Suppression of neuropil aggregates and neurological symptoms by an intracellular antibody implicates the cytoplasmic toxicity of mutant huntingtin

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Mutant huntingtin accumulates in the neuronal nuclei and processes, which suggests that its subcellular localization is critical for the pathology of Huntington’s disease (HD). However, the contribution of cytoplasmic mutant huntingtin and its aggregates in neuronal processes (neuropil aggregates) has not been rigorously explored. We generated an intracellular antibody (intrabody) whose binding to a unique epitope of human huntingtin is enhanced by polyglutamine expansion. This intrabody decreases the cytotoxicity of mutant huntingtin and its distribution in neuronal processes. When expressed in the striatum of HD mice via adenoviral infection, the intrabody reduces neuropil aggregate formation and ameliorates neurological symptoms. Interaction of the intrabody with mutant huntingtin increases the ubiquitination of cytoplasmic huntingtin and its degradation. These findings suggest that the intrabody reduces the specific neurotoxicity of cytoplasmic mutant huntingtin and its associated neurological symptoms by preventing the accumulation of mutant huntingtin in neuronal processes and promoting its clearance in the cytoplasm.

Introduction

The expansion of a polyglutamine (polyQ) tract in mutant proteins causes Huntington’s disease (HD) and eight other known neurodegenerative diseases, including spinocerebellar ataxia and spinobulbar muscular atrophy (Zoghbi and Orr, 2000). The accumulation of expanded polyQ-containing proteins in the nucleus and the subsequent formation of nuclear inclusions are pathological hallmarks of these diseases (Gatchel and Zoghbi 2005; Butler and Bates, 2006). In the majority of polyQ diseases, the mutant proteins carry nuclear localization sequences and are therefore localized primarily in the nucleus. However, huntingtin (htt), a 350-kD protein with a polyQ domain in its N-terminal region, is predominantly localized in the cytoplasm. Generation of polyQ-containing N-terminal htt fragments by proteolysis leads to the accumulation of toxic peptides (Ellerby and Orr, 2006) that also form aggregates in the nucleus and the neuronal processes (neuropil aggregates), which include axons and dendrites (DiFiglia et al., 1997; Gutekunst et al., 1999; Lunkes et al., 2002; Wellington et al., 2002; Graham et al., 2006). Furthermore, polyQ expansion clearly causes protein misfolding and conformational alteration, leading to abnormal protein interactions and transcriptional dysregulation in the nucleus (Zoghbi and Orr, 2000; Sugars and Rubinsztein, 2003; Li and Li, 2004; Butler and Bates, 2006). Notably, the brains of HD patients at the early stage of disease contain more neuropil aggregates than nuclear inclusions (Gutekunst et al., 1999). Also, the progressive formation of neuropil aggregates is correlated with disease progression in transgenic mice (Li et al., 1999, 2000; Schilling et al., 1999; Tallaksen-Greene et al., 2005), and these aggregates are associated with axonal degeneration in HD mouse models (Li et al., 2001; Yu et al., 2003). Given the abundance of neuropil aggregates in HD patient brains, understanding the contribution of cytoplasmic htt to HD pathology is imperative.

Unlike nuclear inclusions, neuropil aggregates have not been studied extensively because of their small size. Although...
Generation of an intrabody that preferentially binds mutant htt

Previously, we used a GST fusion protein containing the first 256 amino acids of human htt as the antigen to generate rabbit antibody EM48 (Fig. 1 A). This antigen lacks the polyQ and polyP domains in this antigen. The black box indicates two glutamines (2Q). Amino acid numbers are indicated. (B) mEM48 immunostaining of the hippocampal sections from HD transgenic (R6/2) and wild-type (WT) mice at 12 wk of age. Note that the antibody specifically reacts with intranuclear mutant htt aggregates and smaller neuropil aggregates outside the nucleus. Bar, 5 μm. (C) 35S-labeled N-terminal htt (aa 1–208) fragments containing various glutamine repeats (23, 44, 75, and 130Q) were synthesized in vitro, resolved by SDS-PAGE, and revealed by autoradiography (24 h exposure). The same blot was then probed with mEM48 and visualized with ECL reagents (1 min exposure). Note that mEM48 preferentially reacts with N-terminal htt containing longer repeats.

