Deleterious and protective properties of an aggregate-prone protein with a polyalanine expansion

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Many aggregate-prone proteins, including proteins with long polyglutamine or polyalanine tracts, cause human diseases. Polyalanine proteins may also be present in the tissue of polyglutamine diseases as a result of frameshifting of the primary polyglutamine-encoding (CAG)n repeat mutation. We have generated a Drosophila model expressing green fluorescent protein tagged to 37 alanines that manifests both toxicity and inclusion formation in various tissues. Surprisingly, we show that this aggregate-prone protein with a polyalanine expansion can also protect against polyglutamine toxicity, which can be explained by induction of a heat-shock response. A heat-shock response was also seen in an oculopharyngeal muscular dystrophy mouse model expressing an authentic polyalanine-expanded protein. We also show that long polyalanines can protect against a pro-apoptotic stimulus or the toxicity caused by the long polyalanines themselves. Thus, overexpression of an aggregate-prone protein without any normal functions can result in both pathogenic and protective effects in cell culture and in vivo.

INTRODUCTION

Codon reiteration diseases are a large group of human conditions caused by abnormally long polyglutamine or polyalanine tracts in different proteins. Polyglutamine expansions are seen in Huntington’s disease (HD) and eight other conditions including spinocerebellar ataxias (SCA) types 1, 2, 3, 6, 7 and 17 (1). HD, the most prevalent polyglutamine disease, is a devastating autosomal dominant neurodegenerative condition that typically strikes in middle age and is characterized by movement disorders, cognitive decline and psychiatric symptoms (2). The pathological hallmark of HD and other polyglutamine diseases is the presence of intracellular inclusions (3,4), although the roles of inclusions are debated. Expansions or duplications of polyglutamine tracts leading to stretches of up to 29 repeats cause nine known diseases (5). In one of these, oculopharyngeal muscular dystrophy (OPMD), polyalanine expansions in the poly-A-binding protein nuclear 1 (PABPN1) cause it to form intranuclear inclusions that correlate with disease pathology (6). In addition, polyalanine expansions in four disease genes have been recently shown to lead to inclusion formation in cell models (7), although we are not aware that this possibility has been tested in appropriate tissues of patients with these diseases.

In order to gain insights into polyalanine toxicity in general, we have tested if polyalanine expansions are toxic when expressed alone and also when expressed with polyglutamine expansions. The polyalanine expansions were co-expressed with N-terminal fragments of polyglutamine-expanded mutant huntingtin, to allow us to directly and unequivocally test the overall toxic or protective properties of long polyalanines. In addition, our findings may also have potential relevance to polyglutamine diseases, since frameshifting of the original product encoding CAG/polyglutamines to GCA/polyalanine has been reported in SCA3 (8,9).

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Here, we show that an aggregate-prone protein with a polyalanine expansion [enhanced green fluorescent protein (EGFP) tagged to 37 alanines] can be toxic in vivo in Drosophila and surprisingly, the same mutant protein could also mediate protective effects against polyglutamine expansions, a distinct pro-apoptotic stimulus and also against polyalanines themselves. Our data suggest that this protective effect is mediated by a heat-shock response. Heat-shock responses were seen both in cell culture models expressing expanded polyalanine proteins as well as in an OPMD mouse model expressing an authentic polyalanine expanded protein. However, no heat-shock response was evident in HD mice. In summary, overexpression of an aggregate-prone protein with no normal functions can result in both pathogenic and protective effects in cell culture and in vivo.

RESULTS

Toxic effects of polyalanine expansions

To determine the effects of polyalanine repeats in vivo, we generated transgenic Drosophila expressing 37 or 7 alanines fused to an SV40 nuclear localization signal (NLS) and EGFP, referred to as A37 and A7, respectively. An NLS was used, as the proteins associated with polyalanine diseases are nuclear (5,6). We used 37 polyalanine repeats, because a number of normal human proteins have at least 20 repeats (thus, toxicity of a given repeat length may be modulated by protein context, as is also suggested with polyglutamine repeats). Our previous studies have shown that A37 is toxic and forms inclusions in cell culture (10). Similar A37 inclusions are detergent-insoluble and have fibrillar morphologies by electron microscopy (11).

We also selected a slightly longer repeat than the longestpolyalanine expansion seen in a human disease, in order to maximize the probability of a reliable phenotype within the short Drosophila lifespan, analogous to what many have used for modelling polyglutamine diseases in vivo (12–14). It is worth noting that the 146 CAG repeat expansion used by Ordway et al. (13) and similar large expansions used in other models are not representative of either adult or even juvenile-onset HD, where expansions of more than 75 repeats are quite rare (2). Although such an expansion size may have been reported previously, these are exceptional and are likely to have extreme phenotypes.

We fused the polyalanine expansions to EGFP to investigate the consequences of polyalanine expansions outside the context of the human disease proteins—this allows us to test if long polyalanine expansions could mediate toxicity by gain-of-function mechanisms (as in polyglutamine diseases), as opposed to dominant-negative or loss-of-function effects. We have based our strategy on approaches used to analyse the toxicity of isolated polyglutamine stretches in flies (12) and polyglutamine expansions inserted into a non-essential non-disease associated protein in mice (13) (HPRT), which have led to major insights into the toxicity of these mutations. Furthermore, a major part of the HD literature involves studies of exon 1 fragments in cells and mice, which may be very similar to isolated polyglutamines, given the small size of this exon (14). The A7 and A37 constructs were expressed in Drosophila under the control of a UAS promoter, which is only activated when crossed with flies expressing the GAL4 transcription factor in the tissue of choice (15). We selected lines expressing similar levels of A37 and A7 for phenotypic analyses (Fig. S1).

