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Characterization of C-terminal adaptors, UFD-2 and UFD-3, of CDC-48 on the polyglutamine aggregation in *C. elegans*

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ABSTRACT

CDC-48 (also called VCP or p97 in mammals and Cdc48p in yeast) is a AAA (ATPases associated with diverse cellular activities) chaperone and participates in a wide range of cellular activities including modulation of protein complexes and protein aggregates. UFD-2 and UFD-3, C-terminal adaptors for CDC-48, reportedly bind to CDC-48 in a mutually exclusive manner and they may modulate the fate of substrates for CDC-48. However, their cellular functions have not yet been elucidated. In this study, we found that CDC-48 preferentially interacts with UFD-3 in *Caenorhabditis elegans*. We also found that the number of polyglutamine (polyQ) aggregates was reduced in the *ufd-3* deletion mutant but not in the *ufd-2* deletion mutant. Furthermore, the lifespan and motility of the *ufd-3* deletion mutant, where polyQ40::GFP was expressed, were greatly decreased. Taken together, we propose that UFD-3 may promote the formation of polyQ aggregates to reduce the polyQ toxicity in *C. elegans*.

1. Introduction

CDC-48 (although it is also called VCP or p97 in mammals and Cdc48p in yeast, we use CDC-48 throughout this study) is a AAA (ATPases associated with diverse cellular activities) chaperone that converts the chemical energy generated from ATP hydrolysis into the mechanical force used for protein conformational changes such as the unfolding of proteins and disassembly of protein complexes. CDC-48 is involved in a wide variety of cellular processes, including the modulation of protein aggregation, cell-cycle control, organelle membrane fusion, endoplasmic reticulum-associated protein degradation and mitochondrial quality control [1–7]. It should be noted that two highly homologous CDC-48s, CDC-48.1 and CDC-48.2, exist in *Caenorhabditis elegans* and that their function is essential and redundant [8].

The functional diversity of CDC-48 is determined by a differential binding of a variety of adaptors. Several adaptors have been identified in *C. elegans*, e.g. NPL-4-UFD-1 and six different UBXN proteins, which bind to the N-terminal domain of CDC-48 [9], and UFD-2 and UFD-3, which bind to the C-terminal motif of CDC-48 [9–11]. In general, it is considered that N-terminal adaptors are involved in the determination of substrate proteins, whereas C-terminal adaptors may determine the fate of these substrate proteins [12,13]. UFD-2 is an E4 ubiquitin ligase, which extends the ubiquitin chains of ubiquitinated substrates to promote proteasomal degradation [13–15]. In contrast, neither enzymatic activity nor cellular function of UFD-3 is clarified yet. UFD-2 and UFD-3 reportedly bind to CDC-48 in a mutually exclusive manner [10,13].

Mutational expansion of CAG repeats encoding polyglutamine (polyQ) stretches is responsible for nine neurodegenerative diseases including Huntington's Disease, spinal and bulbar muscular atrophy, dentatorubral pallidoluysian atrophy and spinocerebellar ataxias types 1, 2, 3, 6, 7 and 17 [16,17]. PolyQ aggregates accumulate in nuclear or cytoplasmic inclusion bodies that are invariably associated with end-stage neurodegenerative disease in patients and model animals. It is previously considered that polyQ aggregates have cytotoxicity [18–22]. On the other hand, recently, it has been increasingly suggested that oligomeric forms of polyQ proteins are more toxic and polyQ aggregates may act as a cellular coping mechanism to sequester levels of potentially toxic soluble monomeric and oligomeric species of polyQ [23–25]. To develop a therapeutic tactics including drugs, it is quite important to know exactly what causes the cytotoxicity, but unfortunately it is still contradictory on this issue.

It has been reported that CDC-48 co-localizes with polyQ aggregates in cultured cells and with intraneuronal inclusions in

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several neurodegenerative diseases [26,27]. In addition, the *Drosophila* CDC-48 homologue was identified as a genetic modifier of polyQ-induced eye degeneration [28]. We have also reported that co-expression of either of CDC-48s with a polyQ protein partially suppressed the aggregation of the polyQ protein in *C. elegans* [8] and that CDC-48 bound Huntingtin fragments containing polyQ repeats directly, and retarded the aggregate formation of HttQ53 [29]. These results clearly suggest that CDC-48, a AAA chaperone, plays an important role in polyQ-associated diseases.

In this study, we analyzed the interaction of CDC-48 with C-terminal adaptors and found that CDC-48 preferentially interacts with UFD-3 *in vivo*. We also analyzed whether UFD-2 and UFD-3 are involved in polyQ aggregate formation processes using the *C. elegans* system as a model of polyQ disease. We found that the number of polyQ40 aggregates was reduced in the *ufd-3* deletion mutant but not in the *ufd-2* deletion mutant and that the lifespan and motility of polyQ40-expressing worms were greatly decreased in the *ufd-3* deletion background. Taken together, we propose that UFD-3 may promote the formation of polyQ aggregates to reduce the polyQ toxicity.

