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Expanded Glutamine Repeat Enhances Complex Formation of Dentatorubral-Pallidoluysian Atrophy (DRPLA) Protein in Human Brains

Ikuru Yazawa,*,† Noriko Hazeki,* and Ichiro Kanazawa*

**Department of Neurology, Division of Neuroscience, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; and †Department of Neurology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan*

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The genetic defect in dentatorubral-pallidoluysian atrophy (DRPLA) is expansion of the CAG repeat. The mutant gene is translated into the protein which carries the expanded glutamine repeat. Immunoblots of human brain tissues with and without reduction show that the DRPLA protein is a disulfide-bond complex and that more of this complex is formed in DRPLA brains than in control brains. This suggests that DRPLA protein undergoes greater complex formation in DRPLA brains and the expanded glutamine repeat may enhance complex formation of untruncated DRPLA protein in DRPLA brains. Immunohistochemical findings show that DRPLA protein is localized in the cytoplasm of the neuron, evidence that it undergoes rare disulfide bonding there. © 1998 Academic Press

Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder characterized clinically by progressive dementia, ataxic gait, and involuntary movements (chorea and myoclonus) [1, 2]. The genetic defect that underlies DRPLA is expansion of a CAG repeat [3, 4]. Similar expansions have been found in seven other neurodegenerative disorders: spinobulbar muscular atrophy, Huntington's disease (HD), spinocerebellar ataxia type 1 (SCA1), SCA2, Machado-Joseph disease (MJD), SCA6, and SCA7 [5-13]. In our previous reports, we identified the DRPLA gene product (DRPLA protein) in human brains and showed that the mutant DRPLA gene with the expanded CAG repeat is translated into protein which carries an expanded glutamine repeat [14, 15]. We also showed that the amount of this mutant protein is similar to that of the wild-type protein in the DRPLA brain, indicative that regulation of the level of translation of the DRPLA gene is not central to the development of the disease [16].

Two important hypotheses regarding the protein interaction of the gene product have been proposed in the glutamine repeat disease. One is that the mutant protein is made up of transglutaminase substrates that form cross-linked products with other proteins [17]. The other is that the expanded glutamine repeats may acquire excessively high affinities for each other or acquire non-specific affinities for other regulatory proteins thereby producing agglomerated products in the neurons [18]. Both hypotheses are based on the idea that the mutant protein aggregates in neurons as a result of the expanded glutamine repeat. Recently, peptides carrying the glutamine repeat were shown to form insoluble aggregates with proteins in rat brain extracts in the presence of transglutaminase [19]. In addition, glutathione S-transferase (GST)-huntingtin fusion protein forms insoluble high molecular weight protein aggregates only when the glutamine repeat is expanded [20], and this expanded repeat caused abnormal insoluble aggregation of the partial gene products in vitro. We present evidence of the complex formation of DRPLA protein in human brains and examine the effect of the expanded glutamine repeat on complex formation in DRPLA brains.

We previously showed that polyclonal antibodies against the C-terminal DRPLA protein stained the cytoplasms of neurons in postmortem brain tissues [14]. Because the antibodies detected an unknown protein with the molecular weight of about 100 kDa (p100) in addition to DRPLA protein on the immunoblots, the localization of the DRPLA protein was not completely clear. As rat DRPLA protein was detected, but not the unknown p100, on the immunoblots of rat brain tissues, by means of an immunohistochemical study we were able to show the localization of DRPLA proteins in rat brains. Moreover, we characterized the DRPLA protein in human brains as a disulfide-bonded complex and compared its formation in human control brains and in DRPLA brains to determine the effect of the expanded glutamine repeat on DRPLA protein.

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MATERIALS AND METHODS

Antibody production against DRPLA protein. Polyclonal antibodies (C580) that had been raised in rabbits against synthetic peptides corresponding to the C-terminal end of the human DRPLA protein (amino acids 1171-1184) [21] were purified by affinity chromatography as described elsewhere [14].

Glutathione S-transferase (GST) fusion protein was generated as follows: Sequences encoding residues 13-168 of human DRPLA protein were amplified by the polymerase chain reaction (PCR) using the primer pair 5′-CGTGGATCCCGAGGAGTGGACGGAAGAAAG-3′ and 5′-ACAGTCGACTCAGGAAGGAGGAAAGAGTGGAG-3′ as described previously [14]. The PCR products were digested with the BamHI and SalI sites and cloned into vector pGEX-5X1 (Pharmacia). The resulting plasmids carried the fusion proteins of GST and the N-terminal residues of human DRPLA protein. The GST fusion protein was expressed in bacteria then fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and designated GST-N01, after which its protein concentration was determined. Approximately 300 μg of GST-N01 combined with complete Freund's adjuvant was injected subcutaneously into rabbits. Two weeks later, the same dose of fusion protein plus incomplete adjuvant was given as a booster, after which booster injections were given every 4 weeks. Ten days after the final booster, antisera were obtained and analyzed by the enzyme-linked immunosorbent assay. The polyclonal antibodies present were purified by affinity chromatography (Multiple Peptide systems) coupled with GST-N01, and designated N01. In the absorption experiment, 50 μg of GST-N01 was added to 1 ml of adequately diluted antibodies. The mixture was incubated overnight at 4°C then centrifuged at 10,000g for 20 min. The supernatant was collected for analysis. The immunoreactive reaction of N01 was abolished by preabsorption with GST-N01 on the immunoblots.

