Huntingtin affinity for partners is not changed by polyglutamine length: aggregation itself triggers aberrant interactions

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Huntington’s disease (HD) is caused by the expansion mutation above a length threshold of a polyglutamine (polyQ) stretch in the huntingtin (Htt) protein. Mutant Htt (mHtt) pathogenicity is proposed to rely on its malfunction and propensity to misfold and aggregate. Htt has scaffolding properties and has been reported to interact with hundreds of partners. Many interactors show apparent increased or decreased affinity (dysinter- action) for mHtt, which may account for selective malfunctions and striatal degeneration in HD. These dysinteractions are proposed to result from mutant polyQ conformational changes that remain elusive. To date, dysinteractions have only been studied using semi-quantitative techniques with their outcome potentially influenced by the presence of mHtt aggregates. Therefore, the molecular mechanism underlying these dysinteractions remains to be determined. Here, we have used purified proteins devoid of aggregates to quantify the interaction of normal and mHtt with two partners: SH3GL3, reported to have increased binding to mHtt, and the 2B4 antibody, a model partner. Using surface plasmon resonance and pull-down techniques, we show that in the absence of aggregation polyQ length has no effect on Htt interactions. We demonstrate that the presence of aggregates affects the spatial distribution and solubility of Htt partners and strongly influences the outcome of pull-down experiments. Our results show that expanded polyQ per se does not alter Htt interactions and suggest that aggregated mHtt form molecular platforms that influence the Htt interacting network. Modulating mHtt aggregation could thus have beneficial effects on specific cellular pathways deregulated in HD.

INTRODUCTION

Polyglutamine (polyQ) diseases are a group of nine genetic diseases that result in degeneration of defined brain areas. They are caused by the abnormal expansion of polymorphic cytosine-adenine-guanine trinucleotide repeats, which code for polyQ sequences in the ubiquitously expressed disease proteins. Expansion above a certain polyQ length threshold, specific to each disease, confers neurotoxic properties to the mutant proteins (1).

PolyQ disorders share a number of common features: they are progressive neurodegenerative diseases; the age at onset and the severity of diseases are function of the polyQ length; they have an apparent polyQ length toxic threshold, above which the disease becomes penetrant; intracellular accumulation of amyloid-like-aggregated protein fragments bearing the polyQ expansion is a hallmark of polyQ diseases, and the toxicity of various aggregation products is established (2,3). A direct neurotoxic role of polyQ, independent of the function of the carrier protein, has been demonstrated (4).
suggested that inherent toxic properties of expanded polyQ account for the common features in polyQ disorders.

Other features are specific to each disease, as the degeneration occurs in different brain regions and causes disease-specific symptoms. Since the nine disease proteins differ in their functions, sizes and domain organizations, it is proposed that dysfunctions of proteins carrying the mutant polyQ participate to disease specificities (5,6).

The most frequent polyQ disease is Huntington’s disease (HD), which is caused by the expansion of a polyQ stretch (beyond 37–40 residues) in a large cytoplasmic protein, named huntingtin (Htt). All along its sequence, Htt bears repetitive structural elements called ‘HEAT repeats’ that have scaffolding properties (7,8). Accordingly, a plethora of Htt partners were reported, supporting a role for Htt in diverse functions, such as endocytosis, vesicle trafficking, post-synaptic signaling, calcium homeostasis and gene regulation (5,6). Many studies proposed that the polyQ expansion mutation increases or decreases the interaction between mutant Htt (mHtt) and some partners. These observations led to the hypothesis that such aberrant interactions—or dysinteractions—of mHtt with its partners can cause neuronal dysfunction and could account for the selective striatal degeneration observed in HD (9–12).

A predominant hypothesis proposes that only polyQ above a certain pathological size threshold undergoes a toxic conformational change, which would influence interactions between mHtt and its partners (5,6,13–15).

This ‘structural toxic threshold’ model has emerged in the late 1990s to accommodate several other observations: (i) the fact that some antibodies display a stronger interaction with expanded polyQ, which could suggest that mutant polyQ adopts a specific conformation (15,16), (ii) the discovery that expanded polyQ form amloid-like sodium dodecyl sulfate (SDS) insoluble aggregates both in vitro and in vivo (17), which at that time seemed to be a property specific to expanded polyQ and (iii) the pioneering work of Max Perutz on the structural properties of polyQ stretches (18), which led to his famous ‘water-filled nanotube’ model, as a plausible conformation of polyQ above the toxic size threshold (19).

Although the structural toxic threshold model constitutes the most frequent attempt found in the literature to provide a molecular explanation for mHtt dysinteractions (5,6), the conformation of expanded polyQ remains elusive. Indeed, several laboratories including ours have challenged the mutant polyQ toxic conformation hypothesis by studying the relationship between the polyQ length, structure, aggregation, interaction and toxicity and showed that both mutant and normal polyQ are toxic and display strikingly similar structural properties (3,20–27).

Demonstration of polyQ-length dependence of Htt interaction relies on semi-quantitative techniques, such as yeast two-hybrid (Y2H), glutathione S-transferase (GST) pull down or immunoprecipitation (IP) (5,6), whose outcome could be influenced by the aggregation of mHtt and partners. It is well known that mHtt, endogenously or overexpressed in a variety of cellular systems (from Escherichia coli to human cells), is prone to proteolysis, accumulation and aggregation. Therefore, the presence of aggregated fragments of Htt could perturb the interaction of Htt with its partners and could constitute a molecular trigger of the reported Htt dysinteractions (5,6).

