Potent inhibition of huntingtin aggregation and cytotoxicity by a disulfide bond-free single-domain intracellular antibody

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Huntington’s disease (HD) is a progressive neurodegenerative disorder caused by an expansion in the number of polyglutamine-encoding CAG repeats in the gene that encodes the huntingtin (htt) protein. A property of the mutant protein that is intimately involved in the development of the disease is the propensity of the glutamine-expanded protein to misfold and generate an N-terminal proteolytic htt fragment that is toxic and prone to aggregation.

Intracellular antibodies (intrabodies) against htt have been shown to reduce htt aggregation by binding to the toxic fragment and inactivating it or preventing its misfolding. Intrabodies may therefore be a useful gene-therapy approach to treatment of the disease. However, high levels of intrabody expression have been required to obtain even limited reductions in aggregation. We have engineered a single-domain intracellular antibody against htt for robust aggregation inhibition at low expression levels by increasing its affinity in the absence of a disulfide bond. Furthermore, the engineered intrabody variable light-chain (VL)12.3, rescued toxicity in a neuronal model of HD. We also found that VL12.3 inhibited aggregation and toxicity in a Saccharomyces cerevisiae model of HD. VL12.3 is significantly more potent than earlier anti-htt intrabodies and is a potential candidate for gene therapy treatment for HD. To our knowledge, this is the first attempt to improve affinity in the absence of a disulfide bond to improve intrabody function. The demonstrated importance of disulfide bond-independent binding for intrabody potency suggests a generally applicable approach to the development of effective intrabodies against other intracellular targets.

Huntington’s disease | neurodegeneration | yeast-surface display | protein engineering | directed evolution

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n Huntington’s disease (HD), a proteolytic fragment of the huntingtin (htt) protein that contains an expanded polyglutamine stretch misfolds and forms β-sheet-rich aggregates. Intracellularly expressed antibodies with specificity for htt have been shown to reduce aggregation and toxicity in cellular and organotypic slice culture models of HD (1–4). However, high intracellular antibody (intrabody) expression levels have been required to obtain moderate reductions in aggregation and toxicity. This occurrence has proven to be a barrier to the development of a treatment for HD with intrabodies by means of gene therapy, given the limited ability of viral vectors to deliver genes to the CNS. Intrabodies are an attractive means of manipulating intracellular protein function. However, their success has been limited largely to use in target validation, rather than experimental therapy in preclinical disease models, in part due to their limited efficacy. A key problem arises from the conditions under which antibodies against intracellular targets are isolated and engineered. With the exception of the yeast two-hybrid approach to intrabody isolation (5), antibodies are isolated and engineered under oxidizing conditions by yeast or phage display (1, 6, 7), where stabilizing disulfide bonds form; however, disulfide bonds do not form as readily in the reducing environment of the cytoplasm, where intrabodies are intended to function. Lead optimization or incremental improvement of intrabody function has not been reported to date with a yeast two-hybrid approach, perhaps due to the qualitative nature of that screening system.

Previously, we reported the isolation of a single-chain antibody (scFv) specific for the first 20 aa of htt, and its reduction to a single variable-light-chain (VL) domain, to enable intracellular expression and mild inhibition of htt aggregation (1). We have now engineered this VL intrabody for robust and effective inhibition of aggregation and cytotoxicity by removing the disulfide bond to make intrabody properties independent of redox environment, whether intracellular or extracellular. First, the cysteines that form the disulfide bond were mutated to hydrophobic residues, a technique shown to be effective for obtaining higher yields of active antibody expressed from Escherichia coli (8). This procedure resulted in an unexpectedly large decrease in the intrabody’s affinity for its antigen. Iterative rounds of mutation and screening were then applied to improve the intrabody’s affinity, a process that mimics affinity maturation in the immune system. We find that the ability to block htt exon 1 aggregation correlates with antigen-binding affinity in the absence of disulfide bonds. Disulfide-independent binding affinity and intrabody expression levels (1, 9–11) appear to be the two essential design variables for the development of highly functional intracellular antibodies.

Yeas-surfacdisplay (YSD) (12) is a technique for isolation of novel antibodies (13), improving protein function (14–17), and analysis of protein properties (1, 18–20). In this system, the gene for a protein of interest is fused to the gene for the yeast-mating protein (Aga2p) and to epitope tags, such as c-myc, for detection. When transformed into an appropriate yeast strain, the protein is displayed on the yeast cell wall, where it is accessible to antigens or other interaction partners and immunofluorescent reagents in solution. In this way, the properties of individual proteins may be analyzed by flow cytometry, or libraries of expressed proteins may be sorted to isolate clones with desired properties by FACS. We have used this technique to engineer an intrabody for high affinity without a disulfide bond, allowing facile transfer of this property to the intracellularly expressed intrabody.

