Molecular chaperone HSP70 prevents formation of inclusion bodies of the 25-kDa C-terminal fragment of TDP-43 by preventing aggregate accumulation

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Abstract

Transactive response DNA/RNA-binding protein 43-kDa (TDP-43) C-terminal fragments, such as a 25-kDa fragment (TDP-25), have been identified as a ubiquitinated and phosphorylated components of inclusion bodies (IBs) in motor neurons from amyotrophic lateral sclerosis patients. Cells contain proteins that function as molecular chaperones and prevent aggregate formation of misfolded and aggregation-prone proteins. Recently, we reported that heat shock protein (HSP)70, an abundant molecular chaperone, binds to TDP-25 in an ATP-dependent manner; however, whether HSP70 can prevent the formation of TDP-25-related IBs remains unknown. Here, we showed that HSP70 prevented TDP-25 aggregation according to green fluorescent protein-tagged TDP-25 (G-TDP-25) colocalization in the cytoplasm with mCherry-tagged HSP70 (HSP70-R). The mobile fraction of HSP70-R in the cytoplasmic IBs associated with G-TDP-25 increased relative to that of G-TDP-25, suggesting that HSP70 strongly bound to G-TDP-25 in the IBs, whereas a portion remained dissociated from the IBs. Importantly, the proportion of G-TDP-25 IBs was significantly decreased by HSP70-R overexpression; however, G-TDP-25 levels in the insoluble fraction remained unchanged by HSP70-R overexpression, suggesting that G-TDP-25 formed aggregated species that cannot be dissolved, even in the presence of strong detergents. These results indicated that HSP70 prevented the accumulation of G-TDP-25 aggregates in cytoplasmic IBs, but was insufficient for G-TDP-25 disassembly and solubilization.

Keywords Proteostasis · Protein aggregation · HSP · FRAP · ALS

Abbreviations

ALS	Amyotrophic lateral sclerosis
FRAP	Fluorescence recovery after photobleaching
FUS/TLS	Fused in sarcoma/translated in liposarcoma
GFP	Green fluorescent protein
HSC	Heat shock cognate
HSP	Heat shock protein
IB	Inclusion body
RFP	Red fluorescent protein

SDS	Sodium dodecyl sulfate
TDP-43	Transactive response element DNA/RNA-binding protein 43-kDa
TDP-25	25-kDa C-terminal fragment of TDP-43

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Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the dysfunction of motor neurons and by muscle atrophy. A common feature of ALS includes the formation of inclusion bodies (IBs) containing protein aggregates (Blokhuis et al. 2013; Kitamura et al. 2015). These IBs often contain proteins encoded by ALS-associated genes, with >20 proteins identified in ALS-associated IBs, including superoxide dismutase 1, transactive response element DNA/RNA-binding protein 43-kDa (*TARDBP*; TDP-43), fused in sarcoma/translated in liposarcoma (FUS/TLS), and optineurin (Renton et al.

2014). The proteins included in the IBs in ALS patients frequently exhibit ubiquitin modifications. Additionally, RNA-binding properties have frequently been identified in ALS-associated proteins, including TDP-43, FUS/TLS, hnRNP A1, and TAF15 (Ito et al. 2017). TDP-43 is the major disease-associated protein associated with ALS and frontotemporal lobar degeneration (Ling et al. 2013), with many ALS-associated missense mutations identified in the *TARDBP* gene and resulting in amino acid substitutions (Ling et al. 2013). These TDP-43 mutants are intimately involved in the onset and severity of ALS.

A hyper-phosphorylated and ubiquitinated form of TDP-43 accumulates in IBs in the motor neurons of ALS patients (Blokhuys et al. 2013; Ling et al. 2013). In ubiquitin-positive IBs, both intact TDP-43 and C-terminal fragments (CTFs) of TDP-43 accumulate (Neumann et al. 2006). Although several CTFs have been identified, that of TDP-43_{220–414} (25-kDa; TDP-25) is highly aggregate-prone (Zhang et al. 2009; Kitamura et al. 2016). TDP-25 can be produced by digestion by caspase 3 and calpain and is prone to aggregation and the formation cytoplasmic IBs in cultured cells (Zhang et al. 2009; Che et al. 2011; Pesiridis et al. 2011; Yamashita et al. 2012; Kitamura et al. 2016). Misfolded and aggregated proteins interfere with cellular functions, including those associated with protein folding, protein degradation, and organelle biogenesis (proteostasis/protein homeostasis) (Labbadia and Morimoto 2015). Therefore, it is important to clarify mechanisms related to the prevention of TDP-43 CTF aggregation, including TDP-25, in ALS pathophysiology.

