down-regulation of pathogenic mutant AChR. By transfection of both siRNA and shRNA into mammalian cells expressing wild-type or mutant AChR subunits, we show, using 125I-α-bungarotoxin binding and immunofluorescence to measure cell surface AChR expression, efficient discrimination between the silencing of αS226F AChR mutant RNA transcripts and the wild-type. In this model we find that selectivity between mutant and wild-type transcripts is optimized with the nucleotide mismatch at position 9 in the shRNA complementary sequence. We also find that allele-specific silencing using shRNA has comparable efficiency to that using siRNA, underlining the general potential of stable expression of shRNA molecules as a long term therapeutic approach for allele-specific silencing of mutant transcripts in dominant genetic disorders.

INTRODUCTION

Dominantly inherited neurological disorders are often both progressive and incurable. The pathogenic agent may be expressed from a mutant allele throughout life and its effects are not ameliorated by expression from the normal allele. A possible therapeutic approach is to suppress expression from the mutant allele by targeting the mutant RNA transcripts, although exquisite accuracy is required, since the difference between wild-type and mutant transcripts is frequently only a single nucleotide substitution.

The concept of using RNA molecules as therapeutic agents is relatively new (1–3), but has been boosted by the discovery of the phenomenon of RNA interference (RNAi) (4). It depends upon two key features of RNA molecules; first, that they can form, or be part of, catalytic centres capable of cleaving multiple RNA substrate molecules; and secondly, that the catalytic core can be precisely targeted through RNA–RNA Watson and Crick pairing with the substrate. Advances in the last two years have enhanced the potential of RNAi as a therapeutic agent. First, it was shown that the mammalian cell antiviral response to exogenous double-stranded RNA could be induced by short hairpin RNAs (shRNA) synthesized from RNA polymerase III promoters in vivo (6–10); and third, that in Drosophila melanogaster embryo extracts and in mammalian cells as little as a single nucleotide change within the target sequence reduces the silencing effect (6,8,11).

To test the ability of siRNA and shRNA to give allele-specific down-regulation of mutant transcripts we used the slow channel congenital myasthenic syndrome (SCCMS) as a model for an excitotoxic autosomal dominant neurological disorder. This disorder has the characteristic clinical feature of fatiguable muscle weakness, and is usually slowly progressive, with a variable age of onset and selective muscle involvement (12). It results from missense mutations in genes encoding the muscle acetylcholine receptor (AChR) subunits (13). Since the disease mechanism that underlies SCCMS is well characterized and the AChR biology can be studied at both expression and functional levels (12), SCCMS provides an excellent model to study allele-specific gene silencing.

Here we investigate selective down-regulation of mutant AChR containing the α-subunit SCCMS mutation αS226F (14) using siRNA and shRNA following transfection into...
mammalian cells. We demonstrate efficient discrimination between mutant and wild-type transcripts when the nucleotide mismatch is at position 9 in the shRNA complementary sequence. We also find that shRNA shows comparable efficiency to siRNA for allele-specific silencing, emphasising the potential of stable expression of shRNA as a long term therapeutic agent in dominant genetic disorders.

RESULTS

Inhibition of aS226F mutant AChR subunit gene expression by siRNA

To determine whether post-transcriptional gene silencing using RNA interference could be exploited for the allele-specific silencing of mutant gene transcripts, we tested this hypothesis in a cellular model of the neurological disorder SCCMS. SCCMS disturbs normal functioning of the neuromuscular junction, and results from missense mutations in genes encoding the muscle AChR subunits. Targeting the aS226F mutation within the AChR α subunit, which results from a C→T transition at nucleotide position 677 (14) (Beeson, unpublished data), we first wanted to determine whether this mutant transcript could be efficiently discriminated from the wild-type transcript using siRNA, and its expression silenced. siRNAs were designed to target the aS226F mutant transcript from nucleotide positions 658–676 (where +1 is the first nucleotide of the coding sequence of the mature α subunit), matching the target mutant transcript at each nucleotide position, but showing a mismatch to the wild-type AChR α subunit transcript at position 10 (Fig. 1A). siRNA or non-specific siRNA (NS-siRNA) were co-transfected into mammalian HEK 293 cells with cDNAs expressing either the mutant aS226F or wild-type AChR transcripts, and the effects of each siRNA upon AChR expression determined using two methods. First, we used an 125I-α-bungarotoxin (125I-α-BuTx) binding assay. 125I-α-BuTx binds specifically and with high affinity to AChR providing a highly sensitive assay that allows cell surface AChR expression to be quantified. Transfected cells were incubated with mAb G3 that binds to the AChR δ subunit (15) and binding detected by FITC-conjugated secondary antibody. Second, we used a surface AChR expression detected by immunofluorescence. Transfected cells were incubated with mAb G3 that binds to the AChR δ subunit (15) and binding detected by FITC-conjugated secondary antibody.