Expression of A37 in the nervous system of adult Drosophila led to a shorter lifespan, compared with Drosophila expressing A7 or the GAL4 driver alone (Fig. 1A). We then looked for behavioural manifestations of nervous system dysfunction. Normal flies display a strong negative geotactic response; when tapped to the bottom of a test tube, they rapidly climb to the top. The climbing response of flies expressing A37 was impaired at 2 days after eclosion compared with flies expressing GAL4 driver alone or A7 (Fig. 1B).

Figure 1. Toxic effects of long polyalanine repeats in Drosophila. (A) Survival curves of two A37 lines and one A7 line expressing the transgene in the nervous system with Nrv2-GAL4 or Nrv2-GAL4-alone without any transgene (GAL4). The two different A37 lines were statistically different from the A7 line and from GAL4 alone (Kaplan–Meier survival test, P < 0.0001 for both A37 lines). Approximately, 200 individuals were used for each genotype. (B) Climbing abilities of two A37 lines and one A7 line expressing the transgene in the nervous system under the control of Nrv2-GAL4 and Nrv2-GAL4-alone (GAL4) 2 days after eclosion. The climbing ability of flies expressing Nrv2-GAL4-alone was arbitrarily set as 100%. Both A37 lines were different from A7 and GAL4-alone (P < 0.001 for both A37, Mann–Whitney U-test). A7 was not different from Nrv2-GAL4. Graph shows summary data and error bars represent standard errors of mean. Approximately 100 flies were used. (C) Survival to adulthood of flies expressing A37 or A7. Flies heterozygous for A7 or A37 and a balancer chromosome were crossed to three GAL4 lines and the proportions of the adult flies with A7 or A37 were determined. The proportion of flies expressing A7 versus the balancer chromosome was arbitrarily set as 1, and the data for A37 lines are expressed as odds ratios relative to 1 (see Materials and Methods). The ratio is decreased in both A37 lines, reflecting the decreased survival of flies with A37 transgene to adulthood (Nrv2-GAL4: P < 0.0001, A37a; P = 0.0003, A37b; MHC-GAL4: P < 0.0001 A37a, P = 0.0009 A37b; Ey-GAL4: P < 0.0001 A37a and A37b). Error bars represent 95% confidence intervals. Data for each genotype are based on three different bottles, each with at least 300 flies. (D) Photographs of Drosophila eyes. Expression of A37 driven by ey-GAL4 led to rough and small eye phenotypes, which were not seen in flies expressing A7b, or two copies of A7 transgene (A7a + A7b), or GAL4-alone. Eyes of females are shown. (E) A37 forms aggregates in the eye imaginal disc of third instar larvae and A7 shows a diffuse distribution inside the nucleus, when expressed under ey-GAL4 control. (F) Congo red rescue the pre-adult lethality caused by expression of A37 under ey-GAL4 control. Progeny of ey-GAL4 crossed to either UAS-A37/TM6B or A7/TM6B were treated with 250 µM or 36 mM Congo red and the number of adult flies with and without the transgene were counted. 36 mM Congo red increased the proportion of flies with the A37 transgene (P < 0.0001, odds ratio: 4.3, 95% confidence interval 2.9–6.3), while it did not have any effect on the proportion of flies with the A7 transgene (proportion of A7 is higher than 50% because of low viability of flies with TM6B). At least three bottles were evaluated per each combination, with approximately 200 flies in each bottle (also applies to g). (G) Congo red decreases the frequency of rough/small eye phenotype caused by expression of A37 under ey-GAL4 control. Treatment with 250 µM and 36 mM Congo red reduced the frequency of rough/small eye phenotype to 70% or 10% of control (untreated), respectively (P = 0.0002 for 250 µM, and P < 0.0001 for 36 mM; odds ratio was used). Treatment was done as described in Fig. 1F. (H) Congo red decreases number of cells with inclusions in cell culture. SK-N-SH cells were transfected with 2 µg of EGFP-NLS-A37 and treated with 100 µg/ml Congo red after transfection. Cells were fixed 48 hours later and scored for the presence of inclusions (P < 0.0001; odds ratio: 0.41; 95% confidence interval 0.33–0.49). Three experiments were done, at least 300 cells were scored in each experiment. Error bars represent standard errors of mean except where otherwise indicated. ***P < 0.001. Flies were raised at 29°C in A-F or at 25°C in g.
results were obtained at 3 and 4 days after eclosion (data not shown).

We also evaluated the effects of A37 on survival to adulthood, using Nrv2-GAL4, MHC-GAL4, ey-GAL4, which drive expression in the nervous system, muscles, and the eye-antennal disc and other tissues, respectively (visualized by EGFP, data not shown). Expression of A37 with each of these drivers led to reduced survival to adulthood compared with an A7 line (Fig. 1C). Line A37a has more dramatic effect than A37b, which is likely due to the lower expression when A37b was driven by GAL4 in larvae (data not shown). The effects on survival are unlikely due to leaky expression, as there is ~4-fold difference in survival between different GAL4 drivers; the lethality of A37 flies crossed to ey-GAL4 may be due to expression seen in the abdomen (visualized by GFP, data not shown).