Molecular chaperones assist protein folding and prevent aggregation by accelerating refolding and/or stabilizing misfolded states (Kim et al. 2013). Families of molecular chaperones include heat shock proteins (HSPs), such as the 70-kDa (HSP70, DnaK), 40-kDa (HSP40, DnaJ), and 90-kDa (HSP90) variants, and chaperonins (Hartl et al. 2011; Kim et al. 2013). HSP70 and HSP40 cooperatively assist protein folding, with this activity well conserved among different species. During the folding process, unfolded proteins that cannot be assisted by HSP70-HSP40 are passed on to HSP90 or chaperonin molecules (Lopez et al. 2015; Schopf et al. 2017). A previous study reported that HSP70 and HSP90 prevent phosphorylation of TDP-43 and TDP-43 CTFs (Zhang et al. 2010), and that HSP70 binding to TDP-25 can be dissociated by ATP in the presence of magnesium ion (Kitamura et al. 2017). However, it remains unclear whether HSP70 can prevent TDP-25-mediated IB formation in the cytoplasm. In this study, we showed that HSP70 overexpression efficiently decreased the proportion of cells containing cytoplasmic TDP-25 IBs.

Materials and methods

Preparation of plasmid DNA

Plasmids encoding monomeric green fluorescent protein (GFP), N-terminal GFP-tagged TDP-25 (G-TDP-25), C-terminal mCherry-tagged HSP70 (HSP70-R), and mCherry (RFP) were used as reported previously (Kitamura et al. 2016, 2017).

Cell culture and transfection

Mouse neuroblastoma Neuro2A cells were maintained as reported previously (Kitamura et al. 2016, 2017; Yahara et al. 2017). Briefly, Neuro2A cells were grown in a glass-based dish (#3910-035; AGC Techno Glass Co., Shizuoka, Japan) or 35-mm plastic dishes (#150460; Thermo Fisher Scientific, Waltham, MA, USA) for 16 h prior to transfection. A plasmid mixture consisting of 1.6 µg of the GFP-TDP-25 plasmid and 400 ng of the HSP70-R or RFP plasmid was transfected into the Neuro2A cells using 5 µL Lipofectamine 2000 (Thermo Fisher Scientific). After incubation for 24 h, experiments were performed.

Confocal laser scanning microscopy and analysis of cells carrying cytoplasmic IBs

Confocal fluorescence images were captured on an LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) through a C-Apochromat × 40/1.2NA W Korr. UV-VIS-IR objective lens (Carl Zeiss) at 37 °C and 5% CO₂. The microscope was operated on the ZEN 2009 software platform (Carl Zeiss). GFP or RFP were excited at 488 or 543 nm, respectively, and the excitation beams were split by an HFT488/543 filter for GFP and RFP. GFP and RFP fluorescent signals were separated by a dichroic mirror (NFT545) and collected through a BP505-530 band-pass filter and an LP585 long-pass filter, respectively. The pinhole size for GFP and RFP was set to 70 and 82 µm, respectively, and the zoom factor was set to × 1. X- and Y-scanning sizes were each 512 pixels.

