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Phenotypic effects of expanded ataxin-1 polyglutamines with interruptions *in vitro*

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ABSTRACT: Spinocerebellar ataxia type 1 is a neurodegenerative disease caused by expansion of an uninterrupted glutamine repeat in ataxin-1 protein. Protein aggregation and immunoreactivity to 1C2 monoclonal antibody are two distinct pathognomonic features of expanded ataxin-1, as well as of other polyglutamine disorders. Rare cases of non-affected elderly subjects carrying expanded ataxin-1 alleles were found in random population. However, in these alleles the glutamine stretch was interrupted by histidines. Due to lack of phenotype, these alleles should be considered "normal". Most importantly, occurrence of these unusual alleles provides a unique opportunity to investigate which molecular properties of expanded ataxin-1 are not coupled to polyglutamine pathogenesis. Towards this goal, we compared *in vitro* the immunoreactivity to 1C2 antibody and the ability to form aggregates of interrupted and uninterrupted alleles. Immunoblotting showed that expanded-interrupted ataxin-1 had an affinity to 1C2 resembling that of normal ataxin-1. On the contrary, filter assay showed that aggregation rate of expanded-interrupted ataxin-1 resembles that of expanded-uninterrupted ataxin-1. These observations indicate that affinity for 1C2 does not directly correlate with self-aggregation of ataxin-1. Moreover, self-aggregation is not directly affected by histidine interruptions. In conclusion, these results support the hypothesis that mechanisms underlying neuronal degeneration are triggered by protein misfolding rather than by protein aggregation. © 2001 Elsevier Science Inc.

KEY WORDS: Spinocerebellar ataxia type 1, SCA1, Trinucleotide expansion, Amyloid-like protein aggregates.

INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1, MIM #164400) is an autosomal dominant disorder characterized by late onset progressive ataxia, dysarthria and loss of cerebellar Purkinje cells and brainstem neurons. The disease is caused by an expansion of a (CAG)n repeat within the SCA1 gene, corresponding to an expansion of a polyglutamine tract within the protein, ataxin-1 [7]. Age at onset usually ranges between 20 and 60 years; thereafter the disease progressively worsens, leading to death in 10–20 years. Juvenile onset cases characterized by a more rapid progression of the disease have also been reported [19,23].

Normal SCA1 alleles contain an uninterrupted (CAG)n repeat when they carry less than 21 units. Instead, the repeat is interrupted by one to three CAT encoding for histidine(s) when there are more than 21 triplets. The interruptions (**CAT**, **CAT**CAG**CAT**, and **CAT**CAG**CAT**CAG**CAT**) are localized approximately in the middle of the (CAG)n stretch. The trinucleotide stretch in the expanded alleles is always made up by an uninterrupted (CAG)n repeat [3]. The majority of normal alleles range from 18 to 38 repeats, while expanded alleles range from 39 to 82 repeats [3]. Only two exceptions of normal alleles with >39 repeats are known at present: an interrupted 44 repeat SCA1 allele carried by a child affected with an early onset unusual cerebellar ataxia (age 2), and her healthy 33-year-old father [11], and a 45 repeat allele carried by a healthy 66-year-old subject. The sequence analysis of this subject showed an internal configuration with two blocks of interruptions [$(CAG)_{12}$**CAT**CAG**CAT**$(CAG)_{12}$**CAT**CAG**CAT**$(CAG)_{15}$] [5].

Expanded, CAG pure, alleles are prone to length variation (instability), during both meiotic and mitotic cell division [2], while the presence of variant trinucleotides interrupting the (CAG)n sequence has been shown to have a stabilizing effect [8].

At the protein level, ataxin-1, the SCA1 gene product, is found predominantly in the nucleus of neurons and in the cytoplasm of peripheral tissues [16]. Despite the wide expression pattern of ataxin-1, a selective degeneration of cerebellar Purkinje cells and brainstem neurons can be found in SCA1. The analyses of the subcellular localization of ataxin-1 in the Purkinje cells of SCA1 transgenic mice, neurons of SCA1 patients, and in transfected COS-1 cells, have revealed that normal ataxin-1 localizes to several nuclear structures and that it associates with the nuclear matrix, whereas the expanded ataxin-1 localizes in a few large structures, in the affected neurons [17].

