

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 (V_L)12.3, rescued toxicity in a neuronal model of HD. We also found that V_L12.3 inhibited aggregation and toxicity in a *Saccharomyces cerevisiae* model of HD. V_L12.3 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

In 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 (V_L) domain, to enable intracellular expression and mild inhibition of htt aggregation (1). We have now engineered this V_L 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.

Yeast-surface display (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 V_L (1) were changed to valine and alanine (C22V and C89A) by site-directed

Abbreviations: HD, Huntington's disease; V_L, variable light chain; intrabody, intracellular antibody; htt, huntingtin; httex1Q_n, 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 (MATLEK-LMKAFESLKSFQQQ-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 $V_L12.3$. FACS sorting was performed by using a Cytomation (Fort Collins, CO) Moflo FACS machine by the staff of the Massachusetts Institute of Technology Flow Cytometry Core Facility. All constructs and clones were sequenced at the Massachusetts Institute of Technology Biopolymers Laboratory.

Mammalian Cell Culture, Aggregation Assay, and Toxicity Assays. ST14A cells (23), human embryonic kidney (HEK)293 cells, and SH-SY5Y cells were cultured according to standard protocols. C-terminal His₆-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 was monitored by using fluorescence microscopy. Transfection efficiencies and expression levels of the first exon of htt (httex1)Q97-GFP were monitored by flow cytometry on a Moflo FACS machine (Cytomation). Cell lysis, preparation of Triton X-100-soluble lysates, and immunoblots were carried out as described (24). Triton X-100-insoluble fractions were prepared by resuspending the Triton X-100-insoluble pellet in water, followed by sonication and centrifugation at $16,000 \times g$ for 10 min; the final pellet was resuspended in SDS gel-loading buffer before processing in immunoblots with a monoclonal anti-htt recognizing the first 17 aa of the htt protein (m445).

Intracellular expression levels of intrabodies were measured by anti-His (antibody from Santa Cruz Biotechnology) Western blot.

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which was used to measure metabolic activity, transiently transfected ST14A cells were sorted 48 h posttransfection, based on GFP signal to collect populations expressing GFP or httex1-GFP transgenes. These cells were sorted directly into 96-well plates, 35,000 cells per well, in 100 μ l of maintenance media. The MTT assay (kit from American Type Culture Collection) was then performed according to the manufacturer's protocol. A FLUOstar OPTIMA 96-well plate reader (BMG Labtechnologies, Durham, NC) was used to measure absorbance of the metabolic product at 570 nm.

Yeast Cell Culture, Aggregation, and Toxicity. Yeast media were prepared, based on standard protocols (25), by using complete supplemental mixtures (Bio 101). Transformation of yeast was

performed as described (26). Yeast-integrating plasmids containing galactose-inducible promoters [pRS303 backbone (27)] for the expression of htt fragments were linearized by digestion with *Bst*XI before transformation. $V_L12.3$ -YFP was subcloned into p414 (American Type Culture Collection), which also contains a galactose-inducible promoter. Filter retardation assays of aggregated material were performed essentially as described (28). For the induction of expression of the htt fragment in yeast, cultures were grown at 30°C in raffinose-containing liquid media and transferred to galactose-containing media. To measure growth, yeast cells were diluted to a final OD₆₀₀ of 0.05 and transferred to a microtiter plate. Yeast cultures were grown at 30°C with intermittent, intensive shaking on the Bioscreen C (Growth Curves USA) for 48 h with OD measurements taken every 2 h. Western blot analysis of $V_L12.3$ -YFP and httex1Q72-CFP with anti-GFP antibodies indicated that the intrabody was present at lower protein concentrations than the htt exon 1 fragment (data not shown).

Results

Elimination of Anti-htt V_L Intrabody's Disulfide Bond Reduces Affinity for Htt. Intracellular expression of antibody fragments leads to incomplete formation of structurally important disulfide bonds. To determine the impact of incomplete disulfide bond formation on V_L expression and affinity for htt, the cysteines of YSD V_L were mutated to valine and alanine (C22V and 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, \blacklozenge and \bullet , 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 $V_L \approx 30$ nM, $V_{L,C22V,C89A} > 10$ μ M), 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 httex1Q97-GFP (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.

