Huntingtin toxicity in yeast model depends on polyglutamine aggregation mediated by a prion-like protein Rnq1

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The cause of Huntington's disease is expansion of polyglutamine (polyQ) domain in huntingtin, which makes this protein both neurotoxic and aggregation prone. Here we developed the first yeast model, which establishes a direct link between aggregation of expanded polyQ domain and its cytotoxicity. Our data indicated that deficiencies in molecular chaperones Sis1 and Hsp104 inhibited seeding of polyQ aggregates, whereas *ssa1*, *ssa2*, and *ydj1–151* mutations inhibited expansion of aggregates. The latter three mutants strongly suppressed the polyQ toxicity. Spontaneous mutants with suppressed aggregation appeared with high frequency, and in all of them the toxicity was relieved. Aggregation defects in these mutants and in *sis1–85* were not complemented in the cross to the *hsp104* mutant, demonstrating an unusual type of inheritance. Since Hsp104 is required for prion maintenance in yeast, this suggested a role for prions in polyQ aggregation and toxicity. We screened a set of deletions of nonessential genes coding for known prions and related proteins and found that deletion of the *RNQ1* gene specifically suppressed aggregation and toxicity of polyQ. Curing of the prion form of Rnq1 from wild-type cells dramatically suppressed both aggregation and toxicity of polyQ. We concluded that aggregation of polyQ is critical for its toxicity and that Rnq1 in its prion conformation plays an essential role in polyQ aggregation leading to the toxicity.

Introduction

Expansion of polyglutamine (polyQ) domains in certain proteins causes several fatal neurodegenerative diseases, for example, Huntington's disease. All of these diseases are inherited as a dominant trait, and their onset and severity closely correlate with the length of abnormal polyQ sequences (Gusella and Macdonald, 1998; Cummings et al., 1999a). These and other observations led to a conclusion that polyQ expansion is a gain-of-function mutation, causing polypeptides with expanded polyQ (for example, huntingtin) to acquire an unusual conformation, which facilitates their aggregation into intracellular inclusion bodies (IBs)* and causes cell toxicity (Difiglia et al., 1997; Scherzinger et al., 1997; Bates et al., 1998).

It was initially assumed that denatured or abnormal proteins aggregate and form IBs simply due to their intrinsic tendency to associate with each other until the aggregates eventually become insoluble. However, recent reports demonstrated that formation of cytoplasmic IBs in mammalian cells may be a complex process involving transport of small protein aggregates from the cell's periphery to the centrosome along microtubules. These IBs contain Hsps, ubiquitin, ubiquitin-conjugating enzymes, and 26S proteasomes forming a large structure termed aggresome (Vidair et al., 1996; Wojcik et al., 1996; Johnston et al., 1998; Anton et al., 1999; Garcia-Mata et al., 1999; Wigley et al., 1999; Fabunmi et al., 2000). Recent findings from our and other groups indicate that IBs formed by polyQ-containing polypeptides have similarities with the aggresome (Stenoien et al., 1999; Wyttenbach et al., 2000; Meriin et al., 2001; Waelter et al., 2001). Furthermore, we have found recently that a stress-activated protein kinase MEKK1 stimulates early stages of IB formation (Meriin et al., 2001), suggesting that various steps of IB formation are tightly controlled by the cell.

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*Abbreviations used in this paper: IB, inclusion body; GFP, green fluorescent protein; GuHCl, guanidine hydrochloride; polyQ, polyglutamine.

Key words: aggregation; polyglutamine; toxicity; prions; yeast

Close correlation between formation of the huntingtin-containing IBs and death of the affected neurons suggests that IB may be involved in neurodegeneration (Davies et al., 1998). However, there is evidence that IB formation may not be necessary for the neuronal death, since artificial inhibition of aggregation of polyQ-containing polypeptides in certain animal and cellular models did not inhibit and even enhanced neuronal apoptosis (Klement et al., 1998; Saudou et al., 1998; Cummings et al., 1999b). Therefore, IB formation may be irrelevant to the neurodegeneration process or may even serve a protective role by capturing toxic soluble polyQ molecules (for review see Sherman and Goldberg, 2001). The question whether soluble abnormal proteins or IBs cause toxicity and neurodegeneration is the focus of ongoing discussion in the field. Because of the complexity of polyQ-induced neuronal death and IB formation, development of adequate cellular and animal models is critical to dissect cellular mechanisms of these processes. The practical application of this research would be identification of proteins involved in neurodegeneration and IB formation in order to use them as targets for drug design.

In mice, *Drosophila* and *Caenorhabditis elegans* expression of extended polyQ polypeptides caused toxicity and neurodegeneration, and IBs in neurons were detectable, whereas polypeptides with polyQ of normal length were not toxic and did not form IBs (Davies et al., 1997; Scherzinger et al., 1997; Kazemi-Esfarjani and Benzer, 2000; Satyal et al., 2000). Notably, in mice model expression of exon 1 of huntingtin, a small NH_2-terminal fragment with extended polyQ domain, was sufficient to cause both neurodegeneration and IB formation (Davies et al., 1997). Yeast *Saccharomyces cerevisiae* has also been used as a model, since it provides a useful tool for screening of genes involved in IB formation and potential polyQ-induced toxicity and for screening of chemical compounds, which inhibit these processes. Short polyQ was shown to be soluble in yeast, whereas long polyQ polypeptide–formed IBs; however, no toxicity of polyQ polypeptides in yeast has been reported so far (Krobitsch and Lindquist, 2000; Muchowski et al., 2000). In these yeast models, the molecular chaperone Hsp104 was reported to be essential for the aggregation of polyQ (Krobitsch and Lindquist, 2000). Overexpression of other molecular chaperones Hsp70 and Hdj1 also affected polyQ aggregation suppressing formation of fibrous aggregates and promoting instead formation of amorphous structures (Muchowski et al., 2000).

