Characterization of the dipeptide repeat protein in the molecular pathogenesis of c9FTD/ALS

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Abstract

The expansion of the GGGGCC hexanucleotide repeat in the non-coding region of the chromosome 9 open-reading frame 72 (C9orf72) gene is the most common cause of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (c9FTD/ALS). Recently, it was reported that an unconventional mechanism of repeat-associated non-ATG (RAN) translation arises from C9orf72 expansion. Sense and anti-sense transcripts of the expanded C9orf72 repeat, i.e. the dipeptide repeat protein (DRP) of glycine–alanine (poly-GA), glycine–proline (poly-GP), glycine–arginine (poly-GR), proline–arginine (poly-PR) and proline–alanine (poly-PA), are deposited in the brains of patients with c9FTD/ALS. However, the pathological significance of RAN-translated peptides remains unknown. We generated synthetic cDNAs encoding 100 repeats of DRP without a GGGGCC repeat and evaluated the effects of these proteins on cultured cells and cortical neurons in vivo. Our results revealed that the poly-GA protein formed highly aggregated ubiquitin/p62-positive inclusion bodies in neuronal cells. In contrast, the highly basic proteins poly-GR and PR also formed unique ubiquitin/p62-negative cytoplasmic inclusions, which co-localized with the components of RNA granules. The evaluation of cytotoxicity revealed that overexpressed poly-GA, poly-GP and poly-GR increased the substrates of the ubiquitin–proteasome system (UPS), including TDP-43, and enhanced the sensitivity to a proteasome inhibitor, indicating that these DRPs are cytotoxic, possibly via UPS dysfunction. The present data indicate that a gain-of-function mechanism of toxic DRPs possibly contributes to pathogenesis in c9FTD/ALS and that DRPs may serve as novel therapeutic targets in c9FTD/ALS.

Introduction

Advances in genetics and pathological studies have revealed that frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) constitute a disease spectrum that shares a common molecular basis (1). Recently, two groups identified an expansion of the GGGGCC hexanucleotide repeat in the non-coding region of the chromosome 9 open-reading frame 72 (C9orf72) gene, which is located on the short arm of chromosome 9, in familial and sporadic FTD and ALS (c9FTD/ALS) (2,3). Based on a large series of patients who were investigated, this pathological mutation was shown to be the most common genetic abnormality in FTD and ALS (4,5). Therefore, understanding the mechanisms that are responsible for c9FTD/ALS is expected to lead to new therapeutic targets against the different forms of FTD and ALS.

Three mechanistic models of c9FTD/ALS have been proposed: reduced levels of C9orf72 transcription (haploinsufficiency), formation of toxic RNA foci and sequestration of RNA-binding proteins (RNA toxicity) and abnormal accumulation of the dipeptide repeat protein (DRP), which is produced via repeat-associated non-ATG (RAN) translation. First, the previously reported reduced levels of C9orf72 transcripts in the brains of patients with c9FTD/ALS (2,4,6) and the motor deficits caused by
C9orf72 knockdown in zebrafish (6) imply that haploinsufficiency is a potential cause of the disease. Second, the transcribed expansion forms nuclear RNA foci that are predicted to contain highly stable G-quadruplex structures (7) and hairpin loops as assessed by biochemical studies and in silico modeling. These conformations could be neurotoxic through excessive sequestration of various RNA-binding proteins, leading to loss of their activity and to RNA dysregulation (8–12). In accordance with the tri- and tetra-nucleotide expansions that are present in myotonic dystrophy type 1 (DM1) and 2, the hexanucleotide repeat may bind profugally to and sequester numerous RNA-binding proteins, including splicing factors as well as factors involved in transcription and RNA trafficking, leading to disturbance of the normal splicing and processing of other RNAs (13). Recent studies revealed that several RNA-binding proteins, such as hnrNP A1, Pur α and ADARB2, are sequestered in C9orf72 RNA foci (9,11,14).

Third, two groups have demonstrated that an unconventional mechanism of RAN translation arises from C9orf72 expansion; this was confirmed by the detection of insoluble material in brain homogenates and neuronal inclusions throughout the central nervous system (CNS) of patients with c9FTD/ALS using antibodies generated against RAN-translated peptides that arise from the putative GGGGCC repeat (15,16). The products translated from six reading frames, namely the dipeptide repeats of glycine–alanine (poly-GA), glycine–proline (poly-GP) and glycine–arginine (poly-GR) from sense repeat transcripts, and proline–glycine (poly-PG), proline–arginine (poly-PR) and proline–alanine (poly-PA) from anti-sense repeat transcripts, are deposited throughout the postmortem brains of patients with c9FTD/ALS as TDP-43-negative and p62-positive inclusions (17,18). These characteristic inclusions are also immunoreactive for ubiquitin and select ubiquitin-binding proteins, most notably ubiquitin-2 (19,20). These same two groups recently reported that poly-GA protein forms a toxic aggregate and is the critical toxic species causing neurodegeneration in c9FTD/ALS, leading to interference of protein homeostasis (21,22). Other groups reported that expression of the highly basic arginine-containing DRPs, poly-GR and poly-PR, causes cellular toxicity, which is independent of RNA toxicity (23,24). However, the biochemical characteristics of DRP and details of the molecular mechanisms involved in the pathogenesis of c9FTD/ALS remain unclear.