Expression of A37 in the developing Drosophila eye using ey-GAL4 led to the formation of abnormal eyes characterized by smaller size, abnormal shape and rough surface, while expression of A7 or GAL4 alone did not have any visible effects on the external eye morphology (Fig. 1D). We identified at least four A37 lines exhibiting abnormal eye phenotypes when A37 was expressed under the control of ey-GAL4. We analysed 11 independent A7 lines, none of which exhibited any visible eye abnormalities (when driven with ey-GAL4). We did not detect any abnormal eyes even when dosage of the A7 transgene was doubled (Fig. 1D). Also, we observed moderately abnormal eyes with the A37 transgene at 25°C (Fig. 1D), while at 29°C (leading to higher expression of the transgene) we observed in some cases a complete loss of eyes in flies expressing A37 (data not shown). In stark comparison, we never observed any abnormalities with any A7 lines even at this higher temperature. A37 expression resulted in inclusion formation, whereas A7 exhibited diffuse fluorescence in the eye-antennal imaginal disc of third instar larvae (Fig. 1E). When A37 was expressed in the nervous system or muscles, similar inclusions were observed while only diffuse fluorescence was seen with A7 (data not shown).

The toxic effects of long polyalanines were verified with at least two independent insertions and the toxicity of long polyalanines was always compared with the toxicity of short polyalanines expressed at similar levels. Thus, these results clearly demonstrate that expression of long polyalanine repeats causes pathology in vivo and is associated with inclusion formation.

Congo red treatment reduces aggregation and toxicity of both long polyglutamines (in cells and in vivo) (16) and long polyalanines (in the context of mutant PABPN1 (in cells) (17). We therefore tested if Congo red could rescue the polyglutamine toxicity in vivo using the survival to adulthood and rough/small eye phenotype as our readouts. In both cases, Congo red significantly rescued A37 toxicity (Fig. 1F and G). We did not quantify the effects of Congo red on aggregation in the Drosophila eye disc because it appears difficult to quantify changes in aggregation in this system unless they are very large, possibly because of high expression levels of the aggregating protein [see (18) for detailed discussion]. For example, while numerous studies observed the effect of heat-shock proteins on polyglutamine aggregation in mouse

and cell models (19–25), such changes were not observed in Drosophila eye disc (26). Similarly, Drosophila myeloid leukaemia factor reduced polyglutamine aggregation in cell culture (18) but no effects were observed in Drosophila eyes (27).

In mammalian cell culture, Congo red decreased the proportion of A37-transfected cells with inclusions using the same EGF-NLS-A37 construct (10) as used in flies (Fig. 1H). (In all subsequent experiments using A7 or A37 in cells, we have used such constructs.) This suggests that Congo red-type compounds may be worth considering as therapeutics for diseases caused by long polyalanine repeats, such as OPMD. While this protective effect of Congo red may be due to direct effects against aggregation, we cannot exclude the possibility that Congo red may act by blocking the accessibility of toxic epitopes on monomeric expanded polyalanine species (28).

Polyalanines protect against polyglutamine toxicity

We initially tested if polyalanines could modulate polyglutamine toxicity using model systems expressing an N-terminal fragment of huntingtin with expanded polyglutamines, which we and others have extensively previously characterized both in cell culture and in vivo (29). Co-transfection of mammalian cells with a 1:1 ratio (1 μg:1 μg) of HD exon 1 construct with 74Q (74 glutamates) and A37 construct resulted in enhanced toxicity—this is not unexpected as both constructs are toxic when transfected alone (Fig. S2 and data not shown).

Could long polyalanines be protective when they are expressed at levels that are not toxic? We decreased the amount of DNA per well so that transfection with A37 construct did not show any obvious toxicity (Fig. S2). Co-transfection of mammalian COS-7 cells with a 1:1/9 ratio (1 μg:111 ng) of HD exon 1 Q74 construct and A37 construct led to decreased toxicity accompanied by decreased percentage of cells with Q74 inclusions (Fig. 2A). A7 did not affect cell death or aggregation (Fig. 2A). Similar results were obtained in a neuronal SK-N-SH cell line using the same ratio (data not shown). Interestingly, co-transfection of mammalian COS-7 cells with a 1:1/3 ratio of HD exon 1 Q74 construct and A37 construct led to an intermediate effect between the 1:1/9 (protective) and 1:1 ratios (enhanced toxicity). The 1:1/3 ratio resulted in no change in toxicity of the Q74 (Odds ratio = 1.084; 95% CI 0.89–1.46, P = 0.7, data not shown).

Next, we tested if long polyalanines could protect against polyglutamine toxicity in vivo in Drosophila. For these experiments, we had initially considered using polyglutamine models driven by the UAS-GAL4 system (15). However, we observed an interaction of A37 with GAL4 that is likely to result in decreased GAL4-dependent transcription (and therefore any modification of polyQ toxicity in such a model may be due to modification of GAL4-dependent transcription of the polyQ transgene) (see Supplementary Data and Fig. S3). The A37-GAL4 interaction necessitated selection of a model where the polyQ transgene was independent of the GAL4 transcription factor. The only published model meeting this criteria expresses the N-terminal part of mutant human...
huntingtin (120Q) (30) placed under the direct control of the gmr promoter. As our cell-based data suggest that A37 could exert protective properties at sub-toxic doses, we chose elav-GAL4 to drive the expression of A37/A7. We have used elav-GAL4C155 (31), which drives expression of the UAS transgene in the photoreceptor cells and the nervous system. We have not observed any effects on the survival of flies expressing elav-GAL4 and A37 at the temperature used for the following experiment (25°C, data not shown).

Expression of A37 (or A7) in the eyes using elav-GAL4 did not lead to any rhabdomere loss in Drosophila eyes up to 20 days (which is much later than the time points of any of the following experiments). In addition, elav-GAL4 alone is not toxic and does not lead to any rhabdomere loss (data not shown). The absence of A37 toxicity in the Drosophila eye when using elav-GAL4 is likely to be due to low levels of expression (judged by GFP fluorescence, data not shown).