Sample preparation. Postmortem brain tissue samples from five DRPLA patients (24-69 years old), whose disease had been diagnosed genetically by the PCR analysis [14, 22] and confirmed pathologically as were samples from four control patients (59-79 years old), and brain tissues of two rats (body weight; 150 and 450 g) were examined. Each cerebral hemisphere was stored at −80°C. Tissue samples (2 g) of cerebral cortices from human control and DRPLA brains and the total rat brain tissue samples (1.2 and 1.84 g) were each homogenized in 5 volumes of tris-saline buffer with protease inhibitors (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 μg/ml aprotinin, 1 mM EDTA, 10 μg/ml Leupeptin, 0.5 mM Pefabloc SC, and 10 μg/ml Pepstatin) with and without 1 mM N-ethylmaleimide (NEM). Samples were stored at −80°C until used for gel electrophoresis.

Reducing/non-reducing electrophoresis and immunoblots. After protein was determined by the bicinchoninic acid (BCA) protein assay (Pierce), samples of the brain tissues were mixed with equal volumes of sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 160 mM Tris-HCl, pH 6.8, 20% glycerol) with and without a reducing agent (10% 2-mercaptoethanol). The acrylamide concentration of the stackings was 5% and that of the running gels 7.5% in 0.1% SDS. Samples (10 μg each) were electrophoresed in the gel, then SDS-polyacrylamide gel electrophoresis (PAGE) was done. After electrophoresis, the gels were subjected to immunoblotting. Proteins were transferred electrophoretically to a polyvinylidene difluoride (Immobilon) membrane (Millipore), which then was blocked with 4% nonfat milk and stained overnight at 4°C with the two affinity-purified polyclonal antibodies (N01 and C580). The Immobilon membrane was incubated for 1 hour at room temperature with anti-rabbit secondary antibodies at 1:300 dilution. The reaction was made visible with an enhanced chemiluminescence (ECL) Western blotting system (Amersham).

Immunohistochemistry. Fresh blocks of the two rat brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 18 hours, after which they were incubated at 4°C in PB containing 25% sucrose then frozen. Cryostat sections of 4% paraformaldehyde-fixed blocks, that had been incubated for 30 minutes in 0.3% hydrogen peroxide in methanol, were incubated overnight at 4°C with the two affinity-purified antibodies. The reaction products were made visible by the avidin-biotin-peroxidase complex method (Vectastain) with hematoxylin as the counterstain.

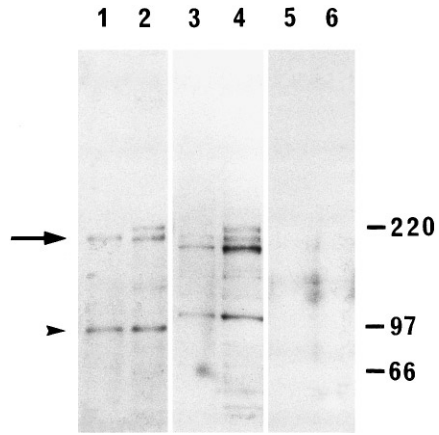

FIG. 1. Immunoblots of the total homogenates of the cerebral cortices of a human control (lanes 1, 3, 5) and brain tissues of a DRPLA patient (lanes 2, 4 ,6) (10 μg of protein per lane) stained with C580 (lanes 1, 2) and N01 (lanes 3-6) under reduced conditions. Both antibodies detected the wild-type and mutant reduced forms of DRPLA proteins with apparent molecular masses of about 195-205 kDa (arrow) and 205-220 kDa. In the absorption experiment with N01, 100 μg of the GST-N01 fusion protein was added to 1 ml of adequately diluted N01, and the mixture was incubated overnight at 4°C, then centrifuged at 10,000*g* for 20 min. The supernatant was collected for analysis. The immunoreactive reactions of N01 on the immunoblots were abolished by preabsorption with GST-N01 (lanes 5, 6). In addition to the DRPLA proteins, C580 detected the unknown 100kDa protein; p100 (arrowhead) (lanes 1, 2) and N01 detected the unknown 180 and 110kDa proteins (lanes 3, 4).

RESULTS

Formation of disulfide bonding in human DRPLA protein. In an earlier study, we raised the polyclonal antibody, C580, against synthetic peptides corresponding to the C-terminus residues (amino acid 1175-1184) of human DRPLA protein [14]. We here generated rabbit polyclonal antibody raised against bacterially produced fusion protein, designated N01, that had N-terminus residues (amino acid 13-168) of human DRPLA protein [21]. The total homogenates of the human control, DRPLA patient, and rat brain tissues were analyzed in SDS-polyacrylamide gels. The gels were immunoblotted using the two rabbit polyclonal antibodies (N01 and C580). On the immunoblots of human control and DRPLA brain tissue samples under reducing conditions, the two antibodies detected the untrucated wild-type DRPLA protein which migrated in a band that had the apparent molecular weight of approximately 195-205kDa (wild-type reduced form) and an additional mutant DRPLA protein with a molecular weight of about 205-220kDa (mutant reduced form) only in DRPLA brains (Fig. 1).