The molecular chaperones Hsp104, Hsp70, and Sis1 were implicated in emergence and maintenance of prion conformation of certain yeast proteins, for example, Sup35, Rnq1, Ure2 (Chernoff et al., 1995, 1999; Derkatch et al., 1997; Newnam et al., 1999; Jung et al., 2000; Moriyama et al., 2000; Sondheimer and Lindquist, 2000; Sondheimer et al., 2001; Wegrzyn et al., 2001). These polypeptides in prion conformation aggregate and convert normal polypeptides of the same type into prion conformation, thus recruiting them into IBs. The phenotypic traits resulting from such aggregation are inherited in a non-Mendelian fashion (Wickner et al., 1999; Serio and Lindquist, 2000). These mechanisms closely resemble aggregation of mammalian prion PrP, a cause of a group of neurodegenerative disorders (Prusiner, 2001).
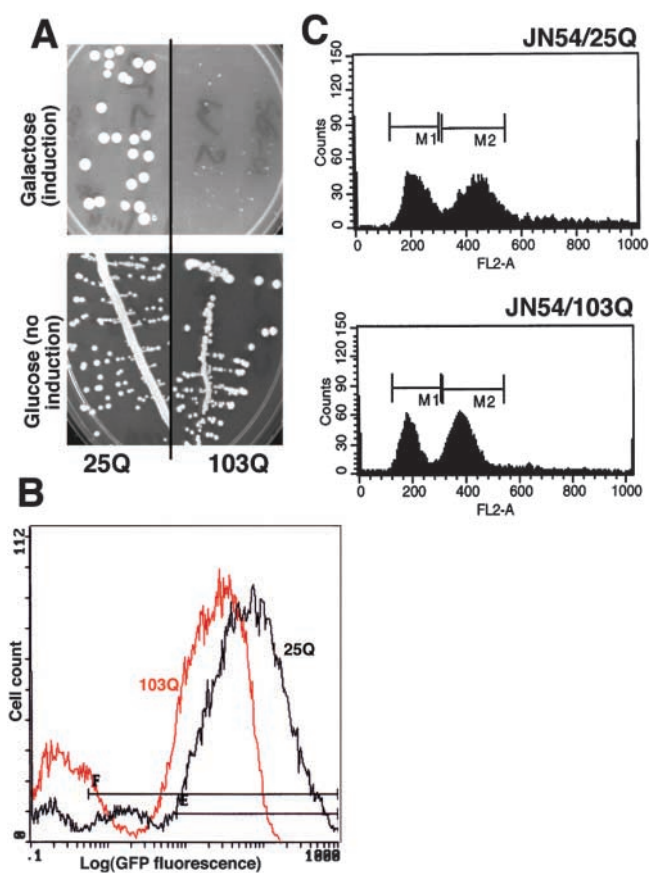

Figure 1. **Accumulation of 103Q in yeast cells inhibits yeast growth.** (A) Cells transformed with 25Q or 103Q expression vector grew on selective glucose plates for 3 d or on galactose plates for 5 d. (B) Flow cytometry analysis of fluorescence of the cells induced for 20 h. The peaks at the left represent cells, which do not express polyQ. Note, the scale of the fluorescence of accumulated GFP fusion is logarithmic. (C) Accumulation of 103Q does not affect cell cycle. FACS® analysis of yeast cultures expressing 25Q or 103Q after 20 h induction in selective galactose medium.

Here, we develop a new yeast model of polyQ expansion diseases, which establishes a direct link between polyQ aggregation and toxicity. Using this model we searched for cellular elements involved in control of polyQ aggregation and toxicity.

Results

Accumulation of 103Q is toxic to yeast cells

To develop the yeast model of the polyQ expansion disease, we expressed in yeast cells the green fluorescent protein (GFP)–tagged polypeptides derived from normal (25Q) and mutant (103Q) (see Materials and methods) forms of huntingtin under a galactose-inducible promoter. In contrast to 25Q, expression of 103Q was toxic to yeast cells, since colonies expressing 103Q ceased to grow within 1–2 d after induction on galactose medium (Fig. 1 A, top). No difference in colony size was seen on glucose-containing medium without polyQ expression (Fig. 1 A, bottom), indicating that accumulation of 103Q was responsible for the growth defect.

Notably, the cellular levels of accumulated 103Q were much lower than the level of 25Q as seen by a decrease in in-

tensity of fluorescence of the cells expressing 103Q (Fig. 1 B). This difference in accumulation of the polyQ polypeptides evidently was not related to 103Q degradation, since both 25Q and 103Q polypeptides were relatively stable in yeast (unpublished data). The difference in expression may be related to reduction of the plasmid copy number in cells expressing 103Q due to plasmid instability. Accordingly, in spite of constantly maintained selection for the plasmid's URA marker cells expressing 103Q were losing this plasmid rapidly. After 3 d on selective galactose plate, only 0.1% of cells expressing 103Q retained the plasmid with the 103Q gene, whereas as much as 25% of cells expressing 25Q still maintained the plasmid (unpublished data). Therefore, the 103Q-induced toxicity appears to be so severe that cells which lost the plasmid have an advantage over 103Q-expressing cells even on the selective medium.