We carefully considered the most appropriate read-out for the effects of A37/A7 on Q120-induced degeneration in this model (see Supplementary data) and selected pseudopupil analysis for subsequent experiments.

Expression of A37 reduced the polyglutamine toxicity by increasing the number of visible rhabdomeres in the eye of the 120Q flies, while A7 did not affect the degeneration at all three time points examined (Fig. 2B and C). A37 increased the visible rhabdomeres by 9% at day 1 after eclosion, 29% at day 2 and by 58% at day 4 (Fig. S4), showing that the protective effect is maintained over a period of time (Fig. 2C). The in vivo data are consistent with cell culture experiments, showing that the A37 aggregate-prone protein rescues the toxicity of polyglutamines, while A7 does not have any effect.

Polyglutamine toxicity may be mediated by aberrant interactions between the polyglutamine protein and other proteins within the cell, such as transcriptional cofactors, heat-shock proteins, or proteasome subunits, leading to their sequestration (2). We therefore wanted to test if the effect of A37 on polyglutamine toxicity was due to a direct interaction between polyalanine and polyglutamine repeats, possibly preventing some of the aberrant interactions. We could not detect co-localization of YFP-A37 and CFP-HD89Q in any of the cells examined, and A37 was always excluded from 89Q inclusions and vice versa (Fig. 3A). This finding was consistent with data from confocal 3D reconstruction and FRET between expressed donor (CFP-HD89Q) and acceptor (YFP-A37) constructs—after expression of both constructs, we were not able to register any donor–acceptor proximity using acceptor bleach (data not shown). Failure of co-localization by immunofluorescence was confirmed with an independent pair of constructs: EGFP-A37 and HA-tagged HD-Q74 in any of the cells examined, and A37 was always excluded from 89Q inclusions and vice versa (Fig. 3A).

Therefore, the protective effect of A37 is not because of the sequestration of the polyglutamine protein.

The properties of polyalanine expansions are not common to all proteins with polyamino acid expansions. Polyserines appeared to exhibit very different properties compared with polyalanines in mammalian cell culture. Transfection of

![Figure 2](https://academic.oup.com/hmg/article-abstract/15/3/453/630552/457)
COS-7 cells with S5 or S25 led to toxicity (even at 1:1/9 dose) without any visible inclusions (Fig. S6). Co-transfection of either S5 or S25 with HD exon 1 74Q in a 1:1 or 1:1/9 ratio resulted in greater toxicity than with 74Q alone, without affecting inclusion formation, both in COS-7 cells (Fig. S6) and SK-N-SH cells (data not shown). In contrast to polyalanines, polyserines are tightly sequestered to polyglutamine inclusions (Fig. S6). Polyserines do not form inclusions, even in the presence of polyglutamine inclusions; hence the polyserines sequestered in polyglutamine inclusions may only represent a small fraction of the mutant polyserine molecules. The consequences of this are difficult to predict.

Polyalanine expansions induce a heat-shock response

Interestingly, while long polyalanines decreased the proportion of cells with polyglutamine inclusions (Fig. 2A and B), they increased the number of polyglutamine inclusions per inclusion-containing cells (Fig. 3B), a phenomenon reminiscent of the protective effect of heat-shock proteins on ataxin-1 (19). In addition, we have generated and characterized a new mouse model of OPMD (A13+1 and non-transgenic littermates) at 9 months of age. Hsp70 levels are increased in mice expressing mutant form of PABP2 (under the control of a muscle promoter) compared to non-transgenic littermates. Western blots were probed with an antibody raised against hsp70 and normalized with an actin antibody. (C) Western blots of muscle lysates from a transgenic mouse model of Huntington’s diseases R6/1 (Tg) and non-transgenic littermates (NT) at 43 weeks of age. Hsp70 levels are not increased in mice expressing fragment of mutant huntingtin compared to non-transgenic littermates. Western blots were done identically to Fig. 3.

These findings prompted us to examine hsp70 induction in our cell model. We observed strong induction of hsp70 by immunocytochemistry in cells transfected with A37 but not in the cells transfected with A7 (Fig. 4A). We further quantified the percentage of transfected cells with high levels of hsp70 (judged by immunocytochemistry) and confirmed that A37 (but not A7) induces hsp70 expression both when transfected alone (Fig. 4B) or when co-transfected with polyglutamine constructs (Fig. 4C). Consistent with these results, A37 (but not A7) increased hsp70 expression on western blots of cells transfected with A37 alone (Fig. 4D) or when co-transfected with a polyglutamine construct (Fig. 4E).

To test if hsp70 induction was mediated by HSF-1 driven transcription, we performed luciferase assays using a HSE-luciferase reporter construct. We observed a 3-fold increase in luciferase activity in the presence of A37 compared with EGFP, while A7 had no effect (Fig. 4F). We observed no increase in HSE-luciferase activity in stable inducible lines expressing mutant versus wild-type huntingtin exon 1 or full-length huntingtin (Sugars and Rubinsztein, unpublished data). The above experiments were performed in the presence of the broad-spectrum caspase inhibitor zVADfmk to avoid potential confounds of cell death (caspase activation) but similar results were also obtained without zVADfmk (data not shown). In addition, we observed increase of hsp70 expression when cells were transfected with a low (Fig. 4A–F) or high dose of long polyalanines (Fig. S5).

These data show that long polyalanines induce a heat-shock response by activating HSF-1-driven transcription, which is likely to protect against polyglutamine-mediated toxicity.