To elucidate the impact of individual DRP, we generated synthetic cDNAs encoding 100 repeats of poly-GA, poly-GP, poly-GR, poly-PR and poly-PA with start codon, avoiding GGGGCC repeats, and evaluated the effects of these proteins on transfected cultured neuronal cell lines and cortical neurons in vitro and in vivo. Our results revealed that among the five DRPs, poly-GA showed a strong tendency for aggregation and inclusion formation in neuronal cell lines, which was dependent on the length of the GA repeats. The poly-GA inclusions were immunoreactive for ubiquitin and p62, but not for TDP-43, which is similar to the characteristic pathological inclusions present in c9FTD/ALS. In contrast, the highly basic proteins poly-GR and poly-PR also formed unique ubiquitin/p62-negative cytoplasmic inclusions; these inclusions co-localized with the components of RNA granules, including TDP-43. In addition, overexpression of poly-GA, poly-GP and poly-GR DRP interfered with the ubiquitin–proteasome system (UPS) of the cell and enhanced the sensitivity to a proteasome inhibitor. These findings demonstrate that DRP affects the protein quality control system (protein homeostasis), resulting in cytotoxicity, and are potential therapeutic targets for c9FTD/ALS.

Results

Characterization of DRP expression in cultured cells

To study the biochemical and cellular characteristics of each c9FTD/ALS RAN-translated DRP, we generated synthetic cDNAs encoding 100 repeats of DRP with N-terminal FLAG and C-terminal V5 tags. We assembled base pairs to encode poly-GA, GP, GR, PR and PA avoiding GGGGCC repeats followed by the optimization of the preferred codon usage. We transfected these cDNAs, which were inserted in a mammalian expression vector with start codon, into various cell lines and analyzed them by western blotting. As shown in Figure 1A, the poly-GA protein (predicted size,
17.4 kDa) was detected at ~18 kDa, and the poly-GP (predicted size, 20.0 kDa) and poly-PR (predicted size, 25.3 kDa) proteins were observed as unexpected bands approximately between 25–50 kDa and ~40 kDa, respectively, suggesting peculiar SDS-binding properties, folding, and/or post-translational modification because of the decreased electromobility of proteins containing highly repeated sequences. Poly-GR protein (predicted size, 25.9 kDa) was detectable at ~22 kDa after long exposure in a lysate from Neuro2a cells. Poly-PA protein (predicted size, 16.8 kDa) was faintly detected as a large band (~90 kDa) in Neuro2a cells (four independent western blotting tests showed the same band). The poly-GA protein and part of the poly-PR protein were also detected within the stacking gel in a lysate from Neuro2a cells, which revealed that poly-GA and poly-PR are prone to aggregation and form a high-molecular-weight complex. Considering that the highly basic properties of poly-GR [estimated isoelectric point (pI), 12.96] might interfere with its transfer to the blotting membrane, a dot blot assay using Neuro2a and 293T cell lysates was performed to detect poly-GR expression as well. As shown in Figure 1B, poly-GR was detected as a stronger signal on the nitrocellulose membrane [Density ratio to poly-GA (mean ± standard deviation), Neuro2a: poly-GR, 0.44 ± 0.56; poly-GR, 0.10 ± 0.01; poly-PR, 0.70 ± 0.09; poly-PA, 0.02 ± 0.016; density ratio to poly-GR, 293T: poly-GR, 1.28 ± 0.98; poly-GR, 0.63 ± 0.48; poly-PR, 1.14 ± 0.20; poly-PA, 0.25 ± 0.40 (n = 3)]. Poly-GA and poly-PR showed a stronger signal than expected from the findings of western blotting, possibly because of the inefficient transfer of high-molecular-weight poly-GA and poly-PR aggregates. We could not detect any signal of poly-PA on dot blot. This finding is consistent with immunocytochemistry, which shows only a few poly-PA expressed cells in Neuro2a cells (Fig. 2), possibly because of inefficient translation or rapid degradation. Next, a filter retardation assay was performed to evaluate the aggregation of DRP, and aggregated poly-GR in Neuro2a cells and poly-PR in both Neuro2a and 293T cells were trapped on the cellulose acetate membrane (Fig. 1B), suggesting that poly-GA and poly-PR form strongly insoluble aggregates, consistent with the results of western blot analysis (16).