At present, seven other disorders have been shown to result from CAG expansion, the so-called polyglutamine (polyQ) disorders (SCA2, 3, 6, 7, spinal and bulbar muscular atrophy [SBMA], Huntington's disease, and dentatorubral-pallidoluysian atrophy (DRPLA) (for a review see [24]). It has been postulated that all of these disorders result from a common gain-of-function, toxic to the cell, due to the expanded polyQ tract [1,9,22]. It has been recently shown, both *in vivo* and *in vitro*, that expanded polyQ stretches lead to the formation of intranuclear inclusions (NIs) containing insoluble ubiquitous aggregates of the protein [12].

Although the disease-causing function of NIs is not yet understood, nuclear expression of polyQ proteins is clearly implicated in pathogenesis. In fact, SCA1 transgenic mice expressing an expanded ataxin-1 targeted to the cytoplasm instead of its normal nuclear location do not develop ataxia and do not show neuronal degeneration [6]. Also, in transfected neurons,

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TABLE 1

FUSION PROTEINS PRODUCED BY THE pGEX CONSTRUCTS DESCRIBED IN THE TEXT

pGEX-poly32Q(H)	GST-◆- Upstream peptide-$Q_{14}HQHQ_{15}$-Downstream peptide
pGEX-poly45Q(H)	GST-◆- Upstream peptide-$Q_{12}HQHQ_{12}HQHQ_{15}$-Downstream peptide
pGEX-poly47Q	GST-◆- Upstream peptide-Q_{47}-Downstream peptide
pGEX-poly82Q(H)	GST-◆- Upstream peptide-$Q_{12}HQHQ_{12}HQHQ_{13}HQ_{19}HQHQ_{16}$-Downstream peptide
pGEX-poly76Q	GST-◆- Upstream peptide-Q_{76}-Downstream peptide

GST, glutathione S-transferase.
Upstream peptide → AGATTPSQRSQLEAYSTLLANMGSLSQTPGHKAE.
Downstream peptide → HLSRAPGLITPGSPPHLSRAPGLITPGSPPPAQQNQYVHISSSPQNTG.
◆, Thrombin cleavage site; (H), polyglutamines interrupted by histidines.

mutant huntingtin induces apoptosis only when the protein is localized to the nucleus [13]. In addition, the NIs pathogenic role has also been questioned following the results obtained by Klement et al. [6] on transgenic mice expressing an expanded ataxin-1 containing a deletion in its self-association region, and thus unable to form visible insoluble aggregates. Despite the lack of NIs these mice developed ataxia.

Two interesting peculiarities arose from previous investigations, that could explain the pathogenic phenomenon of expanded polyglutamines: their increasing ability to form amyloid-like protein aggregates with the elongation of the polyQ stretches [10,14, 15], and the specific capability to be recognized by the 1C2 and 1F8 MoAbs [20,21], Abs whose affinity increases with the elongation of the polyQ stretches.

Here we report the results of the conformational analysis *in vitro* of the SCA1 expanded-interrupted and -uninterrupted polyQ stretches in terms of affinity to 1C2 MoAb and their capability to form insoluble protein aggregates, tested by filter retardation assay [14].

MATERIALS AND METHODS

Plasmid Construction

The DNA of the subject carrying the expanded-interrupted SCA1 allele [5] (genotype 29/45) and the DNA of a SCA1 affected subject carrying an expanded allele with 46 repeats (genotype 29/46) were amplified by polymerase chain reaction (PCR) using the following pair of primers: Rep-for (5′-CCAGCTTCATCCCATCACAG-3′) and Rep-rev (5′-TGAGCGTGTGTGGGATCATC-3′).