Materials and Methods

YSD. The cysteine residues of yeast-displayed VL (1) were changed to valine and alanine (C22V and C89A) by site-directed

Abbreviations: HD, Huntington’s disease; VL, variable light chain; intrabody, intracellular antibody; htt, huntingtin; httex1Q, first exon of htt with n glutamines; YSD, yeast-surface display; HEK, human embryonic kidney; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; mfu, mean fluorescence unit.

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mutagenesis of the V_L gene using QuikChange PCR (Stratagene). YSD labeling experiments to measure expression and binding were conducted as described (21). A peptide consisting of the first 20 aa of htt was used as the antigen (MALTEK-LMKAFESLKSFOOQ-I-biotin, synthesized by the Massachusetts Institute of Technology Biopolymers Laboratory). The antigen was synthesized to contain three glutamines because the beginning of the polyglutamine region would be an ideal target for interfering with the misfolding of htt exon 1. Affinity maturation of V_L,C22V,C89A relied on protocols previously described (14). Briefly, the V_L,C22V,C89A gene was used as the template for the creation of a library of point mutants through error-prone PCR by using nucleotide analogues. The resulting PCR products were amplified and transformed into yeast, along with digested pCT-CON (a YSD vector), to create a library through homologous recombination (22). The library had a diversity of 3 × 10^7 intrabody mutants displayed on the surface of yeast. This library was sorted four times by FACS to isolate mutants with an ~10-fold improvement in affinity, as measured by titration with the 20-aa htt peptide. These mutants were then used as the template in the next round of library generation. The entire process, from library generation to isolation of improved mutants, was repeated three times to yield V_L,C22V,C89A (8) to make mutant V_L,C22V,C89A. Yeast cell-surface protein expression levels, which can be monitored by the presence of a C-terminal c-myc tag detected by immunofluorescence and flow cytometry, have been shown to correlate strongly with protein stability (19, 20). Significantly, yeast cell-surface expression levels of V_L,C22V,C89A were comparable with those of V_L, suggesting that the absence of the disulfide bond did not significantly alter stability of the protein (Fig. 1A). A negative peak can be seen just above a fluorescence value of 10^1, due to cells that have lost the expression plasmid.

We then measured the affinity of the wild-type V_L and mutant V_L,C22V,C89A for a biotinylated peptide antigen consisting of the first 20 aa of htt, by titration of the YSD intrabodies (Fig. 1B, ⬤ and ○, respectively). The mutant lacking a disulfide bond exhibited a binding affinity 2–3 orders of magnitude lower than the wild-type intrabody (approximate affinities are V_L, ~30 nM, V_L,C22V,C89A > 10 μM), indicating the importance of disulfide bond formation in maintaining the structural integrity of the antigen-binding site of the intrabody. Because disulfide bonds are not thermodynamically favored in the reducing environment of the cytoplasm, the intracellular affinity of V_L is expected to be on the order of that of the mutant lacking the disulfide bond.

Elimination of Disulfide Bond Does Not Affect Aggregation Inhibition Properties of Intrabody in Transiently Transfected Mammalian Cell Model of HD. To ensure that mutation of the cysteine residues that form the disulfide bond of the YSD V_L mimics intracellular expression, we measured the effect of disulfide bond elimination on the ability of the intrabody to block htt aggregation when transiently transfected into mammalian cells at a high plasmid ratio relative to htt. ST14A cells were cotransfected with httx1Q97-YFP (also in pcDNA3.1) and an empty vector, V_L or V_L,C22V,C89A, at a 2:1 intrabody:htt plasmid ratio. Twenty-four hours posttransfection, cells with aggregates were counted. Both the wild-type intrabody and the mutant lacking cysteines inhibited aggregation to the same extent (Fig. 1C) when expressed at high levels. The equivalent aggregation inhibition of V_L and V_L,C22V,C89A, despite the almost 1,000-fold difference in affinities of the intrabodies under oxidizing extracellular expression conditions, strongly suggests that the disulfide bond in V_L does not form in the cytoplasm.definitions of aggregation and toxicity assays. ST14A cells (23), human embryonic kidney (HEK)293 cells, and SH-SY5Y cells were cultured according to standard protocols. C-terminal His-tagged intrabody constructs were expressed from a pcDNA3.1 vector under the control of a cytomegalovirus promoter. The method used to quantify the effect of intrabodies on intracellular mutant htt exon 1 aggregation in the three cell lines mentioned above is described in detail elsewhere (1); briefly, cells were transiently transfected by using Lipofectamine (Invitrogen) or similar reagents, and the presence of aggregates before processing in immunoblots with a monoclonal anti-htt c-myc tag detected by immunofluorescence and flow cytometry, have been shown to correlate strongly with protein stability (19, 20). Significantly, yeast cell-surface expression levels of V_L,C22V,C89A were comparable with those of V_L, suggesting that the absence of the disulfide bond did not significantly alter stability of the protein (Fig. 1A). A negative peak can be seen just above a fluorescence value of 10^1, due to cells that have lost the expression plasmid.