Results

Generation of an intrabody that preferentially binds mutant htt

In the present study, we developed an intracellular antibody (intrabody) based on a compelling feature of one unique htt antibody, EM48, which preferentially reacts with mutant htt (Gutekunst et al., 1999; Graham et al., 2006). This intrabody, when expressed in neurons, reduces the cytotoxicity of N-terminal mutant htt and decreases both the formation of neuropil aggregates and the neurological symptoms of HD mice. We further demonstrated that this intrabody promotes the degradation of cytoplasmic mutant htt by increasing its ubiquitination. These findings suggest that the intrabody specifically targets mutant htt with abnormal conformation and can serve as a valuable tool to specifically reduce the cytoplasmic neuropathology of HD.

the role of htt aggregates remains controversial (Saudou et al., 1998; Yamamoto et al., 2000; Arrasate et al., 2004; Chang et al., 2006), subcellular localization seems to be critical for the effects of mutant htt and its aggregates. Given the limited confines of neuronal processes, it is conceivable that neuropil aggregates are sizeable enough to physically block intracellular transport. In any case, the formation of neuropil aggregates does reflect the transport and accumulation of toxic htt fragments in neuronal processes and allows us to investigate the toxic effects of cytoplasmic mutant htt in the unique neuronal structure. The normal function of neuronal processes is dependent on the proper transport of proteins and nutrients from the cell body to nerve terminals and may be more vulnerable than nuclear function to a variety of insults. Understanding the effects of cytoplasmic mutant htt in neuronal processes would be useful in the development of an effective treatment strategy for HD patients.

In the present study, we developed an intracellular antibody (intrabody) based on a compelling feature of one unique htt antibody, EM48, which preferentially reacts with mutant htt (Gutekunst et al., 1999; Graham et al., 2006). This intrabody, when expressed in neurons, reduces the cytotoxicity of N-terminal mutant htt and decreases both the formation of neuropil aggregates and the neurological symptoms of HD mice. We further demonstrated that this intrabody promotes the degradation of cytoplasmic mutant htt by increasing its ubiquitination. These findings suggest that the intrabody specifically targets mutant htt with abnormal conformation and can serve as a valuable tool to specifically reduce the cytoplasmic neuropathology of HD.
more mEM48 immunoreactivity, despite their lesser amounts on the blot (Fig. 1 C).

This increased immunoreaction as a result of polyQ expansion led us to clone the cDNA for mEM48 from its hybridoma cell line, with the aim of expressing this recombinant antibody in cells. We joined cloned cDNAs encoding variable light (V_L) and variable heavy (V_H) chains of mEM48 via an oligonucleotide linker, generating a 750-bp cDNA that encodes a single-chain Fv (scFv) for mEM48. This intrabody (scFv-EM48) was tagged with the HA epitope and expressed under the cytomegalovirus (CMV) promoter (Fig. 2 A).

When coexpressed with an N-terminal mutant htt (1–208 aa) containing 130Q, scFv-EM48 colocalized with htt aggregates in both cultured HEK293 cells (Fig. 2 B) and primary cortical neurons (Fig. 2 C). Coexpression of scFv-EM48 and mutant htt in cultured rat cortical neurons (arrows) also shows the colocalization of scFv-EM48 with mutant htt. In B and C, cells were immunostained with mouse anti-HA (12CA5) and rabbit EM48. Bars, 10 μm. (D) Western blot analysis of immunoprecipitates from transfected HEK293 cells that express scFv-EM48-HA and exon1 htt with 130Q or 20Q. scFv-EM48 [scFv] was precipitated by the antibody (12CA5) to the HA epitope (bottom). The input and precipitates (IP) were then probed with rabbit EM48 (top). The arrow indicates soluble htt-20Q and the arrowhead indicates htt-130Q. The bracket indicates the stacking gel in which aggregated htt is seen. (E) Densitometry of the ratio of precipitated htt to input from a representative experiment. Similar results were seen in two separate experiments.
in transfected HEK293 cells (Fig. 2 D). Some aggregated htt, which remained in the stacking gel, was coprecipitated with the scFv-EM48 as well, a result that is in agreement with our immunocytochemistry data showing that scFv-EM48 is present in some htt aggregates. More importantly, there was very little normal htt in the immunoprecipitates, despite the fact that a substantial level of transfected normal htt could be detected by rabbit EM48. Densitometry of the ratio of precipitated/input amount also shows that scFv-EM48 binds only weakly to normal htt, whereas it preferentially binds to mutant htt.

