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Expression and purification of ataxin-1 protein

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ABSTRACT

Ataxin-1 is part of a larger family of polyglutamine-containing proteins that is linked to nine distinct neurodegenerative disorders. There are no known effective therapies for any of these expanded polyglutamine tract disorders. One possible reason for this is the lack of sufficient amounts of pure polyglutamine-containing proteins suitable for biochemical and conformational studies. Here, we show that we were able to successfully purify a non-pathological, wild-type human ataxin-1 protein containing a 30-glutamine repeat sequence. This ataxin-1 protein was expressed in *Escherichia coli* as a fusion protein with a GST tag at the N-terminus and a double $(His)_6$ tag at the C-terminus. The devised dual affinity tag strategy allowed successful purification of the full-length ataxin-1 fusion protein to 90% homogeneity as confirmed by Western blot analysis using the two monoclonal ataxin-1 antibodies developed in our laboratory. In addition, the GST tag was successfully removed from the purified ataxin-1 fusion protein by treatment with Tobacco etch virus (TEV) protease. Since polyglutamine-containing proteins tend to aggregate, solvents/buffers that minimize aggregation have been used in the purification process. This dual affinity purification protocol could serve as a useful basis for purifying aggregation-prone proteins that are involved in other neurodegenerative diseases.

1. Introduction

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease that is characterized by progressive deterioration of motor function and ataxia resulting from loss of cerebellar Purkinje cells and brain stem neurons (Zoghbi and Orr, 2000). SCA1 belongs to a larger family of neurological disorders that includes Huntington's disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and the spinocerebellar ataxias, SCA2, SCA3, SCA7 and SCA17 (Orr et al., 1993; Chung et al., 1993; Rudnicki and Margolis, 2003). This group of disorders is caused by unstable expansion of the CAG trinucleotide repeat sequence within the coding region of the corresponding gene. This translates to the expression of a pathological altered gene product (the mutant protein) that contains the expanded polyglutamine tract (Orr et al., 1993). The ninth expanded polyglutamine tract disorder, SCA6, is caused by a smaller, stable CAG expansion (Matsuyama et al., 1997).

Interestingly, although the polyglutamine proteins share no homology with each other outside of the polyglutamine tract, they all display two common characteristics. There is a polyglutamine length-dependent threshold above which each disease becomes fully penetrant, and there is selective neuronal loss in spite of ubiquitous expression throughout the central nervous system. Several lines of evidence suggest that expansion of the polyglutamine tract is necessary for the pathology of the disease and that it confers a new toxic property on the mutant protein (Mattila et al., 1998), but what remains elusive is the mechanism by which this polyglutamine tract expansion leads to selective neurodegeneration.

In normal neuronal cells, wild-type ataxin-1 is located in intranuclear structures – <0.5 μm in diameter, whereas in affected neurons of SCA1 patients and transgenic mice, mutant ataxin-1 is found within large heterogeneous intranuclear aggregates – >2 μm in diameter (Skinner et al., 1997). Accumulating evidence indicates that ataxin-1 aggregate formation is regulated by a region outside of the polyglutamine tract. Deletion of this region – the self-associating domain (SAD) – in transgenic mice expressing the mutant ataxin-1 protein prevented aggregation and suppressed the disease progression (Burright et al., 1997; Klement et al., 1998; Skinner et al., 2002). This result suggests that the appearance of aggregates in susceptible neuronal regions correlates with progression of the disease. Other evidence suggests that despite their obvious association with the disease, aggregates may simply be epiphenomena or may even be neuroprotective (Chesselet et al., 2009). The AXH region of ataxin-1 – which overlaps partly with the SAD – is implicated in self-association, in RNA binding and in interaction with several proteins including p80 coilin, ubiquitin protease (USP7) and leucine-rich acid nuclear protein (LANP). USP7 and RNA display weaker affinity for the mutant ataxin-1 protein than for its wild-type counterpart (Yue et al., 2001; Hong et al., 2002). Conversely, LANP has an exaggerated affinity for the mutant

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ataxin-1 protein when compared with the wild-type ataxin-1 protein (Matilla et al., 1997). Another region located outside of the polyglutamine tract which incorporates the nuclear localization signal (NLS) has been found to be necessary for cerebellar Purkinje degeneration (Klement et al., 1998). Furthermore, studies show that polyglutamine tract-containing peptides are more toxic than the full-length mutant proteins and do not elicit selective neuronal degeneration characteristic of the disease (Lin et al., 1999). Taken together, it would appear that although expansion of the polyglutamine tract is necessary for SCA1 pathology, regions outside of the polyglutamine tract appear to play a significant role in modulating neurotoxicity observed in the SCA1 disease. Unfortunately, despite numerous studies carried out on SCA1, no effective therapies have emerged, in part, no doubt because its pathogenic mechanism(s) is/are largely unknown. One approach to solving this problem may be to firstly identify and understand the normal function and turn over of the full-length 98 kDa wild-type ataxin-1 protein. Since the regions outside of the polyglutamine tract are identical for both the wild-type ataxin-1 protein and its mutant form, and it has been shown that these regions modulate neuronal degeneration in SCA1, it follows then that studies carried out on wild-type ataxin-1 protein may inform the efforts to delineate SCA1 pathogenesis. In addition, there seems to be a relationship between the normal function of the wild-type ataxin-1 and the toxicity of the corresponding expanded (mutant) counterpart, given that a Drosophila model over-expressing wild-type ataxin-1 with 30 repeat glutamines develops a very mild form of SCA1 (Fernandez-Funez et al., 2000; Lim et al., 2008). This suggests that reduced protein turnover or protein misfolding may have a part to play in SCA1's pathogenesis (Chen et al., 2004).