Pathological studies revealed that DRP forms TDP-43-negative inclusions throughout the CNS of c9FTD/ALS cases. To examine subcellular localization, we performed immunocytochemistry in various transfected cell lines (Fig. 2 and Supplementary Material, Fig. S1). Poly-GR protein formed characteristic cytosolic and nuclear inclusion bodies (IBs) in Neuro2a [percentage of cells with IBs ± standard deviation, 69.3 ± 15.2% (n = 3) and NSC34 cells [percentage of cells with IBs, 22.5% (45/200 cells, n = 1)]. However, it distributed diffusely throughout the cell body in non-neuronal cell lines such as 293T, HeLa or COS cells. Poly-GP was distributed throughout the cell body in most cells, and poly-GR was distributed diffusely throughout the cytoplasm, with some located in nucleoli. Notably, poly-GR formed some cytoplasmic inclusions in 293T [percentage of cells with IBs, 51.4 ± 20.1% (n = 3)]. HeLa [percentage of cell with IBs, 16.0% (32/200 cells, n = 1)] and COS cells [percentage of cell with IBs, 44.5% (89/200 cells, n = 1)] (Fig. 2B and Supplementary Material, Fig. S1). Poly-PR was located in the cytoplasm and nucleus (predominantly nucleoli), and it also formed cytoplasmic IBs in non-neuronal cells and a few IBs in neuronal cells [Neuro2a, percentage of cells with IBs, 6.7 ± 0.6% (n = 3), 293T [73.0 ± 17.0% (n = 3)], NSC34 [3.00% (6/200 cells, n = 1)], HeLa [12.0% (24/200 cells, n = 1)] and COS cells [43.0% (86/200 cells, n = 1)]. Poly-PA was distributed diffusely throughout the cell body, but the number of expressed cells was remarkably small.

Primary cortical neurons also showed similar findings, with the presence of poly-GA [percentage of cells with IBs, 10.0% (20/200 cells, n = 1)], PR IBs [percentage of cells with IBs, 10.1% (22/200 cells, n = 1)] and rare poly-GR IBs [percentage of cells with IBs, 1.00% (2/200 cells, n = 1)] (Supplementary Material, Fig. S2), suggesting that IB formation by DRP is strongly dependent on the cellular environment of each cell type.

To evaluate the viability of DRP-expressed cells, we transfected cDNAs encoding the DRP or control green fluorescent protein (GFP) followed by internal ribosome entry sites and DsRed as a reporter to assess viable cells. Cells were fixed after a number of days of culture and counted for the proportion of DsRed-positive viable cells in three random fields in each of three experiments. As shown in Supplementary Material, Figure S3, the ratio of DsRed-positive cells expressing all DRPs to control cells expressing GFP declined significantly at 2 and 3 days after transfection. Notably, the viability of poly-GR-transfected cells was markedly lower compared with that of cells transfected with other DRPs, suggesting that expression of GR has cytotoxic properties.

Characterization of IBs formed in DRP-expressing cells

Next, we evaluated the unique IBs formed by poly-GA, poly-GR and poly-PR in Neuro2a and 293T cells, respectively, using various pathological markers. A characteristic feature of c9FTD/ALS is the presence of inclusions that are immunoreactive for ubiquitin and p62/sequestosome 1 but are negative for TDP-43, particularly in the cerebellar cortex, hippocampus and cerebral neocortex (17,18). As shown in Figure 3, almost all poly-GA IBs were positive for both ubiquitin [percentage of ubiquitin-positive IBs, 90.0% (45 ubiquitin + IBs/50 IBs, n = 1)] and p62 [percentage of p62-positive IBs, 94.0% (47 p62 + IBs/50 IBs, n = 1)]. Because the FLAG tag has possible ubiquitination sites (i.e. two lysine residues), we generated an HA-tagged poly-GA, which does not bear any lysine residues. Notably, HA-tagged poly-GR also formed ubiquitin-positive IBs, indicating that these IBs recruit other ubiquitinated protein(s). Poly-GR IBs were negative for TDP-43, even when inclusions were located in the nuclei. Together, these findings indicate that poly-GA can solely form TDP-43-negative ubiquitin/p62-positive IBs, which are similar to those observed in c9FTD/ALS (17,18).

In contrast, IBs formed by the highly basic proteins poly-GR and poly-PR in 293T cells were both ubiquitin and p62 negative (Supplementary Material, Fig. S4). Arginine-rich motifs are known to bind to RNA and are involved in the regulation of RNA metabolism (25); therefore, we examined the distribution of the FTD/ALS-related RNA-binding protein, TDP-43, and that of other RNA granule markers in 293T cells containing poly-GR and poly-PR IBs (Fig. 4). Poly-GR and poly-PR IBs recruited TDP-43 [percentage of TDP-43-positive IBs, poly-GR, 80.5% (161 TDP-43 + IBs/200 IBs, n = 1); poly-PR, 100% (200 TDP-43 + IBs/200 IBs, n = 1)] and co-localized with some components of RNA granules, such as G3BP [percentage of G3BP-positive IBs, poly-GR, 100% (200 G3BP + IBs/200 IBs, n = 1); poly-PR, 100% (200 G3BP + IBs/200 IBs, n = 1)], ataxin-2 and HUr (Supplementary Material, Fig. S5), suggesting that poly-GR and poly-PR affect the distribution of RNA-binding proteins and potentially the RNA quality control system.

