Structural Transformation of the Amyloidogenic Core Region of TDP-43 Protein Initiates Its Aggregation and Cytoplasmic Inclusion*

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Background: The highly flexible C-terminal region of TDP-43 is implicated in disease pathology.

Results: An amyloidogenic core was identified to be critical for TDP-43 aggregation.

Conclusion: Helix-to-sheet structural transformation of the amyloidogenic core initiates TDP-43 aggregation and cytoplasmic inclusion formation.

Significance: This is a potential therapeutic target for mitigating the TDP-43 proteinopathies.

TDP-43 (TAR DNA-binding protein of 43 kDa) is a major deposited protein in amyotrophic lateral sclerosis and frontotemporal dementia with ubiquitin. A great number of genetic mutations identified in the flexible C-terminal region are associated with disease pathologies. We investigated the molecular determinants of TDP-43 aggregation and its underlying mechanisms. We identified a hydrophobic patch (residues 318–343) as the amyloidogenic core essential for TDP-43 aggregation. Biophysical studies demonstrated that the homologous peptide formed a helix-turn-helix structure in solution, whereas it underwent structural transformation from an α-helix to a β-sheet during aggregation. Mutation or deletion of this core region significantly reduced the aggregation and cytoplasmic inclusions of full-length TDP-43 (or TDP-35 fragment) in cells. Thus, structural transformation of the amyloidogenic core initiates the aggregation and cytoplasmic inclusion formation of TDP-43. This particular core region provides a potential therapeutic target to design small-molecule compounds for mitigating TDP-43 proteinopathies.

Amyotrophic lateral sclerosis and frontotemporal dementia with ubiquitin are two types of neurodegenerative diseases with overlapped clinical features (1–3). The clinical association was confirmed by the discovery of TDP-43 (TAR DNA-binding protein of 43 kDa) as the major constituent protein of the cytoplasmic inclusions observed in the motor neurons and glial cells of patients suffering from amyotrophic lateral sclerosis or frontotemporal dementia with ubiquitin (4, 5).

TDP-43 contains two RNA recognition motif domains that bind RNA targets and a long C-terminal glycine-rich region (GRR) that was reported to mediate protein-protein interactions (6–8). TDP-43 is a predominantly nuclear protein; strongly associates with RNA splicing and translation machinery; and functions in transcription, pre-mRNA splicing, mRNA transport, and stability (9–11). Mislocalization of aggregation of the C-terminal fragments of TDP-43 (including ~35- and ~25-kDa fragments) are featured in clinical observations (4, 12, 13). Missense mutations in the TDP-43 gene cluster in exon 6, which encodes the C-terminal GRR (14–19), aggravate cytotoxicity (20–23). A wealth of studies have suggested that the C-terminal region of TDP-43 is aggregation- or inclusion-prone (24–27), whereas a Gln/Asn-rich domain in the C terminus was also proposed to have a strong tendency for self-aggregation (21, 28, 29). The C-terminal GRR domain is composed of ~150 amino acid residues (positions 262–414) and is critical not only for cytoplasmic deposition (30, 31) but also for seeding full-length TDP-43 in vitro or in cells (32, 33). Accumulating evidence suggests that the C-terminal region plays crucial roles in TDP-43 proteinopathies, and it is of great importance to define its core region for aggregation. Recently, two studies showed that several small peptides from the GRR aggregate and form fibrils in vitro (34, 35). However, the detailed mechanism by which TDP-43 forms aggregates and inclusion bodies in cells remains largely unclear.

In this study, we identified an amyloidogenic core in the C-terminal flexible region, which is essential for TDP-43 aggregation and cytoplasmic inclusion formation. Structural transformation of the homologous peptides has been studied by various biophysical techniques, and the importance of the peptide segment in the aggregation and inclusion formation of TDP-43 was also confirmed in vitro and in cells. We propose that the amyloidogenic core segment is the molecular determinant of TDP-43 aggregation, and structural transformation of this core

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The abbreviations used are: GRR, glycine-rich region; HP, hydrophobic patch; FI/pix, fluorescence intensity/pixel; ThT, thioflavin T; AFM, atomic force microscopy; QN, Gln/Asn-rich motif; STS, staurosporine; TFE, trifluoroethanol.
region initiates TDP-43 aggregation and cellular inclusion formation.

