Antisense-Mediated Down-Regulation of the Human Huntingtin Gene

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ABSTRACT

The present study determines whether the expression of the huntingtin gene might be subject to antisense (AS)-mediated down-regulation. A series of AS oligodeoxynucleotides (ODNs) complementary to the huntingtin transcript [i.e., nucleotide (nt) 1 to 15 (ODN III)] were designed and synthesized, and the AS efficacy was investigated by using a combination of in vitro transcription and translation to mimic in vivo conditions. An oligomer directed to nt 1 to 15 (ODN III) markedly reduced the incorporation of [3H]leucine into the huntingtin gene product in a dose-dependent manner (ED_{50} of \approx 11.5 \mu M). ODNs that overlap with ODN III on both 5'- and 3'-flanking regions also produced translation arrest of the huntingtin protein; however, the AS-mediated effect of these ODNs represented \approx 50% of the effect of ODN III. In contrast, an ODN directed to nt 19 to 35 had no AS effect. The efficacy of ODN III also was investigated in an inducible, stably transfected PC-12 cell line expressing a truncated huntingtin exon 1 protein. In accordance with the cell free translation studies, ODN III (1–10 \mu M) markedly decreased the abundance of the huntingtin-green fluorescence fusion protein to 40 to 46% of the control levels. In summary, a series of putative AS candidates were screened for down-regulation of the huntingtin gene, and an ODN molecule directed to the methionine initiation codon was identified with maximum AS effects.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder that causes impairment of cognitive and motor functions, and severe neuronal loss, particularly in the striatum (Martin and Gusella, 1989). The mutation underlying this disease is an expansion of a trinucleotide repeat (CAG) in exon 1 of the gene coding for the huntingtin protein (Huntington’s Disease Collaborative Research Group, 1993). The finding of aggregated polyglutamines in the form of frequently ubiquinated nuclear inclusions in transgenic mice expressing exon 1 of huntingtin with expanded CAG repeats, and in patients with HD, suggests that accumulation of incompletely processed proteins in neurons may play a critical role in the disease (Davies et al., 1995; Dragatsis et al., 2000). This leads to the hypothesis that decreasing the production of huntingtin may delay the accumulation of mutant huntingtin, thereby delaying the onset of the disease and slowing its progression. Although strategies aimed at blocking or decreasing selectively the production of the mutant protein are highly desirable, a more practical approach may be to decrease the production of the product of both alleles. However, although it is known that huntingtin is essential for development (Nasir et al., 1995; Dragatsis et al., 2000), it is unclear whether this protein is essential for neuronal function in adults. The answer to this question is essential in determining whether strategies that reduce levels of both mutant and normal huntingtin are viable therapeutic approaches.

In the present investigation, we have used an antisense (AS)-mediated strategy to determine whether reducing huntingtin protein levels is feasible. Data presented herein suggest that AS molecules directed to the methionine initiation codon of the HD gene down-regulate the expression of the huntingtin transcript.

Experimental Procedures

Materials. The human huntingtin clone 4G4E4.0, containing a normal range CAG repeat of 23, described by Lin et al. (1995), was obtained from Dr. G. Bates (United Medical and Dental School of Guy’s and St. Thomas’s Hospitals, University of London, London, UK). The T3-TNT cell free transcription/translation system, T4 DNA polymerase, T4 DNA ligase, and all restriction endonucleases were...
purchased from Promega (Madison, WI). Oligodeoxynucleotides (ODNs) were custom synthesized at Keystone/Biosource Laboratories (Menlo, CA). The edysine-inducible mammalian expression vectors pIND and pVgRXR, Zeocin, ponasterone (PA), and anti-green fluorescence protein (GFP) antisera were obtained from Invitrogen (San Diego, CA). Horse serum, fetal bovine serum, G418, and Lipofectamine were obtained from Life Technologies, Inc. (Bethesda, MD).