To study whether expression of expanded polyQ inhibits growth by affecting the cell cycle progression, we compared cellular DNA contents in the cultures expressing 25Q and 103Q. Cells induced in selective galactose medium for 20 h were stained with propidium iodide and then analyzed by FACS®. Expression of 103Q in strain W303 caused a slight increase of G2 phase cells in comparison with cells expressing 25Q (unpublished data), whereas in 103Q-expressing JN54 cells no changes in the cell cycle were observed (Fig. 1 C) in spite of significantly decreased growth. This indicates that there is no substantial cell cycle delay, which could account for the growth inhibition upon accumulation of 103Q.

Molecular chaperones are essential for polyQ aggregation and toxicity

Microscopic observation showed that 25Q was diffusely distributed in yeast cells, whereas every cell expressing 103Q formed multiple aggregates of two types (Fig. 2 A): appearing either as large disordered lumps or as small grains (or flakes), some of which were swarming in a cell. A fraction of cells with small aggregates increased in expense of cells with large aggregates upon prolonged 103Q accumulation, suggesting that aggregates are dynamic formations capable of further evolution. Fractionation of cell homogenates in the presence of 1% Triton X-100 provided independent evidence that the majority of 25Q (>75%) were soluble in the cells (Fig. 2 B). Notably, a fraction of the 25Q was found in 800 g pellets, indicating that although visible aggregates were not seen with short polyQ, some of the molecules associated with large detergent-insoluble cellular structures. With 103Q, from 50 up to 95% of the polypeptide was found in pellets, depending on duration of induction and yeast strain.

Because polyQ expression in yeast shows a length-dependent toxicity, this system offers a simple method to study the mechanisms of polyQ aggregation and toxicity genetically. In search of cellular elements controlling the process of aggregation, we screened a set of mutants of chaperones and related proteins, including *hsp104, ssa1 ssa2, ssa1 ssa3, ssa1 ssa4, ssb1 ssb2, sse1, sse2, pdr, ydj1–151, sis1–85, xdj1, zuo1, ypr061c, ynl227c, yfr041c, ynl077w, sti1,* and *sba1.* Among these mutants, *hsp104* deletion, *sis1–85* mutation, *ssa1 ssa2* double deletion (Fig. 2 C) and *ydj1–151* mutation (unpublished data) significantly suppressed 103Q aggregation as assayed by fractionation. Two distinct types of the aggregation
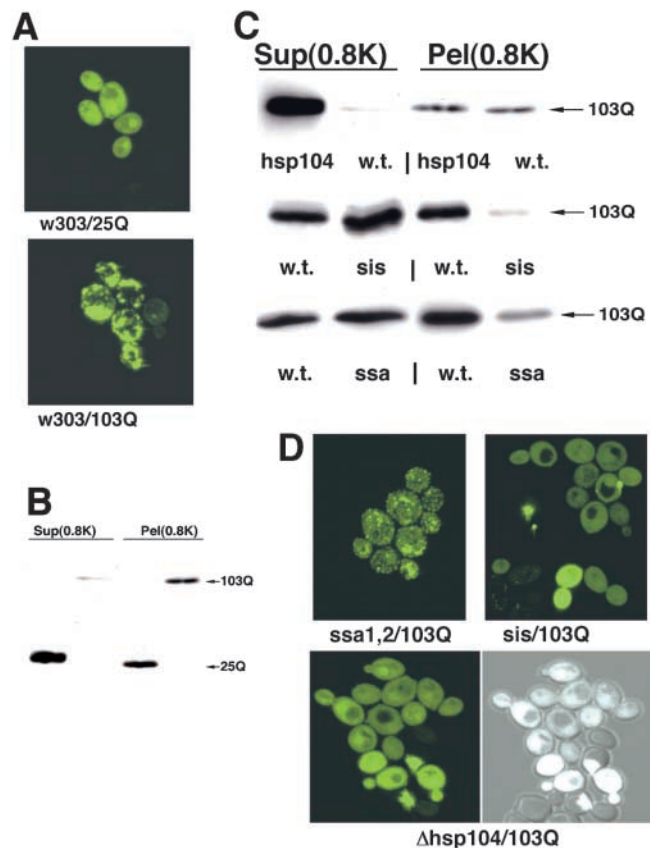

Figure 2. **Molecular chaperones are involved in aggregation of 103Q in yeast cells.** (A) Fluorescent confocal micrographs of yeast cells expressing polyQ polypeptides after induction for 7 h. (B and C) Levels of polyQ polypeptides (20 h induction) in soluble and insoluble fractions of yeast cells lysates. Sup, soluble fraction; Pel, pellets. (D) Confocal micrographs of yeast strains expressing 103Q. *ssa1, 2* and *sis1–85* mutants (top); *hsp104* deletion mutant (bottom). (The picture with combined fluorescent and phase–contrast microscopy on the right makes cells with one large aggregate and no diffused fluorescence visible.)

patterns were seen with these chaperone mutants. In *ssa1 ssa2* (Fig. 2 D) and *ydj1–151* (unpublished data) mutants, 103Q formed a multitude of very small grain-like aggregates against a bright diffused background representing soluble 103Q. (Of note, there was no compensatory induction of other chaperones in this particular strain of *ssa1 ssa2* mutant.) In *hsp104* and *sis1–85* strains, the majority of cells did not form any visible aggregates, and the rest of the cells formed one or few large condensed aggregates (Fig. 2 D). These large aggregates resembled a compressed bundle of filaments, whose appearance was completely different from the IBs seen in the wild-type strain (compare Fig. 2 A).