The preferential binding of the intrabody to mutant htt proves that the immunoreaction of mEM48 with htt is enhanced by polyQ expansion. Because mEM48 does not react with mouse htt and was generated with an antigen (2Q-htt) that lacks the polyQ and polyP tracts, we reasoned that comparison of the N-terminal sequences of 2Q-htt and htt from human and mouse might provide insight regarding the epitope for mEM48 (Fig. 3 A). Because scFv-EM48 reacts with mutant htt exon1, we focused on the amino acid sequences in exon1 and found that two amino acid residues (VA) located after the polyP tract are present in both human htt and 2Q-htt but not mouse htt. To examine whether mouse mutant htt that carries an expanded polyQ tract also fails to react with mEM48, we performed mEM48 Western blotting of brain tissues from YAC128 mice, which express full-length human mutant htt, and from homozygous (Hom) or heterozygous (Het) HD 150Q knock-in (KI) mice, which express full-length human mutant htt. Note that the 1C2 antibody recognizes mouse and human mutant htt, whereas mEM48 only recognizes human mutant htt. (C) Htt sequences shown in A were used to synthesize N-terminal (N), human, mouse, or C-terminal (C) peptides. These peptides were applied to the dot blot at different concentrations (1–100 μM), and the blot was subsequently probed with mEM48. Only the human peptide reacts with mEM48. (D) PC12 cell lysates containing transfected GFP-htt (1–208 aa with 130Q) were immunoprecipitated by mEM48 in the presence of the human or mouse peptide (10 μM). The blot was probed with rabbit EM48. Only the human peptide blocks the immunoprecipitation. Control, immunoprecipitation without peptides. (E) Immunoprecipitation of scFv-EM48-HA in transfected PC12 cells by mouse anti-HA. The blot was probed with mEM48 for the coprecipitated GFP-htt-130Q (top, Htt) and with anti-HA for scFv-EM48 (bottom). The human but not the mouse peptide markedly inhibited the coprecipitation of htt.
approximately 60–70% of HEK293 cells expressed transfected proteins (unpublished data). Using SYTOX green, which labels dying cells, we observed that scFv-EM48 reduced the number of htt-transfected cells stained with SYTOX green compared with cells transfected with mutant htt and vector (Fig. 4, A and B). We also examined the viability of htt-transfected cells using the MTS assay. The expression of htt-130Q reduced cell viability compared with htt-23Q transfection. However, this decrease in viability was improved by coexpression of scFv-EM48 (Fig. 4 B).

Figure 4. scFv-EM48 suppresses cytotoxicity of mutant htt. (A and B) HEK293 cells were transfected with htt (1–208 aa) containing 23Q or 130Q and scFv-EM48 (+scFv). Cotransfection with a vector served as a control. SYTOX green staining of transfected HEK293 cells is shown in A, in which greater fluorescence is correlated with increased cell death. In B, the relative fluorescence values of SYTOX green-containing cells were obtained from six transfection experiments. Cell viability was also measured by a modified MTT assay (MTS) and expressed as absorbance at 490 nm (OD490 nm absorbance values; n = 9, P < 0.05). The decreased MTS correlates with reduced cell viability. (C) Immunofluorescent staining of cultured rat cortical neurons (7 days in vitro) that express transfected htt (htt-23Q or htt-130Q) or scFv-EM48 alone (top). Htt was labeled by rabbit EM48, and scFv-EM48-HA was labeled by antibody to the HA epitope. Note that mutant htt formed neuritic aggregates and caused neuritic fragmentation in transfected cells. Coexpression of scFv-EM48 reduced htt aggregation in neurites and degeneration of cultured neurons (bottom). Nuclei were stained with Hoechst blue dye in the merged images. (D) The percentage of cells showing disrupted neurites and fragmented nuclei. Data were obtained by counting 156–198 transfected cells in three transfection experiments. The data are presented as means ± standard error. *, P < 0.05; **, P < 0.01. Bars: (A) 100 μm; (C) 8 μm.

Intrabody inhibits htt cytotoxicity
If scFv-EM48 indeed binds tightly to mutant human htt, it may prevent htt from binding to other proteins, thereby reducing htt toxicity. We transfected HEK293 cells with N-terminal htt (1–208 aa) that contained either 23Q or 130Q. scFv-EM48 was also coexpressed to assess its protective effect. Approximately 60–70% of HEK293 cells expressed transfected proteins (unpublished data). Using SYTOX green, which labels dying cells, we observed that scFv-EM48 reduced the number of htt-transfected cells stained with SYTOX green compared with cells transfected with mutant htt and vector (Fig. 4, A and B). We also examined the viability of htt-transfected cells using the MTS assay. The expression of htt-130Q reduced cell viability compared with htt-23Q transfection. However, this decrease in viability was improved by coexpression of scFv-EM48 (Fig. 4 B).
Intrabody suppresses neuropil aggregates in HD mouse brain

Although the results in Fig. 4 established the protective effects of scFv-EM48 in cultured cells, whether scFv-EM48 could confer protection in HD mouse brains remained to be seen. To test the effects of scFv-EM48 in the brain, the protein must be delivered via viral infection and microinjection of a small volume (1–2 μl per injection). However, conventional adenoviral and lentiviral vectors failed to yield a sufficiently high titer of viral scFv-EM48. Intrabody instability