The PCR products were purified, digested with EagI and cloned into the EagI site of the expression vector pGEX-4T-2 in frame with the glutathione S-transferase (GST) (Amersham-Pharmacia, USA), yielding pGEX-poly45Q(H) and pGEX-poly47Q, respectively (Table 1). With the same method pGEX-poly32Q(H) and pGEX-poly76Q were obtained from PU31 and PUSCA74 SCA1 cDNAs, respectively. To obtain the pGEX-poly82Q(H) the following procedure was used: (1) the 45Q(H) allele was amplified with Rep-for and AlwNI-rev (5′-GCCCTGCTCAGGTGCTGAT-

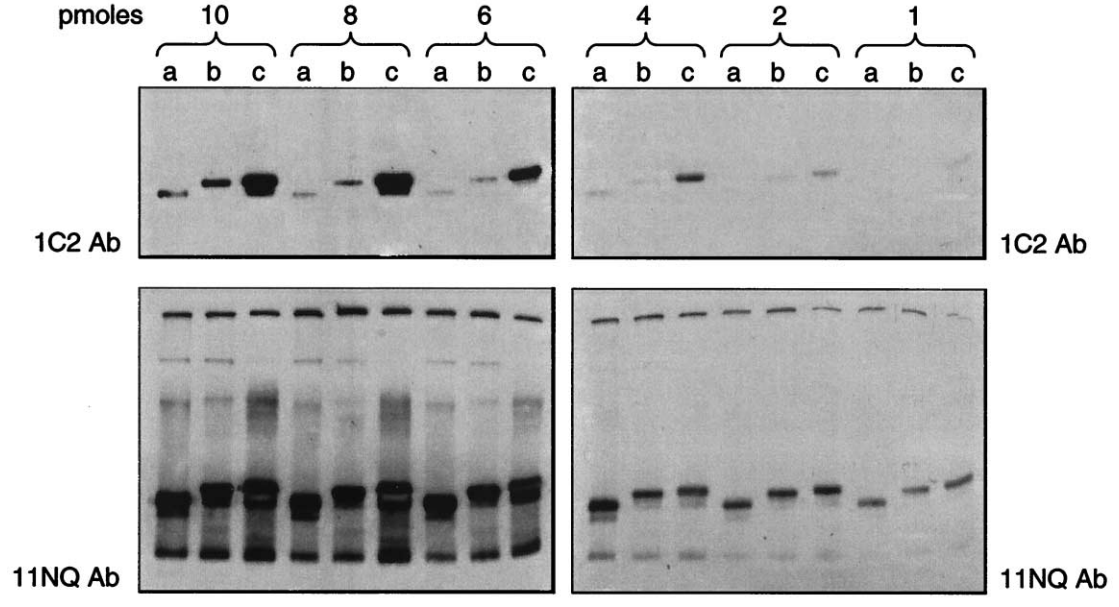

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified glutathione S-transferase (GST)-polyQ and GST-polyQ(H) proteins. a, poly32Q(H); b, poly45Q(H); c, poly47Q. Increasing quantities (pmoles) of fusion proteins were loaded. Top: nitrocellulose filters were probed with 1C2 MoAb. Bottom: after stripping, the same filters were probed with 11NQ Ab.

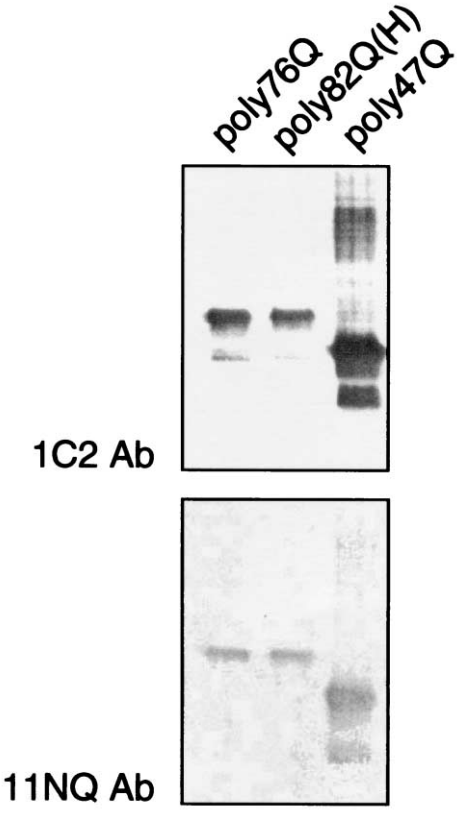

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of not purified glutathione S-transferase (GST)-poly76Q, GST-poly82Q(H), GST-poly47Q. 1 pmole, 1 pmole, and 10 pmoles were loaded, respectively. Top: nitrocellulose filter was probed with 1C2 MoAb. Bottom: after stripping, the same filter was probed with 11NQ Ab.

