Toxicity of expanded polyglutamine-domain proteins in *Escherichia coli*

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Abstract Five neurodegenerative diseases are caused by proteins with expanded polyglutamine domains. Toxicity of these proteins has been previously identified only in mammals, and no simple model systems are available. In this paper, we demonstrate in *E. coli* that long polyglutamine domains (59–81 residues) as GST-fusion proteins inhibit growth while smaller glutamine (10–35 residues) or polyalanine (61 residues) domains have no effect. Analogously in humans, polyglutamine repeats less than 35–40 glutamines produce a normal phenotype, while expansion greater than 40 glutamines is always associated with disease. Expression of polyglutamine proteins in *E. coli* may help identify the molecular mechanism of pathogenesis of CAG trinucleotide repeat diseases and be a useful screen to identify potential therapeutic compounds.

Key words: Polyglutamine; Trinucleotide repeat; CAG repeat; Pathogenesis; Huntington's disease; DRPLA; (*E. coli*)

1. Introduction

Five neurodegenerative diseases are caused by expanded CAG repeats which encode expanded polyglutamine domains in the expressed disease proteins [1–6]. These dominantly inherited diseases, Huntington's disease, dentatorubral pallidoluysian atrophy (DRPLA), spinocerebellar ataxia I (SCA1), spinobulbar muscular atrophy (SBMA) and Machado-Joseph disease (MJD) result from gain-of-function mutations [7,8]. The expanded polyglutamine domain itself is likely to be directly involved in disease pathogenesis since: (a) all five proteins are associated with neurodegenerative diseases with similar phenotype, yet the polyglutamine domain is the only region of homology in the expressed proteins [8]; (b) polyglutamine repeats less than 35–40 glutamines produce a normal phenotype, while expansion greater than 40 glutamines is always associated with disease [9]; (c) the size of the polyglutamine repeat is the major determinant of age-of-onset and clinical phenotype [3,6,10–12]; and (d) expression of a portion of the Machado-Joseph protein containing an expanded polyglutamine repeat causes neuronal degeneration in transgenic mice and kills transfected COS cells [13].

The mechanism by which a polyglutamine domain results in disease pathogenesis is unknown. A potential mechanism of pathogenesis is abnormal protein-polyglutamine interactions [8,14]. To characterize further the interactions between polyglutamine proteins and other proteins, and to obtain a source of polyglutamine domain peptides, we cloned CAG repeats of varying lengths into a GST-fusion protein vector and expressed these proteins in *E. coli*. We unexpectedly observed that expression of GST-fusion proteins with 59 or greater glutamine residues killed *E. coli*, while GST-fusion proteins containing 10–35 glutamines did not. Expression of a GST-fusion protein containing 61 alanine residues was not toxic. Demonstration of length-dependent polyglutamine-induced toxicity in bacteria may simplify examination of the mechanism of pathogenesis and provide a system for screening potential therapeutic compounds.

2. Materials and methods

2.1. Construction of polyglutamine clones

Polyglutamine repeat-GST fusion proteins were synthesized by PCR from the human DRPLA cDNA [15] with modification of codons to those frequently observed in *E. coli*. Each clone was named Q followed by the number of uninterrupted glutamines. A DRPLA cDNA containing 79 or 14 repeats was used as a template to construct clones Q81, Q19 and Q10 using primers 5Q: 5′-TGATGGATCCGTTTCCACCCATCACCATCACCAC and 3Q: 5′-TGATGAATTCCGGCGGACCAGAGTTACCGTGATG. For construction of Q62 and Q58, we used TA62 and TA55 plasmid DNA [3]. For construction of Q35, genomic DNA with 35/14 CAG repeats in the DRPLA gene was amplified using primers 5min: 5′-TGATGGATCCTCTTTCCCTCCCCCAACAAG and 3min: 5′-TGATGAATTCCCCTTGAGAAGTGGAAGAGGAAG. The PCR product was then used as a template for further PCR using primers 5Q and 3Q. Clone Q(−) lacking the polyglutamine tract was constructed by PCR of the sequences adjacent to, but excluding, the CAG repeat using primers 5min and 5Q(−); 5′-GTGGTGATGGTGATGGGTGGAGAC, 3min and 3Q(−); 5′-CATCACGGTAACTCTGGTCCGCCTCCT. The PCR products were then ligated. PCR reaction conditions were as follows: 20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 0.1 mg/ml bovine serum albumin, 5% (v/v) DMSO, 200 μM dNTP, 10 pmol of primer and 2 units of Pfu DNA polymerase (Stratagene). After 3 min denaturation at 94°C, samples were subjected to 25 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C and 45 s extension at 72°C and a final extension at 72°C for 6 min. Fragments were cloned into the *Bam*HI and *Eco*RI site of the pGEX-4T-3 vector which contains an IPTG inducible promoter (Pharmacia). The recombinant DNAs were transformed into TOP 10F′ cells (Invitrogen). The nucleotide sequences of all constructs were confirmed by the dideoxynucleotide chain terminator method. The number of each clone reflects the number of glutamines in the polyglutamine domain. The sequence