2. Materials and methods

2.1. C. elegans strains and general methods

Worms were maintained using standard protocols as described previously [30]. The Bristol strain N2 was used as the wild-type strain. The deletion mutants of *cdc-48.1(tm544)*, *ufd-2(tm1380)* and *ufd-3(tm2915)* were obtained from Dr. Shohei Mitani (Tokyo Women's Medical University). XA7200 *unc-119(ed3) qaIs7200*[*unc-119(+)*] and XA7203 *unc-119(ed3); cdc-48.1(tm544) qaIs7201*[P*cdc-48.1*-FLAG::CDC-48.1, *unc-119(+)*] were reported previously [31]. To construct transgenic worms expressing expanded polyQ stretches, DNA fragment encoding 40 polyQ stretches fused to GFP (polyQ40::GFP) was cloned and microinjected as described previously [8]. Extrachromosomal DNA was integrated into a chromosome of *C. elegans* by UV irradiation as described previously [32]. To exclude unexpected additional mutations due to UV irradiation, integrated worms obtained were out-crossed 3 times, thus yielding XA7249 *qaIs7249*[P*unc-54*-polyQ0::GFP] and XA7250 *qaIs7250*[P*unc-54*-polyQ40::GFP]. Males carrying mutations were generated from mutants and were used to transfer the mutation. We generated the following strains: XA7251 *cdc-48.1(tm544) qaIs7201*, XA7252 *cdc-48.1(tm544) ufd-2*(*tm1380*) *qaIs7201*, XA7253 *cdc-48.1(tm544) ufd-3*(*tm2915*) *qaIs7201*, XA7260 *ufd-2*(*tm1380*) *qaIs7249*, XA7261 *ufd-3*(*tm2915*) *qaIs7249*, XA7262 *ufd-2*(*tm1380*) *ufd-3*(*tm2915*) *qaIs7249*, XA7263 *ufd-2*(*tm1380*) *qaIs7250*, XA7264 *ufd-3*(*tm2915*) *qaIs7250*, XA7265 *ufd-2*(*tm1380*) *ufd-3*(*tm2915*) *qaIs7250*. XA7200, XA7203 and strains carrying *qaIs7201* were maintained and analyzed at 25 °C. Others were maintained and analyzed at 20 °C.

2.2. Immunoprecipitation and immunostaining

Immunoprecipitation and immunostaining assays were performed as described previously [9,31].

2.3. Western blotting

Total lysates of worms and IP samples were resolved on 5–12% SDS gradient gels (Wako) or 10% or 12.5% SDS-PAGE gels, and then proteins were transferred to a nitrocellulose membrane. Prestained Protein Markers (Broad Range) (NACALAI TESQUE, INC.) or Dr. Western (Oriental Yeast CO., LTD.) was used as a Western blotting marker. Signals were detected with anti-GFP (Clontech; 1:1000), anti-UFD-2 (1:1000), anti-UFD-3 (1:1000), anti-CDC-48 (1:5000), anti-FLAG(M2) (Sigma; 1:1000), and anti-α-tubulin (Sigma; 1:1000) antibodies as a primary antibody. Secondary antibodies were anti-mouse IgG horseradish peroxidase–conjugated F(ab′)2 fragment (GE Healthcare; 1:5000) and anti-rabbit IgG horseradish peroxidase–conjugated F(ab′)2 fragment (GE Healthcare; 1:5000). Proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin–Elmer Life Sciences). Chemiluminescent signals were detected with LAS-4000 mini. α-Tubulin was used as a loading control.

2.4. Measurement of polyglutamine aggregates

The number of polyQ::GFP aggregates in body wall muscle cells was counted under the Olympus SZX12 fluorescence microscope. Approximately 10 worms were analyzed per each experimental condition. Young adult worms were defined as Day 1.

2.5. Lifespan analysis

Synchronized young adults were treated with 0.4 mg/ml fluorodeoxyuridine (FUdR) for 24 h to prevent progeny production. Then worms were transferred to fresh 0.2 mg/ml FUdR-containing plates and started recording of survival every other day (at least 70 worms per strain). Lifespan assays were repeated at least two times. Worms were considered dead, when they did not move after repeated taps with a pick. The day when eggs were laid was considered Day 0. Data analysis was performed as described by Yang et al. [33], using the publicly available analysis suite OASIS (http://www.sbi.postech.ac.kr/oasis/).

2.6. Worm motility assay

Motility was measured as described previously [34].

3. Results and discussion

3.1. Preferential binding of CDC-48 to UFD-3 in C. elegans

UFD-2 and UFD-3 reportedly bind to CDC-48 in a mutually exclusive manner in yeast [10,13]. It was also reported that *C. elegans* UFD-2 and UFD-3 bound to the C-terminal motif of CDC-48.1 and CDC-48.2 (hereafter collectively CDC-48s) by using a yeast two-hybrid analysis [9,11] and we confirmed it (Supplemental Fig. S1). We then asked whether UFD-2 and UFD-3 can bind to CDC-48s in *C. elegans in vivo*. We first generated antibodies against *C. elegans* UFD-2 and UFD-3, and confirmed that they well recognized UFD-2 and UFD-3, respectively (Supplemental Fig. S2). Previously we have prepared the *C. elegans* strain XA7203, which expresses FLAG-tagged CDC-48.1 [31]. Lysates were immunoprecipitated with the anti-FLAG(M2) antibody and immunoprecipitates were analyzed by Western blotting by using anti-CDC-48, anti-UFD-2, and anti-UFD-3 antibodies. As shown in Fig. 1A, FLAG::CDC-48.1 was well precipitated. Both FLAG::CDC-48.1 and CDC-48.2 were detected, suggesting that FLAG::CDC-48.1 and CDC-48.2 can form a hetero-hexamer. Note that CDC-48 usually forms a hexameric ring-like structure [35]. UFD-3 was clearly co-precipitated with FLAG::CDC-48.1. It is interesting to mention, however, that UFD-2 was undetectable in the immunoprecipitates under the experimental conditions used, although UFD-2 existed in the total lysates. These results suggest that UFD-3 can form a stable complex with CDC-48 *in vivo*.

So, if UFD-2 and UFD-3 bind to CDC-48s in a mutually exclusive manner and UFD-3 forms a stable complex with CDC-48s, it might be possible that UFD-2 does not show any binding to CDC-48s.