In order to carry out the necessary biochemical and conformational studies, sufficient amounts of pure full-length wild-type ataxin-1 protein must be available. The purification of these polyglutamine proteins can be technically challenging, particularly given the large molecular weight of proteins such as ataxin-1 and 2. As a result, the majority of studies involving polyglutamine proteins have so far been confined to the cellular level, to model systems and to the considerably smaller ataxin-3 (42 kDa) (Chow et al., 2006). These polyglutamine proteins tend to aggregate, they are generally toxic to the system in which they are produced, they are generally insoluble when expressed in a bacterial system, and they tend to accumulate in inclusion bodies in the bacterial vector system in which they are expressed. In fact, it has been demonstrated that expression of model GST fusion proteins with greater than 59 glutamine residues killed *E. coli* whilst model GST fusion proteins containing 61 alanine residues were not toxic (Onodera et al., 1996). In this study, we show that we were able to express a portion of full-length wild-type ataxin-1 protein containing a 30-repeat glutamine sequence in the soluble fraction of the *E. coli* lysate, and hence avoid the denaturing/renaturing cycles required to release the protein from the inclusion bodies. Recently, dual affinity tagging utilizing tags attached to both the N- and C-termini of the expressed protein greatly aided the purification of many difficult proteins (Terpe, 2003; Mccluskey et al., 2007; Yeliseev et al., 2007). By applying two consecutive steps of affinity purification, the contaminating proteins that lack either the N- or C-terminus of the full-length protein will be effectively removed, retaining only the full-length expressed protein that contains both tags (Cass et al., 2005; Yeliseev et al., 2007; Arnau et al., 2006). We have designed a dual affinity tag purification system that resulted in substantial gain in purity of the desired protein in the non-denatured state, with no visible aggregation. The larger of the two affinity tags, which is used to facilitate solubilization of the protein during lysis and purification, was successfully removed after purification to leave the soluble, non-aggregated C-terminally tagged ataxin-1 protein. Finally, we have produced two monoclonal antibodies (mAbs) directed against either the N- or C-terminal region of ataxin-1 which significantly aided the assessment of the purification process. To date, the commercially available antibodies raised to the ataxin-1 protein are only available as polyclonals.

2. Materials and methods

2.1. Ataxin-1 antibody production

2.1.1. Monoclonal antibody production

Two recombinant ataxin-1 proteins, the N-terminal fragment (1–330 aa) and a C-terminal fragment (499–815 aa) of human ataxin-1 protein (Banfi et al., 1994) were synthesized for use as immunogens in the production of monoclonal antibodies against ataxin-1 (Uchihara et al., 2001) The N-terminal gene fragment was amplified using the following primers: forward 5′-CGCGAATTCATGAAATCCAACCAAGAG-3′ and reverse: 5′-AGTAAGCTTTCACTTCTCCATCTCACCGTT-3′. The C-terminal gene fragment was amplified using the following primers: forward 5′-ATAGAATTCATGGAAGCGTCGGGGGCA-3′ and reverse: 5′-GGCAAGCTTCTACTTGCCTACATTAGACCG-3′. The amplified fragments were each cloned in frame, into the *EcoRI*/*HindIII* site of the pET-41 vector (Novagen) to generate a glutathione S transferase (GST)–ataxin-1 fusion protein. The expression vectors were transformed into *E. coli* (BL21 (DE3)) and the proteins expressed and then purified using glutathione resin. Inbred BALB/c mice were immunized with either of these two purified recombinant proteins and their spleens used to generate hybridoma cells as previously described (Uchihara et al., 2001) .The optimal clones were limit diluted twice and the secreted antibodies purified using a HiTrap protein G HP affinity column (GE Healthcare). The resultant antibodies produced included 2D7, an $IgG1_{\kappa}$ monoclonal antibody directed against N-terminal human ataxin-1, and 3E11, an IgG1κ monoclonal antibody directed against C-terminal human ataxin-1.

2.2. Protein expression

For the expression of the GST–TEV–ataxin-1–$(His)_6$-$(His)_6$ protein, full-length ataxin-1 was amplified using the following primers: forward 5′-GAAAACCTGTATTTCCAGGGAATGAAATCCAACCAACAG-3′ and reverse 5′-GGCAAGCTTCTACTTGCCTACATTAGACCG-3′. The forward primer included a TEV recognition sequence upstream of the ataxin-1 start site. The amplified TEV–ataxin-1 product was then used as a template for further amplification using a forward primer containing the *SpeI* recognition sequence 5′-CGCACTAGTAAAACCTGTATTTCCAGGGA-3′ and the same reverse primer. The amplified TEV–ataxin-1 product was cloned into the *SpeI*/*HindIII* site of the pET-41 vector and then transformed into *E. coli* (BL21 (DE3)). The resulting recombinant ataxin-1 protein contained an N-terminal GST followed by a TEV cleavage site and a C-terminal–$(His)_6$-$(His)_6$ tag (Fig. 1). The cells were grown at 37 °C until A_{600} was about 0.7. The temperature was then lowered to 28 °C and the cells were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. Two different concentrations of IPTG (0.5 mM or 1 mM) were tested. An additional set of experiments was carried out with the induction temperature maintained at 37 °C for 3 h. Similarly, for these experiments, two different concentrations of IPTG were investigated. The cells were then harvested by centrifugation, the cell pellet snap frozen in liquid nitrogen and then stored at −70 °C until required for lysis and purification.

2.3. Cell lysis and purification

For lysis, the pellet was resuspended in 1 ml of lysis buffer (150 mM NaCl, 20 mM NaH_2PO_4, pH 7.4, 0.5% Triton X-100) sup-