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Abbreviations: DRPLA, dentatorubral pallidoluysian atrophy; SCA1, spinocerebellar ataxia I; SBMA, spinobulbar muscular atrophy; MJD, Machado-Joseph disease; IPTG, isopropylthio-β-D-galactoside; Q10-81, number of uninterrupted glutamines in clone; GST, glutathione-*S*-transferase; A61-SR, clone containing 61 alanines with a carboxyl terminal serine and arginine; Q62-SR, clone containing 62 glutamines with a carboxyl terminal serine and arginine.

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of polyglutamine stretch of each clone was as follows:

Q10 : $(CAGCAA)_2(CAG)_6$

Q19 : $(CAGCAA)_2(CAG)_{15}$

Q35 : $(CAGCAA)_2(CAG)_{31}$

Q59 : $(CAGCAA)_4(CAG)_{51}$

Q62 : $(CAGCAA)_2(CAG)_{58}$

Q81 : $(CAGCAA)_2(CAG)_{45}CAACAGCAA(CAG)_{29}$

2.2. Construction of polyalanine clones

The polyalanine-GST fusion protein was synthesized by amplifying the Q62 plasmid DNA using 5′-TGATGGATCCTTCCACC-CATCCCCATCCCCACCAGCAGCAGCAGCA and 3Q as primers. To eliminate the effect of C-terminal differences in the fusion proteins produced by the polyalanine and polyglutamine plasmids, we amplified the Q62 plasmid DNA using 5Q and 5′-TGATGAATTCC-GGCGGACCAGAGTTTCAGCGACTCTGCTG as primers. PCR and cloning were performed as described above. The nucleotide sequences of all constructs were confirmed by the dideoxynucleotide chain terminator method. Clone A61-SR contained 61 alanines, and the clone Q62-SR contained 62 glutamines. The carboxyl-terminus of the fusion proteins was identical, serine and arginine.

Summary of clones:

Q(−) : GST – LVPRGSVSTHHHHHHHGNSGPPEFPGRLERPHRD

Q10 to Q81 : GST – LVPRGSVSTHHHHH$(Q)_{10-81}$HHGNSGPPEF–PGRLERPHRD

Q62 – SR : GST – LVPRGSVSTHHHHH$(Q)_{62}$SR

A61 – SR : GST – LVPRGSFHPSPSPP$(A)_{61}$SR

2.3. Culture conditions

Individual clones of TOP10F′ (Invitrogen) or BL21(DE3)pLysS (Novagene) cells containing the indicated plasmid were grown overnight at 30°C in LB medium in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol in the case of pLysS cells. The cells were diluted to an OD_{600} of 0.1 in LB medium supplemented with antibiotics. After 30 min incubation at 37°C, either 0.1 or 1 mM IPTG was added to each culture. At the indicated times, a 200 μl aliquot of each culture was removed. Cell growth was monitored for up to 5 h by measuring cell densities by OD_{600} on a Beckman DU-64 spectrophotometer.

