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Molecular properties of TAR DNA binding protein-43 fragments are dependent upon its cleavage site

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ABSTRACT

Aggregation of TAR DNA binding protein-43 (TDP-43) is a hallmark feature of amyotrophic lateral sclerosis and frontotemporal lobar degeneration. Under pathogenic conditions, abnormal cleavage of TDP-43 produces the phosphorylated C-terminal fragments (CTFs), which are enriched in neuronal inclusions; however, molecular properties of those TDP-43 fragments remain to be characterized. Here we show distinct degrees of solubility and phosphorylation among fragments truncated at different sites of TDP-43. Truncations were tested mainly within a second RNA recognition motif (RRM2) of TDP-43; when the truncation site was more C-terminal in an RRM2 domain, a TDP-43 CTF basically became less soluble and more phosphorylated in differentiated *Neuro2a* cells. We also found that cleavage at the third β-strand in RRM2 leads to the formation of SDS-resistant soluble oligomers. Molecular properties of TDP-43 fragments thus significantly depend upon its cleavage site, which might reflect distinct molecular pathologies among sub-types of TDP-43 proteinopathies.

1. Introduction

Misfolding of protein molecules often leads to the formation of insoluble aggregates, and abnormal accumulation of such aggregates in the affected tissues is a major pathological change observed in neurodegenerative diseases [1]. In pathologically observed inclusion bodies, not only do full-length proteins constitute the insoluble aggregates, but also those truncated fragments have been detected [2–5]. A cellular processing of proteins is thus considered to be mal-functioned, which inhibits the proper folding of those proteins and thereby facilitates the aggregate formation. Such truncated proteins are rarely formed in healthy controls; therefore, truncation-associated changes in molecular properties of proteins would be an important factor in the pathogenesis of neurodegenerative diseases.

Abnormal processing of TAR DNA binding protein-43 (TDP-43) has been recently proposed to be associated with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) [6,7]. TDP-43 is a multi-domain protein: a Tudor-like N-terminal domain, two RNA recognition motif domains (RRM1, RRM2) in the middle, and a C-terminal Gly-rich region [8]. In physiological conditions, TDP-43 functions as a DNA/RNA-binding protein that shuttles between nucleus and cytoplasm and thereby regulates an alternative splicing of mRNA [9]. Under pathogenic conditions, in contrast, cytoplasmic inclusions are observed that contain C-terminal fragments (CTFs) of abnormally cleaved and hyper-phosphorylated TDP-43 [6,7]. Furthermore, it should be noted that those CTFs are distinct in their electrophoretic mobility among different clinicopathological sub-types of TDP-43 proteinopathies [10,11]. Due to significant effects of phosphorylation on the electrophoretic mobility of proteins, it is still in debate whether multiple sites in pathological TDP-43 are susceptible to proteolysis in a disease-specific manner. We nonetheless suspect that multiplicity of the potential cleavage sites in TDP-43 plays roles in diversifying the molecular pathologies of TDP-43 proteinopathies.

Indeed, several cleavage sites have been suggested in an RRM2 domain (Val 193–Ile 257) [12–15]; Igaz et al. have successfully purified TDP-43 fragments from inclusions in an FTLD brain and identified Arg 208 as one of pathological cleavage sites in TDP-43 [13]. In addition, Asp 219 and Asp 247 have been proposed as alternative cleavage sites by Nonaka et al. [14]. A proteolysis by caspase-3 at Asp 221 in RRM2 also seems possible [12,15]. Based upon these results, multiple sites in TDP-43 are susceptible to abnormal cleavage, which could produce a variety of CTFs with different sizes. Any protease(s) responsible for cleaving TDP-43 have not been identified, or even it remains unknown whether the proteolysis of TDP-43 is essential to the disease mechanism. Despite this, several pathological fragments of TDP-43 with distinct sizes are reminiscent of a proteolysis of amyloid precursor protein (APP) in Alzheimer disease, in which diverse sets of fragments, in particular $A\beta_{40}$ and $A\beta_{42}$, are generated [2]. $A\beta_{42}$ is