**EXPERIMENTAL PROCEDURES**

**Constructs**—The intrinsic NdeI restriction site in the human TDP-43 gene was mutated via nonsense mutation. The NdeI/BamHI-digested PCR fragments of TDP-43 and its fragments were ligated into the pET22b-GFP plasmid to make the GFP fusion constructs. The cDNAs coding for TDP-43, its mutants, TDP-1, and TDP-Ins were subcloned into the pET22b vector (Novagen) using NdeI/XhoI cloning sites. The cDNAs of TDP-43 fragments were subcloned into the pET32M vector using BamHI/XhoI cloning sites. TDP-43, TDP-1, and TDP-Ins were cloned into the pcDNA3.1-Myc/His vector (Invitrogen), and TDP-35 (90–414) was cloned into the pCMVtag2B plasmid (Stratagene) for eukaryotic expression. All mutants were generated by site-directed mutagenesis using PCR. The cDNA encoding TDP(311–360) was cloned into the pHGB vector to generate the GB1-TDP(311–360) fusion (where GB1 is the IgG B1-binding domain of streptococcal protein G) with an N-terminal His tag. All constructs were verified by DNA sequencing.

**Peptide Synthesis**—Two peptides, TDP-HP and its mutant A324E/M337E, were obtained by Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid-state chemical synthesis (HD Biosciences Co., Ltd.). Peptide purity (>99%) were determined by HPLC and electrospray mass spectrometry. The peptides were dissolved in alkaline solution at pH 12 to yield a stock and then diluted to the appropriate concentration in buffer A (50 mM Tris-HCl, pH 8.0) for experiments. The concentrations of the peptides were determined spectrophotometrically using their extinction coefficients (ε280).

**Protein Expression and Purification**—TDP-43, its mutants, and TDP-Ins were overexpressed and purified as described previously (32). TDP-1 was purified through Ni2+-nitrilotriacetic acid and desalting with buffer A. For further usage, these proteins were steriley filtered through 0.22-µm filters to remove any granular matter. The concentrations of the proteins were determined using the standard BCA assay, and the proteins were immediately subjected to time course incubation experiments to examine the aggregation properties. The thioredoxin- or GB1-fused TDP-43 fragments were purified through Ni2+-nitrilotriacetic acid followed by a Superdex-75 FPLC column (GE Healthcare), and their concentrations were determined spectrophotometrically using each extinction coefficient (ε280).

**Assaying the Aggregation Abilities of GFP-fused TDP-43 Fragments**—C-terminally GFP-fused TDP-43 and its fragments and mutants were expressed in *Escherichia coli* BL21(DE3) cells (36, 37). 5 ml of LB medium with 100 µg/ml ampicillin was incubated with 2% (v/v) of a seed culture that had already grown overnight at 37°C. Cultures were grown with shaking at 37°C and induced with a final concentration of 0.5 mM isopropyl β-D-thiogalactopyranoside at A600 = 0.6. After further culture for 3.5 h at 37°C, 1 ml of the cell culture was centrifuged at 5000 × rpm for 5 min. The cell pellets were suspended in 20 mM Tris-HCl at pH 7.5 and diluted to an absorbance of 0.2 at 600 nm. A 1-ml sample was subjected to SDS-PAGE analysis, and another 1-ml sample was collected for GFP fluorescence recording. The relative protein amount was quantified by recording the grayscale (pixel) of a band in the gel, and the fluorescence intensity was measured in a Varian Cary Eclipse spectrofluorometer (Agilent Technologies) at an excitation wavelength of 490 nm and an emission wavelength of 510 nm. The ratio of the fluorescence intensity to the protein amount (FI/pix) was used to evaluate the aggregation ability of a GFP-fused fragment. All measurements were performed in triplicate, starting with the bacterial cultivation step.