The repeat range reported for normal individuals to date does not exceed 24 repeats (2–4,26,27), whereas pathological expansions consist of >70 repeats and usually encompass 700–1600 repeats. Recently, a 29-repeat allele was detected in a patient from a Dutch family with c9FTD/ALS (28). To clarify whether the formation of poly-GA IBs depends on repeat length, we generated cDNAs coding 61, 46, 26 and 8 repeats of poly-GA and examined the formation of IBs in Neuro2a cells. As shown in Figure 5A, the percentage of cells with IBs decreased in a repeat
length-dependent manner, and IBs were rarely detected in cells with 8 or 26 poly-GA repeats, which is consistent with the pathological repeat range. We also generated plasmids encoding 15 and 100 repeats of poly-GA with a large tag, GFP, to stabilize short peptide. As expected, 100-repeats poly-GA formed IBs, but not 15-repeats poly-GA (Fig. 5B).

Pathological investigation revealed that all DRPs form IBs in c9FTD/ALS. However, poly-GP protein has a lower tendency to form IBs in cultured cells. To clarify whether poly-GA, poly-GR and poly-PR IBs interact with other DRPs, we examined cells co-expressing two DRP sets. As shown in Figure 6 and Supplementary Material, Figure S6, poly-GA IBs barely recruited poly-GP, poly-GR and poly-PR in Neuro2a cells [percentage of poly-GP-positive IBs in poly-GA IBs, 9.4% (10 poly-GP + IBs/106 IBs, n = 1); poly-GR, 6.0% (6 poly-GR + IBs/100 IBs, n = 1); poly-PR, 7.0% (8 poly-PR + IBs/114 IBs, n = 1)]. In contrast, poly-GR and poly-PR formed IBs with other DRPs in HeLa cells [percentage of poly-GA-positive IBs in poly-GR IBs, 67.0% (67 poly-GA + IBs/100 IBs, n = 1); poly-GR, 79.2% (80 poly-GP + IBs/101 IBs, n = 1); poly-PR, 87.0% (87 poly-GP + IBs/100 IBs, n = 1); poly-GR, 93.0% (93 poly-GR + IBs/100 total IBs, n = 1)]. Therefore, our findings indicate that...
aggregations of poly-GR and poly-PR affect the distribution of other DRPs and are potentially seeds of IB in c9FTD/ALS.

Poly-GA inclusion formation in vivo

*In utero* electroporation is a method that is used to transfect DNA into neuronal stem cells in the ventricular zone of the developing cerebral cortex (29,30). To confirm our findings in vivo, we then used *in utero* electroporation to transfect DRP-encoding plasmids into mouse cerebral cortical cells on embryonic day 15 (Fig. 7). Neuronal cells produced from the transfected progenitor cells migrate radially toward the brain surface and differentiate into glutamatergic pyramidal neurons in the cerebral cortex (31–33). As shown in Figure 7A and B, poly-GA-transfected neurons formed FLAG- and V5-positive inclusions by postnatal day 10 in both the cytosol and nuclei [percentage of cells with IBs, 31.0% (44/142 cells, n = 1)]. Staining for Brn-2, which is expressed in layers II, III and V cortical neurons (32,33), confirmed that electroporated cells are cortical neurons in the upper cortical layers II–III (Fig. 7C). These inclusions were immunoreactive for ubiquitin and p62/sequestosome 1 (Fig. 7D and E), which was consistent with the results of the experiments performed in cultured cells. In contrast, poly-PR formed a few IBs [percentage of cells with IBs, 5.0% (6/119 cells, n = 1)] but poly-GP, poly-GR and poly-PA did not, which was compatible with the expression patterns observed in Neuro2a and NSC34 cells. Collectively, the aggregated state of poly-GA protein was also confirmed in vivo.

Protein degradation system of DRPs of c9FTD/ALS

The two major intracellular protein degradation systems are the UPS and autophagy system in eukaryotic cells. Previous studies of Epstein-Barr Virus nuclear antigen 1 (EBNA-1) containing a 239-amino acid GA repeat revealed that the GA repeat domain prevents interaction with proteasome and inhibits UPS-dependent proteolysis in cis (34,35). Next, we examined whether four DRP degradations depend on these systems (poly-PA is difficult to detect quantitatively on blot analysis). We treated DRP-expressing
cells with a proteasome inhibitor, MG132, and an autophagy inhibitor, 3-methyladenine (3-MA), and assessed the amount of DRP using dot blot or western blot analyses (Fig. 8A). Although both reagents increased the levels of poly-GP, poly-GR and poly-PR, MG132 did not change the levels of poly-GA. Therefore, these findings indicate that autophagy is responsible for the degradation of all DRPs, whereas UPS does not contribute to poly-GA degradation, consistent with the previous studies of EBNA-1 (34,35).

Next, we evaluated the effect of autophagic clearance on poly-GA IB formation. The immunocytochemical analysis mentioned earlier revealed that poly-GA IBs were positive for p62, which is a key molecule in the autophagic clearance of ubiquitinated proteins. As shown in Figure 8B, these IBs were co-localized with the co-transfected marker of autophagosome GFP-LC3. We also found that the formation of poly-GA IBs was drastically increased in ATG5(−/−) mouse embryonic fibroblasts (MEFs), which are defective in autophagy (Fig. 8C). Therefore, these findings indicate that poly-GA IBs are, at least in part, degraded by the autophagy system. Poly-PR formed only a few IBs in ATG5(+++) MEFs [percentage of cells with IBs, 2.73% (10/400 cells)] and ATG5(−/−) [2.75% (11/400 cells)]. In contrast, poly-GP, poly-GR and poly-PA did not form IBs, even in ATG5(−/−) MEFs (data not shown).