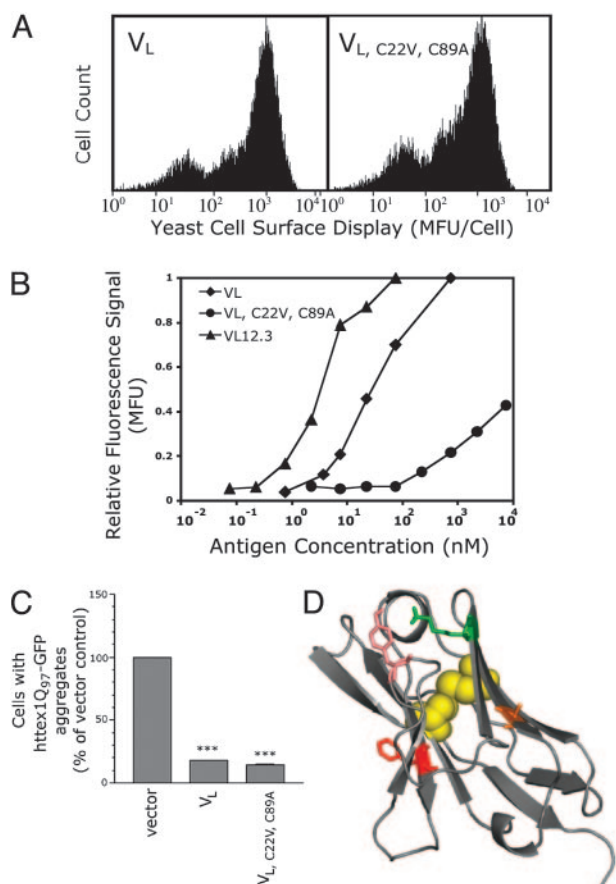


Fig. 1. A single-domain intrabody against htt was engineered for high affinity in the absence of a disulfide bond. (A) Histograms of yeast cell-surface expression levels for V_L and $V_{L,C22V,C89A}$, indicating comparable levels of expression with and without the disulfide bond. mfu, mean fluorescence units. (B) Antigen-binding curves for YSD V_L mutants measured by flow cytometry. Values normalized to maximal intensity were measured, except for $V_{L,C22V,C89A}$, which was normalized to maximal intensity measured for V_L . V_L (◆) has a K_d of ≈ 30 nM, whereas V_L with cysteine mutations ($V_{L,C22V,C89A}$; ●) has significantly lower binding affinity (>10 μ M). Repeated rounds of random mutagenesis of $V_{L,C22V,C89A}$, followed by sorting for improved binding resulted in the mutant $V_{L12.3}$, which has a K_d of ≈ 3 nM. (C) Effect of V_L and $V_{L,C22V,C89A}$ on htt aggregation in ST14A cells transiently transfected with indicated intrabody or vector control and httex1Q97-GFP at a 2:1 plasmid ratio. Both intrabodies are equally capable of partially blocking aggregation when overexpressed at high levels. ***, $P < 0.001$. (D) Homology model showing mutations obtained during engineering; the model contains residues present before mutagenesis. Cysteine residues are yellow. Mutations observed after mutagenesis and sorting are F37I (red), Y51D (pink), K67R (green), and A75T (orange).

Intrabody Lacking Disulfide Bond Engineered for High Affinity by Directed Evolution. Because the intracellular affinity of the V_L was relatively low, we hypothesized that more potent aggregation inhibition could be achieved by engineering $V_{L,C22V,C89A}$ for higher affinity. Random mutagenesis of the $V_{L,C22V,C89A}$ 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 $\approx 3 \times 10^7$ 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 ≈ 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 $V_{L12.3}$ was identified

with significantly improved affinity, (titration shown in Fig. 1B; approximate $K_d \approx 3$ 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 $V_{L12.3}$ Robustly Blocks Aggregation in Transiently Transfected Mammalian Cell Models of HD. To determine whether $V_{L12.3}$ has improved htt aggregation inhibition properties, various cell lines were transiently cotransfected with httex1Q97-GFP and $V_{L12.3}$, 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 controls, and previously reported C4 (3) and V_L (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. $V_{L12.3}$ exhibited the ability to essentially ablate aggregation at these high levels of expression, as shown in Fig. 2A (●), for $V_{L12.3}$ in ST14A cells, compared with C4 (▲) and empty vector (■). Significantly, aggregation inhibition persisted over a period of several days.