Results

Synthesis of Oligonucleotides. Phosphodiester (PO) ODNs complementary to regions containing the methionine initiation codon and other regions of the exon 1 of the huntingtin transcript were investigated in a stably transfected PC-12 cell line expressing an inducible truncated huntingtin-GFP fusion construct (A. Kazantsev, B. L. Apostol, and L. M. Thompson, unpublished data). After clonal selection of cells expressing high expression levels (A. Kazantsev, B. L. Apostol, and L. M. Thompson, unpublished data). This huntingtin construct, subcloned into the edysine-inducible mammalian expression vector pIND (Invitrogen), encodes the first 17 amino-terminal amino acids (Fig. 1). The expression of clone 839 is directed by the T3 promoter, to transcribe an mRNA containing 222 nt of the 5' untranslated region of human huntingtin and an open reading frame that translates a 10,068-Da human HD product (Kazantsev et al., 1999). Briefly, this clonal line named PC12 pIND 17 A.2 was derived from stable transfection of the huntingtin construct into a clonal pVgRXR expressing PC-12 cell line by using lipofectin (Life Technologies, Inc.). This PC-12 cell line had been previously stably transfected with pVgRXR (Invitrogen), a hybrid edysine receptor, and selected for high receptor expression levels (A. Kazantsev, B. L. Apostol, and L. M. Thompson, unpublished data). After clonal selection of cells expressing high inducible levels of huntingtin protein following induction with 5 μM PA, determined by Western blot analysis, cells were maintained in Dulbecco's modified Eagle's medium (high glucose) containing 5% horse serum, 2.5% fetal bovine serum, 0.2 mg/ml Zeocin, and 100 μg/ml G418 (Life Technologies, Inc.). For the AS studies, cells were plated in 6-well cluster dishes at low density, and neurite formation was induced with 50 ng/ml rat recombinant NGF-β (NGF-β) and other molecular biology grade reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Construction of an expression vector containing the human huntingtin exon 1 nt (3345) of the EcoRI 5' fragment of human HD gene clone 4G4E4.0 (under 54°C) was deleted by double digestion with HindIII and SfiI to form a human huntingtin exon 1 transcription plasmid named clone 839 (top right). The expression of clone 839 is directed by the T3 promoter, to transcribe an mRNA containing 222 nt of the 5'-untranslated region of human huntingtin and an open reading frame that translates a 10,068 Da human HD product corresponding to exon 1 (bottom). Number of nt is indicated on the left-hand side. The number 1 indicates the αtG methionine initiation codon, +, the stop codon tga.

Cell Culture Experiments. The efficacy of AS oligonucleotides complementary to the huntingtin transcript was determined by using a combination of in vitro transcription and translation that mimics in vivo conditions, as recently described for the Alzheimer's β-amyloid precursor protein APP695 (Boado et al., 1998). Transcription/translation of huntingtin clone 839 was performed in the presence of T3 RNA polymerase, 2 μCi of [35S]methionine, or 5 μCi of [3H]leucine with 50% v/v rabbit reticulocyte lysate according to the manufacturer's instructions (Promega). After incubation, translated huntingtin exon 1 was analyzed by trichloroacetic acid precipitation and SDS-polyacrylamide gel electrophoresis (Boado et al., 1998). Incubations were performed in triplicates and results expressed as mean ± S.E.

Table 1

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence Nt.</th>
<th>Huntingtin Exon 1</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>5'-TCTCCCGCCCGGCAC-biotin-3'</td>
<td>25 to 6</td>
</tr>
<tr>
<td>II</td>
<td>5'-GCCATGGCGGCTTCC-biotin-3'</td>
<td>10 to 5</td>
</tr>
<tr>
<td>III</td>
<td>5'-TCCAGGCTGGCAGCAG-biotin-3'</td>
<td>1 to 15</td>
</tr>
<tr>
<td>IV</td>
<td>5'-GACAGCTGCTTCCAGG-biotin-3'</td>
<td>8 to 25</td>
</tr>
<tr>
<td>V</td>
<td>5'-GACAGCTGCTTCCAGG-biotin-3'</td>
<td>17 to 35</td>
</tr>
</tbody>
</table>

* Nt. 1 corresponds to A residue of the ATG methionine initiation codon.
sion of huntingtin-GFP, whereas lower concentration (5 and 10 μl/dish) only induced 51 to 54% of the effect seen with 20 μl/dish LipofectAMINE. Cells were rinsed with PBS twice and lysed in 250 μl of sample buffer (2% SDS, 60 mM Tris, pH 6.8). Proteins were measured by using the bicinchoninic acid protein assay system (Pierce, Rockford, IL), and glycerol and 2-mercaptoethanol were added to a final concentration of 10 and 5%, respectively, before loading soluble protein onto gels. Samples were denatured for 2 min in boiling water, cooled down on ice, and resolved in a 12% SDS-polyacrylamide gel electrophoresis, following by electroblotting onto Immobilon P filters for Western blotting analysis. Immunoblot analysis was carried out by using chemiluminescence with an anti-GFP antiserum as primary antibody, and peroxidase-labeled goat anti-rabbit IgG as secondary antibody by using the Phototope-HRP detection system (New England Biolabs, Boston, MA) as described previously (Boado et al., 1999). Blots were quantified by scanning densitometry of films with the NIH Image 1.54 program on a Macintosh PowerBook 3400c microcomputer as previously described (Boado et al., 1996). These studies allow for identification of both the molecular weight and the abundance of the immunoreactive protein corresponding to the huntingtin-GFP.