**Time Course of Aggregation in Vitro**—The thioredoxin-fused TDP-43 fragments were diluted to 100 µM with 25 mM Tris-HCl and 150 mM NaCl (pH 8.0). Thrombin (5 µl) was added to each 500-µl sample, followed by incubation at 37°C with shaking. The time course of the aggregation process was monitored by thioflavin T (ThT) fluorescence assay. Fresh TDP-43, its mutants, TDP-1, and TDP-Ins were diluted to a final concen-

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**FIGURE 1.** Comparison of the inclusion formation properties between human TDP-43 and *C. elegans* TDP-1. A, domain architecture of TDP-43 and TDP-1. G-rich, glycine-rich; G/S-rich, glycine/serine-rich. TDP-Ins is a TDP-1 mutant with an insertion of the HP and QN sequences of TDP-43. B, immunofluorescence microscopy imaging of TDP-43 (left), TDP-1 (middle), and TDP-Ins (right) in STS-treated HeLa cells. Cells were stained with C-terminally Myc-tagged antibody for visualizing TDP-43, TDP-1, or TDP-Ins species (green) and with DAPI for the nucleus (blue). Scale bar = 10 µm. DMSO, dimethyl sulfoxide. C, percentages of the cells with inclusion bodies (IBs) in STS-treated HeLa cells for TDP-43, TDP-1, and TDP-Ins. Cells were counted and statistically analyzed by one-way analysis of variance. Data are means ± S.E. (n = 18). *** p < 0.001. D, time courses showing aggregation of TDP-43, TDP-1, and TDP-Ins in vitro as monitored by ThT fluorescence. The TDP-43, TDP-1 and TDP-Ins proteins were purified by *E. coli* overexpression. The concentration of each protein was 100 µM.
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The time course of the aggregation process was monitored by ThT fluorescence assay or fractionation analysis of the supernatant and pellet as described previously (38). The protein or peptide concentration was 100 μM in buffer A. 20 μL of each of the incubated proteins or peptides was added to 980 μL of solution with 5 μM ThT in 50 mM glycine/NaOH (pH 9.0), and the emission intensities at 482 nm were recorded.

Circular Dichroism Measurements—All CD measurements were performed on a Jasco J-715 spectropolarimeter at room temperature. The parameters for recording the CD spectra were those reported previously (38, 39). Data were further processed for noise reduction, base-line subtraction, and signal averaging when needed. Each spectrum was processed by averaging three scans of the same sample. For solution CD measurements, the peptide samples were diluted to a concentration of 0.25 mg/ml in 20 mM Tris (pH 8.0), and the data are presented as mean residual molar ellipticities (degrees cm²/dmol).

NMR Spectroscopy and Structure Calculation—For solution NMR analysis, the GB1 fusion strategy was applied to improve the solubility (41). 13C/15N-Labeled GB1-TDP(311–360) (~0.6 mM) was dissolved in 20 mM phosphate, 50 mM NaCl (pH 6.5), and 8% D₂O for NMR data acquisitions. All spectra were recorded at 25 °C on a Bruker AVANCE 600-MHz spectrometer.

Atomic Force Microscopy—The morphologies of peptides during aggregation were visualized by atomic force microscopy (AFM) imaging as described previously (38). The samples (250 μM) were incubated in 100 mM phosphate and 100 mM NaCl (pH 7.0) with shaking at 37 °C. One drop of 10 μL of peptide solution was deposited on a freshly cleaved mica surface for adsorption for 30 min, and the sample was then rinsed sev-
eral times with water and dried with an air flow. A commercial AFM system (MultiMode NanoScope IIIa, Veeco) equipped with a J-scanner was employed at a tapping mode. Silicon cantilevers with spring constants of $20$ newtons/m (NSG11, NT-MDT Co.) were used. All imaging was conducted in air at room temperature with a room humidity of $40\%$ or less.