The differences in 103Q aggregation patterns in the chaperone mutants may reflect different roles of the chaperones in polyQ aggregation. We followed the process of aggregation in wild-type and mutant cells in real time by taking pictures every 2 min of several selected cells expressing 103Q. In the wild-type, initially all fluorescent cells contained small aggregates. With time, intensity of fluorescence in aggregates and aggregate size grew, whereas the soluble (diffusely distributed) fluorescent material gradually disappeared (Fig. 3). However, even after 60 min of observation a certain pool of
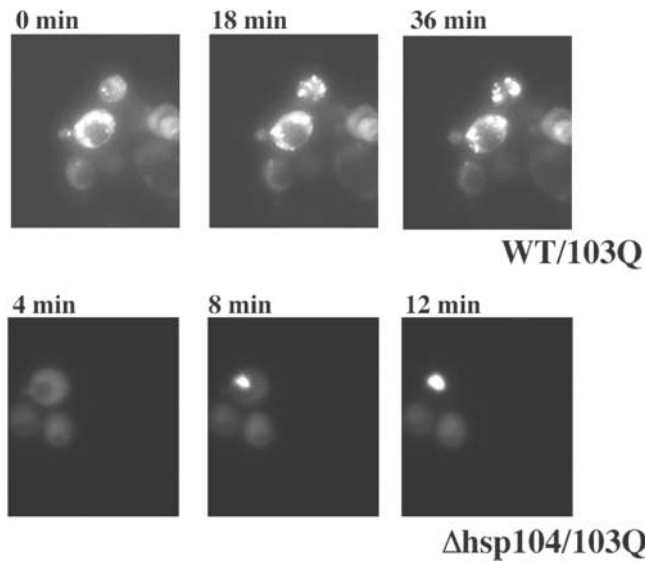
Figure 3. **Deletion in *hsp104* does not impede growth of preformed IBs.** Fluorescent images of wild-type and mutant cells with 103Q (expressed for 3 h before the start of the observation) taken at different time points. The IB formation in the mutant cell was recorded five times; the typical recording is presented.
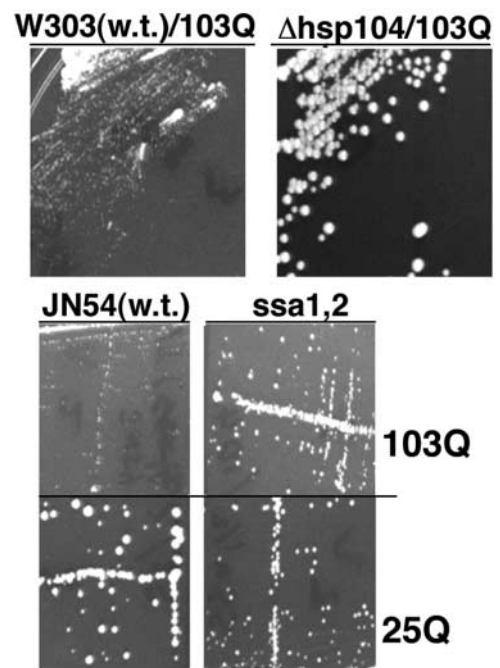

Figure 4. **Deprivation of yeast cells of certain chaperones relieves 103Q-associated toxicity.** Growth (4 d) of *hsp104* mutant and the corresponding wild type (top), both expressing 103Q and *ssa1,ssa2* mutant and the corresponding wild type (bottom) expressing 103Q or 25Q.

103Q still remained soluble. Unlike the wild-type cells, in *ssa1 ssa2* cells no apparent redistribution of soluble 103Q into numerous aggregates was seen within 60 min (unpublished data), indicating that although the nucleation of aggregates is unimpeded in this mutant, further growth of the aggregates is inhibited. We monitored rare events of IB formation in the *hsp104* mutant and found a remarkable difference with polyQ aggregation compared with the wild-type cells (Fig. 3). Once started, the aggregation of 103Q in *hsp104* mutant proceeded rapidly, so 6–12 min after the first appearance of visible seeds almost all soluble polyQ in the cell collapsed into them, forming large IBs (Fig. 3). Therefore, the rate-limiting step in the *hsp104* mutant is not growth of prenucleated aggregates but initiation of the aggregation process. In a small number of cells where aggregates are seeded despite the lack of Hsp104, the consequent growth of the IB proceeds rapidly. Therefore, Hsp104 appears to be involved (possibly indirectly as discussed below) in nucleation of polyQ aggregates, whereas members of the Ssa family appear to be involved in expansion of prenucleated aggregates, which defines the difference in aggregation phenotypes in the chaperone mutants.