GCTG-3′) primers which insert a AlwNI digestion site at the end of the (CAG)n repeat; (2) a 37Q(H) allele from a normal subject was amplified (genotype 30/37) with the PleI-for primer (5′-GGACACGAGTCTGAGCAGCAGCA), which inserts a PleI digestion site at the beginning of the (CAG)n repeat, and Rep-rev; (3) the two PCR products were digested with AlwNI and PleI, respectively, and incubated separately with Klenow Fragment of *Escherichia coli* DNA polymerase I to make their termini blunt; (4) the two resulting fragments were then EagI digested and were cloned together in a pGEX-4T-2, yielding pGEX-poly82Q(H). This clone has a "pure" (CAG)n stretch not exceeding 19 repeats. The constructs were transformed in *E. coli* BL21. All reactions were carried out following standard methods. Table 1 reports the fusion proteins clones described above. Any discrepancy between repeat lengths of the clones obtained and starting alleles is due to the instability of repeat tracts itself.

Purification of GST Fusion Proteins

E. coli BL21 carrying the pGEX expression plasmids of interest were grown to an $O.D._{600}$ of 0.6–1.0 at 30°C (the low incubation temperature reduces the level of instability), and induced with IPTG [1 mM pGEX-poly32Q(H), pGEX-poly45Q(H) and pGEX-poly47Q, and 0.1mM pGEX-poly82Q(H) and pGEX-poly76Q] for 3.5 h as described by the manufacturer (Amersham-Pharmacia). Cultures (30 ml) of induced bacteria were centrifuged for 15 min, and the resulting pellets were resuspended in 1.5 ml of lysis buffer [phosphate-buffered saline (PBS) 1×] containing 0.1 mg/ml lysozyme and 0.01 mg/ml DNase I, and stored overnight at −20°C.

Cells were lysated with 10 freeze/thaw cycles, and the resulting lysates were clarified by centrifugation in a microfuge at 13,000 rpm for 10 min. Cleared lysates were incubated overnight at 4°C with 100 μl of a 50% slur of glutathione sepharose 4B. The beads were washed three times and resuspended in lysis buffer (PBS 1×). The bound GST-polyQ fusion proteins were eluted with 100 μl of glutathione elution buffer (10 mM reduced glutathione). Protein concentrations were determined by the Bio-Rad (Germany) dye binding assay using bovine serum albumin as standard.

Western Blot Analysis

The GST-polyQ fusion proteins were fractionated by using sodium dodecyl sulfate (SDS)/10% polyacrylamide gel electrophoresis (PAGE), and electrotransferred to nitrocellulose (Immunblotting polyvinylidene difluoride membrane, 0.2 μm, Bio-Rad). The blots were incubated with anti-ataxin 11NQ polyclonal antibody (1:5000 dilution) [17] and anti-polyQ 1C2 MoAb (1:6000 dilution) [20], followed by a horseradish peroxidase (HRP) labeled second antibody (1:1000 dilution). Immunodetection was carried out with the enhanced chemiluminescence Western blot system (Amersham Pharmacia).

Filter Assay

For *in vitro* aggregation studies, the GST-polyQ proteins were digested with thrombin, resulting in a release of a polyQ containing peptide. Thrombin digestions of the fusion proteins were carried out at room temperature in glutathione elution buffer or in lysis buffer (PBS 1×) at an enzyme/substrate rate of 0.01U:1 μg for 16 h. Digestion was terminated by adjusting the protein mixtures to 2% SDS and 50 mM dithiothreitol (DTT).