In the present report, to assess the influence of Htt aggregates on interactions, we studied Htt/partners interactions in the presence or the absence of N-terminus of Htt (Nter-Htt) aggregates. To do so, we developed protocols to prepare highly purified protein interactors devoid of aggregates and to provoke Htt aggregation in a controlled manner. We performed for the first time precise affinity measurements of the interactions between Htt and partners using the quantitative biophysical technique surface plasmon resonance (SPR). In the absence of aggregation, SPR as well as classical pull-down experiments reveal that wild-type and mHtt interact with partners with similar affinity, suggesting that the mutant polyQ per se does not alter Htt interactions. Importantly, we demonstrate that the presence of aggregates influences the solubility and spatial distribution of partners, as well as the outcome of pull-down experiments. Together, these results are consistent with a model for disease pathology in which aggregated Htt fragments are a critical molecular entity perturbing Htt interactions and functions in HD.

RESULTS

Choice of Htt and partners domains

For our study, we used the Nter-Htt because most partners identified so far bind to this region. It contains a HEAT-repeats cluster and a proline-rich domain (PRD; Fig. 1A). The PRD is the Htt domain that binds to the largest number of partners, many of which show altered affinity for mHtt (5,6). Moreover, the proximity of PRD to the polyQ stretch may facilitate the observation of polyQ-length-dependent aberrant interactions.

The choice of Htt partners used in the present study (Fig. 1A) was motivated by three criteria: (i) physical and functional interactions between wild-type Htt and the selected partner have been reported, (ii) partners have been shown to display aberrant interaction with mHtt and dysfunction in mHtt expressing cells and (iii) partners and Htt domains of interaction can be produced in amount and quality suitable for SPR analyses. We selected two partners fulfilling the first two criteria: Huntington-associated protein 1 (HAP1) and SH3GL3 (or endophilin-3). HAP1 is the best characterized Htt partner and was reported to be a modifier of age at onset in HD (28). It interacts with the first HEAT-repeat cluster of Htt (Fig. 1A). HAP1/Htt interaction plays a role in microtubular vesicle trafficking and calcium homeostasis. Several studies suggested that HAP1/mHtt increased interaction accounts for defects in vesicle transport and cytosolic calcium signaling in HD (12,29,30). SH3GL3 is highly expressed in the brain, (31), like Htt (32). SH3GL3 was shown to display increased interaction with mHtt and to accelerate mHtt aggregation process (14). This could participate to neuronal dysfunction by perturbing SH3GL3 function in endocytosis and by sequestering proteins involved in vesicle trafficking (14,33). SH3GL3 contains an SH3 domain that binds to the PRD of Htt (Fig. 1A). SH3GL3 can be considered as a model partner representative of other partners that bind to Htt PRD through small specialized domains (14,34–38), such as SH3...
or WW domains that mediate a specific interaction with proline-rich sequences (37,38).

In addition, we took advantage of the in-house 2B4 anti-Htt monoclonal antibody (39), whose interaction domain in the PRD overlaps with that of SH3GL3 (40,41). 2B4 was used as a positive control for interaction with Htt PRD.

**Protein purification and quality assessments**

To perform quantitative interaction studies in the strict absence of aggregates, we verified the solubility and stability of all purified protein samples. Our working scheme (Fig. 1B) includes sample quality controls at different time points after purification to ensure that the proteins remained soluble and non-proteolyzed until the interaction assays. The importance of controlling for the strict absence of aggregates prior to quantitative interaction measurements by SPR was demonstrated when we produced and purified fragments of diverse sizes encompassing Htt and HAP1 minimal domains of interaction (Supplementary Material, Fig. S1A) (13,42,43). Quality-control assessment after purification revealed low yields, absence of monomeric/dimeric species
but high content of soluble aggregates/oligomers (Supplementary Material, Fig. S1B and C and data not shown). Even if they could be used to confirm direct interactions by pull-down experiments (Supplementary Material, Fig. S1D and E), none of these Htt-HEAT and HAP1 fragments were suitable for analysis by SPR.

In contrast, different SH3GL3 domains and Nter-Htt fragments corresponding to the exon-1, fused to various tags, were readily purified in higher quantity and better quality than Htt-HEAT and HAP1. A schematic representation of these recombinant Nter-Htt and SH3GL3 proteins is shown in Figure 1C (further details on amino acid sequence and protein engineering are presented in Supplementary Material, Fig. S2). Following affinity purification and size-exclusion chromatography, we isolated soluble monomeric/dimeric fractions for all these proteins, with the exception of MBP-SH3GL3 that formed soluble oligomers (Supplementary Material, Fig. S3 and data not shown). The final purity of Nter-Htt fragments, SH3GL3 domains and the antigen-binding domain of 2B4 antibody (2B4-Fab) proteins was verified by SDS–polyacrylamide gel electrophoresis (PAGE) and western blot (Fig. 1D and E).