**RESULTS**

**Human TDP-43 Is More Susceptible to Cytoplasmic Inclusions than Its Nematode Ortholog, TDP-1**—Human TDP-43, as well as its *Caenorhabditis elegans* ortholog, TDP-1 (42), is a nuclear splicing factor composed mainly of two RNA recognition motif domains functioning in DNA/RNA recognition and a long C-terminal GRR part (7). Sequence analysis showed that TDP-43 contains a hydrophobic patch (HP; residues 318–343) and a Gln/Asn-rich motif (QN; residues 344–360), whereas TDP-1 lacks this region (Fig. 1A). To understand the role of this

**Cell Culture, Immunocytochemistry, and Confocal Microscopy**—The methods used for cell culture, transfection, Western blotting, immunocytochemistry, and confocal microscopy were reported previously (32).

**FIGURE 3. Aggregation of TDP-43 and its fragments in vitro.** A, time courses showing aggregation of the thioredoxin fusions with various C-terminal fragments of TDP-43 upon thrombin cleavage. The aggregation ability is represented by ThT fluorescence intensity. The concentration of each protein or peptide was $100 \mu M$. B, time courses showing aggregation of the purified C-terminal fragments of TDP-43 and its various mutants. D, aggregation of TDP-43 and its mutants as detected by fractionation and SDS-PAGE analysis. TDP-1 was set as a control. The protein samples at each time point were centrifuged to separate the supernatant (S) and pellet (P) and were analyzed by SDS-PAGE (12% gel). The concentration of each protein was $80 \mu M$. E, time courses of the supernatant fractions of TDP-43 and its mutants. Data were obtained by recording the grayscale in the gels. Three independent experiments were performed ($n = 3$).
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unique region in inclusion formation of TDP-43, we visualized the cellular inclusion bodies by confocal microscopy. As reported previously, staurosporine (STS) can induce fragmentation and deposition of TDP-43 (13, 32), which can mimic the pathological condition. Overexpression of TDP-43 gave rise to a number of cells with cytoplasmic inclusion bodies upon STS treatment; however, under similar conditions, overexpression of TDP-1 generated only a small number of cells with inclusions (Fig. 1, B and C). This suggests that TDP-43 is more susceptible to cytoplasmic inclusions compared with TDP-1, implying that the HP and QN regions play a critical role in TDP-43 aggregation and inclusion formation. Interestingly, insertion of the HP and QN sequences into TDP-1 (TDP-Ins) gave it the ability to form inclusions in cells, as in the case of wild-type TDP-43. To further understand the aggregation properties of TDP-43 and TDP-1, we performed ThT fluorescence assay on these purified proteins in vitro (32). Purified TDP-43 showed high fluorescence intensity due to ThT binding (Fig. 1D), indicating that the protein contains a partial fraction of aggregates that binds ThT efficiently. The fluorescence enhancement upon incubation suggests that TDP-43 is prone to aggregation, whereas TDP-1 is unlikely to form aggregates due to the lack of the HP and QN regions. However, TDP-Ins easily aggregated in solution, as the purified protein formed mostly soluble aggregates that strongly bound ThT. Thus, the HP and QN regions, named the amyloidogenic core (residues 318–360), are responsible for aggregation and inclusion formation of TDP-43.