Most importantly, suppression of aggregation in the *hsp104*, *ssa1 ssa2*, and *ydj1–151* chaperone mutants, independently on the mechanism, correlated with suppression of polyQ toxicity. The *ssa1ssa2* and *ydj1–151* mutants grew slower than the parental strain as seen with control cells expressing 25Q. By contrast, expression of 103Q, although dramatically reducing growth of wild-type colonies, did not significantly affect growth of these mutants (Fig. 4, bottom). It was difficult to make a conclusion about the effect of *sis1–85* mutation on polyQ toxicity, since *SIS1* is an essential gene, and this mutation by itself dramatically slowed growth of yeast. The *hsp104* deletion, which did not affect yeast growth (unpublished data), relieved 103Q toxicity (Fig. 4,

top), so colonies of cells lacking Hsp104 transformed with either 103Q or 25Q were large and did not differ in size on galactose plate (unpublished data). We studied whether relief of polyQ toxicity in the *hsp104* mutant was caused by suppression of aggregation. Since 103Q aggregated in a fraction of *hsp104* cells, we investigated whether in these cells aggregation was toxic. Accordingly, individual cells of the *hsp104* strain induced in the galactose medium for 16 h were placed on a grid on galactose-selective plates using a micromanipulator. Cells containing IBs were identified, and emergence of colonies was monitored. Although 14 out of 18 cells without aggregates gave rise to colonies, only 1 out of 9 cells with aggregates formed a colony, suggesting that even for *hsp104* mutant cells IBs are toxic. These data indicate that the growth defect in yeast cells expressing expanded polyQ is caused by its aggregation.

Yeast prions are required for 103Q aggregation

To find additional cellular components involved in polyQ aggregation and toxicity, we selected spontaneous mutants in which aggregation of 103Q was suppressed. We expected that 103Q would not be toxic in the cells defective in aggregation; hence, the block on the polyQ expression (assayed as cell fluorescence) would be relieved. Accordingly, using a cell sorter we isolated a fraction of cells with the highest fluorescence, which was expected to be enriched with the aggregation mutants. Two consequent cycles of cell sorting yielded >85% of cells without visible aggregates, indicating that the enhanced fluorescence indeed was associated with the reduced aggregation. The calculated frequency of the spontaneous mutations was unexpectedly high (close to
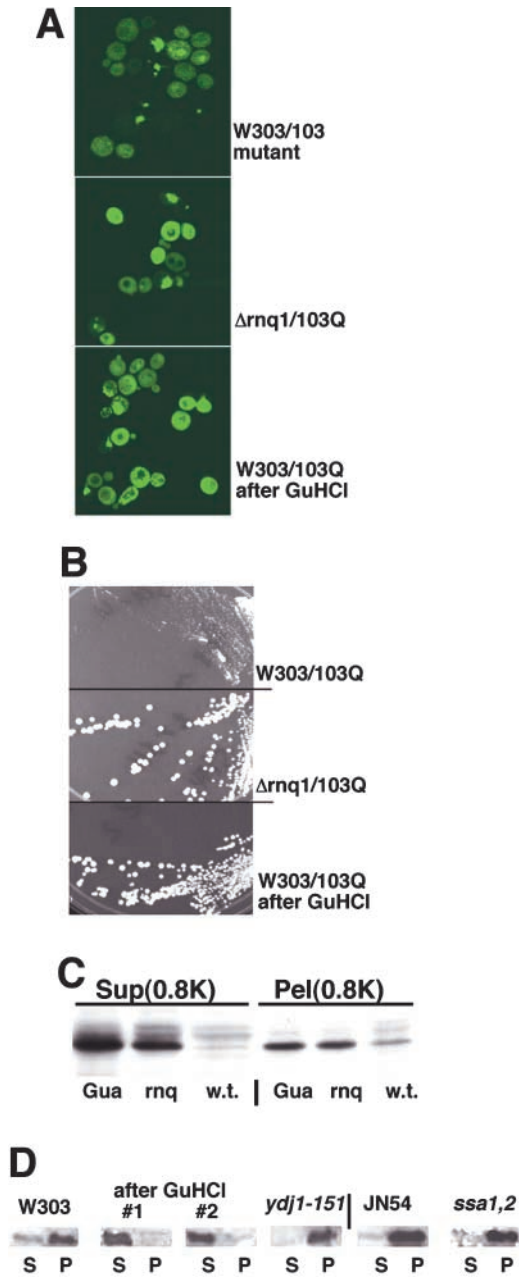
Figure 5. 103Q aggregation and toxicity depend on yeast prions. (A) Fluorescent confocal micrographs of yeast strains expressing 103Q. (B) Growth (4 d) of wild-type, guanidine-pretreated and *rnq1*mutant cells expressing103Q. (C) Increase in the cellular levels and solubility of 103Q (expressed for 24 h) in the cells presented in Fig. 5 B; Gua, GuHCl pretreated; rnq, *rnq1* mutant. Note that (a) 103Q is represented by the lowest band and (b) most of insoluble 103Q in GuHCl-treated and *rnq1* cells represents the polypeptide from the fraction of cells with IBs. (D) Distribution of Rnq1 between supernatant (S) and pellet (P) after a centrifugation at 100,000 *g* of homogenates of the original W303 and JN54 strains and their derivatives.

10^{-3}). The aggregation pattern in 25 randomly chosen independent clones resembled that of *hsp104* and *sis1–85* mutants (Fig. 5 A, top). Importantly, in all of these clones 103Q-associated growth defect was suppressed (unpublished data), emphasizing the correlation between aggregation of polyQ and its toxicity.