Given the strong capability of $V_{L12.3}$ 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, $V_{L12.3}$ 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 $V_{L12.3}$ are shown in Fig. 2C.

Flow cytometry was used to determine whether expression levels of httex1Q97-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 httex1Q97-GFP expression.

Efficacy of $V_{L12.3}$ was characterized and compared with two previously described intrabodies (C4 and V_L) in other cell lines, both by fluorescence microscopy and Western blotting analysis. In SH-SY5Y human neuroblastoma cells at a 1:1 intrabody:htt ratio only $V_{L12.3}$, and not earlier intrabodies, effectively reduced aggregation (Fig. 2E). Aggregation inhibition properties of $V_{L12.3}$ in HEK293 cells (Fig. 2F) were comparable with those observed in ST14A and SH-SY5Y cells. Partial dose–response curves are shown for each intrabody. Especially noteworthy is the ability of $V_{L12.3}$ 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 $V_{L12.3}$, 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 $V_{L12.3}$ and httex1Q97-GFP, whereas

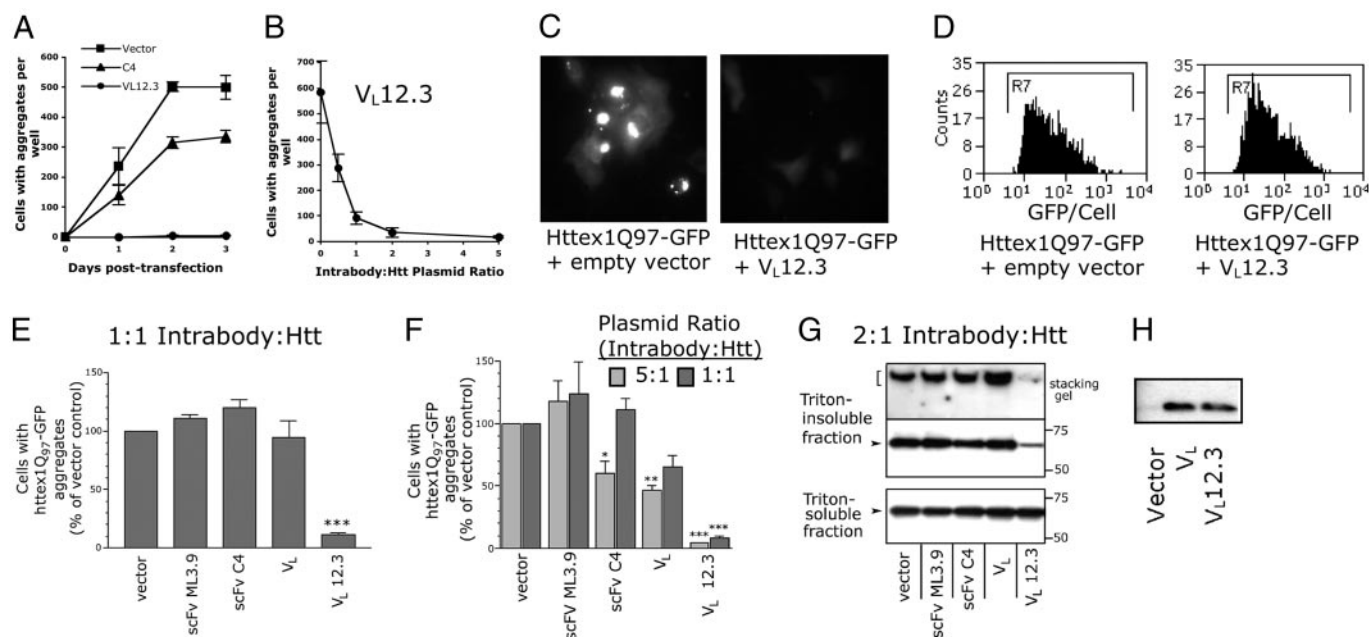


Fig. 2. Engineered $V_L12.3$ robustly blocks htt aggregation in several different cell lines. (A) ST14A cells were transiently cotransfected with $httex1Q97$ -GFP and either an intrabody [C4 (3) or $V_L12.3$] or an empty control vector, and cells with visible aggregates were counted 1, 2, and 3 days posttransfection (5:1 intrabody:htt plasmid ratio, $n = 3$). $V_L12.3$ (●) persistently eliminated htt aggregation over 3 days. (B) Dose–response of $V_L12.3$ was measured at 2 days by using various intrabody:htt plasmid ratios ($n = 3$). (C) Fluorescence microscopy images of typical cells. (D) Flow cytometry histograms showing expression level per cell of $httex1Q97$ -GFP in transfected cells in the presence of intrabody compared with empty vector (mean fluorescence intensity 82 vs. 76 mfu, respectively; transfection efficiencies were comparable in both samples, at 13% and 11%, respectively). (E) Comparison of intrabody activity for the intrabodies mentioned above and a non-htt binding intrabody (scFv ML3.9) and wild-type V_L at a 1:1 intrabody:htt plasmid ratio (***, $P < 0.001$) in SH-SY5Y human neuroblastoma cells. (F) Partial dose–response for the same intrabodies in HEK293 cells. (G) Western blot of Triton X-100-soluble and -insoluble fractions of cells lysed 24 h after cotransfection at a 2:1 intrabody:htt ratio. (H) Anti- His_6 Western blot of intrabody expression levels in transiently transfected HEK293 cells.

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.

V_L was expressed at levels equivalent to or slightly higher than $V_L12.3$, as measured by anti- His_6 Western blot (Fig. 2H).