A Hydrophobic Patch in the Flexible C Terminus Is Essential for TDP-43 Aggregation—To clarify which region of TDP-43 is essential for the initiation of its self-aggregation, we prepared a series of GFP-tagged constructs at their C termini and overexpressed them in E. coli to report the aggregation properties of the fusion proteins (Fig. 2A). Aggregation of the fused portion can prevent folding of the GFP reporter and thus significantly suppresses its fluorescence emission (36). So, the ratio of the fluorescence intensity to the protein amount (FI/pix) represents the aggregation ability of a GFP-fused fragment. GFP alone and GB1-fused GFP were set as controls for the background fluorescence. Compared with GB1-GFP, most N-terminally truncated fragments were prone to aggregation, except for C-terminal fragment 344–414, which was less aggregation-prone (Fig. 2B). Of them, four fragments harboring the hydrophobic patch of residues 318–343 readily aggregated (Fig. 2C), suggesting that this region is essential for aggregation of various TDP-43 fragments. The hydropathy profile of C-terminal fragment 262–414 of TDP-43 also indicates that this region is highly hydrophobic (data not shown). Moreover, charged mutations (A324E or M337E) in this fragment significantly reduced the aggregation tendencies, whereas the double mutation completely abolished aggregate formation (Fig. 2D). This observation was also verified by mutation or deletion of this sequence in the full-length TDP-43 protein (Fig. 2E). Taken together, these results demonstrate that the hydrophobic patch (residues 318–343) is essential for TDP-43 aggregation, implying that this HP region initiates the aggregation of full-length TDP-43.

Aggregation of TDP-43 and Its Fragments in Vitro—To further investigate the importance of the aggregation-prone region in determining TDP-43 aggregation, we compared the aggregation properties of various C-terminal fragments of TDP-43 in vitro by ThT assay. First, we analyzed the time course of a thioredoxin fusion with various C-terminal fragments of TDP-43 upon thrombin cleavage (Fig. 3A). As a result, TDP(311–414) and TDP(311–360) could cause fluorescence enhancement of the ThT dye to completion within 2 h, suggesting that these two fragments are susceptible to aggregation. Interestingly, TDP(311–360) gave rise to a typical sigmoidal curve with a lag phase of ~0.5 h. However, TDP(361–414) gave rather low fluorescence enhancement during incubation, indicating that it cannot form aggregates. Upon deletion of the hydrophobic patch (TDP(344–414)) or the Gln/Asn-rich motif (Δ(344–360)) from TDP(311–414), it still had the ability to cause fluorescence enhancement but without a sigmoidal curve, suggesting that the Gln/Asn-rich motif is not determinant but something important to aggregation. We then purified these proteins and peptide fragments and measured their aggregation abilities by ThT assay. The data reconfirmed that TDP(311–360) readily aggregated (Fig. 3B). Similarly, compared with full-length TDP-43, which was prone to aggregation, mutation in the HP region (A324E/M337E) and deletion of the hydrophobic patch or Gln/Asn-rich motif significantly reduced the aggregation abilities of TDP-43 to a different extent (Fig. 3C).

We also carried out fractionation analysis on various TDP-43 forms to demonstrate the role of the amyloidogenic core in determining the aggregation of TDP-43. By separating the supernatant and pellet from the aggregated proteins at different incubation times, aggregation of wild-type TDP-43 was so rapid that most of the protein molecules formed aggregates within 24 h (Fig. 3D, first row). However, the A324E/M337E and HP-deleted mutants of TDP-43, as well as TDP-1, retained high amounts of proteins in the supernatant under similar incubation conditions. The QN-deleted mutant still had the ability to
form aggregates, as did wild-type TDP-43 (Fig. 3, D and E). Collectively, these data demonstrate that the HP motif is determinative to TDP-43 aggregation, whereas the Gln/Asn-rich motif may play a role in assisting with aggregation and fibril formation.

Solution Structure of the Amyloidogenic Core Fragment—To gain insights into the molecular mechanism of TDP-43 aggregation, we solved the solution structure of the amyloidogenic core fragment (TDP(311–360)) using NMR techniques. Because the peptide is highly hydrophobic in sequence and insoluble in aqueous solution (Fig. 3), we fused the peptide sequence to the C terminus of GB1 to obtain NMR data (41). As predicted from the resonance peaks in the NMR spectra (data not shown), the fusion protein formed a monomer in solution, so the resonance and NOE assignments of the TDP(311–360) portion could be obtained for

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**Figure 5. Structural transformation and aggregation of the TDP(311–360) peptide.** A, time course of the CD spectrum changes of TDP(311–360) in solution during incubation. B, secondary structure content changes of TDP(311–360) at various incubation times. The data were obtained by analyzing the CD spectra in A with a computer program. C, time course of the CD spectrum changes of TDP(361–414) in solution during incubation. 