Mating of all 25 spontaneous mutants and *hsp104* and *sis1–85* mutants with the wild-type haploid strain (BY4742) demonstrated that the aggregation defects were recessive (unpublished data). Surprisingly, all diploids obtained by mating the spontaneous mutants with the *hsp104* deletion strain displayed defects in aggregation similar to that of parental mutants (unpublished data), indicating the lack of complementation. Since a frequency of spontaneous mutations of 10^{-3} in the *hsp104* gene is improbable, these data implied an unusual type of inheritance. Crossing of the *hsp104* deletion mutant with *sis1–85* also yielded diploid cells with defects in aggregation similar to that of parental mutants (unpublished data), indicating that defects in polyQ aggregation were inherited in a non-Mendelian manner.

The conclusive proof of such type of inheritance came from sporulation/dissection of diploids resulting from mating of the mutants with the wild-type strain. All four spores in a tetrad usually displayed wild-type–like aggregation and toxicity of 103Q, indicating a non-Mendelian mode of inheritance. In contrast, sporulation and dissection of diploids originating from a mating of wild-type strain and *hsp104* mutant resulted in 2:2 segregation of aggregation/toxicity pattern. In these tetrads, suppressed aggregation was always associated with the *hsp104* deletion. The non-Mendelian mode of inheritance of polyQ and toxicity was reminiscent of inheritance of prion elements in yeast (Wickner et al., 1999; Sondheimer and Lindquist, 2000). Since both Hsp104 and Sis1 have been implicated in prion propagation (Chernoff et al., 1995; Derkatch et al., 1997; Moriyama et al., 2000; Sondheimer and Lindquist, 2000; Sondheimer et al., 2001), we suggested that some protein(s) in prion conformation may be essential for toxicity and aggregation of polyQ polypeptides.

To test this possibility, we screened a set of deletion mutations in nonessential prion genes and in genes involved in aggregation of prion proteins (i.e., *mks1*, *new1*, *sla1*, *rnq1*, *ure2*, and *ybr016w*) for their effects on polyQ aggregation. Of all of the mutants tested, only cells with the *rnq1* deletion displayed an aggregation defect. The 103Q aggregation pattern in these cells was similar to that of *hsp104* and *sis1–85* mutants (Fig. 5 A, middle). No 103Q-related toxicity was detected in *rnq1* mutant cells (Fig. 5 B) despite the fact that 103Q was accumulated to higher levels than in the wild-type strain (Fig. 5 C). Mating of the *rnq1* mutant with the isogenic wild-type strain followed by sporulation and tetrad dissection resulted in a 2:2 segregation of suppressed aggregation to toxicity as was shown before with the *hsp104* mutant. Suppressed aggregation was always associated with *rnq1* deletion. These data strongly indicate that Rnq1 is essential for aggregation and toxicity of polyQ polypeptides in yeast. Using a differential centrifugation of cell homogenates, we found that in W303 and JN54, the wild-type strains used in this study, all Rnq1 protein was associated with 100,000 g pellets, indicating that this protein exists in prion form (Fig. 5 D).

To investigate whether prion conformation of Rnq1 is critical for polyQ aggregation, we employed a method of curing yeast cells of prions by growing them in the presence of guanidine hydrochloride (GuHCl), which prevents propagation of prion conformation (for review see Chernoff, 2001). Accordingly, yeast cells were grown for three passages on glucose-containing medium (to block 103Q induction)

in the presence of 5 mM GuHCl. Expression of 103Q was induced in 22 randomly chosen individual clones originating from this procedure, and 21 of them displayed the aggregation phenotype similar to that of the spontaneous mutants described above (Fig. 5 A, bottom). Moreover, none of the 21 clones demonstrated a growth defect in response to accumulation of 103Q (Fig. 5 B) despite 103Q levels being significantly higher than the levels in the original cells (Fig. 5 C), which underscores the importance of polyQ aggregation for its toxicity. In two randomly chosen clones with no polyQ aggregation, all Rnq1 was found in the 100,000 g supernatant fraction in contrast to the original strain where most of Rnq1 was found in the insoluble fraction (Fig. 5 D). This confirms that the GuHCl treatment indeed cured cells of prion form of Rnq1 ([RNQ$^+$]). These data strongly suggest that prion conformation of Rnq1 is essential for aggregation and toxicity of polyQ.

Since *hsp104* and *sis1* mutations are known to cure cells of [RNQ$^+$] (Derkatch et al., 1997; Sondheimer et al., 2001), it appears that suppression of polyQ aggregation and toxicity by these mutations is caused primarily by the loss of Rnq1 prion. On the other hand, the *ssa1ssa2* and *ydj1–151* mutations, which also relieved polyQ-related toxicity (Fig. 4), did not affect prion state of Rnq1, since all Rnq1 remain associated with the insoluble fraction in the *ssa1ssa2* or *ydj1–151* cells homogenates (Fig. 5 D). Therefore, effects of *ssa1ssa2* and *ydj1–151* mutations appear to be unrelated to [RNQ$^+$]. These data support the notion that Ssa and Ydj1 apparently affect a step in IB formation different from one controlled by Hsp104 and Sis1.