Figure 1. Allele-specific suppression of aS226F mutant AChR by siRNA. (A) Sequence and prediced duplex of siRNA targeting the aS226F transcript from nucleotide positions 658–676 (+1 is the first nucleotide of coding sequence of the mature α subunit) showing the mismatch with the wild-type sequence at position 10. (B) Expression of AChR containing aS226F mutant and wild-type α subunits in HEK 293 cells co-transfected with siRNA or non-specific siRNA (NS-siRNA). Total 125I-α-BuTx binding to surface of HEK 293 cells was measured 48 h post-transfection. Results are normalized for 125I-α-BuTx binding to aS226F and α wild-type, respectively, and represent the mean ± SD of five experiments. Bars in the chart represent the following experiments: C, control non-specific 125I-α-BuTx binding to transfected cells; M, aS226F-AChR transfected cells; M + siRNA, aS226F-AChR transfected cells + siRNA; M + NS-siRNA, aS226F-AChR transfected cells + NS-siRNA; WT, wild-type AChR transfected cells; WT + siRNA, wild-type AChR transfected cells + siRNA; WT + NS-siRNA, wild-type AChR transfected cells + NS-siRNA. (C) Surface AChR expression detected by immunofluorescence. Transfected cells were incubated with mAb G3 that binds to the AChR δ subunit (15) and binding detected by FITC-conjugated secondary antibody. (a) Untransfected cells; (b) aS226F-AChR transfected cells; (c) aS226F-AChR transfected cells + siRNA; (d) aS226F-AChR transfected cells + NS-siRNA; (e) wild-type AChR transfected cells; (f) wild-type AChR transfected cells + siRNA.
with respect to the wild-type AChR α subunit transcript (Fig. 1B). Expression of mutant αS226F transcripts was reduced to 28 ± 13.79% of normalized surface levels, whilst wild-type AChR α subunit expression was significantly less reduced to 71 ± 9.84% of normalized surface levels. Second, we also determined AChR surface expression by immunofluorescence. Transfected cells were incubated with a monoclonal antibody (mAb) G3 that binds to the AChR δ subunit (15), and binding was detected by FITC-conjugated secondary antibody (Fig. 1C). The selective down-regulation of the mutant transcript was confirmed qualitatively using this approach.

Synthesis of shRNA by in vitro transcription

Like siRNA duplexes, hairpin siRNAs have been shown effectively to inhibit the expression of complementary mRNAs (8,16,17). We therefore next wanted to determine whether or not preferential allele-specific silencing of the mutant αS226F transcript could also be effected by short hairpin RNA (shRNA), and if so whether or not allele-specific silencing using this approach was of comparable efficiency to that already demonstrated using siRNA. We therefore designed oligonucleotides and used a T7 in vitro transcription system to generate expression of shRNA constructs to test this hypothesis (8,18).

shRNA expression constructs were transcribed from the T7 promoter as shown in Fig. 2A. shRNAs were matched to a 19 nucleotide sequence of the target transcript, using a three-nucleotide loop and four terminal uridines simulating the RNA polymerase III termination signal. To demonstrate that this T7 expression system yielded the predicted shRNA expression, the shRNA transcripts were size fractionated on a 7% agarose/TBE gel following digestion by DNase I (Fig. 2B). Efficient transcription of shRNA transcripts of the expected size was observed. It is likely that the weak upper band is due to some dimerization, since the RNA was size fractionated before the annealing reaction to hairpin formation.