Engineered $V_L12.3$ Inhibits Toxicity in Neuronal Cell Culture 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 $V_L12.3$ 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 $httex1Q97$ -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 $httex1Q97$ -GFP exhibit an attenuated ability to reduce MTT (Fig. 3). Cotransfection with $V_L12.3$ at a 2:1 ratio resulted in completely restored ability to metabolize MTT, indicating normal levels of mitochondrial activity.

Engineered $V_L12.3$ 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 $V_L12.3$ -yellow fluorescent protein fusion

($V_L12.3$ -YFP) on galactose-inducible promoters were made. Negative control strains were also constructed with an empty vector in place of $V_L12.3$ -YFP.

The aggregation state of htt in the presence and absence of $V_L12.3$ -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- μ m 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 $V_L12.3$ -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 $V_L12.3$ -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 OD_{600} of cultures as a function of time (Fig. 4C). The inhibition of aggregation and toxicity observed in the yeast system upon expression of $V_L12.3$ 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

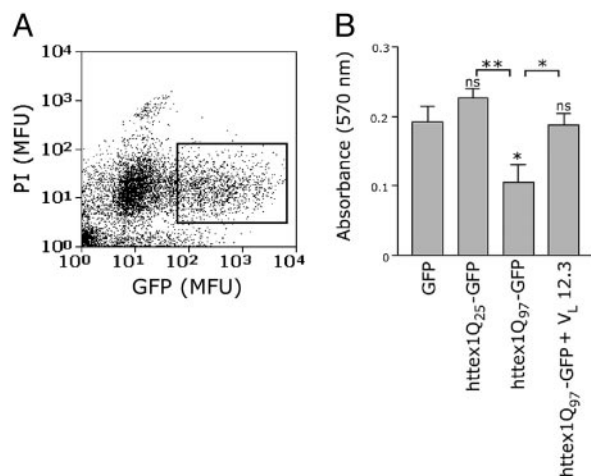


Fig. 3. Engineered intrabody $V_L12.3$ inhibits metabolic dysfunction in neuronal model of HD. ST14A cells were transfected with a plasmid encoding GFP, $httex1Q_{25}$ -GFP, $httex1Q_{97}$ -GFP, or $httex1Q_{97}$ -GFP with $V_L12.3$ in a 2:1 ratio. (A) Live GFP-positive cells were collected by FACS in 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 A_{570} was measured; mean values from three separate experiments containing all four samples are shown. Statistics directly over error bars are for comparison with GFP. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. Statistics over brackets are comparisons between the two samples indicated. Four additional pairwise comparisons may be made between $httex1Q_{97}$ -GFP and $httex1Q_{97}$ -GFP plus $V_L12.3$; the pooled results indicate a $56 \pm 25\%$ increase in A_{570} ; $P < 0.001$. Expression of $httex1Q_{97}$ -GFP significantly reduced the ability of cells to reduce MTT, but this effect was reversed by the coexpression of $V_L12.3$.

intracellular aggregates in medium spiny neurons of the striatum. This intrabody, $V_L12.3$, 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, V_L (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 aggregation of mutant htt exon1 protein almost completely. Given the relative inefficiency of viral gene delivery to the CNS, it is essential that in any proposed gene therapy, the therapeutic protein whose gene is delivered should work as efficiently as possible. For this reason, $V_L12.3$ may prove useful in treating HD through gene therapy, in addition to use as a research tool in further studies of the role of htt aggregation in HD pathogenesis.