Impairment of the ubiquitin–proteasome system by DRPs of c9FTD/ALS

There is ample evidence showing that the toxic aggregation of proteins associated with neurodegeneration leads to UPS dysfunction (36). We wondered whether DRPs impair ubiquitin-mediated proteasomal degradation. To address this question, we examined UPS using reporter constructs, Ub-G76V-GFP, which are rapidly degraded by the UPS. As shown in Figure 9A,
UPS reporters were increased in the presence of three DRPs (ratio of Ub-G76V-GFP levels in DRP-expressing cell to that in empty vector-expressing cells: poly-GA, 1.93 ± 0.53; poly-GP, 2.59 ± 0.97 and poly-GR, 2.51 ± 1.84), whereas the native GFP level was unaffected in the presence of any DRP. Surprisingly, poly-PR-expressed cells significantly decreased the levels of Ub-G76V-GFP (0.39 ± 0.59), and this effect was canceled by treatment with the proteasome inhibitor MG132 (Supplementary Material, Fig. S7). Therefore, poly-PR may have a different effect(s) on UPS reporters than other DRPs. Because, as described earlier, the expression of poly-PA is too low for quantitative investigation in Neuro2a cells (Fig. 1), we excluded poly-PA from this analysis.

**Figure 5.** Repeat length dependency of poly-GA IB formation. (A) Neuro2a cells were transiently transfected with poly-GA plasmids (100, 61, 46, 26 and 8 repeats) (red). In ~300 transfected cells from 3 independent experiments, the percentage of cells harboring poly-GA IBs was calculated. Note that IB formation was rare in cells expressing 26 or 8 poly-GA repeats. Scale bar, 10 µm. Error bars represent standard deviations. Asterisks indicate significant differences versus 100 poly-GA repeats (P < 0.005, Dunnett’s t test). (B) IB formation in 200 transfected cells with poly-GA-GFP plasmids (100 and 15 repeats) (green) from 3 independent experiments was counted. Asterisks indicate significant differences versus 100 poly-GA repeats (P < 0.001, Dunnett’s t test).
Figure 6. Intracellular interaction between poly-GR or poly-PR and other DRPs. Representative micrographs of HeLa cells following transfection with poly-GR or poly-PR tagged with T7 (green) and poly-GA, poly-GP, poly-PR and poly-PA tagged with FLAG (red). Poly-GR and poly-PR IBs recruited other DRPs into IBs. Co-expressing poly-GR or poly-PR and poly-PA were undetectable. Scale bar, 10 µm.
The C9orf72 expansion causes TDP-43 accumulation in affected brains and spinal cords (15,16,37). Therefore, we examined the effect of DRPs on the amount of TDP-43, which is a well-known substrate of the proteasome (38,39). Predictably, TDP-43 was upregulated after the co-transfection of these three DRPs into Neuro2a cells (ratio of overexpressed TDP-43 levels in DRP-expressing cell to that in empty vector-expressing cells: poly-GA, 1.53 ± 0.27; poly-GP, 1.92 ± 0.22 and poly-GR, 1.72 ± 0.29), indicating that DRPs affect the level of TDP-43. In contrast, poly-PR expression did not affect TDP-43 levels (0.97 ± 0.34).

Finally, to determine whether DRP expression leads to cell death, we assessed sensitivity to a proteasome inhibitor, as determined by immunostaining of cleaved caspase-3 (Fig. 9B). In the absence of treatment, the proportion of dying poly-GR-expressing...
cells was significantly greater than that of other cells (GFP, 1.24 ± 0.42%; poly-GA, 0.29 ± 0.51%; poly-GP, 1.09 ± 0.47%; poly-GR, 4.47 ± 1.34% and poly-PR, 0.44 ± 0.76%), suggesting that poly-GR is essentially toxic to cells, consistent with the results shown in Supplementary Material, Figure S3. We also found that these three DRPs enhanced the susceptibility to the proteasome inhibitor MG132 compared with mock (GFP) expression (GFP, 3.96 ± 1.44%; poly-GA, 12.17 ± 1.61%; poly-GP, 8.98 ± 2.07% and poly-GR, 10.93 ± 2.64%). In contrast, poly-PR expression did not affect sensitivity to a proteasome inhibitor (3.89 ± 1.95%), compatible with the result of experiment with UPS reporter (Fig. 9A). Taken together, our findings demonstrated that the expression of poly-GA, poly-GP and poly-GR is potentially cytotoxic and that it is at least partly linked to UPS impairment.