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structural analysis. The solution structure exhibits two canonical α-helices from residues 321 to 330 and from residues 335 to 343 (Fig. 4). There are a series of NOEs observed between side chains of Gln-327 and Trp-334, indicating that the two helices have restricted orientation. Actually, the S31QSSW334 tetrapeptide forms a typical turn connecting the two helices. As expected, the Gln/Asn-rich region forms a flexible random-coil structure. Thus, NMR analysis revealed that the overall structure of the TDP(311–360) peptide is composed mainly of a helix-turn-helix supersecondary structure in solution, providing the possibility of a structural transformation from an α-helix to a hairpin-like β-sheet.

Structural Transformation of the Amyloidogenic Core Peptide of TDP-43—We next investigated the structural transformation of the TDP(311–360) peptide during the aggregation process by CD spectroscopy (Fig. 5). TDP(311–360) shows a negative peak at 206 nm and a shoulder at ~220 nm in the CD spectrum (Fig. 5A), indicating that the peptide forms partially α-helical secondary structure (~15%) in solution. Interestingly, when aggregation occurred during incubation, the double negative peaks decreased dramatically to completion within 2 h (Fig. 5A), as in the case of ThT detection (Fig. 3, A and B). By secondary structure estimation, the percentage of the α-helix, as well as the turn and random coil, is decreased, whereas the content of the β-sheet is significantly increased to an extent of ~80% (Fig. 5B). This suggests that, during aggregation, TDP(311–360) undergoes a transformation from an α-helix to a β-sheet-rich conformation and possibly to an amorphous aggregate form. As a comparison, TDP(361–414) shows a strong negative peak at ~201 nm, indicating a random-coil dominant structure in solution, but its disordered structure remained unchanged during incubation (Fig. 5C). We also recorded the CD spectra in solid state to characterize the secondary structures of these peptides under amyloid conditions (40). The solid-state CD spectrum of TDP(311–360) shows a negative peak at 225 nm and a strong positive peak at 196 nm, suggesting that this peptide also forms a β-sheet-rich structure in the solid amyloid form (Fig. 5D). However, TDP(361–414) seems to have a propensity to form α-helical structure in solid state. AFM revealed that TDP(311–360) could form abundant filaments after incubation within 12 h (Fig. 5E), but TDP(361–414) could not (Fig. 5F). This suggests that the TDP(311–360) peptide has a strong propensity to structurally transform from an α-helix to a β-sheet-rich structure and regular fibrils during aggregation.

Structural Transformation of the HP Peptides—We chemically synthesized two peptides, TDP-HP (residues 318–343) and its mutant A324E/M337E (Fig. 6A), and monitored the secondary structural transformation during aggregation. TDP-HP bound ThT dye and strongly enhanced the fluorescence intensity even without incubation of the peptide (data not shown), suggesting that the hydrophobic TDP-HP peptide is prone to aggregation in solution. However, the mutant peptide formed by introducing charged residues could not bind ThT and did not cause any fluorescence enhancement. The CD spectrum of TDP-HP exhibits a broad negative peak at ~220 nm (Fig. 6B), indicating that the peptide contains mixed secondary structures, probably due to formation of partial oligomers or soluble aggregates even just as it is being dissolved. During incubation, the negative peak of TDP-HP decreased dramatically (Fig. 6B). By secondary structure estimation, the percentage of the α-helix is decreased a bit, whereas the β-sheet content is significantly increased (Fig. 6C). This suggests that, during aggregation, TDP-HP undergoes a transformation to a β-sheet-rich conformation and possibly to an amorphous aggregate form. On the other hand, the A324E/M337E mutant peptide shows a strong negative peak at ~202 nm and a shoulder at 220 nm in the CD spectrum, indicating a random-coil dominant structure with a marginally stable helix in solution, but its partially disordered structure remained unchanged during incubation (Fig. 6D). This is consistent with the observation that the charged mutant was unlikely to undergo aggregation and structural transformation. The solid-state CD spectrum of TDP-HP is indistinguishable from that in solution (Fig. 6E), suggesting that TDP-HP also forms a β-sheet-rich structure in the solid amyloid form. Compared with the spectrum in solution, the mutant peptide exhibits double negative peaks at 222 and 208 nm in solid state, indicating formation of a typical α-helix dominant structure. Together, these data strongly suggest that the hydrophobic patch is determinative not only in amyloidogenic aggregation but also in secondary structural transformation of TDP-43.