shRNA gene silencing of DsRed2 expression: optimizing mismatch discrimination

shRNA can effectively inhibit the expression of RNAs complementary to either the sense or antisense sequence (7,8,16,17,19). However to be efficacious for allele-specific gene silencing it is necessary for the shRNA to discriminate effectively single nucleotide mismatches between the mutant and wild-type target sequences. Therefore in order to determine the optimal position of the nucleotide mismatch to discriminate between mutant and wild-type transcripts we tested the effects of altering the position of the nucleotide mismatch between shRNA and wild-type target, using the reporter gene DsRed2 as a model system.

shRNA constructs were designed to test the effects of nucleotide mismatches at several positions between the shRNA and the target DsRed2 transcript, the sequences and predicted structures of which are shown in Figure 3A. These included: R NS-shRNA, a non-specific shRNA (detailed in methods); R shRNA, matched to the target DsRed2 sequence; R M9-shRNA, mismatch at position 9; R M10-shRNA, mismatch at position 10; R M10.11-shRNA, double mismatch at positions 10 and 11. shRNAs targeted the DsRed2 transcript from nucleotide positions 5–24, where +1 is the first A of the initiating ATG codon.

The effects of the mismatches upon efficient silencing of DsRed2 expression were evaluated following co-transfection into mammalian HEK 293 cells of pDsRed2-N1 and respective shRNA transcripts. Five days following co-transfection HEK 293 cells were stained for DAPI, and the numbers of cells expressing fluorescent DsRed2 or DAPI were analysed by microscopy. Representative images under each experimental condition are shown in Figure 3B, including control untransfected cells and control non-specific shRNA (NS-shRNA) transfected cells. DsRed2 expression was quantified as a measure of gene silencing, by determining the number of DsRed2 expressing cells as a percentage of total cells, with results normalized to the proportion of DsRed2 expressing cells in the presence of R NS-shRNA to control for variations in transfection efficiency (Fig. 3C). Strong silencing of DsRed2 expression was observed with the fully matched shRNA (R shRNA) as expected, with an observed reduction in expression of 81 ± 3.83%. A single nucleotide mismatch at position 10 in the antisense strand of the shRNA (R M10-shRNA) resulted in a lesser degree of transcript silencing, 37 ± 9.96%, but some silencing of DsRed2 expression was still observed. However, a mismatch placed at nucleotide position 9 (R M9-shRNA) was...
found to almost completely abolish the ability of the shRNA to silence DsRed2 expression, with a reduction in expression of only 7%.

Double nucleotide mismatches at positions 10 and 11 (R M10.11-shRNA) were also highly effective at abolishing silencing activity of the shRNA.

Inhibition of αS226F mutant AChR subunit gene expression by shRNA

Having evaluated the effects of various nucleotide mismatches on the ability of T7 synthesised shRNAs to silence a reporter gene target, DsRed2, in mammalian cells, we then sought to use this knowledge of the effects of nucleotide mismatches to test whether T7 transcribed shRNAs could inhibit the expression of a mutant AChR allele. The position of the nucleotide mismatch that had demonstrated the greatest effect in abolishing the silencing activity of shRNA on the DsRed2 target transcript was located at position 9 in the antisense strand of the shRNA. Therefore, we designed shRNA constructs targeting the αS226F mutant AChR subunit from nucleotide positions 659–677, that were perfectly matched to the target αS226F mutant transcript but that differed by a single nucleotide mismatch from the wild-type AChR α subunit transcript, with the nucleotide mismatch placed at position 9 in the antisense shRNA strand (Fig. 4A).