As hsp-induction is known to be protective against apoptosis (35), we tested if transfection with A37 could also protect against staurosporine-induced cell death. Indeed, A37 decreased the percentage of dead cells compared with cells...
transfected with A7 construct (Fig. 4G). This raises the possibility that enhanced HSF-driven transcription may allow polyalanines to protect against some of their inherent toxicity. Indeed, Drosophila with one loss-of-function Hsf allele had enhanced toxicity of the polyalanine mutation (using both abnormal eyes and survival as our read-outs), compared to flies with two wild-type Hsf alleles (Fig. 4H–J). Therefore, whether polyalanines are toxic or not may be a balance between deleterious effects (increased at high expression) and protective effects (HSF induction).

It would not have been informative to test whether A37 expression still protected against polyglutamine toxicity in mutant flies containing a loss-of-function allele of Hsf (like the flies analysed in Fig. 4H–J). Hsf knockdown and dominant-negative hsp70 (or hsp40) significantly enhance the toxicity of polyglutamines in flies (36–38). This is consistent with Hsf enhancing the levels of Hsps even under basal conditions (39). Thus, polyglutamine flies with knockdown of hsp70/Hsf are likely to have more toxicity irrespective of whether polyalanines are expressed or not, particularly since our data suggest that HSF-1 knockdown enhances polyalanine toxicity (Fig. 4H–J). Thus, enhanced toxicity because of HSF-1 knockdown in polyQ + polyA flies will not be interpretable.

**DISCUSSION**

**Mechanism of polyalanine toxicity**

We have generated a Drosophila model expressing EGFP tagged with 37 alanines, to provide insights into the consequences of such mutations in vivo. Long polyalanines are toxic and form inclusions in Drosophila when expressed in a variety of tissues. The mode by which expanded polyalanines cause toxicity has been widely discussed and the possibilities include gain-of-function, loss-of-function and dominant-negative models (5,7). The use of a model EGFP protein tagged with polyalanines allows us to provide the first in vivo evidence that polyalanines can cause the disease by a gain-of-function mechanism in vivo. We believe that this conclusion is valid for our EGFP-polyalanine model, as it is a non-native protein that allows us to dispense with the loss-of-function and dominant-negative components of the argument. However, any extension to polyalanine diseases requires the assumption that 37 alanines tagged to EGFP behave in a way that is qualitatively similar to 17 alanines in the protein causing OPMD, or the larger expansions of up to 29 alanines in various transcription factors causing some of the other polyalanine diseases (5,7). In these diseases, we cannot exclude the possibility that loss-of-function/dominant-negative effects may occur, even if there is gain-of-function toxicity.

**Protective properties of expanded polyalanines**

Surprisingly, the same polyalanine expansion mutation could also rescue polyglutamine toxicity in two different cell lines and in vivo in Drosophila. In addition, long polyalanines also protect against a pro-apoptotic stimulus (staurosporine) in cells and against long polyalanines themselves. The protective effects induced by long polyalanines could be explained by their ability to induce a heat-shock response even at non-toxic concentrations. Thus, using model proteins (mutant huntingtin fragments) or toxic insults, we have demonstrated that expanded polyalanines can exhibit protective properties.

Our study shows that the same aggregate-prone protein can have pathogenic and protective effects in cell culture and in vivo. This situation is different and complementary to what has been reported before (40), where full-length mutant ataxin 3 protein had protective effects. Our protein is non-native and the wild-type counterpart had no obvious protective effect, whereas the protective effect of the mutant ataxin 3 appeared to be a consequence of the wild-type protein function—the wild-type ataxin 3 appeared to protect at least as well as, if not better than, the mutant protein (40). Thus, our data suggest that the polyalanine mutation itself (as opposed to the surrounding protein context) has both toxic and protective effects. This is an important principle to consider and the experimental set-up that is required to make this point necessarily has to use a non-native protein.

**Expanded polyalanines exhibit protective properties because of induction of a heat-shock response**

Long polyalanines induced a heat-shock response that was consistent with the altered aggregation of polyglutamines and the protection against cell death (19). Raised levels of hsps were induced both by polyalanines tagged to EGFP and in our OPMD mouse model that expresses an authentic polyalanine expansion mutation with a repeat length seen in patients (41). Increased HSF-1 activity leads to increased levels of hsp70 and other inducible hsps like hsp27. While this manuscript was being written, we noted that hsp70 mRNA was one of many transcripts up-regulated in another OPMD cell model (42)—however, this study did not confirm upregulation of hsp70 in independent experiments (false positive results occur frequently with microarray studies).

It is important to stress that hsp70 accumulation in inclusions (25,43,44) or in disease cell models exposed to proteasome inhibitors (45) does not mean that the relevant proteins are inducing a heat-shock response. Hsps can accumulate in inclusions even when heat-shock responses are compromised [e.g. in the R6/2 HD mice (25) and proteasome inhibition by itself leads to a heat-shock response (46)]. Furthermore, even though some cell studies may suggest that polyglutamines can induce a heat-shock response, the converse occurs in vivo in mammalian models (25,47), (this paper).

HSF-1 inducible hsps like hsp70 and HSF-1 itself have been shown to protect against polyglutamine toxicity in cell models (19), in flies (26) and in mice (48). Hsp70 reduces aggregation in cell models but both hsp70 and hsp27 may protect against polyglutamine toxicity via additional aggregation-independent mechanisms, like inhibition of caspase-9 activation (23).