The GST-polyQ proteins (before or after thrombin digestion) were diluted into 200 μl of 0.1% SDS and filtered through a cellulose acetate membrane (Schleicher and Schuell, 0.2 μm pore size) and a nitrocellulose membrane (Bio-Rad, 0.2 μm pore size), using a BRL dot blot filtration unit [14]. Filters were washed with water, and the proteins retained on the filters detected by incubation with the anti-ataxin 11NQ Ab, followed by an HRP labeled anti-rabbit second antibody.

RESULTS

Five SCA1 constructs (consisting of 108 nucleotides 5′ to the poly-CAG segment, the poly-CAG segment itself and 96 nucleotides 3′), were obtained for the production of GST-SCA1 polyQ fusion proteins in *E. coli*. The structures of GST-SCA1 polyQ proteins are shown in Table 1 (the H between parentheses indicates that the polyQ is interrupted by histidines). SDS-PAGE of the purified GST-poly32Q(H), GST-poly45Q(H), GST-poly47Q, GST-poly76Q, and GST-poly82Q(H) proteins revealed that all recombinant proteins migrated at a size corresponding to nearly that predicted from their amino acid sequence. These bands were also detected when the various protein fractions were subjected to immunoblot analysis using the anti-ataxin 11NQ Ab. 11NQ Ab specifically detects the GST-polyQ fusion proteins on immunoblots, whereas the GST tag alone is not recognized. Interestingly, the GST-poly45Q(H) and GST-poly47Q migrate at different sizes, not justified by their length difference consisting of only two amino acids. Although PAGE was performed under denaturing