To examine whether scFv-EM48 also reduces the neurotoxicity of mutant htt, we cotransfected scFv-EM48 with htt-130Q in cultured rat cortical neurons. Similar to previously published findings (Saudou et al., 1998; Li et al., 2001), mutant htt could lead to neuritic disruption (Fig. 4C) and pyknotic nuclei (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200710158/DC1). Expression of scFv-EM48 alone did not affect the morphology of cultured neurons, but its coexpression with htt-130Q significantly reduced the number of htt-transfected neurons with disrupted neurites or fragmented nuclei (Fig. 4, C and D; and S3).
in cells and viral toxicity very likely impose a considerable obstacle to using these viral vectors. Next, we tried a helper-dependent adenoviral vector in which the viral coding sequences have been replaced with noncoding human genomic DNA (Toietta et al., 2002, 2005). The elimination of all viral protein-coding sequences in this vector results in less cytotoxicity and long-term expression of transgenes compared with the earlier-generation adenoviral vectors (Toietta et al., 2002, 2005). Western blot analysis confirmed that this adenoviral vector expressed scFv-EM48 protein of the correct size in cultured cells (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200710158/DC1).

Using immunofluorescence double-labeling with antibodies to the HA epitope and to a neuronal marker (NeuN), we first confirmed that scFv-EM48 was expressed in neurons after microinjection into the mouse brain (Fig. 5 A). Unlike transfected cells, in which mutant htt can form large inclusions in the cell bodies, neurons in HD brains show aggregates in the nucleus and neuronal processes (DiFiglia et al., 1997; Gutekunst et al., 1999; Li et al., 1999). After confirmation of neuronal scFv-EM48 expression, we next injected adenoviral scFv-EM48 into the striatum of 7-wk-old R6/2 mice. At this age, R6/2 mice begin to develop prominent htt nuclear and neuropil aggregates, and stereotaxic injection into the mouse striatum becomes feasible. 4 wk after the procedure, we found that the number of neuropil aggregates in the injected area had been reduced dramatically when compared with the noninjected region (Fig. 5 B). There appeared to be a slight reduction of nuclear staining as well, but this difference was difficult to quantify by immunostaining.

We also injected adenoviral scFv-EM48 into N171-82Q mice. This HD mouse model expresses the first 171 amino acids of human htt with 82Q and also demonstrates obvious neuropil aggregates, and stereotaxic injection into the mouse striatum becomes feasible. 4 wk after the procedure, we found that the number of neuropil aggregates in the injected area had been reduced dramatically when compared with the noninjected region (Fig. 5 B). There appeared to be a slight reduction of nuclear staining as well, but this difference was difficult to quantify by immunostaining.

To verify the specificity of adenoviral scFv-EM48, we also injected mice from both strains with a control virus that does not express scFv-EM48. We quantified the neuropil aggregate density per image (630×) and normalized this quantity by the density of neuropil aggregates in the noninjected contralateral region. This method allowed us to mitigate the influence of variations in immunostaining and brain section preparation. Compared with control virus, injection of adenoviral scFv-EM48 significantly reduced neuropil aggregates in R6/2 and N171-82Q mouse brains (P < 0.01; n = 10 for adenoviral scFv-EM48 vs. n = 6 for control; Fig. 5 D).

Both R6/2 and N171-82Q mice widely express mutant htt in various brain regions. As a result, they develop severe neurological symptoms and early death by the age of 3–6 mo (Davies et al., 1997; Schilling et al., 1999). However, inhibiting the expression of transgenic htt via siRNA in the striatum of N171-82Q mice, which exhibit a slower disease progression than R6/2 mice, leads to an improvement in their motor function (Harper et al., 2005). Therefore, we speculated that N171-82Q mice would also be good candidates for testing the in vivo protective effects of scFv-EM48 after its delivery to the striatum. To this end, we bilaterally injected adenoviral scFv-EM48 into the striatum (1.25 μl per site) of 10-wk-old N171-82Q mice. As a control, HD mice were injected with the same amount of adenoviral vector. After 8 wk, we observed significant improvements in gait function for the HD mice that had been injected with adenoviral scFv-EM48 (Fig. 6, A and B). Measures included stride length (50.82 ± 1.95 mm [n = 11] for scFv-EM48 vs. 42.8 ± 3.1 mm [n = 9] for control virus; P < 0.05). Also, the RotaRod performance of HD mice was improved. For example, the times on the RotaRod were 93.7 ± 4.32 s (n = 10) for scFv-EM48–injected mice and 59.8 ± 1.39 s (n = 9) for control virus–injected mice (P < 0.05) on trial day 3 (Fig. 6 C). However, we did not observe a significant improvement in body weight or survival of N171-82Q mice injected with adenoviral scFv-EM48 (Fig. 6 D). The selective protection of scFv-EM48 against motor deficits is likely caused by the limited expression of this intrabody in the striatum and its selective effect on cytoplasmic mutant htt. This finding also suggests that the expression of mutant htt in the nucleus and in brain regions other than the striatum can contribute to severe neurological phenotypes.

