Neuroscience Letters 552 (2013) 156–161

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Increased aggregation of polyleucine compared with that of polyglutamine in dentatorubral-pallidoluysian atrophy protein

Yasuyo Suzuki, Chenghua Jin, Ikuru Yazawa*

Laboratory of Research Resources, Research Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi 474-8511, Japan

HIGHLIGHTS

- We compared the aggregation of ATN1 by polyQ tracts with that of ATN1 by polyL tracts.
- ATN1 with polyL tract showed a higher aggregation than that with polyQ tract.
- Intracellular distribution of ATN1 with polyL tract was different from that with polyQ tract.
- The aberrant localization was caused by the pathological degradation of ATN1.

ARTICLE INFO

Article history:
Received 13 May 2013
Received in revised form 11 June 2013
Accepted 29 July 2013

Keywords:
Dentatorubral-pallidoluysian atrophy
Atrophin-1
Neurodegeneration
Polyglutamine
Polyleucine

ABSTRACT

Polyglutamine (polyQ) diseases result from expansion of CAG trinucleotide repeats in their responsible genes. Although gene products with polyQ expansions undergo conformational changes to aggregate in neurons, the relationship between inclusions and neurotoxicity remains unclear. Dentatorubral-pallidoluysian atrophy (DRPLA) is a polyQ disease, and DRPLA protein, also known as atrophin-1 (ATN1), carries an expanded polyQ tract. To investigate how an expanded polyQ tract influences ATN1 aggregation and localization, we compared the aggregation of ATN1 with a polyQ tract to that of ATN1 with a polyleucine (polyL) tract. In COS-7 cells, polyL-ATN1 triggered more aggregation than polyQ-ATN1 of similar repeat sizes. Immunocytochemical and biochemical studies revealed that replacement of the polyQ tract with polyL alters ATN1 localization, leading to retention of polyL-ATN1 in the cytoplasm. Despite this change in localization, polyL-ATN1 and polyQ-ATN1 demonstrate comparable repeat length dependent toxicity. These results suggest that expanded polyQ repeats in ATN1 may contribute to neurodegeneration via alterations in both protein aggregation and intracellular localization.

1. Introduction

Polyglutamine (polyQ) diseases are a group of hereditary neurodegenerative disorders that include Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy, and several forms of spinocerebellar ataxia (SCA) [13,27,29]. Neuronal intranuclear inclusions containing polyQ were first observed in HD transgenic mice and in the brain tissues of HD patients [3,4]. It was surmised that the HD gene product huntingtin aggregated in HD neurons. Similar neuronal intranuclear inclusions with the gene product have also been reported in the brain tissues of patients with other polyQ diseases and mice in models of other polyQ diseases [7,9,10,14,21]. These findings suggest that the mechanism of pathogenesis of HD is derived from the aggregation of proteins or peptide inclusions with expanded polyQ tracts. In contrast, the onset of a neurological phenotype or cell dysfunction mediated by the expanded polyQ tract in the responsible gene product was independent of the formation of inclusions [8,9,19]. One study showed that the presence of inclusion bodies reduced the risk of neuronal death due to polyQ expansion [1]. Thus, the relationship between inclusions and neurotoxicity remains controversial [17].

The polyQ diseases show progressive and refractory neurological symptoms caused by neuronal cell loss in selective regions of the central nervous system (CNS). This selective neuronal damage gives rise to the specific features of each disease. DRPLA is caused by the expansion of the polyQ tract within the DRPLA protein, which is also known as atrophin-1 (ATN1). A number of studies have demonstrated that ATN1 is localized in both the nucleus and cytoplasm of

Abbreviations: ATN1, atrophin-1; CNS, central nervous system; DRPLA, dentatorubral-pallidoluysian atrophy; HD, Huntington's disease; polyQ, polyglutamine; polyL, polyleucine; SCA, spinocerebellar ataxia; TPEN, *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone.

* Corresponding author at: Laboratory of Research Resources, Research Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka-cho, Obu-shi, Aichi 474-7511, Japan. Tel.: +81 562 46 2311; fax: +81 562 46 8319.
E-mail address: yazawaik@ncgg.go.jp (I. Yazawa).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2013.07.043

neurons in the human CNS [24–26]. We recently reported that novel C-terminal fragments containing the polyQ tract accumulated at the nucleus and cytoplasm in cellular models of DRPLA and were specifically found in the brain tissues of DRPLA patients [22]. The study demonstrated that proteolytic processing of ATN1 regulated the intracellular localization of the cleaved fragments. Furthermore, a general caspase inhibitor increased the accumulation of the cytoplasmic fragments [22]. The polyQ expansion produces abnormal aggregation of full-length and fragments of ATN1, and the fragment may play an important role in neurodegeneration.

Some investigators have hypothesized that proteins with expanded polyQ tracts misfold and aggregate as antiparallel β-strands [18]. The proteins interact between polar residues and join protein molecules together in a way that is similar to leucine zippers termed "polar zippers" [15]. Previous reports have shown that long homopolymeric leucine tracts interacted with themselves [12], and polyleucine (polyL) tracts displayed a higher propensity for aggregation and toxicity in cells compared with polyQ tracts [5]. Oligomerization of polyL tracts may be caused by leucine zipper-like associations [2]. In the present study, we expressed ATN1 in which a polyQ tract was replaced by a polyL tract and assessed the aggregation of ATN1s. We determined the pathological role of polyQ aggregation in DRPLA neurodegeneration.