Discussion

Here, we describe the first yeast model of polyQ expansion diseases in which accumulation of the polyQ domain was cytotoxic. The fact that 103Q is toxic for yeast cells may imply that the deleterious effects of polyQ extension are more general than they appeared before and that the mechanisms of toxicity could be conserved through evolution. The novel yeast model sheds light on the debated connection between aggregation of polyQ and its toxicity. We present several lines of evidence that the toxicity strongly associates with polyQ aggregation: (a) short polyglutamine, which did not form aggregates, was not toxic for the cells in spite of being accumulated at higher levels, whereas long polyglutamine aggregated and was toxic; (b) impairment of aggregation at apparently two distinct steps in chaperone mutants *hsp104* and *ssa1ssa2* or *ydj1–151* led to suppression of 103Q toxicity, whereas in the rare *hsp104* mutant cells, which formed IBs, 103Q remained toxic; (c) deletion of the *RNQ1* gene encoding a prion protein suppressed both aggregation and toxicity; and (d) curing the wild-type cells of prions suppressed 103Q aggregation and was sufficient to relieve the cytotoxicity in the course of subsequent 103Q induction. The most plausible conclusion from these data is that 103Q aggregation is essential for its toxicity.

Our data indicate that in addition to genetic factors the presence of prions determines polyQ aggregation and consequently cytotoxicity in yeast cells. It is possible that the lack of polyQ toxicity in previously reported yeast models may be related to the lack of [RNQ$^+$] prion in the strains used in those experiments (Krobitsch and Lindquist, 2000; Hughes et al., 2001). This suggestion is supported by the morphology of aggregates in these reports, which resembled that in our clones cured of [RNQ$^+$] prion. Another critical factor in toxicity appears to be the intracellular level of expanded polyQ (unpublished data).

Our data demonstrate that, counterintuitively and in contrast to in vitro results, aggregation of polyQ in vivo is strongly dependent on cellular factors. We found that among chaperones, in addition to Hsp104 (Krobitsch and Lindquist, 2000), Sis1, Ssa1/2, and Ydj1 also play an essential role in formation of the IBs. The majority of cells mutated in either *hsp104* or *sis1* genes were unable to form any visible 103Q aggregates. In rare *hsp104* mutant cells in which nucleation of aggregates occurred, IBs grew efficiently. These data suggest that Hsp104 (and probably Sis1) is not essential for expansion of aggregates but rather for seeding of IBs. Likely, these chaperones control seeding of aggregates via maintenance of the [RNQ$^+$] prion (Derkatch et al., 1997; Sondheimer et al., 2001; Wegrzyn et al., 2001). In addition, Hsp104 could be directly involved in shearing of polyQ aggregates. Such a role for Hsp104 would be consistent with its involvement in shearing of Sup35 aggregates (Patino et al., 1996; Paushkin et al., 1996), which is critical for maintenance of prion form of Sup35 ([PSI$^+$]) (for review see Chernoff, 2001; Wegrzyn et al., 2001).

Ssa1/Ssa2 and Ydj1 were also essential for formation of IBs but at a different step. Real-time observation of this process indicated that Ssa1/Ssa2 (possibly with the help of Ydj1) might be involved in expansion of preformed seeds. This agrees with the previously proposed role of Ssa in the growth of prion aggregates in yeast (for review see Chernoff, 2001). In cells with decreased levels of Ssa (or deprived of Ydj1), growth of aggregates might become a limiting step, and some 103Q molecules unable to join IBs would remain soluble, which in turn would allow more nucleation centers to emerge in a cell and a much higher number of small aggregates to be formed. Together these data indicate a novel and unexpected role of molecular chaperones in promoting formation of protein aggregates. Furthermore, different chaperones facilitate two distinct steps in the aggregation process. Interestingly, although physiological levels of chaperones are necessary for the aggregation, overexpression of Hsp104, Hsp70, and Hsp40 was reported to keep polyQ polypeptides in a soluble form (Chan et al., 2000; Satyal et al., 2000). However, it should be noted that it is not clear whether strains used in these studies contained the [RNQ$^+$] prion.

Our data that the polypeptide with expanded polyQ forms inclusions only in the presence of the [RNQ$^+$] prion are consistent with the latest report showing that aggregation of the MJD protein with polyQ expansion is facilitated in so-called [PIN$^+$] cells. [PIN$^+$] is a non-Mendelian genetic trait promoting emergence of [PSI$^+$] (Derkatch et al., 1997). It was shown that [PIN$^+$] may be caused by a number of potential prion-forming proteins, for example, Rnq1 and New1 (Derkatch et al., 2001). Interestingly, deletion of the *NEW1* gene did not significantly affect aggregation of 103Q polypeptide, suggesting the major role of [RNQ$^+$] prion in this process. Whether aggregates of prion form of

Table I. **Strains used**

Strain	Genotype
W303	*MATa ade2-1 trp1-1 leu2-3,112 his 3-11,15 ura3-52 can1-100 ssd1-d*
hsp104Δ (YS483)	Isogenic to W303, *hsp104::LEU2*
ydj1-151 (ACY17b)	Isogenic to W3031b (W303 used as a wild type), *ydj1-2::HIS3 LEU2::ydj1-151*
WT *SIS1* (CY736)	Isogenic to W303, *sis1Δ::HIS3* (*SIS1 on LEU2/CEN* plasmid)
sis1-85 (CY732)	Isogenic to W303, *sis1Δ::HIS3* (NH$_2$–HA-tagged *sis1-85* on *LEU2/CEN* plasmid)
JN54	*MATa leu2-3,112 trp1Δ his3-11,15 ura3-52 lys2*
ssa1 ssa2 (MW123)	*MATα leu2-3,112 trp1Δ his3-11,15 ura3-52 lys2 ssa1::HIS3 ssa2::LEU2*
ssa1 ssa3 (MW328)	*MATα leu2-3,112 trp1Δ his3-11,15 ura3-52 lys2 ssa1::HIS3 ssa3::TRP1*
ssa1 ssa4 (MW329)	*MATα leu2-3,112 trp1Δ his3-11,15 ura3-52 lys2 ssa1::HIS3 ssa4::LYS2*
ssb1 ssb2 (JN212)	Isogenic to JN54, *ssb1-1::LEU2 ssb2-1::HIS3*

Rnq1, which has a QN-rich domain, could directly serve as a nucleation site for polyQ, or Rnq1 acts on the polyQ aggregation indirectly is yet to be established.