PolyQ length does not affect Htt interactions

We first asked whether the affinity of Nter-Htt for the SH3 domain of SH3GL3 or the antigen-binding domain of 2B4 (2B4-Fab) is modified by the polyQ length. To do this, we quantified the interaction of SH3-His and 2B4-Fab with MBP-Htt-Q_{16} or with MBP-Htt-Q_{46} proteins by SPR.

In SPR experiments, dynamic interaction parameters are measured in real time between a protein that is fixed on a sensor surface (ligand) and its partner that is injected in solution (analyte). Four parallel SPR measurements can be performed simultaneously in separated microfluidic channels. We captured MBP-Htt-Q_{n} ligands on SPR chip channels via an anti-MBP antibody. SH3-His and 2B4-Fab analytes were injected in increasing amounts, and classical kinetic measurements were performed (Fig. 2A). We found that interactions of Nter-Htt with 2B4-Fab or SH3-His are not affected by the polyQ length since the shape of binding curves recorded on MBP-Htt-Q_{16} and MBP-Htt-Q_{46} surfaces are highly similar. Kinetic data for binding of the 2B4-Fab could be fitted to a 1:1 model, yielding comparable affinities of \( \sim 70 \) nM, regardless of the polyQ size. Kinetic data for binding of SH3-His could not be fitted to a 1:1 model, which suggests a more complex interaction with Nter-Htt. However, visual examination of the kinetic curves indicates similar binding modes. The quality control of protein samples at the end of the SPR session revealed that they were still monomeric, as shown for MBP-Htt-Q_{46} and SH3-His (Supplementary Material, Fig. S3D).

In other sets of experiments, we used different combinations of carrier proteins or different methods of ligand immobilization on sensor channels to rule out the influence of these parameters on the SH3/HttQ_{n} interaction and to confirm the absence of polyQ length effect. When the MBP-SH3-His analyte was injected on TRX-Htt-Q_{16} or TRX-Htt-Q_{46} ligands covalently bound to sensor channels, we observed again that the interaction of SH3 with Nter-Htt-Q_{n} is not affected by the polyQ length (Supplementary Material, Fig. S4A). Interestingly, when the GST-SH3-His analyte was injected on MBP-Htt-Q_{n} ligands bound on the chip via an anti-MBP antibody, the interaction profiles showed slower dissociation kinetics (Supplementary Material, Fig. S4B). This may be partly due to the dimeric nature of the GST tag, which allows the dimeric GST-SH3-His analyte to interact simultaneously with two PRDs bound to the SPR chip, a phenomenon called ‘avidity’. The same effect was observed when the monovalent 2B4-Fab was replaced by the bivalent 2B4 analyte on Nter-Htt-Q_{n} ligands (data not shown). Importantly, even though these results suggest that tags can influence the nature of an interaction, the SPR interaction patterns of GST-SH3-His with MBP-Htt-Q_{16} or MBP-Htt-Q_{46} were again very similar and did not reveal any polyQ length effect (Supplementary Material, Fig. S4B).

Since our SPR results strikingly differ from earlier studies demonstrating the polyQ length influence on the interaction between Nter-Htt-Q_{n} and SH3GL3 (14) by pull down, we performed a similar pull down study using our GST-SH3 and MBP-Htt-Q_{n} protein samples devoid of aggregates. Figure 2B shows that, consistent with SPR data, there was no effect of the polyQ length on interactions by pull down.

To make a more direct comparison with data reporting aberrant interactions between Nter-Htt and SH3GL3 domains (14), we used MBP-SH3GL3 that mostly behaves like soluble oligomers (Supplementary Material, Fig. S3C). Using amylose pull down of MBP-SH3GL3 and monomeric and aggregate-free TRX-Htt-Q_{n} proteins, we observed that SH3GL3 interacts similarly with Htt-Q_{16} and Htt-Q_{46} (Fig. 2C). Our results (Fig. 2C) differ from those of Sittler et al. (14) (Fig. 1C), although the two studies were performed with the same interacting domains of Htt and SH3GL3, the same expression system (E. coli) and similar pull-down techniques. The one important difference between both studies consists in the highly purified protein preparation devoid of aggregates that we used.

All together, SPR and pull-down results demonstrate that, in the absence of Htt aggregation, the polyQ length does not affect the binding of SH3, SH3GL3 or 2B4-Fab on the Htt PRD domain. We thus decided to investigate the impact of aggregates on Htt/partners interaction assays and on the partners’ localization and function.

Nter-Htt aggregates dramatically affect the spatial distribution of 2B4, through native and specific interaction with PRD

During purification and quality control of the proteins used in the present study, we could observe aggregation events in several situations: (i) spontaneous proteolysis was sometimes observed in Nter-Htt samples kept at 4°C, which results in the aggregation of the released polyQ fragments (data not shown) (23,44); (ii) thawing frozen samples of Nter-Htt-Q_{46} often led to partial aggregation (data not shown); (iii) as shown in Supplementary Material, Figure S3, large fractions of soluble oligomeric SH3 and SH3GL3 were systematically observed during purification, and these proteins had a tendency to further aggregate when conserved at 4°C and (iv) spontaneous aggregation was observed upon concentration.
of protein samples. These observations illustrate the difficulty to produce, purify and handle recombinant proteins and highlight the importance to assess their quality prior to perform in vitro interaction studies.