Colony forming ability was assayed 1 and 3 h after adding 0.1 mM IPTG. Aliquots of cell culture were removed, diluted, and plated on LB plated containing ampicillin. After overnight incubation, viable colonies were counted and expressed as colony forming units (CFU)/μl of culture.

2.4. Protein analysis

100 μl of cell culture was centrifuged and the cell pellet collected. The cell pellet was suspended in Laemmli buffer containing SDS and 2-mercaptoethanol and boiled for 3 min. The samples were electrophoresed on a 10% polyacrylamide gel containing 2% SDS. Proteins were transferred to Immobilon P membrane (Millipore) by standard Western transfer techniques. After transfer, the membrane was incubated in blotto (5% dried milk in Tris-buffered saline (pH 7.6) with 0.1% Tween 20 (Pierce)) at room temperature for 1 h. The membrane was incubated with goat polyclonal anti-GST antibody (Pharmacia) at 1:10000 dilution in blotto overnight at 4°C, then washed five times in blotto. The membrane was exposed to rabbit anti-goat secondary antibody conjugated with horseradish peroxidase (1:2000 dilution) for 1 h at room temperature, then washed seven times in blotto. Horseradish peroxidase was visualized with an enhanced chemiluminescence detection kit (Amersham) and exposed to Hyperfilm ECL (Amersham).

3. Results

3.1. Expression of glutamine-GST fusion proteins in E. coli

Expression of GST-fusion proteins with 10–81 glutamines in *E. coli* is shown in Fig. 1. Isopropylthio-β-D-galactoside (IPTG) induction increased expression of GST-fusion proteins containing 10–35 glutamines. Unexpectedly, identical treatment of bacteria transfected with plasmids containing 81 glutamines decreased GST-fusion protein expression. Polyglutamine-containing proteins migrate on polyacrylamide gels slightly slower than predicted from molecular mass; aberrant migration of these proteins on SDS-polyacrylamide gels has been reported [16,17].

To determine whether the induction of GST fusion proteins containing a polyglutamine domain was toxic to bacteria we assayed cell growth. Prior to addition of IPTG, the growth rate of *E. coli* containing plasmids encoding GST-fusion proteins with 10–81 polyglutamines was equivalent to the growth rate of *E. coli* transformed with the Q(−) construct lacking a polyglutamine domain (Fig. 2A). The flanking peptides encoded in these plasmids were identical. After induction with IPTG, the growth rate of *E. coli* expressing GST-fusion proteins greater than 59 glutamines was markedly reduced compared to *E. coli* expressing GST-fusion proteins with smaller glutamine domains (0.1 mM IPTG, Fig. 2B; 1.0 mM IPTG, Fig. 2C). In addition, the toxicity of polyglutamine domain proteins increased quantitatively with the size of the expansion when the repeat contained more than 59 glutamines (Fig. 2). Fig. 2 also demonstrates that increasing the concentration of IPTG increases toxicity.

The inhibition in growth rate shown in Fig. 2 could be caused by slowing of growth or by cell death. To address this question, we assayed *E. coli* survival by colony forming ability. Between 1 and 3 h after induction with 0.1 mM IPTG, *E. coli* transformed with Q10-GST showed a 500% increase in colony forming units. In contrast, under identical conditions, *E. coli* transformed with Q62-GST had a 75% reduction in colony forming units. Growth inhibition, therefore, was due

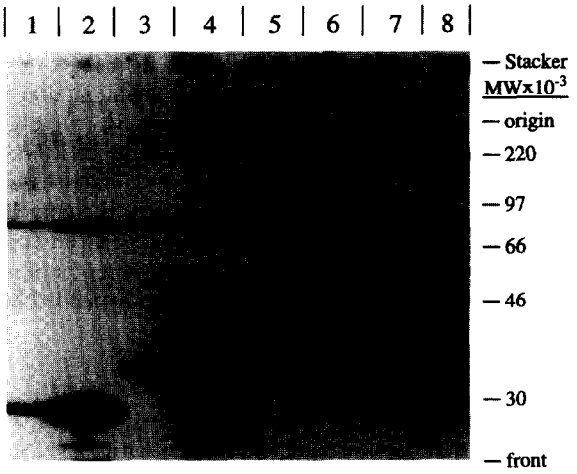

Fig. 1. Expression of GST-polyglutamine fusion proteins. *E. coli* transformed with plasmids encoding GST-fusion proteins Q10 (lanes 1,2); Q35 (lanes 3,4); Q59 (lanes 5,6) and Q81 (lanes 7,8) were grown in the absence (odd lanes) or presence (even lanes) of 1.0 mM IPTG.