In summary, we demonstrate that long polyalanines induce a heat-shock response and this is a feature of both our model protein and an authentic disease protein containing polyalanine expansion mutation. Our data and those of others (see above) show that this is not a general property of all aggregate-prone proteins.
Effect of polyalanine frameshift products in polyglutamine diseases

Our findings may have disease relevance in SCA3 where polyQ to polyA frameshifts have been reported (8). Our data show that low doses of polyalanines can protect against polyglutamine-induced toxicity, while high expression of polyalanines exacerbates polyglutamine toxicity. In order to address the outcome of the polyalanine frameshifts in polyglutamine diseases, it is necessary to know the relative load of polyalanine products in specific neurons in human disease brains. This is technically extremely difficult and is currently unknown. Also, there will be polyalanine products of different lengths, depending on whether the frameshift occurred close to the N-terminus of the CAG tract (when polyalanine tracts will be long), or closer to the C-terminus (when products may be short and inert like A7). So, one would have to somehow quantify the size distributions of such events. Note that the total amount of these products in the whole brain would not be informative as there may be populations of cells with high and low levels of the polyalanine frameshift products leading to overall toxic and protective effects.

A recent study suggested that polyalanine frameshifts exacerbate polyglutamine toxicity in cell models of SCA3 (9). A crucial finding of this study was that anisomycin reduced both frameshifts and toxicity, suggesting a causal effect. However, anisomycin reduces protein synthesis (9) and the attenuation of toxicity could have been simply because of a reduction in dose of the toxic transgene product. Also, the analyses of cell death comparing stable inducible cell lines expressing frameshift-competent and -incompetent clonal cell lines was difficult to interpret, as it appears that only one clonal cell line of each was reported. It is not uncommon for different clones of a particular stable cell line to exhibit very different properties. Most importantly, the SCA3 study did not directly test (as we did in cell and Drosophila models) whether polyalanine expansions could modulate polyglutamine toxicity when expressed together—their data were largely inferential.

Thus, our data suggest a new paradigm for the effects of these frameshift products, although this phenomenon is not a primary focus of the paper and we do not address their final outcome in polyglutamine disease pathogenesis.

Expanded polyalanines can exhibit both toxic and protective properties simultaneously

Irrespective of the consequences of these frameshifts or even if they are important in vivo, our experiments enabled us to reach the conclusion that the same disease-causing mutation can have both deleterious and protective properties at the same time (Figs. 3, 4 and S5). It is important to emphasize that we observe induction of a heat-shock response both at high and low levels of expression, that is when polyalanines exhibit either overall toxic or protective properties (Figs. 3, 4 and S5).

The protective polyalanine-induced heat-shock response was also observed in vivo with an authentic polyalanine expanded protein—mutant PABPN1 in an OPMD mouse model. Furthermore, we have shown that overexpression of HSP70 can protect against mutant PABPN1 toxicity in cell models (17). Thus, our data with model proteins are reflected with authentic disease proteins in vivo—we speculate that the

Figure 4. Expanded polyalanines induce heat-shock response. (A) COS-7 cells were transiently transfected with 111 ng A37 or A7 (green) and 48 h later cells were fixed and immunolabelled with hsp70 antibody (magenta). Cells expressing A7 or untransfected cells have very low levels of hsp70 staining (just above or equal to background, not visible on the photograph). In contrast, a significant proportion of cells expressing A37 have very bright hsp70 signals. These very brightly staining cells were considered immuno-positive in experiments described in Fig. 4B and C and Fig. S5. Bright fluorescence was seen in cells with and without visible inclusions. (B) Transfected cells from (A) were scored for the presence of high levels of hsp70 (shown by immunocytochemistry). Transfection with A37 construct dramatically increases proportion of cells with high levels of hsp70. (Comparison of C1 and A7, P < 0.001; A7 and A37, P < 0.001; odds ratio was used). Approximately 150 cells were scored per experiment and this was done at least in duplicate (also applies to C). (C) COS-7 cells were co-transfected with Q74 and EGFP, A7 or A37 constructs (1 μg:111 ng) and immunolabelled as in (A). The percentage of transfected cells with high levels of hsp70 expression (shown by immunocytochemistry), was recorded. The A37 construct increased the proportion of cells with high levels of hsp70 in the presence of Q74. Please note that the experiments in (B) and (C) are separate experiments that were performed on different days and therefore the difference in the percentage of cells with high levels of hsp70 might represent a variation in baseline between experiments. (Comparison of C1 and A7, P = 0.894; C1 and A37, P < 0.001; A7 and A37, P < 0.001; odds ratio was used). (D) COS-7 cells were transfected with 111 ng of EGFP, A7 or A37 construct. After 48 h, transfected (GFP+) cells were sorted using FACS and probed with hsp70 and actin antibody. A37 construct induces expression of hsp70. Approximately 20,000 GFP+ cells were used for the western blot analysis (three independent experiments were done with similar results were). (E) COS-7 cells were co-transfected with Q74 and EGFP, A7 or A37 construct in a ratio 1:1(9 (1 μg:111 ng). After 48 h, transfected cells (GFP+) were sorted using FACS and probed with hsp70 and GFP antibody. A37 construct induces expression of hsp70. Approximately 20,000 GFP+ cells were used for the western blot analysis (three independent experiments were done with similar results were). (F) Luciferase reporter assay using a HSE-luciferase construct in the presence of EGFP, A7 or A37 (111 ng). A37 caused activation of the heat-shock element. Mean relative luciferase activity with EGFP was set to 100% with the mean response of A7 and A37 transfected cells expressed as comparative percentages. A representative experiment is shown from six experiments with similar results. Error bars represent the standard error of the mean. (Comparison of C1 and A37, P = 0.002; C1 and A7, P = 0.7465; A7 and A37, P = 0.0001; unpaired T-test). (G) COS-7 cells were transfected with 111 ng of A7 or A37 construct and 48 h later exposed to 2 μM staurosporine for 4 h. Cells were subsequently fixed and number of dead transfected cells (with fragmented nuclei) was counted. Three experiments were done and 200 cells were counted for each experiment. Experiments in Fig. 4A–F were done in presence of pan-caspase inhibitor zVAD.fmk. (H) Toxicity of long polyalanines is enhanced by loss of one copy of functional Drosophila Heat-shock factor (Hsf). Flies of genotype ey-GAL4/UAS-A37/+ (control) or ey-GAL4/Hsff (UAS-NLS-A37/+ (Hsf++)) were scored for the presence of abnormal eyes at 25°C. Hsf(f) alone or in the presence of ey-GAL4 does not lead to any eye abnormalities (data not shown). Flies with Hsf(f) allele alone, in the presence ey-GAL4 or in the presence of ey-GAL4 and A7 never exhibit any visible eye abnormalities. We evaluated 16 bottles (approximately 900 flies). (P < 0.001; odds ratio: 4.287, 95% confidence interval 3.194–5.755). (I) Experiment was done as described in (H) and survival to adulthood was evaluated. We evaluated 10 bottles (approximately 1000 flies). (P < 0.001; odds ratio: 0.321, 95% confidence interval 0.195–0.515). (J) Loss of one copy of functional Drosophila Hsf does not affect survival to adulthood of flies expressing non-toxic short stretches of alanines. Flies of genotype ey-GAL4/CyO; UAS-A7 were crossed to flies with Hsf(f) allele and the survival was evaluated (see Materials and Methods for details). We evaluated 10 bottles (approximately 1500 flies). (P = 0.614; odds ratio = 1.055; 95% confidence interval 0.856–1.301). *P < 0.05; **P < 0.001; NS not significant.
induction of a protective heat-shock response by a polyalanine expansion may be one reason for the late onset and mild pathological effects of this mutation in OPMD. These protective consequences may explain the lack of a degenerative phenotype in certain polyalanine diseases associated with mutations in transcription factors, even though these appear to aggregate in cell models (7, 49). However, further experiments will be necessary to confirm that heat-shock protein induction occurs in animal models and people with other polyalanine diseases.