Quality-control assessment was rarely performed in previous studies reporting on mHtt dysinteraction. Since aggregation of mHtt is commonly observed both in vitro and in vivo, we then asked what could be the influence of mHtt aggregates on Htt partners interaction. SPR could not be used to answer this question, because its microfluidic system is not adapted to the injection of aggregated material. Therefore, we designed the experimental assay depicted in Fig. 3A to study the behavior of Htt partners in the presence of Nter-mHtt aggregates. We assessed whether aggregates affect the solubility and spatial localization of partners, and if so, whether this depends on specific interaction with PRD or on non-specific sequestration by aggregated polyQ. We chose 2B4 and 2B4-Fab for their high affinity to PRD and because they undergo little self aggregation, even if used at high concentration.

The assay showed that 2B4 was very efficiently recruited to Nter-Htt aggregates (Fig. 3B). Addition of competitor peptide corresponding to 2B4 epitope abolished the recruitment to aggregates. Similar results were obtained when 2B4 was replaced by 2B4-Fab (Fig. 3C). Moreover, 2B4-Fab molecules recruited within mHtt aggregates were very efficiently resolubilized by the classical SDS/heating treatment [Fig. 3C, compare lanes total extract (TE)-24 h and supernatant (SN)-24 h], in contrast to the aggregated polyQ that are extremely poorly resolubilized by this treatment (23). Taken together, these results show that 2B4 and 2B4-Fab, which are soluble in the absence of aggregates, are depleted from the soluble fraction and relocalized in the aggregated fraction via native interaction with Htt PRD.

**PolyQ aggregation can strongly bias pull-down interaction assays**

We further asked what could be the influence of Nter-Htt aggregation on the outcome of semi-quantitative techniques classically used to study polyQ-length influence on Htt interactions. To address this question, we performed pull-down experiments with MBP-SH3GL3 and Trx-Htt-Q46, in conditions whereby Trx-Htt-Q46 aggregation is progressively induced. Normal-repeat-length polyglutamine peptides were shown to accelerate aggregation of expanded polyglutamine proteins (45). To induce Trx-Htt-Q46 aggregation in a controlled manner, we thus used a small peptide bearing an 11-glutamine sequence (Q11) that forms amyloid-like aggregates by itself (26) and provokes the aggregation of expanded polyglutamine proteins (45). To induce Trx-Htt-Q46 aggregation in a controlled manner, we thus used a small peptide bearing an 11-glutamine sequence (Q11) that forms amyloid-like aggregates by itself (26) and provokes the aggregation of expanded polyglutamine proteins (45). To induce Trx-Htt-Q46 aggregation in a controlled manner, we thus used a small peptide bearing an 11-glutamine sequence (Q11) that forms amyloid-like aggregates by itself (26).
Figure 3. Aggregates of Nter-Htt recruit 2B4 through specific interaction with PRD. (A) Experimental setup. TRX-Htt-Q46 was incubated with sufficient amount of enterokinase to ensure complete cleavage in 30'. Once released, Htt-Q46 can aggregate after 6 h incubation. 2B4 or 2B4-Fab was added 30' after the beginning of the cleavage. After 24 h incubation, the quantities of 2B4 or 2B4-Fab in the vortexted TE or in the SN after centrifugation were analyzed by WB to evaluate the recruitment within the aggregate. In parallel settings, specific peptide corresponding to the 2B4 epitope or non-specific peptide was added as competitor. (B) Quantification of 2B4 in the SN in the absence or the presence of peptide competitors. The SN was analyzed by WB with goat anti-mouse IgGs (GAM) coupled to peroxidase, and quantification was normalized with the control 2B4 added 30' after proteolysis of TRX-Htt-Q46, prepared extemporaneously. Mean values ± SD of three independent experiments are shown. The results show that in the absence of peptide or in the presence of non-specific peptide, 2B4 was depleted from the SN, whereas in the presence of specific peptide competitor, 2B4 remained soluble and non-aggregated. (C) Quantification of 2B4-Fab in the SN or the TE after Htt-Q46 aggregation. After 2B4-Fab was recruited within Htt-Q46 aggregates, the SN and TE fractions were analyzed by WB with GAM peroxidase, and quantification was normalized with the control 2B4-Fab added 30' after proteolysis of TRX-Htt-Q46, prepared extemporaneously. Mean values ± SD of three independent experiments are shown. A larger amount 2B4-Fab was solubilized in the TE fraction than in the SN by SDS/heating treatment, indicating that the 2B4-Fab recruitment in Htt-Q46 aggregates does not result in strong SDS/heating resistant sequestration.