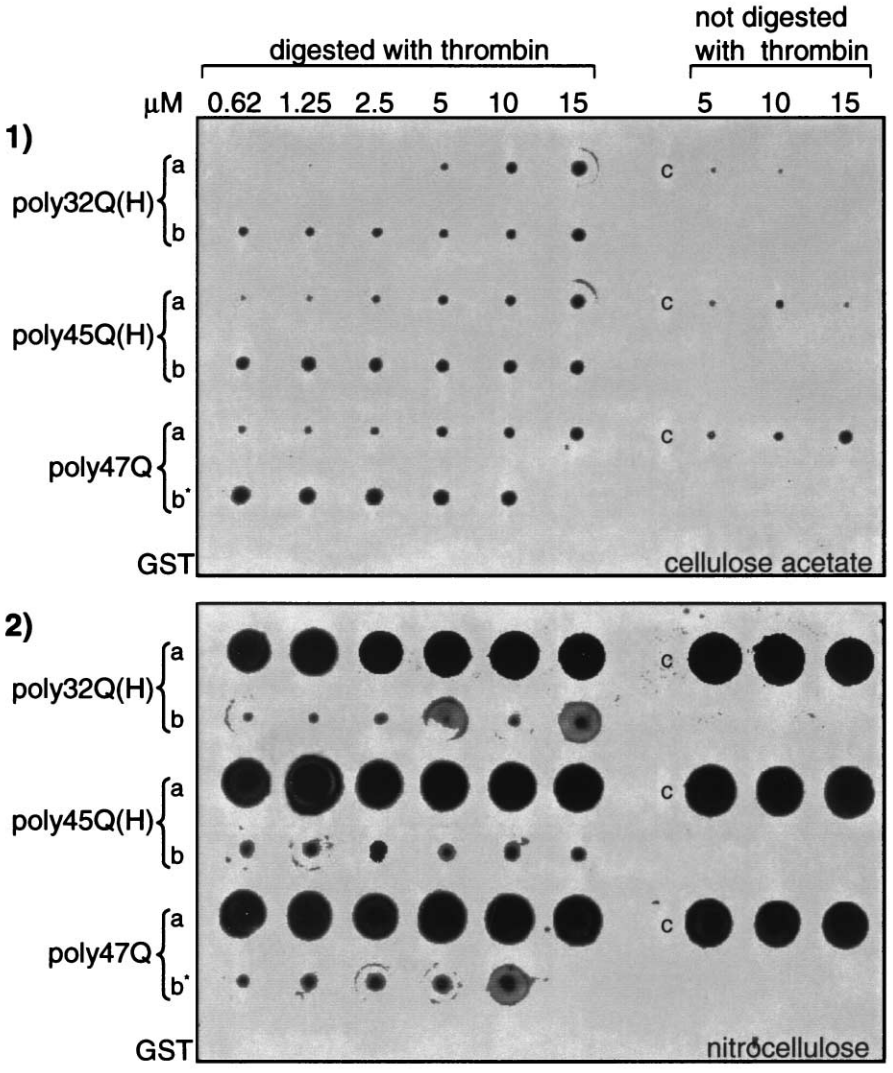

FIG. 3. Filter retardation assay of glutathione S-transferase (GST)-polyQ and GST-polyQ(H) proteins. (1) 200 ng of purified fusion proteins (c) and their thrombin cleavage products [(a) digested but not isolated from GST; (b) digested and isolated from GST] were incubated at different concentrations (μM) and filtered through cellulose acetate membranes. (2) 50 ng of the same products were filtered through nitrocellulose membranes. The proteins retained by the two membranes were detected by incubation with the 11NQ Ab. GST was loaded as negative control. (*The well corresponding to poly47Q(b)/15 μM was not loaded.)

conditions, residual conformation could have been retained thus suggesting a different conformation of the two proteins.

Increasing quantities of GST-poly32Q(H), GST-poly45Q(H) and GST-poly47Q were loaded on SDS-PAGE (Fig. 1). After Western blotting the membranes were hybridized with 11NQ Ab. Figure 1, bottom, shows that the same decreasing amount of the three proteins was charged.

After stripping, the same membranes were hybridized with 1C2 MoAb (Fig. 1, top). This hybridization confirmed an increasing affinity of 1C2 MoAb in parallel with the increasing size of polyQ stretches, but showed an affinity degree of the 1C2 MoAb to GST-poly45Q(H) higher than to GST-poly32Q(H) but much lower than to GST-poly47Q.

Figure 2 shows Western blotting of the two longest fusion proteins [GST-poly76Q and GST-poly82Q(H)], both hybridized with 1C2 and 11NQ Abs. It was not possible to perform the above described experiment on different quantities of proteins since the protein yield was very poor after induction of bacteria, probably because of the toxicity of the protein causing cell death. Also in this case, against the same quantity of the proteins (Fig. 2, bottom), the affinity of 1C2 MoAb was higher for GST-poly76Q, i.e., for the uninterrupted polyglutamine.

The GST-poly32Q(H), GST-poly45Q(H), and GST-poly47Q fusion proteins were digested with thrombin. An aliquot of the digestions was isolated from GST. Aliquots of GST-fusion proteins, digested proteins and digested/isolated proteins were incubated at increasing concentrations for 16 h at room temperature. After incubation, 200 ng and 50 ng of each protein at each

concentration were filtered through cellulose acetate and nitrocellulose membranes, respectively. Nitrocellulose membranes hold all proteins whereas cellulose acetate holds only insoluble aggregates. Figure 3 shows these membranes hybridized with 11NQ Ab. On both filters proteins digested with thrombin but not isolated from GST were loaded in rows a; digested and purified proteins were loaded in rows b; non-digested fusion protein were loaded in rows c; GST tag alone was also loaded as negative control. Hybridization of nitrocellulose membrane confirms that comparable amounts of proteins were charged in rows a, whereas rows b show that such purified proteins cannot be loaded after incubation even at very low concentrations, probably because they aggregate to the extent that they precipitate and/or stick very tightly to the tube wall. This finding has been confirmed by various replications of the same experiment (data not shown). Hybridization of cellulose acetate membrane points out three different aspects of the aggregation: (1) As already reported [14,18], the solubility of polyQ peptides is enhanced by their fusion with GST tag (Fig. 3, rows c). The present results show that the presence of free GST in the medium also enhances the solubility of polyQ peptides (Fig. 3, rows a). (2) In presence of GST, either fused with the protein or free in the medium (Fig. 3; rows a and c), aggregation depends on both concentration and polyQ stretches length. In this case the behavior of interrupted poly45Q(H) is frankly intermediate between poly32Q(H) and poly47Q, tending to be more similar to uninterrupted stretches than to poly32Q(H). (3) The purified polyQs, either interrupted or not, aggregate tightly even at very low concentrations (Fig. 3, rows b). Also under this condition a greater difference in aggregation between longer and shorter polyQs than between interrupted and uninterrupted proteins of comparable length can be seen.