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Intrabody Lacking Disulfide Bond Engineered for High Affinity by Directed Evolution. Because the intracellular affinity of the VL was relatively low, we hypothesized that more potent aggregation inhibition could be achieved by engineering VL,C22V,C89A for higher affinity. Random mutagenesis of the VL,C22V,C89A gene was carried out by using error-prone PCR. The resulting PCR fragments were transformed into yeast along with a YSD vector to create a library through homologous recombination (22). This library had a diversity of \( \sim 3 \times 10^6 \) intrabody mutants displayed on the surface of yeast. Iterative rounds of FACS sorting were used to isolate new mutants with improved affinity. The process of mutagenesis and sorting resulted in an \( \sim 10 \)-fold improvement in binding affinity. The improved mutants obtained were used as the template for the next round of library creation; the entire mutagenesis and sorting process was repeated three times. After the third round, one mutant designated VL12.3 was identified with significantly improved affinity, (titration shown in Fig. 1B; approximate \( K_d \sim 3 \text{ nM} \)).

The improved mutant was sequenced and found to have gained four mutations (F37I, Y51D, K67R, and A75T); continued absence of the cysteine residues was also confirmed. Three of the four mutations were in framework positions (residues in antibody-variable domains that do not generally form contacts with antigens); only one was in a complementarity determining region (Y51D in CDR L2). The locations of the mutations are shown in a homology model (Fig. 1D; homology model generated at the Web Antibody Modeling database, which can be accessed at antibody.bath.ac.uk/index.html).

**Engineered Intrabody VL12.3 Robustly Blocks Aggregation in Transiently Transfected Mammalian Cell Models of HD.** To determine whether VL12.3 has improved htt aggregation inhibition properties, various cell lines were transiently cotransfected with htt\(1Q_{97}\)-GFP and VL12.3, and the formation of aggregates was monitored by fluorescence microscopy and Western blotting. In some experiments, an intrabody that lacked specificity for htt (ML3.9) and an empty control vector were tested as a negative control, and previously reported C4 (3) and VL (1) were included for comparison. First, experiments were performed by using intrabody to htt plasmid ratios of 5:1. In previous work (1–3), such high levels of intrabody overexpression were required to accomplish moderate reduction of aggregate formation. VL12.3 exhibited the ability to essentially ablate aggregation at these high levels of expression, as shown in Fig. 2A ( ), for VL12.3 in ST14A cells, compared with C4 ( ) and empty vector ( ). Significantly, aggregation inhibition persisted over a period of several days.

Given the strong capability of VL12.3 to reduce the formation of aggregates at high expression levels, we then studied the dose–response of aggregate formation by varying the ratio of intrabody to htt plasmid. As shown in Fig. 2B, VL12.3 blocked aggregation significantly even when expressed at very low levels (0.5:1 intrabody: htt plasmid ratio). The formation of aggregates was reduced by nearly 80% when the intrabody plasmid was present in a 1:1 ratio with htt plasmid, and >90% when present at higher levels. Sample images with and without VL12.3 are shown in Fig. 2C.

Flow cytometry was used to determine whether expression levels of htt\(1Q_{97}\)-GFP were different in the presence of the intrabody; expression levels were comparable for samples with intrabody compared with empty vector (Fig. 2D). Therefore, the decrease in aggregation did not occur simply as a result of inhibiting htt\(1Q_{97}\)-GFP expression.

Efficacy of VL12.3 was characterized and compared with two previously described intrabodies (C4 and VL) in other cell lines, both by fluorescence microscopy and Western blotting analysis. In SH-SYSY human neuroblastoma cells at a 1:1 intrabody: htt ratio only VL12.3, and not earlier intrabodies, effectively reduced aggregation (Fig. 2E). Aggregation inhibition properties of VL12.3 in HEK293 cells (Fig. 2F) were comparable with those observed in ST14A and SH-SYSY cells. Partial dose–response curves are shown for each intrabody. Especially noteworthy is the ability of VL12.3 to inhibit aggregation when used at a plasmid ratio (1:1 intrabody to htt) that was completely ineffective with previously reported intrabodies.