Results

To monitor incorporation of [35S]methionine or [3H]leucine into huntingtin protein, dose-response studies with increasing amounts of plasmid encoding the huntingtin exon 1 pro-

Fig. 2. Translation arrest of the human wild-type huntingtin transcript with AS ODNs. Transcription/translation of huntingtin clone 839 (Fig. 1) was performed in the presence of T3 RNA polymerase, 2 μCi of [35S]methionine, or 5 μCi of [3H]leucine with 50% v/v rabbit reticuloocyte lysate in the presence or absence of ODNs shown in Table 1 followed by trichloroacetic acid precipitation. A, dose-response studies with [35S]methionine (Met) or [3H]leucine (Leu) indicating that these amino acids are incorporated into the huntingtin exon 1. B, dose-response and time course studies with clone 839 and [3H]Leu demonstrate that Leu is incorporated in a dose-dependent manner. Symbols indicate DNA concentrations used. C and D, translation arrest of huntingtin exon 1 with AS ODNs by using 0.5 μg of expression plasmid clone 839. AS ODN III directed to nt −1 to 15 (Table 1; Fig. 1) markedly reduced the incorporation of [3H]leucine into the HD gene product in a dose-dependent manner (ED50 of ~11.5 μM; Fig. 2, C and D). In contrast, ODN V had no inhibitory effect on the translation of
huntingtin protein (Fig. 2, C and D). ODNs directed against regions I, II, and IV also produced translation arrest of huntingtin exon 1 (Fig. 2C). However, the AS effect of these latter ODNs represented ~50% of the effect of ODN III (Fig. 2C). The results with ODN III also were reproduced by using film autoradiography (data not shown).

The efficacy of AS oligonucleotides complementary to the huntingtin transcript in arresting translation of the HD gene product also was investigated in an inducible PC-12 cell model that expresses a truncated huntingtin exon 1 with 25 glutamines (Fig. 3). PC-12 cells are derived from a rat pheochromocytoma and develop a neuronal phenotype upon addition of growth factors. The production of a single band of ~34 kDa corresponding to the huntingtin-GFP fusion protein was observed after induction of differentiated cells with PA (Fig. 3A). In contrast, the huntingtin-GFP fusion protein was undetected in cells incubated in the absence of PA (Fig. 3A). The calculated molecular weight of the fusion protein correlates with the predicted molecular weight based on the amino acid sequence (mol. wt. = 32.2 kDa). ODN III markedly decreased the abundance of the huntingtin-GFP fusion protein to 40 to 46% of the control levels by using scanning densitometry of Western blots (Fig. 3, B and C). The AS effect of ODN III upon protein levels was seen with concentrations of oligomer ranging from 1 to 10 µM (Fig. 3C). In accordance with the cell free translation studies, ODN V produced no AS effect on the expression of the huntingtin-GFP (data not shown), indicating that protein levels are not affected by the presence of ODNs in a nonspecific manner. PA does not induce nonspecific gene expression (No et al., 1996) and incubation of the clonal line PC12 pIND 17 A.2 with this agent for 48 h had no effect on total cellular protein levels (e.g., -PA, 725 ± 125; +PA, 750 ± 50 µg of protein/dish, respectively; mean ± S.E., n = 4 and 12, respectively)). Similarly, in another representative experiment, lipofection of cells with ODN III produced no changes in either cell density or in total cellular proteins (~ODN, 475 ± 0; +ODN, 500 ± 15 µg of protein/dish, n = 3, respectively).

**Discussion**

The data presented herein suggest that the expression of the HD gene may be down-regulated with AS oligomers directed to the methionine initiation codon of huntingtin (Figs. 2 and 3). This conclusion is consistent with the following observations. First, ODN III, which is complementary to nt -1 to 15 surrounding the ATG initiation codon, produced translation arrest of the HD gene with an ED_{50} of ~11.5 µM. Second, this effect of ODN III was unrelated to nonspecific effects on translation because ODN V directed to nt 19 to 35 did not down-regulate the expression of huntingtin exon 1 (Fig. 2), suggesting that ODN III exerts translation arrest of the HD gene in a sequence-specific manner. Third, ODNs directed to regions II and IV (nt -10 to 5 and 8 to 25, respectively), which possess 8 and 9 nt overlap with ODN III, respectively, produced approximately 50% of the translation arrest produced by ODN III. Fourth, ODN I, which targets a region 5’ upstream from ODN III (nt -25 to -6), also induced partial inhibition of huntingtin expression compared with ODN III (~50%).

The expression of the huntingtin-GFP fusion protein in a clonal PC-12 cell line may represent an interesting model to investigate the etiology of the HD and for development of potential therapeutics. For example, approximately 10% of these cells show aggregate formation in the form of perinuclear, cytoplasmic, and nuclear inclusions 3 to 5 days after PA induction (A. Kazantsev, B. L. Apostol, and L. M. Thompson, unpublished data). Stimulation of ecdysone-inducible gene expression with PA produces levels of induction that are 4 orders of magnitude higher than that of uninduced samples (No et al., 1996). These high levels of gene induction likely do not reflect the activity of the HD promoter activity in vivo. Therefore, it is hypothesized that the AS effect demonstrated in this cell culture model of HD (Fig. 3) may be even more effective in vivo on the expression of an endogenous HD gene.