Abbreviations: TDP-43, TAR DNA binding protein-43; CTF, C-terminal fragment; RRM2, 2nd RNA recognition motif in TDP-43; ALS, amyotrophic lateral sclerosis; FTLD, frontotemporal lobar degeneration

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only two amino acids longer than $A\beta_{40}$, but each of these Aβ peptides possesses distinct molecular properties; compared to $A\beta_{40}$, $A\beta_{42}$ exhibits higher aggregation propensity [16] with reproduction of amyloid pathologies in model mice [17]. Accordingly, the site(s) to be proteolyzed in a disease-associated protein will be a relevant factor in a pathomechanism; indeed, ratio of $A\beta_{42}$ over $A\beta_{40}$, rather than total amounts of Aβ, correlates with the load of pathological plaques in the brain [18,19]. Given that multiple sites in TDP-43 are considered to be susceptible to proteolysis in TDP-43 proteinopathies, it is important to characterize the cleavage-site-dependent changes in molecular properties of TDP-43 fragments.

In this study, we have examined the transient expression of TDP-43 fragments in mouse neuroblastoma cells, *Neuro2a*. Based upon molecular weights (20–25 kDa) of pathologically detected CTFs, particular attention has been paid on RRM2 as potential cleavage sites of TDP-43. We have then shown that each of those fragments possesses distinct molecular properties including intracellular localization, RIPA-solubility, and phosphorylation and proposed a mechanism in which multiplicity of the cleavage sites in TDP-43 relates to the pathological diversity of TDP-43 proteinopathies.

2. Material and methods

2.1. DNA constructs, cell culture, and transfection

A vector, pIRESneo3 (Clontech), was used for the construction of plasmids expressing human TDP-43 proteins tagged with a C-terminal HA. Truncated products were amplified by PCR using full-length human TDP-43 cDNA as a template. Mutations were introduced by Quikchange mutagenesis (Stratagene), and all constructs used in this study were confirmed by DNA sequencing.

Mouse neuroblastoma *Neuro2a* cells were maintained in DMEM (Wako) with 10% fatal bovine serum (FBS) in 5% (v/v) CO_2 at 37 °C. For immunostaining experiments, cells were seeded on a 4-well chamber slide (Lab-Tek II Chamber Slide w/ cover CC2 glass slide, Nalge Nunc international), while 6-well plates coated with polyethyleneimine were used for Western blotting analysis. Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 4 h, culture media were replaced with DMEM with 1% FBS supplemented with 5 mM N^6,2′-*O*-dibutyryl cAMP (dbcAMP, Nacalai Tesque) for differentiating *Neuro2a* cells. Following further incubation for 36 h, cells were fixed or lysed as mentioned below.

2.2. Immunocytochemistry

For immunostaining of HA-tagged proteins, cells were fixed with 4% paraformaldehyde containing 0.12 M sucrose in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with 0.1% bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated with anti-HA-Fluorescein, High Affinity (3F10) (1:100 dilution, Roche) in PBS containing 0.1% BSA for an hour, washed once with 0.1% Triton X-100 in PBS and twice with 0.1% BSA in PBS. Nuclei were then counter-stained with Hoechst 33342. Images were obtained by using a fluorescence microscope (Olympus IX70, LCPlan Fl ×40).

2.3. Cell lysis and electrophoresis

Cells were washed twice with PBS and harvested by a RIPA buffer containing a protease inhibitor cocktail, Complete (EDTA-free, Roche Diagnostics). In order to suppress the protein dephosphorylation during cell lysis, 2 mM sodium fluoride and 4 mM imidazole were also added. After sonication, cell lysates were ultracentrifuged at 110,000 × *g* for 30 min to separate into supernatant (a soluble fraction) and pellets (an insoluble fraction). The pellets were re-dissolved in a 50 mM Tris buffer (pH 8.0) containing 2% SDS with the same volume with that of the corresponding supernatant. The protein concentration in supernatant was determined by BCA assay using BSA as a standard.