In a cell-based assay, we explored the ability of $V_L12.3$ to eliminate intracellular aggregates of mutant htt exon1. Recently, there has been some discussion of the role that htt aggregates and aggregation might play in HD (34). We used the formation of large inclusions in the presence of overexpressed htt exon1 as a measure of intrabody potency, although smaller intermediates in the aggregation process may be responsible for toxicity, or other abnormal protein interactions involving misfolded htt exon1 may be involved. It is therefore noteworthy that when $V_L12.3$ was expressed along with $httex1Q_{97}$ -GFP, >90% of both aggregation and cell toxicity were prevented.

This study also illustrates the impact of disulfide bond formation (or lack thereof) in the cytoplasm on intracellular binding

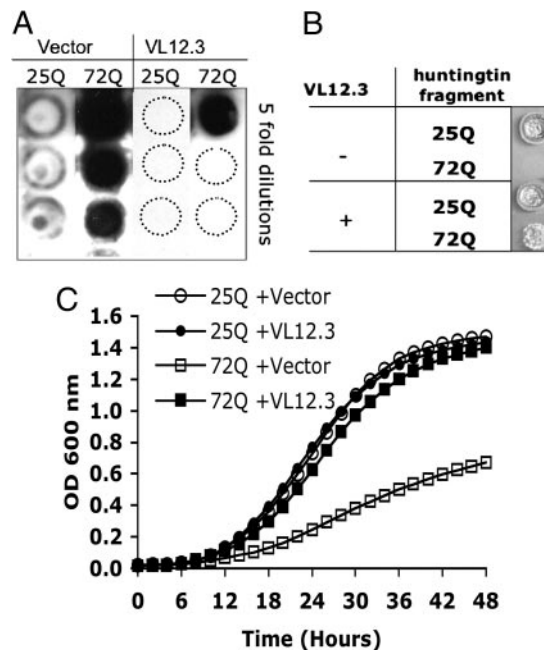


Fig. 4. $V_L12.3$ suppresses aggregation and rescues toxicity in an *S. cerevisiae* model of HD. (A) Filter retardation assay showing $httex1Q_{72}$ -CFP aggregates (dark) from lysates of cells expressing $httex1Q_{25}$ -CFP or $httex1Q_{72}$ -CFP with either $V_L12.3$ or an empty vector control. Dashed circles indicate where insoluble material would appear. Difference between 25Q with and without $V_L12.3$ is insignificant and within the variance usually observed for the assay. (B) Spotting of yeast strains, indicating ability to grow on solid media. (C) Growth curves obtained by measuring the OD_{600} of yeast cultures. Yeast expressing $V_L12.3$ -YFP along with $httex1Q_{72}$ -CFP grow at rates comparable with those expressing htt with nonpathological polyglutamine repeat lengths, in contrast to those carrying an empty vector only.

affinity in intrabody–antigen interactions. Conventional wisdom suggests that disulfide bonds do not form in the cytoplasm. 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, V_L is expressed at levels equivalent to or even above $V_L12.3$ (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:

$$\frac{[\text{Intrabody} \cdot \text{Antigen}]}{[\text{Antigen}]} = \frac{[\text{Intrabody}]}{K_d}, \quad [1]$$

where $[\text{Intrabody} \cdot \text{Antigen}]$ 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 V_L intrabody and $V_{L,C22V,C89A}$. 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*. $V_L12.3$, 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 V_L 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 $V_L12.3$ 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, cotrans-

fection of $V_L12.3$ with httex1Q97-GFP did not decrease httex1Q97-GFP expression levels. Also, the precise binding epitope within the first 20 aa recognized by $V_L12.3$ is unknown, and subtle changes in the epitope may have occurred during affinity maturation.

Single-domain intrabodies without disulfide bonds, such as $V_L12.3$, 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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