Whereas microscopy confirmed that fewer cells contain visible aggregates when cotransfected with VL12.3, we also sought to confirm a reduction in total aggregated htt protein. Western blotting analysis of Triton X-100-soluble and -insoluble htt fractions was performed on cell lysates obtained from HEK293 cells (Fig. 2G), transiently transfected using a 2:1 ratio of intrabody: htt plasmid. Significantly reduced levels of aggregated material were detected in the Triton X-100-insoluble fractions for cells cotransfected with VL12.3 and htt\(1Q_{97}\)-GFP, whereas
cotransfection of httex1Q97-GFP with any of the other intrabodies resulted in amounts of aggregated material comparable with negative control. Coexpression of intrabodies did not decrease the amount of material in the Triton X-100-soluble fraction.

V12.3 was expressed at levels equivalent to or slightly higher than V112.3, as measured by anti-His6 Western blot (Fig. 2H).

**Engineered V12.3 Blocks Aggregation and Cytotoxicity in a Yeast Model of HD.** Energy metabolism impairment and mitochondrial dysfunction have been described in cellular models of HD as well as in HD patients (29, 30). To see whether the engineered V12.3 intrabody could reduce toxicity in mammalian cells in addition to blocking aggregation, the MTT assay was used to measure the mitochondrial activity of transiently transfected ST14A cells (31). ST14A cells were transfected with GFP, httex1Q25-GFP, or httex1Q72-GFP. Forty-eight hours posttransfection, live GFP-positive cells were sorted by FACS. The ability of the cells to metabolize MTT during an additional 4 h of culture was measured. Compared with cells expressing GFP or httex1Q25-GFP, cells expressing httex1Q72-GFP exhibit an attenuated ability to reduce MTT (Fig. 3). Cotransfection with V12.3 at a 2:1 ratio resulted in completely restored ability to metabolize MTT, indicating normal levels of mitochondrial activity.

**Engineered V12.3 Blocks Aggregation and Cytotoxicity in a Yeast Model of HD.** Saccharomyces cerevisiae is likely the simplest in vivo model of HD, exhibiting both htt aggregation and cytotoxicity (32, 33). To determine whether the engineered intrabody could prevent these HD phenotypes in yeast, S. cerevisiae strains expressing both a htt exon 1 protein (with either Q25 or Q72) fused to cyan fluorescent protein (httex1Q25-CFP and httex1Q72-CFP) and a V12.3-yellow fluorescent protein fusion (V12.3-YFP) on galactose-inducible promoters were made. Negative control strains were also constructed with an empty vector in place of V12.3-YFP.

The aggregation state of htt in the presence and absence of V12.3-YFP was measured 8 h after induction by a filter retardation assay. This assay consists of lysing cells and passing the lysate through a filter with 0.2-μm pores, trapping aggregates. The amount of aggregated httex1Q72-CFP is then visualized by CFP fluorescence. As shown in Fig. 4A, cells expressing the intrabody had much less aggregated httex1Q72-CFP. This result was confirmed by fluorescence microscopy; expression of V12.3-YFP resulted in significantly reduced aggregation when measured by this method as well (data not shown).

Finally, we tested the ability of the intrabody to inhibit HD-related cytotoxicity in yeast. S. cerevisiae expressing htt with long polyglutamine tracts have been shown to grow slower than those expressing htt with shorter polyglutamine tracts (33). Growth assays were performed on the cell lines mentioned above. The cell line expressing both V12.3-YFP and httex1Q72-CFP grew at a significantly faster rate than that expressing the empty vector and httex1Q72-CFP, as demonstrated by a spotting assay in which the cells were plated on solid media (Fig. 4B). Growth curves were also collected by measuring the OD600 of cultures as a function of time (Fig. 4C). The inhibition of aggregation and toxicity observed in the yeast system upon expression of V12.3 suggests a conserved mechanism for htt toxicity in mammalian and yeast HD models. This finding confirms the value of S. cerevisiae models in screening and testing potential therapeutic molecules.

**Discussion**

We have developed a highly potent intrabody against the N-terminal 20 aa of the htt protein is mutated in HD and forms...
intracellular aggregates in medium spiny neurons of the striatum. This intrabody, Vi12.3, efficiently prevents the aggregation and toxicity of mutant htt exon1 and may therefore be useful in the treatment of HD by gene therapy. We removed the disulfide bond of the single-domain antibody, VL (1), by site-directed mutagenesis to make its properties, such as stability and affinity, independent of the oxidation state of its environment. We next greatly improved the binding affinity of the antibody by mutagenesis and screening for improved binding. In comparison with previously described intrabodies against htt (1–3), this intrabody effectively prevented aggregation at 10-fold lower expression levels or plasmid ratios, and was able to reduce intracellular aggregates in medium spiny neurons of the striatum.