Confocal super-resolution microscopy

Confocal super-resolution fluorescence images were captured on an LSM510 META confocal microscope with the ConfoCor 3 system (Carl Zeiss, Jena, Germany) through a C-Apochromat × 40/1.2NA W Korr. UV-VIS-IR or Plan-Apochromat × 63/1.4NA oil-immersion objective lens (Carl Zeiss) at 37 °C and 5% CO₂. The microscope was operated on the ZEN 2009 software platform (Carl Zeiss). GFP or RFP were excited at 488 or 594 nm, respectively, and the excitation beams were split by an HFT488/594 filter for GFP and RFP. GFP and RFP fluorescent signals were separated by a dichroic

mirror (NFT545) and collected through a BP50-540 and a BP615-680 band-pass filter, respectively. The pinhole size was set to 20 μm , and the zoom factor was set to $\times 10$. X- and Y-scanning sizes were each 1024 pixels. Fluorescence signals were acquired using avalanche photodiode in the ConfoCor3 system. For the observation using the oil-immersion objective, Z-direction stacks were acquired at 0.22- μm intervals after 4% paraformaldehyde fixation followed by a washing step using Tris-buffered saline. The stack was processed using a two-dimensional Gaussian filter ($\sigma = 1$ pixel) using ImageJ 1.50i (National Institutes of Health, Bethesda, MD, USA).

Assessment of TDP-25 solubility and western blot analysis

Cells expressing G-TDP-25 were solubilized in a modified radioimmunoprecipitation assay (RIPA) lysis buffer consisting of 25 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.01 U/ μL benzonase, and 1% protease-inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), as reported previously (Kitamura et al. 2016). After the lysates were centrifuged at 20,400g for 10 min at 4 $^{\circ}\text{C}$, the supernatant was recovered as the RIPA-soluble fraction. The pellets were solubilized in 20 μL of 1 M urea buffered with phosphate-buffered saline (RIPA-insoluble fraction). Samples were applied to a 10% polyacrylamide gel and subjected to electrophoresis in SDS-containing buffer. G-TDP-25, HSP70, heat shock cognate protein 70-kDa (HSC70), and α -tubulin were detected with anti-GFP (GF200; Nacalai Tesque, Kyoto, Japan), anti-HSC70 (EP1531Y; Abcam, Cambridge, UK), anti-HSP70 (5A5; Abcam), anti-mCherry (632543; Clontech, Mountain View, CA, USA), and anti- α -tubulin (DM1A; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies, respectively.

Fluorescence recovery after photobleaching

Photobleaching experiments were performed on an LSM 510 META system through a C-Apochromat $\times 40/1.2\text{NA}$ W Korr. UV-VIS-IR M27 objective lens (Carl Zeiss), as previously reported (Kitamura et al. 2016, 2017). GFP and RFP were simultaneously excited and photobleached at 488 and 594 nm, respectively, and fluorescence was separated using a dichroic mirror (NFT545). GFP and RFP fluorescence were collected through a band-pass filter (BP505-530) and a long-pass filter (LP615), respectively. The pinhole size was set to 200 μm , the zoom factor was set to $\times 6$, and the interval time for image acquisition was set to 10 s. The X- and Y-scanning sizes were each 512 pixels, the photobleaching period was 0.75 s, and relative fluorescence intensity was measured using the ZEN 2009 software platform (Carl Zeiss) and calculated as

reported previously (Kitamura et al. 2017). To calculate the maximum recovery proportion, recovery curves associated with the relative fluorescence intensity were fitted using Origin 2017 software (OriginLab Corp., Northampton, MA, USA) as previously reported (Kitamura et al. 2017).

Results

Confocal super-resolution microscopy was used to evaluate TDP-25 and HSP70 colocalization in cytoplasmic IBs. The localization of HSP70-R was mainly in the cytoplasm. G-TDP-25 IBs in the cytoplasm were colocalized with transiently expressed HSP70-R, but not with RFP used as a negative control (Fig. 1a and ESM 1), which agreed with a previous report (Kitamura et al. 2017). To investigate whether HSP70-R was functionally associated with G-TDP-25 in the IBs, we performed fluorescence recovery after photobleaching (FRAP) analysis in the cells expressing both G-TDP25 and HSP70-R to quantify mobile components of the fluorescent proteins in live cells, followed by the recording of time-dependent recovery of fluorescence intensity (Matsuda and Nagai 2014; Kitamura et al. 2015; Kitamura and Kinjo 2018). The proportion of fluorescence recovery related to G-TDP-25 during the 280 s after photobleaching was $13.0 \pm 6.1\%$ [mean $\pm$ standard error of mean (SEM)], indicating that 87% of G-TDP-25 was immobile in the IBs (Fig. 1b, c). By contrast, that of HSP70-R showed $52.9 \pm 7.0\%$ recovery (Fig. 1b, c), indicating that 40% of HSP70-R dynamically interacted with G-TDP-25 in the IBs, whereas 60% of HSP70-R was immobile. The proportion of fluorescence recovery of G-TDP25 and HSP70-R in the cytoplasm where IBs were not formed was 97.1 and 95.2%, respectively. Moreover, the proportion of fluorescence recovery of GFP monomer and HSP70-R in the cytoplasm was 100 and 95.0%, respectively. These results suggested sequestration of HSP70 in TDP-25 IBs.