A soluble fraction containing 10 μg of proteins and an insoluble fraction with the same volume with the corresponding soluble fraction were mixed with an SDS-PAGE sample buffer containing β-mercaptoethanol and loaded on a 12.5% SDS-PAGE gel (PAGEL, ATTO) after boiling for 5 min. Proteins were then electroblotted on a 0.2-μm PVDF membrane (Bio-Rad) and analyzed by Western blotting using rabbit polyclonal anti-HA (Y-11, Santa Cruz Biotechnology, 1:1000), mouse anti-TDP-43 phospho-Ser409/410 antibody (Cosmo Bio, 1:1000). Corresponding secondary antibodies that are conjugated with HRP were used as follows; anti-rabbit (1:5000) and anti-mouse (1:1000) antibodies. Blots were developed with the SuperSignal West Dura chemiluminescent substrate (Pierce), and images were obtained using LAS1000 (Fujifilm).

3. Results and discussion

It has been shown that pathological TDP-43 fragments are intact in the C-terminal region and exhibit electrophoretic mobility corresponding to 20–25 kDa [20]. A simple calculation thus implies that an abnormal cleavage occurs within or near RRM2 (Val 193–Ile 250) of TDP-43 (shown in red, Fig. 1A). Indeed, Arg 208, Asp 219, and Asp 247 have been identified as potential cleavage sites under pathological conditions (shown as a bold style in Fig. 1A) [13,14]. While it remains unclear whether the other cleavage sites exist in TDP-43, we suspect that molecular properties of CTF depend upon where its N-terminus starts. To test this idea, we have prepared artificial CTFs of TDP-43 ranging from 17 to 26 kDa in the molecular weight (Fig. 1A); for specification of the truncation site, a CTF is denoted as TDP-43^{X}, in which a superscript, X, indicates a starting amino residue at its N-terminus. Moreover, to facilitate immunodetection of TDP-43 proteins both in cells and cell lysates, all TDP-43 variants in this study are fused with an HA tag at the C-terminus and can thus be denoted as TDP-43^{X}-HA.

3.1. Intracellular localization and inclusion formation of TDP-43 CTFs

As shown in Fig. 1B, full-length TDP-43 (TDP-43^{FL}-HA) was localized in the nucleus of a differentiated *Neuro2a* cell, which can be described by the presence of a bipartite nuclear localization signal (NLS, Lys 82–Lys 84 and Lys 95–Arg 98) in TDP-43 [21]. In contrast, TDP-43^{Ala90}-HA corresponding to the caspase-generated 35 kDa fragment (Fig. 1A) remained mostly nuclear but was also detectable in the cytoplasm (Fig. 1C). This is explained by the lack of one cluster (Lys 82–Lys 84) of bipartite NLS in TDP-43^{Ala90}-HA. When CTF started from the amino residue near or within RRM2, such TDP-43 CTFs (17–26 kDa) were distributed throughout the cell (Fig. 1D–H) due to the complete absence of bipartite NLS. In fact, a dominant role of the bipartite NLS in cellular localization of TDP-43 variants is consistent with previous results published so far [13,21].

In addition to the changes in intracellular localization, truncation of TDP-43 near or within RRM2 has been reported to produce inclusions in the cell [13,14]. Notably, we found significant differences in propensity for inclusion formation among our artificial 17–26 kDa fragments of TDP-43. As represented in Fig. 1D, which shows the cell expressing TDP-43^{Cys173}-HA, formation of intracellular inclusion bodies was rare in TDP-$43^{Met167,\ Cys173,\ Pro178,\ Ser183}$-HA. In contrast, as represented in Fig. 1E (TDP-43^{Val193}-HA) and F (TDP-43^{Arg208}-HA), inclusions became evident in TDP-$43^{Leu188,\ Val193,\ Cys198,\ Thr203,\ Arg208,\ Gln213,\ Val220,\ Pro225}$-HA. Furthermore, a closer comparison among those eight inclusion-forming fragments has shown that morphologies of inclusions fall into two categories; a relatively large single inclusion at the nucleus as shown in Fig. 1E (TDP-$43^{Leu188,\ Val193,\ Cys198,\ Thr203}$-HA) and multiple small dot-like inclusions at the cytoplasm as shown in Fig. 1F (TDP-$43^{Arg208,\ Gln213,\ Val220,\ Pro225}$-HA). When the