Finally, we analyzed the amount of TRX-Htt-Q46 retained by pull down. The results show that, as the proportion of aggregates in the samples increased, increasing amounts of TRX-Htt-Q46 were recovered by pull down (Fig. 4C). For highly aggregated samples (conditions h and i), in which aggregated material was visible in the test tube after incubation, TRX-Htt-Q46 was probably at least partly retained in a non-specific manner by the amylose beads mesh. However, in conditions where the solubility of TRX-Htt-Q46 observed on native–PAGE and the binding of MBP-SH3GL3 to the amylose beads seemed unaffected (Fig. 4A and B, samples a–e), quantities of TRX-Htt-Q46 recovered by pull down were nevertheless increased (Fig. 4C, samples a–e compared with sample 0). Interestingly, the heterogeneous patterns of multimeric Nter-Htt entities are also observed on WB at various [Q11/Nter-Htt] ratios (Fig. 4C). These likely represent diverse intermediates of Nter-Htt aggregation. The increased amount of Nter-Htt recovered by pull down could thus also reflect an increased interaction between soluble aggregation intermediates and MBP-SH3GL3. Importantly, the experiment depicted in Fig. 4 demonstrates that even minute quantities of aggregates can modify the outcome of pull-down experiments.

**DISCUSSION**

The molecular mechanisms underlying brain-specific degeneration in HD are yet unknown. During the past years, much attention has been paid to Htt interacting network and the disruption of specific Htt/partner functions in HD. The observation that Htt interacts aberrantly with numerous partners led to the concept that polyQ expansion undergoes a toxic conformational change that affects interactions between Htt and partners, thereby providing a first mechanism to support a role for mHtt dysinteractions in the pathogenesis.

However, the polyQ-length-dependent conformational change in Htt remains elusive. Moreover, there has been no accurate study to quantitatively assess the effect of the polyQ length on interaction between Htt and partners. The methods used so far to study Htt interactions were semi-quantitative and often relied on overexpressed proteins and/or pull-down procedures, which are prone to produce protein aggregation that could alter the outcome of interaction studies. In the present study, to understand the molecular mechanism underlying mHtt dysinteractions, we studied Htt/partner interactions, in the presence or the absence of Nter-Htt aggregates, with two partners: SH3GL3, a representative partner of Htt-PRD, which was reported to interact aberrantly with mHtt, and the model 2B4 antibody, whose binding sequence on Htt-PRD overlaps with that of SH3GL3 (Fig. 1A).
Expanded polyQ per se does not alter Htt interactions

To assess the Htt interaction in the absence of aggregates, we developed a specific protocol to prepare highly purified recombinant proteins and to carefully remove aggregates contamination from protein samples. We then performed for the first time a quantitative measurement of the interactions between aggregate-free Htt and partners by SPR. The SPR results clearly indicate that Nter-Htt fragments bearing either a non-pathological (Q16) or a pathological (Q46) polyQ interact with similar affinity with SH3GL3 or its SH3 domain and with 2B4-Fab (Fig.2, Supplementary Material, Fig. S4). Similarly, classical pull-down experiments revealed no effect of the polyQ length on the interaction between aggregate-free Nter-Htt fragments and SH3GL3 or its SH3 domain. Together, these results demonstrate that, in the absence of aggregation, mHtt does not interact aberrantly with this set of partners and call for further re-investigation into the effect of the polyQ length of Htt on interaction with other reported partners in conditions where the absence of aggregates is controlled.

The fact that Htt with WT and expanded polyQ length interact identically with partners suggests that the conformation of mutant polyQ per se is not responsible for altered mHtt interactions and thereby disagrees with the structural threshold model. Our results are consistent with numerous reports that challenged the structural threshold model and showed that mutant and normal polyQ display strikingly similar structural properties (3,20–24,26,27): notably, it was shown that (i) the strong interaction of expanded polyQ with some antibodies is not due to a specific mutant polyQ conformation but to a so called ‘avidity effect’ of bivalent antibodies on repeated homopolymeric epitopes (21,23), (ii) normal size polyQ bear intrinsic toxicity (3,25) and can also form amyloid-like fibers in vitro that are resistant to chemical and heating treatments (18,22,23) and (iii) the ‘water-filled nanotube’ model, which derives from the analysis of X-ray diffraction patterns of Q15-aggregated peptides (19), was contradicted after re-analysis of the original diffraction data (27) as well as after new X-ray analysis of aggregated polyQ peptides (26). Moreover, aggregates of polyQ of various lengths (from Q8 to Q45) provide very similar diffraction patterns and the core of amyloid-like-aggregated polyQ consists in a short stretch of 5–7 glutamines (26). All these studies support another pathomechanistic model (23), in which toxicity gradually increases with the polyQ length and aggregation kinetics and is modulated by the protein and cellular context. In this context-dependent model, the polyQ toxicity would manifest when the protein clearance machinery of the cell, which contributes to prevent proteotoxicity by recycling misfolded proteins and declines with aging, is overwhelmed.

Nter-Htt aggregates influence the outcome of interaction assays and affect the spatial distribution and solubility of partners

The present study also provides evidence that Nter-Htt aggregates influence the outcome of interaction assays and strongly affect the spatial distribution and solubility of partners in vitro. The production and handling of recombinant proteins often require careful monitoring of their solubility, especially if...
they are prone to aggregate. During our study, we could observe the aggregation of Htt fragments and other recombinant proteins in a number of conditions (storage, proteolysis, concentration etc.). This led us to suspect that aggregated Htt material was likely present in most interaction experiments published so far. Indeed, in many interaction studies conducted in vitro by pull down, recombinant proteins were overexpressed and their soluble nature was not verified by size-exclusion chromatography or other adapted methods. Moreover, ex vivo Y2H experiments using overexpressed proteins, and IP experiments that rely on the same principle as pull down, could also be influenced by the presence of mHtt aggregates (46–50).