Discussion

Two groups recently reported that poly-GA protein expression using synthetic genes lacking extensive GGGGCC repeats leads to toxic aggregation and causes neuronal toxicity (21,22). Both studies show that poly-GA is critical for neurodegeneration in c9FTD/ALS, leading to interference of protein homeostasis. Our
study also evaluated the effects of these proteins on cultured cells and cortical neurons in vivo using cDNAs encoding 100 repeats of DRP without a GGGGCC repeat and provided several important insights into RAN translation in c9FTD/ALS. First, poly-GA, poly-GR and poly-PR proteins formed characteristic IBs depending on cell type. Poly-GA formed highly aggregated IBs in neuronal cells (Figs 2 and 3), consistent with the studies mentioned earlier (21,22). One possible explanation for this is...
that as poly-GA protein has a higher hydrophobicity (grand average of hydrophaticity, 0.336) compared with poly-GP (−1.080), poly-GR (−2.289), poly-PR (−3.050) and poly-PA (0.100) (40), it may have a strong tendency to self-assemble into compact IBs. In the present study, the formation of these IBs was repeat length dependent, and IBs with fewer than 26 poly-GA repeats were rare (Fig. 5). In addition to poly-GA, poly-GR and poly-PR formed another type of cytoplasmic IB (Fig. 2). These IBs recruited TDP-43 and RNA granule components (including stress granule markers) (Fig. 4 and Supplementary Material, Fig. S5), which are suggested to play critical roles in the pathogenesis of FTD/ALS via RNA regulation (1,41,42). Given that poly-GR and poly-PR have highly basic properties (theoretical pI, 12.96 and 14.00, respectively) and that basic arginine-rich motifs play a key role in RNA-binding proteins (25), the poly-GR and poly-PR protein itself may have the potential to affect RNA regulation via the derangement of RNA granules. Together with recent evidence showing that RNA dysregulation is part of the pathological cascade in TDP-43 proteinopathy via RNA granule formation (1,43), these results provide a possible pathogenic mechanism that underlies neurodegeneration in c9FTD/ALS.

In fragile X-associated tremor ataxia syndrome (FXTAS), which results from the expansion of a CGG repeat located in the 5’ UTR of FMR1, RAN translation of a polyglycine-containing protein (FMRPolyG) was also detected (44). A Drosophila model provided evidence that CGG repeat toxicity is suppressed by eliminating RAN translation, indicating that FMRPolyG contributes to neurodegeneration directly. Recently, two groups reported that expression of the highly basic arginine-containing DRPs, poly-GR and poly-PR, exhibits cellular toxicity (23,24). Mizielska et al. (23) showed that the expression of the C9orf72 GGGGCC repeat in Drosophila caused adult-onset neurodegeneration, and poly-GR and poly-PR proteins were the major toxic species of C9orf72 GGGGCC repeat toxicity, independent of RNA toxicity. Kwon et al. (24) demonstrated that exposure of cultured cells to the poly-GR and poly-PR peptides, which migrated to the nucleus and bound to nucleoli, impaired both premRNA splicing and the biogenesis of ribosomal RNA, resulting in cell death. However, our study showed that the expression of poly-GR, but not poly-PR, was cytotoxic in Neuro2a cells (Fig. 9B). The reason for this discrepancy may reflect the different cell types and experimental conditions. Another possible explanation is that poly-PR forms high-molecular-weight complexes and IBs in neuronal cells more readily than poly-GR. Several lines of experimental evidence have indicated that IBs reduce intracellular levels of diffuse toxic proteins, such as the overexpression of polyglutamine, suggesting that they may serve a neuroprotective function (45,46). Furthermore, soluble oligomers, such as β-amyloid and α-synuclein, are reported more cytotoxic than fibrillar aggregates in general (47–51). Therefore, the aggregation of poly-PR possibly attenuated cellular toxicity in our experiment.

Previous studies have shown that the poly-GA of the EBNA-1, which inhibits ubiquitin-dependent proteolysis in cis (34,35), also exhibits trans-inhibition through a yet unknown mechanism (52,53). Recently, Zhang et al. (21) reported that expression of poly-GA proteins inhibited proteasome activity and resulted in accumulation of ubiquitinated proteins. Another important finding of the present study is that the expression of three DRPs, poly-GA, poly-GP and poly-GR, interfered with UPS and enhanced the sensitivity to a proteasome inhibitor, demonstrating the cytotoxicity of DRPs via UPS dysfunction (Fig. 9). As shown in Figure 8A, the contribution of the UPS to DRP degradation was limited. As DRPs consisted only of dipeptide repeats, few endopeptidases recognized DRPs, which may lead to the delay in the degradation of these proteins, possibly affecting normal protein homeostasis. Notably, our study demonstrated that DRP expression potentially interferes with the homeostasis of the TDP-43 protein, which is a critical protein that is strongly associated with the degree of degeneration in c9FTD/ALS and accumulates in the brains and spinal cords of those affected (37). Because evidence of an interaction between C9orf72 expansion and TDP-43 pathology is lacking so far, our findings provided the novel insight that UPS dysfunction induced by DRPs may contribute to the development of TDP-43 proteinopathy in c9FTD/ALS. Surprisingly, poly-PR decreased the level of UPS reporter. Although one recent study demonstrated that poly-PR peptide impaired the biogenesis of ribosomal RNA and led to interference of translation (24), our research showed that the level of control of native GFP did not change from that of the control. Furthermore, proteasome inhibitor markedly increased UPS reporter in cells with poly-PR to the same level extent as in cells with empty vector (Supplementary Material, Fig. S7), indicating that this effect on reporter by poly-PR is dependent on UPS function. Although the molecular basis of the interaction between poly-PR and the UPS remains unknown, the present findings suggest that poly-PR exhibits a different effect on the UPS compared with other DRPs.