We speculate that the emergence of overall toxicity at higher polyalanine expression levels may be due to dose-dependent increase in the toxic properties overwhelming the protective responses—it is likely that heat-shock responses are saturable as they are physiologically subject to feedback control (50). As polyalanine expansions cause dominant diseases, our data suggest that decreased expression levels of such toxic proteins below normal physiological levels may be protective in certain contexts (Fig. 5). In our studies, the same disease-causing mutation exhibits thus both toxic and protective properties. This situation is distinct from other studies where the protective property is a feature of the protein context and not the disease-causing mutation.

Protective effect of polyalanines is independent of inclusions

Our data address a distinct issue from a recent report (51) finding that cells with inclusions were less likely to die compared with cells expressing the same model constructs without obvious inclusions. We report findings in cells and in vivo and focus on aggregate-prone proteins, as distinct from the inclusions themselves. It is likely that the heat-shock responses we observed are independent of inclusions, as these responses are not obviously attenuated by Congo red treatment (data not shown). Also, the protective responses we report are seen with polyalanine but not with polyglutamine (HD) constructs (either in cells or in vivo).

CONCLUSION

In summary, our data suggest that certain disease-associated aggregate-prone proteins simultaneously mediate a diversity of consequences, some deleterious and some protective (Fig. 5). Irrespective of the specific disease context, our findings have important consequences for the way we view the roles of such so-called ‘toxic’ proteins.

MATERIALS AND METHODS

Mammalian cell culture

African green monkey kidney (COS-7) and human neuroblastoma cell line (SK-N-SH) cells were maintained and transfected as described previously (10, 52). See supplementary methods for details on serine constructs. Other plasmids used were pHM6(CAG)74 and pHM6(CAG)21 (53), A7 and A37 (10). Forty-eight hours post-transfection, cells were fixed with 4% paraformaldehyde in 0.1M PBS of pH 7.6 and pHM6(CAG)74 expression detected by fluorescent immunocytochemistry using an anti-HA antibody (1:200; Covance), or HSP70 antibody (1:200, Stressgen) and secondary antibody Alexa Fluor red anti-mouse (1:1000; Molecular Probes).

Double-expressing cells with both red (polyglutamines) and green (EGFP-tagged alanines or serines) fluorescence were scored for cell death and aggregation; 200 cells per coverslip were counted with the viewer blinded to slide identity. Pooled estimates for aggregation and cell death (assessed by apoptotic nuclear morphology from multiple (×6) experiments were calculated as odds ratios (OR; the ratios of the proportion of abnormal/normal cells in different experimental conditions) with 95% confidence intervals, as described previously (10, 52). OR and P-values were determined by unconditional logistical regression analysis using the general log-linear analysis option of SPSS Version 6.1 (SPSS, Chicago, IL). The specificity and sensitivity of our methods is reported elsewhere (23).

For detergent resistance experiments, transfected cells were viewed under an inverted confocal microscope and SDS and Triton added to a final concentration of 0.1% (v/v). YFP-A37 was co-transfected with CFP HD exon 1 89Q in COS-7 cells, 1 μg each and fixed after 48 h. FRET analysis (acceptor bleach/increase in donor fluorescence) between donor (CFP HD exon 1 89Q) and acceptor (YFP-A37) was performed with a Zeiss Axiovert 200 microscope, using in-built external laser λ = 530 nm for YFP bleach (54).