We thus carried out two types of experiment to demonstrate how Nter-Htt aggregates can influence classical interaction studies. First, we show that in the presence of Nter-Htt aggregates, 2B4 and 2B4-Fab are efficiently depleted from the soluble fraction and recruited in the aggregates (Fig. 3B and C). This recruitment depends on a specific interaction with the PRD and not on unspecified sequestration. Second, we used a classical pull-down setup to study the interaction between SH3GL3 and aggregate-free Nter-Htt fragments as in Figure 2C, but this time we provoked the aggregation of Nter-Htt in a controlled manner (Fig. 4). We show that, compared with control in the absence of aggregates, increased aggregation of Nter-Htt strikingly correlates with increased Nter-Htt retention on MBP-SH3GL3 pull down. Importantly, the pull down of Nter-Htt by MBP-SH3GL3 was increased even in conditions where minute amounts of aggregates were present, demonstrating the vulnerability of pull-down assay to assess interaction with proteins contaminated with aggregates. During this experiment, we also noticed that the efficiency of MBP-SH3GL3 binding on amylose beads was reduced in conditions where Nter-Htt was highly aggregated. This suggests that Nter-Htt aggregates affect the solubility of MBP-SH3GL3, likely through interaction between SH3 and Htt PRD (14) and prevent its proper binding to amylose beads. Together, these results suggest that Nter-Htt aggregates behave like a ‘molecular platform’ that concentrates a large number of PRD sequences in a small physical territory, which affects the localization and solubility of Htt-PRD partners and thereby could influence their function.

A role for Nter-Htt-aggregated species in Htt/partners dysinteractions and dysfunctions in HD

Our in vitro experiments showing aggregates-dependent relocation and dysfunction of partners (Figs 3 and 4) highlight conditions that were proposed to occur in vivo. Indeed, a large number of proteins are associated with large inclusion bodies. They can be subdivided in at least three categories: first, chaperones and proteasomes associate with the inclusion bodies, likely as an attempt to clear protein deposits; second, proteins bearing a polyQ sequence are stably sequestered through co-aggregation with mutant proteins and display loss of function (51–53); finally, natural partners of Htt such as SH3GL3 are also recruited into large inclusion bodies (14,33), likely through the high concentration of binding sites present in aggregates, as suggested by our results (Fig. 3). Loss of function or dysfunction is proposed to be a consequence of partners’ trapping and subcellular delocalization, which is supported by our in vitro experiments (Figs 3 and 4).

In addition, Nter-mHtt forms numerous aggregated structures in vitro, including soluble oligomers that are thought to be highly toxic (2,54–56) and that precede the formation of water insoluble entities (57). There are now growing evidence that Nter-mHtt aggregates formed in cultured cells or in brain tissue from HD mouse models are also complex and that their structure and properties resemble that of in vitro aggregates (58,59). In particular, the presence of small oligomers was detected before any behavioral symptom in HD R6/2 and knock-in mouse models (50). Consistently, in HD knock-in mice, a major proteolytic fragment of Htt corresponding in size to the aggregation prone Htt exon-1 of R6/2 mice was detected as a soluble cytoplasmic form for many months, until it accumulates and aggregates in the nuclei prior to disease onset (49). If some aggregated forms of Nter-mHtt that are small, soluble and free to diffuse in the cell are present in HD cells a long time before their actual conversion in inclusion bodies, one could speculate that they can also be very efficient molecular platforms that perturb Htt’s interactions and functions. These perturbations could be direct, if the domain of interaction of a given partner is present in the aggregated fragment—like the PRD for instance. Perturbations could also be indirect if Nter-mHtt aggregates affect the solubility of Htt and prevent Htt to interact properly with partners, as proposed earlier (45,58,60). The latter mechanism is supported by our observations that small amounts of aggregated polyQ strongly affect the solubility of TRX-Nter-Htt (Fig. 4A). Whether perturbation is direct or indirect, interactions between a partner and Htt could appear enhanced or decreased, depending on the accessibility and conformation of the Htt interacting domains present into the aggregated-Htt platform.

Aggregation-mediated dysfunction of Htt/partners complexes could then result in specific HD dysfunctions (9–12). During aging, increased protein aggregation and decreased proteostasis efficiency (61) would result in increased Nter-Htt aggregation, dysinteractions and dysfunctions, which would participate to the progressive worsening of the HD symptoms. Up to now, there is no effective treatment to prevent or slow down the disease process in HD or in any other polyQ disease. If aggregated polyQ species influence Htt’s and its partners’ functions, then modulating mHtt aggregation could have positive effects on specific cellular pathways deregulated in HD.