shRNA molecules were synthesized using the T7 expression system described above and co-transfected into mammalian HEK 293 cells with cDNAs that express either mutant αS226F-AChR or wild-type AChR transcripts. The 125I-α-BuTx binding assay was again used to determine surface AChR expression. Total 125I-α-BuTx binding to the surface of HEK 293 cells was measured 48 h post transfection, with results normalized for 125I-α-BuTx binding to αS226Fβδε or αβδε, respectively. We found that the shRNA fully matched to the mutant αS226F
Figure 4. Allele-specific suppression of αS226F mutant AChR by shRNA. (A) Sequence and predicted structure of shRNA targeting the αS226F transcript from nucleotide positions 659–677, showing the mismatch with the wild-type sequence at position 9. (B) Expression of AChR containing αS226F mutant and wild-type α subunits in HEK 293 cells co-transfected with shRNA or non-specific shRNA (NS-shRNA). Total 125I-α-BuTx binding to surface of HEK 293 cells was measured 48 h post-transfection. Results are normalised for 125I-α-BuTx binding to αS226F bd or αbd, respectively, and represent the mean ± SD of five experiments. Bars in the chart represent the following experiments: C, control non-specific 125I-α-BuTx binding to untransfected cells; M, αS226F-AChR transfected cells; M + shRNA, αS226F-AChR transfected cells + shRNA; M + NS-shRNA, αS226F-AChR transfected cells + NS-shRNA; WT, wild-type AChR transfected cells; WT + shRNA, wild-type AChR transfected cells + shRNA. (C) Surface AChR expression detected by immunofluorescence. Transfected cells were incubated with mAb G3 and binding detected by FITC-conjugated secondary antibody. Panels show representative images as follows: (a) untransfected cells; (b) αS226F-AChR transfected cells; (c) αS226F-AChR transfected cells + shRNA; (d) αS226F-AChR transfected cells + NS-shRNA; (e) wild-type AChR transfected cells; (f) wild-type AChR transfected cells + shRNA.
transcript silenced this transcript selectively with respect to the wild-type AChR α subunit transcript with which it had a single nucleotide mismatch at position 9 (Fig. 4B). As determined by $^{125}$I-$\alpha$-BuTx binding, the expression of mutant $\alpha$S226F transcripts was reduced to 37 ± 7.93% of normalized surface levels, whilst wild-type AChR α subunit expression was only reduced to 83 ± 14.83% of normalized surface levels. This indicated that selective allele-specific silencing of mutant $\alpha$S226F was possible with respect to the wild-type AChR transcript, and that this silencing was of comparable efficiency to that observed earlier using siRNA. Surface AChR expression was also determined by immunofluorescence using mAb G3 that binds to the AChR δ subunit. Immunofluorescence showed a readily detectable reduction in mutant $\alpha$S226F surface expression, and again confirmed qualitatively the observed selective down-regulation of the mutant $\alpha$S226F transcript with respect to the wild-type AChR α subunit (Fig. 4C).

**DISCUSSION**

We demonstrate allele-specific down-regulation of a pathogenic mutant AChR subunit in HEK 293 cells by both synthetic siRNA and transcribed shRNA. A 19 nucleotide targeting sequence was used to direct the gene silencing. We compared silencing of DsRed2 by shRNA with mismatches with respect to the wild-type sequence at positions 9 and 10 and found that the mismatch at position 9 was more efficient at inhibiting silencing. shRNA was therefore designed to target SCCMS mutant mRNA transcript, $\alpha$S226F, with a mismatch for the wild-type sequence at position 9. Co-transfection of AChR cDNAs with this shRNA into mammalian HEK 293 cells resulted in a severe reduction in mutant AChR expression, but with only modest reduction of the wild-type AChR. Our findings demonstrate a comparable efficiency of allele-specific silencing by siRNA or shRNA, illustrating the potential for long-term shRNA expressed in vivo as a therapy in a neurological disorder.

The T7 transcription system has previously been used for the synthesis of shRNA (8,16,18). We show that this simple method can be used for generating shRNA optimised for the silencing of mutant transcripts. Chemical synthesis of siRNA is still costly, and therefore optimizing the silencing efficiency using a T7 driven shRNA system would be cost-effective. This approach is also highly advantageous as it simulates the shRNA transcript that could be generated from a stable expression vector. In our example we were able to extrapolate from the silencing effects found for mismatches in a sequence of DsRed2 to design an efficient shRNA to target the $\alpha$S226F mutation.