We also performed immunofluorescence double-labeling of viral-infected PC12 cells and found that scFv-EM48 decreased the number of htt aggregates in neurites (Fig. 7, A and B). To verify that scFv-EM48 reduces the distribution of soluble mutant htt in the neurites, we measured the ratio of htt’s neuritic signal to its cell body signal (McGuire et al., 2006). As expected, scFv-EM48 significantly decreased the number of cells with neuritic aggregates (38.8 ± 3.7% for vector control vs. 9.2 ± 0.5% for scFv-EM48; n = 3; P < 0.01). Also, the relative neuritic distribution of mutant htt in PC12 cells was reduced by scFv-EM48 (ratio of neuritic signal/cell body signal: 0.35 ± 0.06 for scFv-EM48 vs. 0.82 ± 0.04 for vector control; n = 10; P < 0.01; Fig. 7 C). These findings are consistent with the in vivo inhibition of neuropil aggregates by scFv-EM48 in HD mouse brains (Fig. 5) and in cultured cortical neurons (Fig. 3).

Intrabody promotes the degradation of mutant htt

The significant reduction of neuropil aggregates and the improvement in neurological symptoms associated with scFv-EM48 led us to explore the mechanism underlying this protection. The decreased amount of neuropil aggregates in HD mouse brains could be caused by the reduced distribution of mutant htt to neuronal processes and/or increased htt clearance in the cytoplasm. We therefore fractionated synaptosomes from the striatal tissue of N171-82Q mice, in which the protein level of mutant htt is comparable to endogenous htt (Schilling et al., 1999). As shown previously (Schilling et al., 1999), a cleavage product smaller than transgenic mutant htt (∼47 kD) is visible in the brain lysates of HD mice. In HD striatal tissues expressing scFv-EM48, this cleaved product and two additional smaller products (Fig. 8 A, arrowheads) are more abundant than in those brains injected with control adenovirus. This difference suggests that the interaction of the intrabody with mutant htt in the cytosolic region may facilitate the degradation
The increased htt degradation in synaptosomes of HD mice injected with adenoviral scFv-EM48 led us to test the possibility that scFv-EM48 can promote the degradation of mutant htt. We coexpressed adenoviral vector or scFv-EM48 with GFP htt-130Q (1 – 208 aa with 130Q) in cultured PC12 cells. GFP htt-130Q is mainly distributed in the cytoplasm and forms cytoplasmic aggregates. Thus, cytoplasmic proteins of PC12 cells were isolated as soluble and nonsoluble or pellet fractions, with the expectation that the latter would be enriched for aggregated htt or htt that is bound tightly to the intrabody. Total cell lysates containing scFv-EM48 show more degraded htt products than control cell lysates (Fig. 8 B). Consistent with the fact that more scFv-EM48 was present in the pellet fraction than in the soluble fraction, more degraded htt products were also of mutant htt. Importantly, compared with the samples from HD mice that were injected with adenoviral vector, the ratio (mean ± SEM, n = 3) of either soluble (control vs. scFv-EM48: 0.528 ± 0.04 vs. 0.279 ± 0.03, respectively) or oligomeric (1.28 ± 0.11 vs. 0.77 ± 0.06) htt to the synaptic protein syntaxin is decreased in the synaptosomal fractions of HD mice that had been injected with adenoviral scFv-EM48 (Fig. 8 A, bottom). Because the intrabody cannot enter the nucleus to affect nuclear mutant htt, there was no significant difference in the levels of aggregated htt in nuclear fractions of HD mice injected with adenoviral control vector or scFv-EM48 (Fig. 8 A). Thus, this in vivo evidence also indicates that scFv-EM48 can reduce the distribution of mutant htt in nerve terminals in HD mouse brains.
detected in the pellet fraction, which suggests that the association of scFv-EM48 with mutant htt is required for the degradation of mutant htt. We then performed pulse-chase experiments to measure the half-life of mutant htt. In the presence of scFv-EM48, the half-life of mutant htt is obviously shorter than in the absence of scFv-EM48 (Fig. 8 C). For example, at 4 h after chasing, 46.9 ± 2.62% of radiolabeled mutant htt remained in the presence of scFv-EM48 compared with 74.1 ± 3.2% (P < 0.05, n = 3) of mutant htt in the absence of scFv-EM48 (Fig. 8 C). It is possible that the binding of scFv-EM48 alters the conformation of mutant htt and leads to its degradation via the ubiquitin–proteasome system. If true, we should see increased ubiquitination of mutant htt in the presence of scFv-EM48. Because the cytoplasmic nonsoluble or pellet fraction is enriched in mutant htt and scFv-EM48 without detectable tubulin (Fig. 8 B), we probed this fraction with an antibody to ubiquitin and observed an obvious increase in the amount of ubiquitinated products (Fig. 8 D, left). Immunoprecipitation of htt by EM48 also shows more ubiquitinated products in the presence of scFv-EM48 (Fig. 8 D, right). These findings are consistent with the increased degradation of mutant htt in the pellet fraction seen in Fig. 8 B. To provide direct evidence that scFv-EM48 increases the ubiquitination of mutant htt, we performed immunofluorescent staining of PC12 cells showing neuritic aggregates and the ratios of neuritic htt signals to cell body signals. The data are presented as means ± standard error. **, P < 0.01.
In control cells that only expressed mutant htt, htt aggregates, which were either ubiquitin negative or weakly labeled by anti-ubiquitin, were also found in neurites, in contrast to scFv-EM48–containing cells in which most htt aggregates were ubiquitinated and remained in the cell body. Using a control intrabody (NAC32) against α-synuclein (Lynch et al., 2008), we found that this control intrabody neither associated with htt aggregates nor reduced htt levels in transfected cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200710158/DC1). Collectively, these results indicate that the intrabody scFv-EM48
can reduce the accumulation of mutant htt in neuronal processes while promoting the ubiquitination and degradation of mutant htt in the cytoplasm.