When interpreting the results of the present study, several points should be kept in mind. First, DRP-immunoreactive inclusions were frequent in granular neurons of the cerebellum in c9FTD/ALS (17,18). Although patients with c9FTD/ALS exhibit cerebellar atrophy, which is not typically observed in other types of FTD, clinical cerebellar ataxia is rare (54,55). A recent report revealed that DRP pathology is not correlated with TDP-43 pathology and the degree of neurodegeneration (37). Hence, while it may be premature to conclude that DRP expression is the core pathological mechanism of c9FTD/ALS, future studies are needed to examine neurodegeneration and motor neuron-dominant phenotypes in DRP-transgenic animals.

Second, poly-GA IBs were TDP-43 negative and p62/ubiquitin positive (Fig. 3), similar to the characteristic DRP inclusions observed in patients with c9FTD/ALS (17,18). However, investigation of DRP co-expression indicated that poly-GA IBs did not recruit other DRPs (Supplementary Material, Fig. S6). Rather, poly-GR and poly-PR IBs, which are TDP-43 positive and p62/ubiquitin negative (Fig. 4, Supplementary Material, Figs S4 and 5), attract other DRPs and are potentially seeds for mixed-DRP IBs (Fig. 6). Pathological observations in patients with c9FTD/ALS indicate that PR inclusions, which co-aggregate with poly-GR (56,57), are p62-positive and show TDP-43-negative immunoreactivity (17,18). Therefore, the DRP IBs detected in our study do not completely reflect the features of the pathological inclusions in patients with c9FTD/ALS. Further detailed studies are required to identify the molecular processes underlying IB formation in vitro, in vivo and also in patient samples, and to determine the pathological significance of DRP IBs.

Finally, several lines of evidence provided by induced pluripotent stem cells (iPSCs) derived from patients with c9FTD/ALS revealed the existence of toxic RNA gain-of-function mechanisms based on repeat-containing RNA foci as a cause of c9ALS and have suggested candidate anti-sense oligonucleotide therapeutic agents (8,9). The present study revealed that DRPs potentially interfere with protein homeostasis, including TDP-43, exhibit cytotoxicity, independent of GC-rich RNAs, and may thus serve as novel therapeutic targets in c9FTD/ALS.

Materials and Methods

Cell culture

HeLa human carcinoma cells, human embryonic kidney 293 cells (293T cells), COS African monkey kidney cells and ATG5(−/−)
MEFs (58) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum. Neuro2a neuroblastoma cells were cultured in αMEM (Gibco) supplemented with 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) whereas Lipofectamine and Plus reagent (Life Technologies) was used for Neuro2a cells according to the manufacturer’s instructions. Autophagy was inhibited with 3-MA (Sigma, St. Louis, MO, USA), and the proteasome was inhibited with MG132 (Sigma).

**Neuronal cell culture**

All animal experiments were performed in accordance with the Institutional Guidelines on Animal Experimentation at Keio University and approved by the Keio University Institutional Animal Care and Use Committee. Primary neuronal cultures were prepared from the cortices of mice embryos as described previously (59) with some modifications. Briefly, cortices of ICR mice embryos at embryonic day 14 (E14) were dissociated into single cells in 10 U/ml papain solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and resuspended in fresh culture medium. Nerve culture medium was prepared from Neurobasal medium (Life Technologies Corporation, CA, USA) with 2% B-27 supplement (Life Technologies Corporation) and 1% penicillin/streptomycin (Life Technologies Corporation). Transfections were performed with Lipofectamine 2000. Neurons were seeded onto 12-mm poly-L-lysine-coated glass coverslips.

**cDNAs and plasmids**

cDNAs encoding poly-GA, poly-GP and poly-GR (sequences are shown in Supplementary information) were synthesized (Life Technologies Corporation) and subcloned into pcDNA (3.1)/V5-His (Life Technologies Corporation), pEGFP-N1 (Clontech, CA, USA) or pRES2-DoRed2 (Clontech). For in utero electroporation, cDNAs were subcloned into pT2K-CAGGSY. Repeat number variants of poly-GA and poly-GR were generated using a KOD-Plus-site-directed mutagenesis kit (Toyobo, Osaka, Japan). GFP-LC3, and pT2K-CAGGSY, Ub-G76V-GFP (Addgene #11941) plasmids were kindly provided from Dr Mamoru Shibata (Department of Neurology, Keio University), Dr Koichi Kawakami (Division of Molecular and Developmental Biology, National Institute of Genetics) and Dr Nico Dantuma (Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden).