DISCUSSION

Here we report analyses *in vitro* of an allelic form of ataxin-1 that, with respect to the repeat length, it is within the range of pathological alleles (i.e., this allele has 45 repeats and the pathological range is 39–82), but, with respect to the internal conformation, it is interrupted by histidines as for normal alleles. A 66-year-old healthy subject carries this allele, hence it can be regarded as a non-pathological allele [5].

Results of affinity tests to 1C2 MoAb, whose specificity was reported to be dependent on the polyQ length, between: (1) a normal allele with a 32 units interrupted polyQ stretch [poly32Q(H)]; (2) an expanded-uninterrupted allele with a 47 units "pure" polyQ (poly47Q); and 3) an expanded-interrupted SCA1 allele [poly45Q(H)], showed that the expanded-interrupted poly45Q(H) allele has an affinity to the 1C2 MoAb much lower than that of the expanded-uninterrupted allele, poly47Q. The present results suggest that histidines interrupting the polyQ stretch of ataxin-1 suppress the misfolding of the expanded polyQ specifically recognized by the 1C2 MoAb. This finding is consistent with the hypothesis formulated by Fernandez-Funez et al. [4] upon the observation that also normal ataxin-1 is toxic if expressed in excess. According to this hypothesis, ataxin-1 has more than one stable conformation, and even a low percentage of the normal protein misfolds towards a pathogenic conformation. Polyglutamine tract expansion increases the likelihood that the protein will adopt a misfolded, pathogenic conformation. On the basis of our observations on the expanded-interrupted polyQs, we can also speculate that the mechanism leading to the adoption of the misfolded conformation is suppressed or mitigated by the interruption of the polyQ by histidine(s).

On the other hand, experiments on protein aggregation showed a level of aggregation of the expanded-interrupted allele frankly intermediate between that of the expanded-uninterrupted poly47Q and the normal poly32Q(H) alleles, if not more similar to the expanded-uninterrupted allele. This suggests that protein aggregation is not directly associated with pathogenicity.

These results suggest that the two biochemical characteristics, affinity to 1C2 MoAb and aggregation, peculiar of expanded polyglutamines are affected by histidine interruption(s) differently. Consequently, this also suggests that these two characteristics are distinct phenomena. The first is peculiar to the expanded-uninterrupted polyglutamines, and therefore most likely associated to the pathogenic pathway, whereas the second seems independent from the pathogenicity of the allele.

The exact role of misfolding as well as of aggregation in polyQ neuropathogenesis is still very controversial. Nevertheless, the present observations provide insights into the understanding of the molecular mechanisms ultimately causing or preventing the neurotoxic effects of ataxin-1 and polyQ in general. Indeed, these results concern basic biophysical properties of polyQ blocks that are common to all diseases of this class.

Although our *in vitro* model is limited to measuring two simple features, it provides parameters and reagents directly suitable for drug screening as well as for subsequent *in vivo* analyses. For instance, *in vivo* comparison experiments between expanded-uninterrupted alleles (pathologic) and similar alleles whose pathogenicity should be suppressed by histidines, would help to identify more precisely the molecular mechanisms originating the disease. In a recent work, Klement et al. [6] have demonstrated uncoupling of SCA1 phenotype from formation of intranuclear aggregates in transgenic mice. Replication of our approach *in vivo*, in transfected cells and transgenic animals, should reinforce the exclusion of a primary involvement of nuclear insoluble aggregates in prompting the disease. On the other hand, it would also be interesting to investigate several more parameters that are supposed to be affected by expansion of polyQ stretches. Such as chaperone effects on expanded but interrupted polyQ, protein clearance by proteasome complex, and binding to nuclear matrix.

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