Fig. 3. Engineered intrabody Vi12.3 inhibits metabolic dysfunction in neuronal model of HD. ST14A cells were transfected with a plasmid encoding GFP, htt exon1Q25-GFP, htt exon1Q72-GFP, or htt exon1Q97-GFP with Vi12.3 in a 2:1 ratio. (A) Live GFP-positive cells were collected by FACS in a 96-well plate, 35,000 cells per well, at 48 h posttransfection; typical dot plot is shown for a GFP sample. Other samples showed a similar pattern, and the sorting gate (boxed area) was the same in all instances. (B) Cells were incubated with MTT reagent for 3 h, solubilized, and the absorbance measured; mean values from three separate experiments containing all four samples are shown. Statistics directly over error bars are for comparison with GFP. **P < 0.05; ***P < 0.01. Statistics over brackets are comparisons between the two samples indicated. Four additional pairwise comparisons may be made between htt exon1Q25-GFP and htt exon1Q72-GFP plus Vi12.3; the pooled results indicate a 56 ± 25% increase in A[570]P < 0.001. Expression of htt exon1Q72-GFP significantly reduced the ability of cells to reduce MTT, but this effect was reversed by the coexpression of Vi12.3.

However, disulfide bond formation has been observed after oxidative stress (35), fueling debate within the intrabody research community about whether such bonds form in cytoplasmically expressed antibody fragments. We found a dramatic lowering of the in vitro affinity when the cysteines were replaced by the hydrophobic residues alanine and valine (Fig. 1B). However, these mutations did not alter intracellular intrabody potency, as measured when the intrabody was present at a high plasmid ratio (Fig. 1C). This result strongly implies that the disulfide bond does not form even when the cysteine residues are present in this case, given the dramatic effect of cysteine mutation on in vitro affinity. It is also interesting to note that mutation of the cysteine residues did not significantly alter antibody expression (on the yeast surface in this case; Fig. 1A), in contrast to other published reports (15, 36).

Several reports have brought into question the relevance of antibody affinity in predicting efficacy of intracellular antibodies (9, 10), suggesting that only expression levels are relevant. However, Vi1 is expressed at levels equivalent to or even above Vi12.3 (Fig. 2H), but is significantly less active. Therefore, affinity is clearly a key determinant in intrabody efficacy in the present case, consistent with the equilibrium relationship:

$$\frac{[\text{Intrabody-Antigen}]}{[\text{Antigen}]} = \frac{[\text{Intrabody}]}{K_d}.$$  \[1\]

where [Intrabody-Antigen] is the concentration of the bound complex. From this relationship, it is clear that high-level intrabody overexpression can at least partially compensate for
diminished intracellular affinity, as we demonstrate here for the wild-type V1 intrabody and V1CLK22V,C89A. For a micromolar affinity intrabody, however, micromolar expression levels are necessary even when antigen concentration is much lower than micromolar, because it is likely to be in striatal neurons in vivo. V1CLK23.3, with 3 nM affinity, should be effective at nanomolar level concentrations. In earlier reports, the role of affinity was obscured by measuring antibody affinity in oxidizing (extracellular) environments, where disulfide bonds will form, for comparison with intracellular assays for activity, in which disulfide bonds are unlikely to form. By mutating the cysteines of V1 so that no disulfide bond will form, we assessed the protein’s properties (and improved its affinity) under oxidizing, extracellular conditions, while maintaining the structurally relevant cytoplasmic form.

Will the V1CLK23.3 intrabody also bind to wild-type htt in HD heterozygote, and will that alter wild-type function? Unfortunately, this answer cannot be determined presently because the function or functions of wild-type htt are still being investigated and are not conclusively known at present. However, cotransfection of V1CLK23.3 with httex1Q97-GFP did not decrease httex1Q97-GFP expression levels. Also, the precise binding epitope within the first 20 aa recognized by V1CLK23.3 is unknown, and subtle changes in the epitope may have occurred during affinity maturation.

Single-domain intrabodies without disulfide bonds, such as V1CLK23.3, are a minimal and versatile unit for antigen recognition. Single-domain antibodies (37) and structurally analogous domains (38) are increasingly being exploited as alternatives to single-chain antibodies for molecular recognition. The approach demonstrated here may find application in engineering existing intrabodies for increased potency against other disease targets, including Parkinson’s (39) and Alzheimer’s diseases, HIV, and cancer.

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