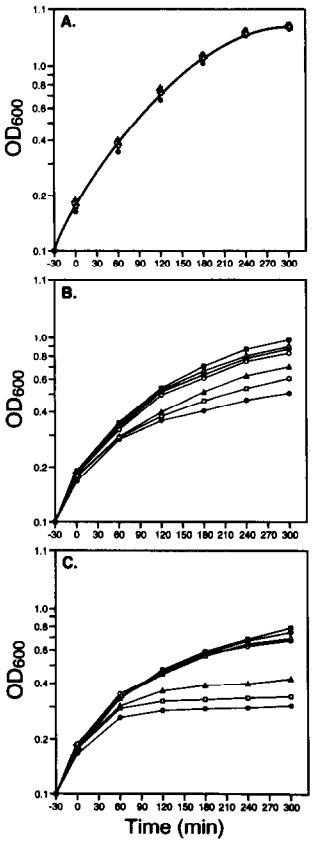

Fig. 2. Viability of *E. coli* expressing GST-polyglutamine fusion proteins. *E. coli* transformed with clones Q(−) (♦); Q10 (■); Q19 (△); Q35 (○); Q59 (▲); Q62 (□); or Q81 (●) were incubated in the absence (A) or presence of 0.1 mM IPTG (B) or 1.0 mM IPTG (C).

to inability of bacteria to replicate. Inability of bacteria to replicate and form colonies is synonymous with cell death.

Toxicity is not due to selective loss of longer polyglutamine plasmids [18,19] since cell growth is inhibited with induction of the polyglutamine fusion protein in either the presence or absence of ampicillin. Length-dependent toxicity of polyglutamine-GST fusion proteins was also observed in another strain of *E. coli* (BL21(DE3)pLysS). Toxicity is not due to depletion of glutamine, since supplementation of the media with glutamine (up to 100 mM) did not effect toxicity.

3.2. Expression of polyalanine-GST fusion protein

To determine whether the toxicity of these proteins is specific for polyglutamine or occurs with any homopolymer of equivalent length, we constructed a polyalanine-GST fusion protein containing 61 alanines. Alanine translation results from shifting the reading frame of the plasmid from CAG to GCA. To minimize variability at the carboxy-terminus the sequence following the repeat was truncated. Plasmids were constructed which produce either 62 glutamine residues (clone Q62-SR) or 61 alanine residues (clone A61-SR) with serine and arginine at the carboxy-terminus. *E. coli* transformed with Q62-SR or A61-SR produced GST-fusion proteins migrating on polyacrylamide gels as shown in Fig. 3. IPTG increased expression of both GST-Q62-SR and the GST-polyalanine fusion protein (A61-SR), but the effects on bacterial growth of the two constructs differed. Prior to incubation with IPTG, *E. coli* transformed with Q62-SR or A61-SR grew at the same rate (Fig. 4A). After induction by IPTG, *E. coli* expressing the A61-SR continued growing, while growth of bacteria expressing Q62-SR was inhibited (Fig. 4B). The growth curve of A61-SR was identical to the Q(−)-GST construct.

4. Discussion

Although proteins containing expanded polyglutamine domains are expressed in virtually every tissue, they appear to cause cell death only in the central nervous system [16,17] and in a transiently transfected mammalian cell line [13]. In this paper we demonstrate that expression of long polyglutamine repeats (59–81 residues) in GST-fusion proteins causes length- and concentration-dependent inhibition of cell growth in *E. coli*. In contrast, expression of smaller glutamine repeat peptides (10–35 residues) has no adverse effect on bacterial cell growth. This toxicity is not indiscriminate since expression of an equivalent length polyalanine-GST fusion protein has no effect on bacterial growth. The inhibition of growth is not simply a change in the rate of growth. The colony forming unit assay demonstrates that *E. coli* expressing long polyglutamine repeats are unable to replicate. It is also unlikely that toxicity is due to glutamine depletion because supplementation of the media with glutamine had no effect. Similarly, glutamyl t-RNA depletion is unlikely because large amounts of Q35-GST protein can be made with no effect on growth.