Figure 5. Properties of long polyalanines. Long polyalanines exhibit both protective and toxic properties at the same time. Overall effect depends on expression levels as toxicity is dose-dependent.
anti-GAL4 antibody against DNA binding domain (Clontech, 1:500) and secondary antibody Alexa Fluor red anti-mouse (1:1000; Molecular Probes).

Congo red was dissolved in DMEM and used at concentration 100 μg/ml. Congo red has been successfully used as an aggregation inhibitor when dissolved in aqueous solution on both cell-based and Drosophila studies (17,55–57).

Western blotting
Mouse muscle protein lysates were prepared and analysed as described in Davies et al. (32). Flies that eclosed within 24 h at 29°C were homogenized in a 1.5 ml microtube containing 1× SDS-PAGE sample buffer and treated as for muscle samples. Membranes were probed with primary antibodies raised against hsp70 (StressGen; 1:1000) or GFP (Clontech 1:10000) and, as a loading control, actin (Sigma–Aldrich; 1:10000). HRP-conjugated antibodies (Amersham Biosciences; 1:5000) were then added to the blots. Immunooreactive bands were detected with enhanced chemiluminescence reagent (ECL; Amersham Biosciences) and the signal was visualized by exposing the membrane to the ECL Hyperfilm (Amersham Biosciences). Densitometry analysis was performed using Scion Image Beta 4.02 software.

Heat-shock element reporter assay
COS-7 cells were transfected in 24-well plates with 200 ng of a reporter plasmid comprising three tandem copies of the heat-shock element (HSE) consensus sequence fused to a minimal thymidine kinase promoter (P_{TAL}) and firefly luciferase gene (pHSE-Luc; BD Biosciences), 200 ng of a plasmid containing the β-galactosidase gene driven by a cytomegalovirus promoter (pCMVβ BD Biosciences) and either 200 ng (ratio 1) or 25 ng (ratio 1/9) of the EGFP-linked polyalanine constructs (EGFP-C1, A7, A37). Cells were harvested 48 h post-transfection and the levels of luciferase and β-galactosidase activity were determined. Luciferase activity from each well was normalized to its β-galactosidase activity to control for transfection efficiency. To determine luciferase activity specifically caused by activation of the HSE, the activity obtained from an empty control vector [pTAL-Luc; firefly luciferase gene under the control of a minimal TK promoter (pTAL); BD Biosciences] was subtracted from the test-wells. Experiments were carried out in the presence or absence of a pan-caspase inhibitor (ZVADfmk; 20 μM; Calbiochem).

For each experiment the mean luciferase activities in the presence of A7 and A37 were expressed as a percentage of the luciferase activity in the presence of EGFP-C1, which was set to 100.

Drosophila stocks and crosses
Flies were grown on standard corn meal molasses medium supplemented with dry yeast (1.2 l water, 12.5 g agar, 105 g dextrose, 105 g maize, 21 g yeast, 35 g Nipagin) at 25°C or 29°C with 40–70% humidity, with 12/12 h light/dark cycle. Seven or 37 alanines were fused to EGFP and the NLS from SV40 T-antigen as described (10). EGFP-NLS-A7 and EGFP-NLS-A37 were subcloned into the pPUAST vector (15). Previously described Nrv2-GAL4 (58), MHC-GAL4 (59), ey-GAL4 (60), HSF[1] (61) were used.

Lifespan determination
Flies were allowed to lay eggs for one day at 25°C and subsequently reared at 29°C. Flies eclosed within 24 h were used for lifespan determination, 20 individuals of the same sex were placed in each vial; approximately 200 individuals were used for each genotype. Flies were transferred to fresh medium and dead individuals were counted every 2 days.

Climbing assay
Flies were raised as described for lifespan determination. Climbing assays were performed in a set-up originally described for phototaxis experiments with the score reflecting how quickly the fly can climb in a tube in different trials (62). Approximately 100 flies were used (same number of males and females). The assay was performed at 25°C with 70% humidity; flies were allowed to acclimatize for approximately 2 h before the assay.

Survival to adulthood
Lines with polyalanine insertions on the third chromosome heterozygous with a TM6B balancer chromosome were used. Flies eclosing as adults with either the transgene (A7 or A37) or with the balancer were counted. The ratio A7:TM6B was arbitrarily taken as 1 and the A37:TM6B ratio was compared to that value. Similarly, in order to quantify the effect of Hsf1 on survival, the abovementioned ratio of A37:TM6B was compared with ratio of: ey-GAL4/Hsf1; A37++; ey-GAL4/Hsf1; TM6B/+ using the same statistical comparison. Odds ratios were determined as described earlier in mammalian cell culture section.

Pseudopupil analysis
Flies were raised at 25°C and rhabdомерes were counted at indicated times. Values for each genotype were obtained from at least 100 ommatidia counted in 5–10 flies and this was done at least twice (see text), with observer blinded to the identity of the slides. Controls were always done at the same time under exactly the same conditions. For pseudopupil analysis of gmr-GAL4, a gmr-GAL4 homozygous strain was crossed with w[1118] (stock isogenized for the X chromosome and two major autosomes which has been used as the genetics background for the European Drosophila Deletion Kit (63). For pseudopupil analysis of gmr-Q102, a stock of elav-GAL4: +; gmr-Q102/TM6B was crossed either with w[1118], A7 or A37 line; w[1118] was used as a parental stock for injection of A7/A37 transgenic lines.

Transgenic mice
Mice were cared for following UK Home Office regulations. Transgenic mice lines used in this study were the HD model R6/1(33) (muscle lysates) and a model of OPMD (A17-1) (32).
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors declare that they have no competing financial interests.

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