The design of AS molecules to specifically target the expanded repeat in huntingtin and not the normal short repeat may represent an ideal approach for AS-based therapeutics for HD. Data presented herein provide evidence for AS-mediated down-regulation of the HD gene, providing the basis

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**Fig. 3.** AS-mediated down-regulation of huntingtin exon 1 in PC-12 cells. The clonal line PC12 pIND 17 A.2 stably transfected with an inducible plasmid encoding a truncated huntingtin-GFP fusion protein was cultured and neurite formation was induced with 50 ng/ml rat recombinant NGF-β for 5 days. ODNs were delivered to the cells by lipofection by using LipofectAMINE. The expression of the huntingtin-GFP fusion protein was induced with 5 µM PA in the culture medium 6 h after lipofection. The abundance of the huntingtin-GFP protein was determined 20 h after lipofection by Western blot analysis by using an anti-GFP antisera as primary antibody and chemiluminescence followed by scanning densitometry. A, 25 µg of lysate from uninduced (-) cells and cells PA-induced for 48 h were loaded per lane. Kodak X-Omat film was exposed for 30 s at 22°C. The production of a single band of ~34 kDa corresponding to the induced huntingtin-GFP fusion protein is seen after induction of cells with PA. B, cells were transfected and incubated for 20 h with 0 or 10 µM ODN III. The expression of huntingtin-GFP was induced with 5 µM PA as described above. Western blot analysis was performed with 25 µg of protein lysate and the film was exposed for 1 min at 22°C (top). ODN III markedly decreased the abundance of the 34-kDa huntingtin-GFP fusion protein to 40 to 46% of the control levels. Each column represents mean ± S.E. (n = 4). C, dose-response study with ODN III. Cells were transfected with graded doses of ODN III and induced with PA as described in B. ODN III markedly reduced the abundance of the huntingtin-GFP fusion protein, and concentrations of 1 µM produced maximum effect. Results are expressed as percentage of control (0 µM ODN), and each point represents mean ± S.E., n = 6. * indicates P < .01 versus control (ANOVA, Scheffe’s test).
for further development of AS therapeutics for HD. The delivery of AS-based therapeutics to the brain, however, is compromised by the poor stability of ODN in vivo, rapid renal clearance, and by the presence of the blood-brain barrier (BBB), which is only permeable to lipophilic molecules of <600 Da (Boado, 1995). Progress in the development of stable AS oligomer delivery to the brain through the BBB was recently reviewed (Boado, 1995; Boado et al., 1998). Biotinylation of PO-ODN at the 3’ terminus has been shown to produce complete protection of ODN against serum and cellular 3’ exonuclease-mediated degradation (Wu et al., 1996). Although stable, PO-ODNs are poorly transported across the BBB into the brain by using a delivery vector such as the OX26 anti-rat transferrin receptor antibody conjugated to streptavidin (SA), due to the strong binding of these oligomers to plasma protein (Wu et al., 1996). When administered intracerebrally, PO-ODNs are neurotoxic (Wojcik et al., 1996; Boado et al., 1998), and they failed to produce sequence specific effects on the HD gene in CD-1 mice (Haque and Isacson, 1997), probably due to non-specific binding to cellular proteins (Boado et al., 1998). Replacement of the deoxyribose/phosphate linkage by a polyamid backbone produces peptide nucleic acids (PNAs), molecules that are resistant to protease degradation and possess high affinity for RNA or single-stranded DNA compared with conventional ODNs (Nielsen et al., 1994). PNAs biotinylated at the amino-terminal group can be transported into the brain by the OX26-SA delivery system with levels of brain uptake that are comparable to that of morphine (Partridge et al., 1995). In addition, biotinylated PNAs recognize and inactivate target mRNA while bound to the delivery vector, suggesting that PNA-OX26-SA conjugates represent optimal AS molecules for further development of AS therapeutics for HD (Boado, 1995; Boado et al., 1998).

In summary, the present study has developed tools for investigating the effect of AS oligonucleotides on the expression of the HD gene, and demonstrated that this gene is susceptible to translational arrest in a cell free translation system. A series of putative AS candidates have been screened for their ability to down-regulate the HD gene, and an ODN molecule directed against the methionine initiation codon of huntingtin producing maximal AS effects has been identified. The AS effect of this molecule also was shown to reduce the production of a truncated huntingtin-GFP fusion protein by transfection into an edcysone-inducible tissue culture model of HD.

References


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