MATERIALS AND METHODS

Plasmid engineering, protein production and purification

The TRX-Htt-Q16 and TRX-Htt-Q46 expression vectors were described elsewhere (21); MBP-Htt-Q16 and MBP-Htt-Q46 were amplified by polymerase chain reaction (PCR) using, respectively, TRX-Htt-Q16 and TRX-Htt-Q46 as source cDNA and cloned into pET-22b(+) (Novagen). All other expression constructs were generated by PCR and recombination with the Gateway cloning system (Invitrogen), and the method and vectors described by Busso et al. (62).
Fusion proteins were produced in *E. coli* BL21DE3, grown either at 16°C (overnight) or 37°C (3 h) and induced with isopropyl-thiogalactopyranoside (0.1 mM). TRX-Htt-Q16, TRX-Htt-Q46, MBP-Htt-Q16 and MBP-Htt-Q46 were released by osmotic shock as in Bennett et al. (22). For other fusion proteins, cell pellets were resuspended in buffer A (10 mM Tris, pH 7.4, 50 mM NaCl, 50 mM KCl and 10% glycerol) containing a complete protease inhibitor (CPI) mixture (Complete, EDTA-free; Roche Diagnostics) and cells were broken by sonication. Cell debris was spun down by centrifugation (200 000g for 1 h at 4°C). The SN was loaded on cognate affinity chromatography columns: Glutathione Sepharose™ 4B affinity beads (GE-Healthcare Biacore, Uppsala, Sweden); Amylose affinity beads (BioLabs® Inc.); TALON® affinity beads (CloneTech); GSTrap™ 4B 5 ml columns; HiTrap™ IMAC FF 5 ml columns or MBTrap HP 5 ml columns (GE-Healthcare Biacore). After washing with buffer A, bound proteins were eluted following the manufacturer’s instructions specific for each column. Affinity-purified proteins were passed through a 16/900 or a 10/300 gel filtration (GF) column, containing either Superose 75 (S75) or Superose 200 (S200) beads, pre-equilibrated in buffer A. Fractions were analyzed by SDS–PAGE and/or WB.

SH3-His was obtained by TEV proteolysis of GST-SH3-His (3 mg) incubated, overnight at room temperature (RT) with 0.15 mg TEV protease in TEV protease buffer [50 mM Tris–HCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), pH 8.0] in 400 μl final reaction volume. GST was retained on Glutathione affinity beads and SH3-His was further purified via GF and used in SPR experiments.

2B4 antibody was purified from ascitic fluids by affinity chromatography using protein A Sepharose beads (Amersham Biosciences), eluted with 0.1 M glycine buffer (pH 3.0) and dialyzed against 20 mM phosphate buffer (pH 7.0). 2B4-Fab was prepared by incubating purified 2B4 with papain agarose (Sigma) at 37°C for 17 h (0.7 U papain/mg 2B4; 0.1 mM DTT). Fc fragments were removed by iterative protein A affinity chromatography steps until complete depletion of Fc (and traces of uncleaved 2B4) from the Fab sample.

All proteins were concentrated by centrifugation on Amicon Ultra-15 Centrifugal Filter Units (Millipore™) of various cutoff. Protein concentration was calculated using the Epsilon-M value specific of each protein, after absorption spectrum scanning between 220 and 320 nm on NanoDrop™ (Thermo Scientific). To ensure optimal proteolysis resistance, CPI was added to the purified proteins (except for 2B4 and 2B4-Fab) immediately after concentration, before storage at 4 or −20°C.

**Surface plasmon resonance**

Affinity measurements were performed either with freshly prepared proteins or with aliquoted/frozen samples whose stability to freezing/thawing was verified. In all cases, samples were centrifuged three times (10; 16 000g; 4°C) and the aggregates-free SN was dialyzed (3 × 1 h) against the running buffer [HBS-EP: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10 mM), pH 7.4; NaCl (150 mM); EDTA (3.4 mM), 0.005% (v/v) surfactant P20] immediately before the experiments.

The same buffer was used for cascade-dilution of proteins. Interaction studies were performed using a BIACORE 2000 instrument (GE-Healthcare Biacore). The research grade CM5 sensorchips (BR-1000-14), amine coupling kit (BR-1000-50) and anti-GST capture kit (BR-1002-23) were purchased from GE-Healthcare Biacore. In SPR experiments, one of the interaction partners—called the ligand—is fixed on the sensor surface, and the other partner—called the analyte—is injected over the surface in a continuous flow. MBP-Htt and GST-SH3 proteins were captured in an oriented manner via, respectively, anti-MBP and anti-GST antibodies that were immobilized on the sensor surface using the standard amine coupling procedures, following the manufacturer’s instructions. TRX-Htt proteins were immobilized covalently through amine coupling. Reference channels contained MBP, GST and TRX, respectively. To perform binding experiments, analytes were injected over ligands and reference surfaces at 25°C, at a flow rate of 30 μl/min, with 10 min dissociation periods in the case of kinetic measurements. The initial reaction rate on the MBP-Htt, GST-SH3 and TRX-Htt surfaces was not affected by flow variations between 30 and 100 μl/min indicating the absence of mass transport (data not shown). For data evaluation, data collected on the reference channel loaded with either MBP, GST or TRX were subtracted from data collected on, respectively, MBP-Htt, GST-SH3 and TRX-Htt channels. Binding curves (PRD/2B4-Fab interaction) were fit to simple 1:1 Langmuir binding models in order to evaluate kinetic and affinity parameters, respectively, using the BiaEvaluation 4.1 software (GE-Healthcare Biacore).