Mismatches in several positions within the 19 nucleotide sequence targeted by the siRNA duplex have been reported to eliminate gene silencing. In an initial study a mismatch at position 10 was shown to completely eliminate gene silencing, correlating with evidence that cleavage of the mRNA target sequence occurs at this point (11). However, in subsequent reports mismatches at this position were found to have only a modest effect (8,16,20,21). In addition, double mismatches in the centre of duplex dramatically reduce silencing effects (16,21). In another report mismatches at positions 2 and 9 dramatically reduced the silencing effects (6). Based upon these reports we first showed discrimination between the mutant and wild-type allele by placing the mismatch at position 10 in the siRNA duplex, and then subsequently looked to enhance discrimination through comparing the effects of mismatches at positions 9 or 10.

Testing mismatches at positions 9 or 10 targeting the reporter gene transcript DsRed2 showed that, although both reduce gene silencing, a mismatch at position 9 had the greater effect. Accordingly, a hairpin was designed to target the AChR αS226F mutation and create a mismatch at position 9 with the wild-type sequence. The data from our different experiments suggests that the optimised shRNA with a mismatch at position 9 has enhanced selectivity over the siRNA with a mismatch at position 10 with respect to silencing the mutant versus wild-type sequence.

This study focuses on a mutation underlying the neuromuscular disorder SCCMS as a model system. In this disorder missense mutations prolong AChR ion channel activations causing excess entry of calcium at the postsynaptic side of the neuromuscular synapse. The excess calcium has an excitotoxic effect leading to an ‘endplate myopathy’ with degeneration of the postsynaptic membrane and loss of AChRs (22). In many cases disability is mild, although slowly progressive. A reduction in AChR levels to below 30% of normal is thought to be required to induce muscle weakness (23), therefore a therapeutic approach that resulted in a slight reduction in wild-type expression would be likely to be tolerated. Moreover, even a modest reduction in the levels of the pathogenic subunit is likely to show therapeutic benefits.

Our findings can be potentially extrapolated to allele-specific gene silencing in other dominantly inherited neurological and other disorders, such as familial forms of Parkinson’s disease due to dominantly inherited missense mutations in the alpha synuclein gene (24). Indeed, during submission of this manuscript we note two reports showing allele-specific silencing of mutations in other neurological disorders (25,26). siRNA was used to target mutant TorsinA RNA transcripts containing a three nucleotide (GAG) deletion that commonly underlies DYT1 dystonia (25). Specific silencing for single nucleotide substitutions were assessed by targeting a single-nucleotide polymorphism (SNP) in the MIDI gene (spinocerebellar ataxia type 3), or disease-causing missense mutations in Tau (frontotemporal dementia with parkinsonism linked to chromosome 17) (26). As in our study, the positioning of the mismatch at central positions within the siRNA-generated sequence resulted in specific silencing of the mutant allele, and together these studies provide robust support for the potential of therapy based on siRNA to inherited dominant disorders. Moreover, this approach may also be frequently applicable to the targeting and silencing of RNA transcripts containing an SNP whose presence may correlate with particular dominantly inherited missense mutations, a so-called mutation-independent approach (27). In addition, SNP silencing may be applicable where the SNP itself is linked to increased susceptibility to a particular disease, such as ApoE4 allele in Alzheimer’s disease (28).

**MATERIALS AND METHODS**

**siRNA synthesis**

An siRNA duplex targeted to a 19 nucleotide sequence surrounding the SCCMS mutation $\alpha$S226F ($\alpha$667C→T) was
designed to create a mismatch at position 10 with the wild-type sequence. A two dT overhang was added at the 3’ prime end to stabilise the duplex. Sense and antisense strands, 5’ UGCUUCUUCUUCCUUAC dTdT 3’ and 5’ GUUAAG-AAGAGAAGAGGAC dTdT 3’, were chemically synthesized and obtained ready annealed (Dharmacon Research).