2. Materials and methods

2.1. Plasmid constructions

The *ATN1* gene was fused to a T7-tag at the 5′-end and to a *Strep*-tagII at the 3′-end (Fig. 1A). To produce mutant proteins with various number of glutamine repeats, we established a method for making the intended CAG/CTG repeat a stable PCR product [22]. ATN1s with polyL tracts were generated by the replacement of a CAG repeat with a CTG repeat in *ATN1*. All ATN1 constructs were cloned into pcDNA3.1 (Life Technologies, Carlsbad, CA, USA). Each expressed protein was represented by adding the number of leucine or glutamine repeats included after ATN1, for example ATN1(L12).

2.2. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum. FuGENE 6 (Roche Diagnostics, Basel, Switzerland) was used for the introduction of exogenous DNA into COS-7 cells according to the manufacturer's instructions. In brief, 1×10^5 cells were plated on 35-mm dishes and 24 h later, each dish was transfected with 2 μg of DNA and 6 μL of FuGENE6 and incubated at 37 °C for 48 h. Whole cell lysates were prepared with 20 mM of HEPES-buffered saline (20 mM HEPES and 150 mM NaCl, pH 7.4) and 1% sodium dodecyl sulfate with protease inhibitors.

2.3. Immunocytochemistry

COS-7 cells were plated on 35-mm dishes and 24 h later, each dish was transfected. Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and blocked with 2% bovine serum albumen and 2% goat serum. Cells were incubated with monoclonal anti-*Strep*-tagII antibody (IBA GmbH, Gottingen, Germany) and polyclonal 55-2 antibody [22]. Alexa 594 anti-mouse IgG or Alexa 488 anti-rabbit IgG was used as secondary antibodies. The nuclei of cells were visualized by staining with 4′-6-diamidino-2-phenylindole (DAPI).

2.4. Treatment with protease inhibitors

Twenty-four hours after transfection, the medium was replaced with serum-free medium, and the cells were incubated with proteasome or protease inhibitors for 24 h. Cells were incubated with an equivalent amount of vehicle, dimethyl sulfoxide used as a control. The cells were treated with 10 μM of the proteasome inhibitor MG-132 (Peptide Institute, Inc., Osaka, Japan), 50 μM of the pan caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD-FMK) (Peptide Institute, Inc.), and 0.5 μM of the intracellular zinc chelator *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Sigma–Aldrich Co., St. Louis, MO, USA).

2.5. Transfection of Neuro2a cells and apoptosis assay

Neuro2a cells were maintained in DMEM supplemented with 10% fetal bovine serum. FuGENE 6 was used for the introduction of exogenous DNA into Neuro2a cells as previously described [22]. In situ labeling of fragmented genomic DNA in Neuro2a cells was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics).

3. Results

3.1. Long polyleucine tracts induced ATN1 aggregation

To assess the effects of polyL tracts on the aggregation of ATN1, ATN1s with polyL tracts (L12, L41, and L50) fused with *Strep-tag*II and T7-tag were expressed in COS-7 cells (Fig. 1B). ATN1s were directly detected with the *Strep*-Tactin HRP conjugate by western blotting (Fig. 1C). On western blots of ATN1(L12) expressed in COS-7 cells, *Strep*-Tactin detected the full-length and an abundant fragment at about 80 kDa. The T7-tag antibody detected apparent bands of the full-length and the 75-kDa fragment of ATN1(L12). ATN1s with long polyL tracts of L41, ATN1(L41), and L50, ATN1(L50), had higher-molecular-weight bands than the full-length ATN1, indicating that ATN1s with long polyL tracts formed an aggregated conformation. In contrast, on western blots of expressed ATN1s with expanded and normal polyQ tracts in COS-7 cells, *Strep*-Tactin showed two C-terminal fragments of ATN1 in addition to the full-length ATN1. The ATN1s with expanded polyQ tracts had no high-molecular-weight bands on the blots, but 1C2, an antibody that recognizes an aggregated conformation, detected the ATN1-Q47 and -Q54 (Fig. 1C). 1C2 detected no immunoreactivity on the blots of the cells expressed ATN1-Q19. To clarify the proteolytic processing of ATN1 containing polyL tract, COS-7 cells that expressed ATN1 were treated with protease inhibitors (Fig. 2 and Supplementary Fig. 1). The proteasome inhibitor MG-132 increased the reactivities of the full-length and fragments of ATN1 on the blots. The increase in reactivity was similar to that of ATN1 with polyQ tracts. These findings indicate that the ubiquitin–proteasome pathway was involved in the processing of ATN1 regardless of the type of homopolymeric amino acid present. When the COS-7 cells were treated with either the Zn chelator TPEN or the pan caspase inhibitor Z-VAD-FMK, which affected the processing of ATN1s with polyQ tracts, the blot showed no change in the band for ATN1s containing the polyL tract. The cleavage of ATN1s with polyL tracts was regulated by a different processing pathway than that of ATN1s with polyQ tracts.

3.2. Subcellular localization of ATN1 containing short and long polyleucine tracts

To investigate the intracellular localization of ATN1s that contained polyL tracts, we immunocytochemically examined COS-7