**Pull-down assays**

*Between GST-SH3-His and MBP-Htt.* GST-SH3-His and GST (0.1 mg each) were incubated with 200 μl glutathione-Sepharose 4B beads (Pharmacia) pre-equilibrated in buffer A and washed three times with ice-cold buffer A. Beads were then incubated with 0.1 mg of purified MBP-Htt-Q16 proteins for 5 min and washed five times with a total volume of 6 ml buffer A. Beads were boiled in Laemmli buffer and analyzed by western blotting. The membrane was probed with an in-house monoclonal MBP antibody (1:5000).

*Between MBP-SH3GL3-His and TRX-Htt.* (1) Figure 2C: MBP-SH3GL3-His proteins (0.1 mg each) were incubated with 200 μl amylase affinity beads (BioLabs® Inc.) pre-equilibrated in buffer B (10 mM Tris, pH 7.0, 200 mM NaCl, 200 mM KCl, 0.1% NP40 and washed three times with ice-cold buffer B). Beads were then incubated for 5 min with TRX-Htt-Q16 protein (1 mg), then washed in buffer B (10 × 1.5 ml). Beads were boiled in Laemmli buffer and analyzed by western blotting. The membrane was probed with the monoclonal 2B4 antibody (1:200). (2) Figure 4: MBP-SH3GL3 and TRX-HD-Q46 (247.5 μl in buffer A) were added to 2.5 μl H2O or 2.5 μl Q11 peptide (exact sequence: RRSQ11 SRR) resuspended at various stock concentrations in H2O (stock concentrations of Q11: a = 4 μM; b = 12 μM; c = 40 μM; d = 120 μM; e = 400 μM; f = 1.2 μM; g = 4 μM; i = 20 mM). Q11 stocks are acidic (pH < 3).
Because of traces of trifluoroacetic acid resulting from peptide synthesis, no aggregation is visible over long periods of time, but once diluted at pH 7 Q11 starts to aggregate, in agreement with previous observations made on a Q15 peptide (22). MBP-SH3GL3 and TRX-HD-Q46 final concentration are, respectively, 1.1 and 30 μM. The molar ratio [Q11]/[TRX-HD-Q46] varies between conditions a to i: a = 0.0013; b = 0.004; c = 0.013; d = 0.04; e = 0.13; f = 0.4; g = 1.33; h = 4; i = 6.7. MBP-SH3GL3, TRX-HD-Q46 and Q11 were incubated for 16 h at 4°C. After incubation MBP-SH3GL3 and TRX-HD-Q46 aggregate pellets were visible in samples containing the two highest quantities of Q11. All samples were vortexed and small aliquots (2 μl) were analyzed by native-PAGE (8/25 Phast System, Pharmacia). Samples were further loaded on amylose bead columns (Ozyme, 200 μl beads per sample) to retain MBP-SH3GL3 by affinity. Beads were washed with 15 ml buffer B, boiled in Laemmli buffer and analyzed by western blotting. The membrane was probed with the monoclonal 2B4 antibody (1:200).

Between HAP1 (143–425) and Htt (75–241). Equal amount of GST fusion and GST proteins were incubated with 80 μl glutathione-Sepharose 4B beads (Pharmacia) pre-equilibrated in buffer A. Beads were washed two times with buffer A, incubated with equal amount of MBP-fusion proteins for 1 h and washed three times with buffer A containing (0.1% NP40 and 1 mM DTT). Beads were boiled in Laemmli buffer and analyzed by western blotting. The membrane was probed with in-house monoclonal MBP antibody (1:5000).

Western blotting were developed with the ECL system (Amersham Biosciences). For quantifications, acquisitions were made on photographic films or directly on Fusion-FX7 system (Vilber-Lourmat). The BIO1D software was used for quantifications.

Recruitment assay

The essay was optimized to ensure full cleavage of TRX-Htt-Q46 before 30’, and that complete aggregation of Htt-Qn is complete after 6 h. The cleavage liberates Htt-Q46 as well as a second product lacking the 15 first amino acids of Htt. Both fragments encompass the polyQ and the PRD, as well as a second product lacking the 15 first amino acids of Htt-Qn is complete after 6 h. The cleavage liberates Htt-Q46 before 30 min. Recruitment assay quantifications.

Figure 3B: TRX-Htt-Q46 (4.48 nmol) and enterokinase (EKMax™, Invitrogen Life Science Technologies—10 U) were incubated for 30 min at 25°C in a volume of 22 μl to separate initially soluble Htt-Q46 from the Nter GST moiety. About 6 μl of 2B4 (9.6 pmol) was added as well as either 6 μl of buffer B [phosphate (50 mM) pH 6.9] or 6 μl of buffer B containing 2B4 epitope peptide (sequence: PPQLPQPPQAQPLLPPQPP; 10.65 nmol) or 6 μl of buffer B containing non-specific peptide (sequence: P106; final concentration 250 μM). Samples were incubated for either 30 min (control and reference for quantification) or 24 h at 25°C, in a total volume of 34 μl. Ten minutes prior to SDS–PAGE analysis, aggregates were pelleted by centrifugation (16 000g, 5 min at RT), and SNs were mixed with Laemmli buffer and boiled. We used SDS–PAGE 4–20% precast gels (Pierce™) following the manufacturer’s instructions. Western blotting was developed with the ECL system (Amersham Biosciences). Acquisition and quantification were done as for GST pull-down assays.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of interest statement. None declared.

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REFERENCES