To elucidate whether HSP70 could effectively decrease the formation of cytoplasmic TDP-25 IBs, we compared the proportion of cells carrying TDP-25 IBs in the cytoplasm in the presence or absence of HSP70-R overexpression. The proportion of cells containing of G-TDP-25 IBs in the presence of RFP (control) expression decreased significantly along with increase in the HSP70-R expression plasmid (0.4, 0.8, and 1.2 μg) (Fig. 2b), indicating that HSP70 played a preventative role in TDP-25 IB formation in the cytoplasm. The proportions of cells containing large IBs of > 2 μm as a diameter or multiple IBs were significantly decreased upon HSP70-R expression (Fig. 2c, d). These results suggested that HSP70 overexpression might have prevented the generation and expansion of TDP-25 IBs.

We then determined whether HSP70 overexpression could reduce the amount of insoluble TDP-25 following

Fig. 1 Association of HSP70 with TDP-25 in IBs. **a** Confocal super-resolution fluorescence images of Neuro2A cells expressing G-TDP-25 (green) and HSP70-R or RFP (magenta; negative control) through a water immersion objective lens. Bar, 5 μ m. **b** Representative images of the fluorescence intensities of G-TDP-25 and HSP70-R in Neuro2A cells during FRAP experiments. Times after photobleaching are indicated above

the images. The white arrow indicates the FRAP photobleached spot in the IBs. Scale bar (left), 5 μ m. **c** Fluorescence recovery curves for G-TDP-25 (green line) and HSP70-R (magenta line) in cytoplasmic IBs. The numbers on the graph indicate plateau values for the recovered proportion (mean $\pm$ SEM.; analyzed cell number = 12). *** p < 0.001, Student's t test

Fig. 2 Cells containing cytoplasmic G-TDP-25 IBs. **a** Representative confocal fluorescence and bright field images of cells expressing G-TDP-25 and HSP70-R or RFP (negative control). The white arrow indicates cells containing cytoplasmic IBs. Bar, 50 μm. **b–d** The proportion of cells containing cytoplasmic G-TDP-25 IBs in the presence of HSP70-R (0.4, 0.8, and 1.2 μg plasmid) or RFP (0.4 μg plasmid) expression. Bars indicate the mean ± SEM. ($n = 3$). * $p < 0.05$,

** $p < 0.01$, *** $p < 0.001$, Student's t test. The population of cells harboring **b** cytoplasmic IBs, **c** large (> 2 μm) IBs, and **d** multiple IBs. Total cell numbers for the analysis (1st, 2nd, and 3rd trials, respectively): 244, 202, and 195 for RFP expression; 217, 260, and 227 for HSP70-R expression (0.4 μg); 253, 215, and 210 for HSP70-R expression (0.8 μg); and 231, 253, and 196 for HSP70-R expression (1.2 μg)

sedimentation of the cell lysate and western blot analysis. Although efficient expression of HSP70-R was observed, amounts of RIPA-soluble G-TDP-25 was increased along with increases in HSP70-R expression, suggesting that HSP70 might maintain soluble state of G-TDP-25 (Fig. 3). Analysis of the diffusion state using fluorescence correlation spectroscopy in live cells showed that the estimated molecular weight of G-TDP-25 in the cytoplasm did not indicate monomeric forms of the protein variant (Kitamura et al. 2017). Therefore, these findings suggested that HSP70 efficiently suppressed the accumulation of TDP-25 oligomers/aggregates specifically in IBs. Furthermore, overexpression of HSP70-R and endogenous HSC70, a constitutively expressed HSP70 subtype, was observed primarily in the RIPA-soluble fraction (Fig. 3), suggesting that HSP70 interactions with TDP-25 in cytoplasmic IBs could be dissociated by detergent in RIPA buffer.