Numerous recombinant proteins are toxic in *E. coli* (rat insulin [20]; HIV protease [21]; *Streptococcus pneumoniae* mal and ami loci [22]; cytochrome c_2 [23] and mutations in tRNA synthetase [24]). These proteins are hypothesized to cause toxicity by (a) impairing export machinery for secreted proteins [22], (b) by the overexpression of secreted proteins [20,22], (c) by proteolysis [21] and (d) by depletion of tRNA [24]. The mechanism of polyglutamine-mediated cell death is unknown and the polyglutamine constructs produce intracellular proteins with no known enzymatic function. The GST

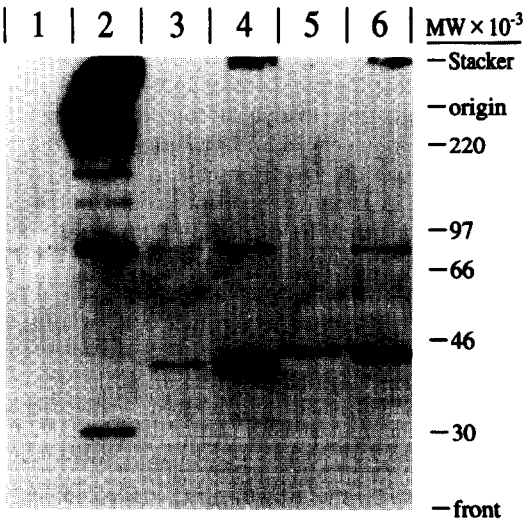

Fig. 3. Expression of GST-polyglutamine or GST-polyalanine fusion proteins. *E. coli* transformed with plasmids encoding GST-fusion proteins A61-SR (lanes 1,2); Q62-SR (lanes 3,4) or Q62 (lanes 5,6) were grown in the absence (odd lanes) or presence (even lanes) of 1.0 mM IPTG.

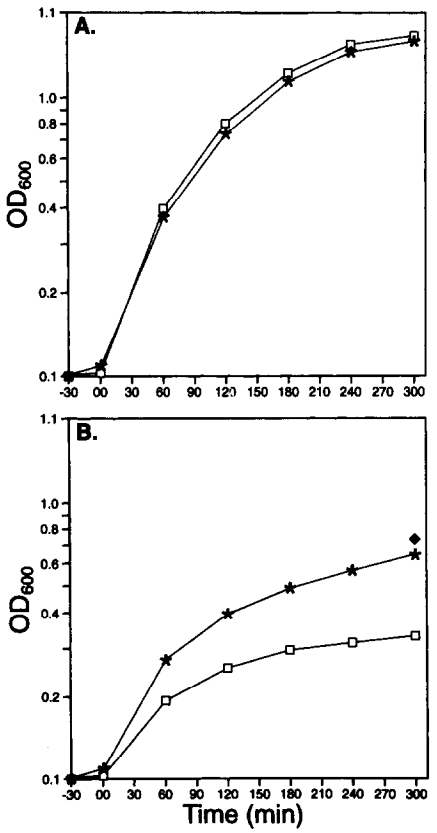

Fig. 4. Viability of *E. coli* expressing GST-polyglutamine or GST-polyalanine fusion proteins. *E. coli* transformed with clones A61-SR (★) or Q62-SR (□) were incubated in the absence (A) or presence of 1.0 mM IPTG (B). Bacterial number was assayed by measuring OD_{600} at the times indicated. OD_{600} for *E. coli* transformed with Q(−) (♦) at the 300 min time point is also shown in (B).

domain of the fusion protein alone, or associated with polyglutamine domains up to 35 glutamines or 61 alanines, has no effects on survival.