**Discussion**

Here, we demonstrate that the intrabody scFv-EM48 preferentially binds mutant htt and inhibits the specific neuropathology of HD. This intrabody provides a useful tool for suppressing the cytoplasmic toxicity of mutant htt, thereby allowing us to differentiate the cytoplasmic from the nuclear effects of mutant htt. The present study also suggests that the abnormal conformation of mutant htt itself makes an effective therapeutic target that is highly specific.

Normal levels of htt are critical for neurogenesis and embryonic development (White et al., 1997), and conditional knockout mice, in which htt expression has been inactivated, show degeneration of neuronal cells (Dragatis et al., 2000). Although these findings suggest that a loss of function may be involved in HD pathology, there is considerable evidence that the polyQ expansion in mutant htt results in a gain of toxic function. For example, even in those HD mouse models expressing endogenous htt at normal levels, transgenic mutant htt leads to neuropathology and neurological symptoms (Davies et al., 1997; Schilling et al., 1999; Graham et al., 2006). The gain-of-toxic-function theory provides a rationale for suppressing the expression of the transgene in HD mice (Yamamoto et al., 2000; Harper et al., 2005). However, such suppression cannot distinguish the effects of mutant htt in the nucleus versus the cytoplasm. An alternative approach has been targeting nuclear export sequences to transgenic polyQ proteins, but expanded polyQ proteins could not be completely directed to the cytoplasm (Jackson et al., 2003; Benn et al., 2005).

Intracellular expression of intrabodies has proved successful at blocking the toxic effects of mutated proteins or pathogenic agents with high selectivity (Hudson and Souriau, 2003; Lobato and Rabbitts, 2004). The interaction of intrabodies with their targets can prevent the binding of these targets to other proteins (Dorai et al., 1994). Furthermore, intrabodies in both cellular and *Drosophila melanogaster* HD models have shown encouraging protective effects (Lecerf et al., 2001; Khoshnan et al., 2002; Colby et al., 2004; Miller et al., 2005; Wolfgang et al., 2005), but such studies have not been extended to HD mouse brains. Unlike most intrabodies used previously, our intrabody scFv-EM48 binds to a unique epitope in human htt and preferentially interacts with mutant htt. Because the polyQ domain is not the epitope for this interaction, the expanded polyQ-mediated conformational alteration must lead to the increased binding of mutant htt to scFv-EM48. This is also consistent with the notion that polyQ expansion enhances the interactions of mutant htt with other molecules. This unique property allows for the selective reduction of mutant htt and its toxic effects, without interfering with the critical function of normal htt. In support of this idea, we provide in vivo evidence that scFv-EM48 can suppress formation of neuropil aggregates in the brain and ameliorate neurological symptoms in N171-82Q mice, in which transgenic mutant htt forms abundant neuropil aggregates (Schilling et al., 1999). In addition, the in vivo effect of scFv-EM48 on mutant htt is persuasively indicated by its preferential binding to mutant htt and its in vitro protection against htt toxicity.