Discussion

In this study, we showed that HSP70 overexpression efficiently decreased the formation of TDP-25 IBs in the cytoplasm (Fig. 2). FRAP analysis also showed that approximately 50% HSP70-R was stationary in G-TDP-25 IBs (Fig. 1), suggesting that a portion of HSP70 might tightly bind to TDP-25 aggregates in IBs. By contrast, HSP70 was solubilized in RIPA buffer, whereas TDP-25 was not. These results suggested that TDP-25 aggregates form a tightly assembled and RIPA-insoluble structure, similar to a piece of grape, and that portion containing TDP-25 aggregates might accumulate in the IBs similar to a tassel (Fig. 4). Additionally, HSP70 might bind to the surface of portions of the TDP-25 aggregates (Fig. 4). As the pieces of TDP-25 aggregates can be dissociated but not be solubilized in RIPA buffer, the bound HSP70 can be solubilized in RIPA buffer. Previous studies reported that the

Fig. 3 Comparison of insoluble G-TDP-25 populations in the presence and absence HSP70-R overexpression. Fractionation of cell lysates and western blot analysis using anti-GFP, HSP70, RFP, HSC70, and α -tubulin antibodies in the presence or absence of HSP70-R overexpression. S and P indicate RIPA-soluble supernatant and -insoluble pellet fractions, respectively. Arrows indicate the position of

endogenous HSP70 and exogenous HSP70-R. P/S ratio (G-TDP25) indicates the amount of G-TDP25 in the pellet per that in soluble fraction. Ratio (HSP70-R) indicates fold-increase of HSP70-R abundance along with increase in the amount of transfected plasmid DNA. Total amount (HSP70) indicates the total amount of both HSP70-R and endogenous HSP70 in the soluble fraction

proportion of mobile HSP70 decreases dependent upon the tightness of the aggregates in IBs (Kim et al. 2002; Matsumoto et al. 2005); therefore, the aggregated state of TDP-25 in IBs might promote HSP70 immobilization. Moreover, TDP-25 aggregates are difficult to disassemble, even in the presence of detergents. This situation could disturb cellular function, leading to cell death. Soluble TDP-25 can be degraded via the ubiquitin-proteasome and/or the autophagy/lysosome systems (Zhang et al. 2010; Brady et al. 2011); however, HSP70 is still required to maintain the diffuse state of TDP-25 oligomers and aggregates in the cytoplasm in order to promote their efficient degradation by these processes (Kitamura et al. 2017).

We were unable to completely prevent the formation of TDP-25 IBs, even in the presence of HSP70-R overexpression. This might be due to the insufficiency of HSP70 abundance to prevent G-TDP-25 aggregation. HSP70 inhibits the aggregation of misfolded proteins in concert with other cofactors, such as HSP40 and nucleotide exchange factors (NEFs), as co-chaperones (Kampinga and Craig 2010; Hartl et al. 2011). Other chaperones, including those associated with cytosolic chaperonins and HSP90, are also capable of preventing aggregation of misfolded neurodegenerative-disease-associated proteins (Kitamura et al. 2006; Brehme et al. 2014; He et al. 2017). It is likely that other chaperones in addition to HSP70 are required to prevent TDP-25 aggregation.

Fig. 4 Model of TDP-25 aggregation in IBs and HSP70 chaperone function against TDP-25 aggregation. Green and magenta circles indicate TDP-25 and HSP70, respectively. Accumulation of TDP-25 and HSP70 interactions are shown

Our findings indicated that HSP70 effectively prevented the formation of TDP-25 IBs in the cytoplasm. These results suggest that HSP70 is closely involved in prevention of IB formation in ALS pathogenesis.

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