In humans the size of the polyglutamine repeat determines whether disease occurs. The size of the polyglutamine domain which causes bacterial toxicity (greater than 35 glutamines) is also the size causing human disease (approx. 40 or more glutamines) [9]. In addition, toxicity of the polyglutamine fusion proteins in *E. coli* increases with length, analogous to the effect of repeat size on age-of-onset and disease severity in humans [3,6,10–12]. Length-dependent toxicity in the nervous system and bacteria may be caused by these large polyglutamine domains adopting a novel conformation. A unique structure for polyglutamine domains greater than 40 residues in length has been suggested by Trottier et al. who isolated a monoclonal antibody which specifically identifies proteins containing enlarged polyglutamine repeats [25].

Another factor contributing to toxicity is the quantity of GST-polyglutamine protein. The extent of toxicity is a direct function of the concentration of the inducer IPTG. Prior to induction with IPTG, the growth of *E. coli* producing an 81 repeat polyglutamine protein was identical to *E. coli* producing smaller polyglutamine repeat proteins despite 'leaky' production of a small amount of protein. Increased concentrations of IPTG caused greater inhibition of *E. coli* growth. A similar effect of protein expression was found in transgenic mouse models of SBMA and SCA1 [26,27]. In a model of SBMA, low level expression of an androgen receptor containing an expanded repeat did not cause clinical or pathologic signs of disease [26]. A transgenic model of SCA1 demonstrated the disease phenotype only in mice with a high level of expression of the ataxin 1 transgene [27].

The mechanism by which expanded polyglutamine domain proteins cause toxicity in bacteria, transfected fibroblasts or in the nervous system is not known. Abnormal energy metabolism has been proposed in the pathogenesis of HD [28]. Chronic, systemic administration of the TCA cycle inhibitor 3-nitropropionic acid (3-NP) in primates, for example, produces a disease phenotype clinically and pathologically similar to HD [29]. 3-NP inhibits succinate dehydrogenase, a metabolic enzyme present in all cells, yet pathology is restricted to a subset of neurons in the central nervous system. Analogously, the polyglutamine repeat proteins are widely expressed throughout the body, yet pathology is restricted to certain neuronal populations. The pathology of the polyglutamine repeat diseases may be restricted to the nervous system because neurons are long-lived, post-mitotic cells with high energy requirements. Cell death may occur in other tissues, but the low death rate allows replacement by mitosis. Alternatively, neuron-specific target proteins may mediate degeneration. The latter possibility seems less likely in view of the toxicity of polyglutamine in COS cells [13] and in *E. coli*.

A potential mechanism of toxicity is a quantitative or qualitative change in protein-polyglutamine domain interactions which vary as a function of polyglutamine length [30]. Polyglutamine domains can mediate protein-protein interactions by forming polar zippers. Perutz has demonstrated that polyglutamine peptides form hydrogen bonded β-pleated sheets, but little is known about the stability of these interactions as a function of chain length [14,31]. Our observation that polyalanine is not toxic in *E. coli* suggests that structural motifs may be critical in toxicity because polyalanine tends to form alpha helices rather than β-pleated sheets [32].

RNA-protein interactions have recently been demonstrated between CAG repeat RNA and RNA binding proteins [33], but our data suggest that this is an unlikely cause of toxicity in *E. coli*. The polyalanine construct A61-SR contains a long CAG repeat, but it is frame-shifted to GCA so that it is translated as alanine instead of glutamine. If pathogenesis were mediated by RNA-protein interactions, A61-SR would have equal toxicity with Q61-SR because of the equal number of CAG repeats in their RNA, but Q61-SR inhibits growth while A61-SR has no effect. A similar absence of toxicity despite RNA expression has also been reported in a transgenic mouse with a frame-shift in the huntington gene [34].

As shown here, expanded polyglutamine domain proteins are toxic in prokaryotic cells. The polyglutamine toxicity in bacteria mimics eukaryotic systems in its length and concentration dependence. *E. coli* expressing expanded polyglutamine proteins may prove to be a useful model system to identify other molecules which interact with polyglutamine proteins, to characterize protein interactions, and to screen for therapeutic compounds.

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