**Antibodies and reagents**

Rabbit polyclonal anti-HA (Y-11), mouse monoclonal anti-ubiquitin, HuR and Bnn-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-α tubulin and anti-V5 were from Invitrogen. Mouse monoclonal anti-FLAG antibody M2 was from Sigma. Rabbit polyclonal anti-V5 (C464.6) and anti-N-terminus fus was from Bethyl Labs (Montgomery, TX, USA). Anti-TDP-43 and G3BP antibodies were from Proteintech (Chicago, IL, USA). Rabbit polyclonal anti-red fluorescent protein (RFP) antibody was from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). Anti-p62/SQSTM1 was obtained from Medical & Biological Laboratories (Nagoya, Japan). Anti-active caspase-3 antibody was purchased from BD Pharmsing (San Jose, CA, USA). Mouse Anti-Ataxin-2 was purchased from BD Biosciences (Sparks, MD, USA). MG132 was purchased from Sigma. Rabbit polyclonal anti-fibrillarin antibodies were from Cell Signaling Technology (Beverly, MA, USA).

**Immunoblot analysis and filter retardation assay**

Cells were briefly sonicated in cold lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.25% sodium dodecyl sulfate, 5 mM EDTA and protease inhibitor cocktail (Sigma)]. Cell lysates were separated using SDS–PAGE with 10% Tris–glycine gel, after which proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Dot blot was performed applying lysates on nitrocellulose membrane (Bio-Rad). For filter retardation assays, cell lysates [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA and protease inhibitor cocktail (Sigma)] were filtered through 0.2-µm cellulose acetate membrane (Sartorius Stedim Biotech, NY, USA) filters using a slot blot apparatus (Bio-Rad), as described previously (60). The membrane was incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies and then visualized using enhanced chemiluminescence reagents (PerkinElmer Life Sciences, Boston, MA, USA). Protein levels in Figures 8 and 9 were determined by densitometry using an Epson ES-2000 scanner and Image J (National Institutes of Health, Bethesda, MD, USA).

**Immunofluorescence with cultured cells**

At 48 h after transfection, cells were fixed with 4% paraformaldehyde on ice for 10 min and then permeabilized in 0.2% Triton X-100 in room temperature for 10 min. Post-fixation with 4% paraformaldehyde on ice for 5 min followed. After blocking of nonspecific binding, coverslips were incubated with primary antibodies and then incubated with Alexa 488 and/or Alexa 586-conjugated secondary antibodies (Invitrogen). Immunofluorescent staining was examined using a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany).

**In utero electroporation and immunohistochemistry of brain slices**

Pregnant ICR mice (Japan SLC) on embryonic day 15 (E15) were deeply anesthetized with pentobarbital sodium (Nembutal), and their intrauterine embryos were surgically manipulated as described previously (30,61). One- to two-microliter volumes of plasmid solutions at concentrations of 1–2 mg/ml were injected into lateral ventricles and in utero electroporations were performed. ACAG-driven tdTomato protein expression vector (tdTomato/CAGGS1) was co-transfected at a concentration of 1 mg/ml as a reporter. DRP expression vector (pCAGGSY-poly-VA/pT2K-CAGGS1) was co-transfected at a concentration of 2 mg/ml.

Coronal slices of the cerebral cortex at postnatal day 10 were prepared as described previously (29). Briefly, the brains were fixed with 4% paraformaldehyde and cut into 50-µm sections with a cryostat. After blocking of nonspecific binding, brain slices were incubated with mouse monoclonal anti-V5, rabbit polyclonal anti-RFP, rabbit polyclonal anti-FLAG, mouse monoclonal anti-ubiquitin and mouse monoclonal anti-p62 antibodies diluted in phosphate-buffered saline containing 0.1% Triton X-100 and 5% normal donkey serum. After three washes, brain slices were incubated with Alexa 488-conjugated donkey anti-mouse, Alexa 555-conjugated anti-rabbit and Alexa 647-conjugated anti-rabbit secondary antibodies (Invitrogen). The nuclei in sections were labeled with 4′,6-diamindino-2-phenylindole (DAPI; Invitrogen, Molecular Probes). Images were acquired through confocal microscopes (FV300 and FV1000, Olympus Optical).
Statistical analysis

A statistical analysis of the data was performed using the Student’s t-test or Dunnett’s t-test using the Statview 5.0 system (Statview, Berkley, CA, USA) or JMP 8 (SAS Institute, Inc., NC, USA).

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We are grateful to Ms Tamao Kitamura (Department of Neurology, School of Medicine, Keio University) for technical assistance. Dr Jun-ichi Miyazaki (Division of Stem Cell Regulation Research, Osaka University, Osaka, Japan) for providing pCAGGS, Dr Koichi Kawakami (Division of Molecular and Developmental Biology, National Institute of Genetics) for providing pCAGGS, Dr Koichi Kawakami (Division of Molecular and Developmental Biology, The University of Tokyo) for providing ATG5(−/−) MEFs. We thank the Collaborative Research Resources, School of Medicine, Keio University, for technical assistance.

Conflict of interest statement: None declared.

Funding

This work was supported by Eisai Co. Ltd. and the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 23591254, 22111004, and 22240041).

References