We found that scFv-EM48 did not significantly reduce the formation of nuclear inclusions. It is conceivable that cytoplasmic scFv-EM48 cannot enter the nucleus, where these htt fragments have already accumulated. Alternatively, small htt fragments can readily enter the nucleus, and expanded polyQ tracts prevent their export from the nucleus (Cornett et al., 2005), whereas the transport of mutant htt to neuronal processes is dependent on its interactions with trafficking proteins. Although scFv-EM48 binds an epitope that may not be involved in interactions with other proteins, its interaction with mutant htt can cause a conformational change that could alter the association of htt with other proteins, such as trafficking proteins. Importantly, interactions of intrabody with htt can alter the stability of htt, as our studies demonstrate that the interaction of scFv-EM48 with mutant htt increases the ubiquitination of mutant htt and reduces its half-life. It has been shown previously that some intrabodies can reduce htt aggregation (Lecerf et al., 2001; Khoshnan et al., 2002; Colby et al., 2004) or decrease the level of exon1 mutant htt (Wolfgang et al., 2005). Our findings suggest that the interaction of scFv-EM48 with mutant htt could alter htt’s conformation and/or make mutant htt more accessible to the ubiquitin–proteasome system, thereby promoting its degradation. It would be interesting to investigate whether previously reported htt intrabodies also act on htt via this mechanism.

It is known that cytoplasmic mutant htt is transported within neuronal processes (Gunawardena et al., 2003; Szefeney et al., 2003). In the present study, we present the first in vivo evidence that the interaction of intrabody with cytoplasmic mutant htt reduces its distribution in the neuronal processes and ameliorates neurological symptoms in HD mice. Decreasing the distribution of mutant htt in neuronal processes can reduce the cytoplasmic effects of htt on mitochondria, trafficking, and synaptic transmission to improve neuronal function. Suppressing neuropil aggregate formation may be particularly relevant in treating the specific neuropathology of both HD and spinobulbar muscular atrophy, in which neuropil aggregates have also been observed (Parker et al., 2001; Piccioni et al., 2002). Although soluble mutant htt can affect intracellular trafficking (Gunawardena et al., 2003; Szefeney et al., 2003; Gauthier et al., 2004; Trushina et al., 2004), polyQ aggregates in the neuronal processes can also physically block intracellular transport (Piccioni et al., 2002; Lee et al., 2004; Chang et al., 2006) and are associated with axonal degeneration in polyQ disease models (Li et al., 2001; Parker et al., 2001; Yu et al., 2003). Because neuropil aggregates are more abundant than nuclear inclusions in the brains of presymptomatic and early-stage HD patients (Gutekunst et al., 1999), suppressing their formation could be beneficial in terms of reducing neuronal dysfunction before degeneration occurs. Because we have shown that scFv-EM48 is specific to mutant htt, the present findings suggest that intracellular antibodies to specific polyQ proteins can make useful tools for unraveling pathological events caused by polyQ proteins, and may also pave the way for development of effective polyQ disease therapies.
Materials and methods

Reagents and animals

Transgenic mice (R6/2 and N171-82Q) were obtained from The Jackson Laboratory and maintained in the animal facility at Emory University. Cerebral cortical tissues from YAC128 transgenic mice were provided by M. Hayden (University of British Columbia, Vancouver, Canada). Brain tissues from HD 150Q knock-in mice were obtained as described previously (Zhou et al., 2003). htt peptides (14–18 residues) from different regions of N-terminal human and mouse htt were synthesized by the Biochemical Core Facility at Emory University. In vitro synthesized htt was generated in 50 μl of in vitro SP6-Transcription/Translation kit (Roche) with [35S]methionine and PKR plasmids (1 μg each) encoding different forms of htt. The reaction was incubated at 37°C for 1 h. 20 μl of reaction was subjected to SDS-PAGE. The rabbit and mouse antibodies to htt were generated as described previously (Zhou et al., 2003). Other antibodies used include mouse monoclonal antibodies to γ-tubulin (Sigma-Aldrich), NeuN (Millipore), syntaxin (Sigma-Aldrich), TBP (Santa Cruz Biotechnology, Inc.), GAPDH (Millipore), and ubiquitin (Dako). PKR expression vectors encoding N-terminal htt (208 aa) with 23Q or 130Q and adenoaviral htt constructs encoding GFP–N-terminal htt (1–208 aa) with 23Q or 130Q were described previously (Shin et al., 2005). FLAG-tagged pcDNA–ataxin-1 82Q plasmid was provided by H.T. Orr (University of Minnesota, Minneapolis, MN). Myc-tagged CMV–ataxin-7 92Q plasmid was provided by A. La Spada (University of Washington, Seattle, WA).