Because the TDP-HP peptide formed a mixed structure of α-helix and β-sheet and it underwent structural transformation during aggregation, we applied trifluoroethanol (TFE), an α-helix inducer, to trigger the secondary structural transformation as monitored by CD spectroscopy (39). With an increase in TFE
concentration, TDP-HP underwent a structural transition from an α/β mixture to an α-helical structure (Fig. 7A). Apparently, the transition presents a three-state process with an equilibrium intermediate of partial α-helix and random-coil structures (Fig. 7B). However, the mutant peptide underwent a classical two-state transition from a random coil to an α-helix (Fig. 7, C and D). Thus, to some extent, TFE can reverse the aggregation process of TDP-HP in solution, corroborating the occurrence of structural transformation during aggregation of the peptide.

The Hydrophobic Patch Is Essential for TDP-43 Aggregation and Cytoplasmic Inclusion Formation—To address the importance of the HP segment in aggregation and inclusion body formation of TDP-43, we observed the cytoplasmic inclusion bodies of two HP mutants in cells by confocal microscopy. Unlike TDP-43, the TDP-35 fragment readily aggregates and forms cytoplasmic inclusions (32). We constructed FLAG-tagged TDP-35 and mutants A324E/M337E and Δ(318–343) and overexpressed them in HEK293T cells. Mutation in the HP region of TDP-35 caused formation of only a few punctate foci in cells, whereas deletion of the hydrophobic patch dramatically abrogated the formation of inclusions (Fig. 8A). We also constructed C-terminally Myc-tagged TDP-43 and mutants A324E/M337E and Δ(318–343), overexpressed them in HeLa cells, and treated the cells with STS. Although both wild-type TDP-43 and its mutants localized well in the nucleus, they exhibited different properties of cytoplasmic inclusion formation in STS-treated cells (Fig. 8B). The percentages of the cells with inclusion bodies for both mutants were significantly decreased compared with that for wild-type TDP-43 (Fig. 8C). The reduced aggregation abilities for the two mutants were also confirmed by analyzing the pellet fraction of the TDP-43 fragments from the STS-treated cells (Fig. 8D). Compared with TDP-43, whose proteolytic fragment TDP-35 was mostly deposited in the precipitate fraction, the A324E/M337E mutant showed a decreased amount of the deposited fragment, and the HP-deleted mutant had its C-terminal fragment predominantly present in the supernatant. These results demonstrate that the

![FIGURE 7. Secondary structural transformation of TDP-HP and its A324E/M337E mutant in TFE as monitored by CD spectroscopy.](image-url)
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HP segment (residues 318–343) is essential for aggregation and inclusion body formation of TDP-43 in STS-treated cells, as well as the TDP-35 fragment in cells.