Our data show that the toxicity of expanded polyQ polypeptide in yeast cells depends on the presence of prion in a cell. This fact suggests that in mammalian tissues some (possibly other than PrP) prion-like proteins may be required for aggregation and neuronal pathology of mutant polypeptides with expansion of the polyglutamine tract. This consideration establishes a link between the two seemingly distant groups of neurodegenerative diseases.

Presented here is a yeast model of cytotoxicity caused by polyQ aggregation, which can be further used in a search for yeast genetic modifiers of aggregation and viability. The identification of such cellular factors will ultimately facilitate the quest for genetic modifiers in humans and be crucial for understanding the mechanisms of the development of polyQ expansion-related pathology.

Materials and methods

Strains and plasmids
Deletion mutants of the *S. cerevisiae* wild-type strains BY4739 (*MATα leu2Δ lys2Δ ura3Δ*) and BY4742 (*MATα his3Δ leu2Δ lys2Δ ura3Δ*) were obtained from the deletion library (ResGen; Invitrogen) of yeast nonessential genes (Winzeler et al., 1999). Table I lists all other strains used in this work.

Vectors for expression of polyQ constructs under control of Gal1 promoter were a gift from A. Kazantsev (Massachusetts Institute of Technology). DNA constructs for mammalian expression of huntingtin with alternating CAG/CAA repeats of different length have been described previously (Kazantsev et al., 1999). Exon1 sequences containing the first 17 amino acids followed by 25 or 103 glutamines were fused in frame with a GFP tag at the COOH terminus of each construct, and a FLAG tag was attached to their NH$_2$ termini (resulting in 25Q or 103Q). Such constructs were subcloned into the pYES2 vector (Invitrogen).

Observation of yeast growth and induction
Cells were routinely grown at 30°C on selective SD medium and then transferred into SG liquid or solid medium for induction of 25Q or 103Q expression.

To stain cells with propidium iodide for flow cytometry, 0.5–5 ml of cells grown in SG medium overnight were harvested, washed with 50 mM Tris-HCl (pH 7.5), and treated as follows. Cells were fixed in 70% ethanol for 1 h, washed, and treated with 1 mg/ml RNase A in 50 mM Tris-HCl (pH 7.5) for 1 h with rotation. Then, cells were treated with 0.5% pepsin in 55 mM HCl for 5 min and incubated with 0.05 mg/ml propidium iodide in 180 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 7 mM MgCl$_2$ overnight at 4°C with rotation.

Microscopy
Confocal microscopy was performed with laser scanning system Radiance 2000 (Bio-Rad Laboratories). To follow a process of aggregation in real time, 103Q was induced for 3 h, cells were harvested, mounted on glass slides covered with solid (1.5% agarose) galactose medium, and observed under the deconvolution epifluorescent microscope (Deltavision; Applied Precision, Inc.). Every 2 min, 10–15 serial sections (0.25 mm/section, 0.5 s exposure/section) were taken to cover the whole thickness of the cell for a total of 1 h.

Analysis of solubility of polyQ polypeptides and Rnq1 protein in cell lysates
Collected cells were disrupted by vortexing with 425–600 μm acid-washed glass beads in 40 mM Hepes, pH 7.5, 50 mM KCl, 1% Triton X-100, 2 mM DTT, 1 mM Na$_3$VO$_4$, 50 mM β-glycerophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin. Disintegration by lyticase treatment provided similar results (unpublished data). After disintegration, cell lysates were left in narrow tubes for 1 h to allow ∼95% of unbroken cells to sediment by the gravity force, whereas almost all of the aggregates released from the broken cells stayed in the solution (unpublished data). The upper portion of supernatant was carefully removed and used for fractionation. Samples were normalized by the amount of total protein. Lysates were subjected to centrifugation at 800 *g* for 10 min. The pellets were washed once with the lysis buffer and resuspended in a volume of water equal to the volume of the supernatant. To assess intracellular aggregation of Rnq1, cells were disrupted by vortexing with glass beads in the buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM KCl, 100 mM EDTA, 1 mM DTT, 1.0% Triton X-100, and 0.2% SDS. Then cell homogenate was cleared from debris by 10 min centrifugation at 3,000 *g* and subjected to centrifugation at 100,000 *g* to separate soluble and insoluble forms of the protein. All samples were supplemented with loading SDS-PAGE buffer containing 2% SDS and boiled for 3 min before being subjected to immunoblotting. GFP-tagged polyQ polypeptides were visualized with anti-GFP antibody (polyclonal) (CLONTECH Laboratories, Inc.). Rnq1 antibody was a gift from S. Lindquist (Whitehead Institute, Cambridge, MA).

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