Generation and expression of scfV-EM48

RNAs from hybridoma cells were used for RTPCR with primers complementarily matching 3′ and 5′ ends of the constant sequences flanking the variable region heavy and light (VH and VL) chains of mouse IgG (GE Healthcare). Cloned scfV cDNAs encoding VH (320 bp) and VL (340 bp) chains of mEM48 were linked by a 45-bp oligonucleotide encoding Gly-Ser (Khoshnan et al., 2002), generating 750-bp cDNA that encodes scfV-EM48. The cDNA was linked with the influenza HA or FLAG epitope and inserted into the PRK vector containing a 750-bp cDNA that encodes scFv-EM48. The cDNA was linked with the control intrabody (NAC32-myc) was provided by A. Messer (Wadsworth Center, New York State Department of Health, Albany, NY). HEK293 cells and cultured primary neurons from the cerebral cortex of embryonic day 17 or 18 rat fetuses were transfected with scfV-EM48 using Lipofectamine 2000 (Invitrogen).

For expression of scfV-EM48 via adenoviral vector, we used a helper-dependent adenoviral vector that contained the CMV promoter, scfV-EM48 cDNA, the woodchuck hepatitis virus posttranscriptional regulatory element, and the bovine-globin polyadenylation site. The expression cassette was cloned into PBS Shuttle, and the corresponding adenovirus backbone was generated by homologous recombination in Escherichia coli, as described previously (Shin et al., 2005). Generation and purification of virus were performed as described previously (Toietta et al., 2002, 2005). We were able to generate viral scfV-EM48 with a titer of 10^12 viral particles per milliliter. Adenoviral vector that did not express scfV-EM48 served as a control.

Cell viability studies

HEK293 cells were grown in 6-well plates to 60–70% confluence. After 48 h transfection, cells were collected and then resuspended in serum-free medium. We dispersed 50 μl of the cell suspension (5,000 cells) into a 96-well plate. Cell viability was determined by counting the number of htt-positive aggregates per image, and the brightness/contrast adjustment function.

Microscopy

All imaging was done at room temperature, ~26°C. Immunocytochemical analysis of cultured cells and mouse brain tissues was performed as described previously (Li et al., 2000; Shin et al., 2005). Light micrographs were taken using a microscope (Axioskop 2 MEO) equipped with a digital camera (Orra-100) and image acquisition software (Openlab). A 20x LD-Achromplan 0.4 NA or 63x 0.75 NA oil immersion objective lens (both from Carl Zeiss, Inc.) was used for light microscopy. For immunofluorescent staining, species-specific fluorescent- or Texas red–conjugated secondary antibodies (Jackson Immunoresearch Laboratories) were applied for 1 h at room temperature followed by counterstaining with Hoechst dye. Enhanced GFP was imaged using 488-nm excitation and a 500–530-nm band-pass filter, RFP was imaged using 543-nm excitation and a 565–615-nm band-pass filter. The figures were created using Photoshop 7.0 software (Adobe) and, in some cases where the brightness and contrast of the whole image needed adjustment, we used the brightness/contrast adjustment function.

Behavioral analysis

To examine gait function, we measured stride length. Mice injected bilaterally with adenoviral vector at 10 wk of age were analyzed after 8 wk of injection. Mice were allowed to walk across a paper-lined chamber and into an enclosed box. After one practice run, tracings for front and rear footprints for each mouse were measured. Measurements were averaged and the data were presented as stride length in millimeters. To measure RotaRod performance, mice were trained for 5 min on three separate days on a RotaRod device (Rotamex 4/8; Columbus Instruments) at 5 rpm and tested at speeds of 4–40 rpm over a 5-min period. Latency to fall was recorded. Mice were allowed 10–20 min to recover between trials. Survival and body weight of mice were also monitored. Uninfected WT mice, n = 11; N171-82Q mice injected with adenoviral scfV-EM48, n = 10; N171-82Q mice injected with adenoviral vector control, n = 9; uninfected HD-N171-82Q mice, n = 9.

Dot blot analysis

A Hybond-P membrane (GE Healthcare) was rinsed in methanol and soaked in PBS. 2 μl of peptide solution in PBS were spotted on the x axis, with different dilutions (0, 1, 5, 25, 50, and 100 μM) on the y axis. The membrane was dried and blocked sequentially in 3% BSA and 5% milk in PBS for 1 h and then incubated overnight with mEM48 antibody at 4°C. The membrane was then washed with 5% milk/PBS three times and incubated with peroxidase-conjugated anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) at a 1:10,000 dilution in 5% milk/PBS for 1 h. The membrane was developed using ECL Plus reagents (GE Healthcare).

Protein binding assays and fractionation

We performed immunoprecipitation of transfected HEK293 and infected PC12 cells using the same method described previously (McGuire et al., 2006).
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