DISCUSSION

A characteristic pathological feature of most neurodegenerative disorders is the aggregation of a particular protein. These disease-associated proteins often possess a amyloidogenic core that has a high tendency to form aggregates and cellular inclusions and to experience conformational changes in the pathogenesis (43). As for TDP-43, the long highly flexible C terminus is generally considered to play important roles in its misfolding, aggregation, and cytoplasmic inclusion formation and even in disease pathology (25, 27, 31). Recently, research on the homologous peptide fragments has suggested that Ala-315 and its flanking region (residues 311–320), which is largely different from the helical hydrophobic patch (residues 318–343), are crucial for TDP-43 aggregation and neurotoxicity (34, 35), but the mechanism by which these peptide segments lead to TDP-43 aggregation has yet to be explored.

Our findings demonstrate that TDP-43 contains an amyloidogenic core within the C-terminal flexible GRR, that the hydrophobic patch in this region plays a critical role in TDP-43 aggregation and inclusion formation, and that the Gln/Asn-rich motif is beneficial to these processes. The hydrophobic patch forms a helix-turn-helix structure in solution, and this peptide segment is prone to structural transformation from an \( \alpha \)-helix to a \( \beta \)-sheet. In contrast, the Gln/Asn-rich motif is a flexible stretch that is beneficial to structural transformation and \( \beta \)-strand expansion. We propose that this structural transformation from a helix-turn-helix to an antiparallel hairpin-like \( \beta \)-sheet initiates aggregation of full-length TDP-43 (Fig. 9).

Although TDP-43 (or TDP-35) harbors mostly GRRs and a Gln/Asn-rich motif in its highly flexible C-terminal tail, only this hydrophobic patch can form ordered \( \alpha \)-helical structure. When structural transformation occurs in this core region during TDP-43 aggregation, \( \beta \)-sheet formation will occur in this region and extend to the flanking regions in sequence. When \( \beta \)-strand expansion occurs through intermolecular interactions, large oligomers, particles, and even disease-related inclusion bodies will form in cells (4, 31). Thus, we conclude that the structural transformation of the hydrophobic patch of TDP-43 triggers its misfolding, aggregation, and cytoplasmic inclusion formation, which are implicated in disease pathology (44). This particular core region may provide a potential target to design small-molecule compounds that can interfere with \( \alpha \)-to-\( \beta \) structural transformation.

FIGURE 8. Aggregation and inclusion formation of TDP-43 and its mutants. A, immunofluorescence microscopy imaging showing inclusion formation of TDP-35 and its mutants in HEK293T cells. TDP-35 formed inclusion bodies in most cells, whereas its mutant A324E/M337E had a moderately reduced ability to form inclusion bodies. Deletion of the core sequence (A(318–343)) completely eliminated the formation of inclusion bodies. Scale bar = 10 \( \mu \)m. B, immunofluorescence microscopy imaging of TDP-43 and its mutants in STS-treated HeLa cells. Cells were stained with C-terminally Myc-tagged antibody for visualizing TDP-43 species (green) and DAPI for the nucleus (blue). Scale bar = 10 \( \mu \)m. DMSO, dimethyl sulfoxide. C, percentages of the cells with inclusion bodies (IBs) in STS-treated HeLa cells for TDP-43 and its mutants. Cells were counted and statistically analyzed by one-way analysis of variance. Data are means ± S.E. (n = 8–12). ***p < 0.001. D, Western blot analysis showing the deposited TDP-43 species in the pellet (P) fraction from STS-treated cells. The results from quantitative analysis of the band density for each degraded fragment are also included. S, supernatant; IB, immunoblot.

FIGURE 9. Schematic representation of the \( \alpha \)-to-\( \beta \) structural transformation of TDP-43 during its aggregation process. The model proposes that TDP-43 forms aggregates through structural transformation of the core sequence. During aggregation, the structure of the HP region transforms from a helix-turn-helix to an antiparallel hairpin-like \( \beta \)-sheet. The intermolecular \( \beta \)-stand interaction of one TDP-43 molecule with another initiates aggregation of full-length TDP-43 and further promotes TDP-43 to form large aggregates and inclusion bodies.
transformation for therapeutic treatment of the TDP-43 proteinopathies (45, 46).

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