Design of oligonucleotides for the in vitro transcription of shRNA

Oligonucleotides were designed for in vitro transcription of RNA from the T7 promoter. Each was engineered to have a cytosine following the T7 core sequence since T7 initiates transcription with a G. DNA template oligonucleotides (Invitrogen) were 63mers containing a 19 nucleotide sequence homologous to the respective target mRNA. Thus, for example, the oligonucleotide used to synthesise zS226F-shRNA that targets the mutant zS226F is 5’ AAAA GCTCTTCCTCTCTTAACTTGTAGTAAAGAAGAAGAGGAGCTATAGTGAGTCGTTAATAGTGAGTCGTATTA 3’.

In vitro transcription of shRNA

Antisense strand oligonucleotides were mixed with equal molar ratio (100 pmol) of a T7 oligonucleotide primer, 5’ TAATAACGACTCACTATAG 3’. The mix was denatured at 94°C for 5 min, annealed at 37°C for 15 min and the shRNA transcribed using the T7 mMessage mMACHINETM (Ambion), to generate 5’ capped RNA products according to the manufacturer’s instructions. RNA concentrations were estimated through visualization on 7% agarose/TBE gels (see Fig. 2). The RNA product was mixed with an equal volume of annealing buffer [100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM MgCl2 (Dharmacon)] in order to allow the formation of the hairpin.

Cell culture and transfection

HEK 293 cells were grown in six-well tissue culture plates, or six-well tissue culture plates containing 25 mm diameter glass cover slips. A total 3 μg of AChR β-, δ- and γ-subunit cDNAs in combination with either mutant (zS226F) or wild-type AChR γ-subunit cDNAs, mixed at a ratio of 1:1:1:2, were co-transfected with 100 pmol of siRNA or shRNA into HEK 293 cells using polyethyleneimine. A similar protocol was used to co-transfect 1 μg pDsRed2 with ~100 pmol of respective shRNAs.

125I-ß-bungarotoxin binding assay

Surface AChR expression was determined 48 h post-transfection by overlaying the cells in PBS containing 10 nM 125I-ß-bungarotoxin (125I-ß-BuTx) and 1 mg/ml BSA for 30 min. Cells were washed four times with PBS and removed from the plate in 1.25% Triton X-100, in 60 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride and the amount of bound 125I-ß-BuTx determined by gamma counter.

Immunofluorescence histochemistry and microscopy

Cells transfected with the AChR subunits (mutant and wild-type) were immunolabelled with a mAbG3 directed against the AChR δ subunit (15). Forty-eight hours post-transfection cells were washed in PBS, incubated with the primary antibody (1:1000) at room temperature for 1 h. After washing in PBS, cells were incubated in the secondary antibody (1:500), FITC-conjugated goat anti-mouse IgG (Molecular Probes), for 1 h at room temperature. The coverslips were mounted onto microscope slides and surface expression visualised using a Bio-Rad Radiance 2000 MP microscope. The microscopic parameters were fixed for analysing all experiments in order to be able to get a reliable comparison between different expression levels. The conditions were: 10% laser beam, Iris 3, Gain 5, Offset 0.0.

For the pDsRedN2 experiments, cells were grown on cover slips and co-transfected with pDsRedN2 and shRNA as described earlier. Five days after transfection, cells were mounted onto slides with DAPI-containing mounting medium and visualised by fluorescence microscopy. A low powered lens was used to scan for the red fluorescent cells. To compare the difference in expression, we performed semi-quantitative analyses calculating the red fluorescent ratio by counting the cells. A microscopic field was divided into 25 squares. Five squares were selected at random and the number of red and blue fluorescent cells were counted separately using Adobe Photoshop 5 software. The mean of number of fluorescent cells within each of the five squares was multiplied by 5 giving the total number of fluorescent cells per field. The percentage of red fluorescing cells was calculated as: number of reds/numbers of reds + blues × 100. Measurements of the shRNA-transfected cells were normalised to the transfection with the non-specific shRNA in order to control for variation in transfection efficiency.

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