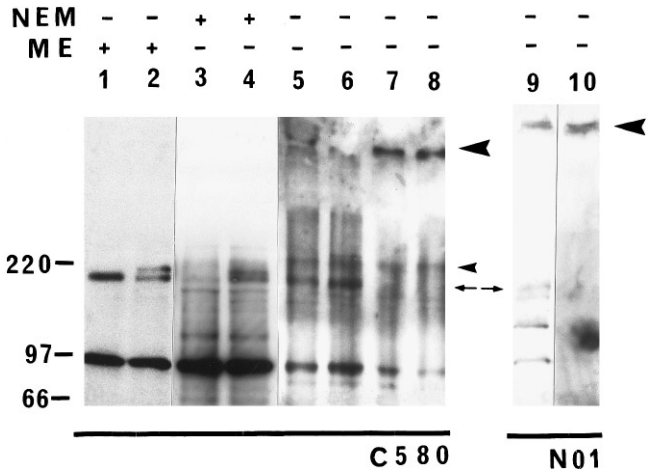

FIG. 2. Immunoblots of the total homogenates of the cerebral cortices of 2 human controls (lanes 1, 3, 5, 6, 9) and brain tissues of 2 DRPLA patients (lanes 2, 4, 7, 8, 10) with (lanes 3, 4) and without (lanes 1, 2, 5-10) N-ethylmaleimide (NEM) (10 μg protein per lane), stained with C580 and N01 under the reduced (lanes 1, 2) and non-reduced (lanes 3-10) conditions. Immunoblots of the NEM-treated human control and DRPLA brain tissues showed the non-reduced form (small arrowhead) in addition to the reduced forms of the DRPLA protein (arrow). C580 and N01 both detected the complex (large arrowhead) located at the top of the stacking gel and the non-reduced form of the DRPLA protein (small arrowhead) on immunoblots of the human control and DRPLA brain tissue samples without NEM treatment under non-reduced conditons. Moreover, the immunoblots of the NEM-untreated DRPLA brain tissue samples showed no immunoreactivity of the reduced wild-type or mutant forms of the DRPLA protein, and the immunoreactivity of the complex form of the protein was higher than that of the control brain tissue samples. ME is the abbreviation for β-mercaptoethanol. The molecular weight markers are indicated on the left.

On the immunoblots of the human control and DRPLA brain tissue samples under non-reducing conditions, the DRPLA proteins migrated differently when treated or not treated with N-ethylmaleimide (NEM), a reagent that blocks free sulfhydryls. The DRPLA proteins, however, did not migrate differently on the immunoblots when treated or not treated with NEM under reducing conditions. In the NEM-treated samples of the human control brain tissues under non-reducing conditions, immunoblots stained with the two antibodies showed the reduced wild-type form and another band with an apparent molecular weight larger than 250kDa, which we designated the non-reduced form (Fig. 2). In contrast, on the immunoblots of human control brain tissue samples without NEM treatment and under non-reducing conditions the reduced wild-type form was replaced by three bands; the reduced form, the non-reduced form, and one of higher apparent molecular weight at the top of the stacking gel, designated the complex form (Fig. 2). These three forms were detected by both antibodies, indicatve that the bands contained the untruncated DRPLA protein. The latter two forms show that the wild-type protein forms disulfide-bond complexes, evidence of the presence of disulfide bonds formed by the cysteines, which are stable in SDS but broken when treated with 2-mercaptoethanol. As shown on the immunoblots of the NEM-treated control brain tissue samples, the non-reduced form of DRPLA protein indicates that human DRPLA protein undergoes disulfide bonding in vivo. In addition, the complex form of the DRPLA protein on the immunoblots of the NEM-untreated control brain tissue samples indicates that human DRPLA protein forms disulfide-bonded high molecular weight complexes. The immunoblots of the NEM-treated DRPLA brain tissue samples under non-reducing conditions showed bands similar to those of the human control brain tissue samples, except for an additional band of the reduced mutant form of the DRPLA protein. The immunoblots of the DRPLA brain tissue samples without NEM treatment under non-reducing conditions differed markedly from those of the control brain tissue samples. These immunoblots showed only the non-reduced form and the complex form at the top of the gel. There was no immunoreactivity of the reduced mutant and wild-type forms, evidence that the extent of disulfide bond formation by DRPLA protein differed in the human control and diseased brains and that DRPLA protein formed more disulfide bonds in the DRPLA brains (Fig. 2).

Localization of DRPLA protein in rat brain. Immunoblots of rat brain tissue samples showed N01 and C580 as a single band with the same apparent molecular mass, about 195kDa, as that of the human untruncated wild-type DRPLA protein (Fig. 3A). Because the rat DRPLA gene is reported to have an amino acid sequence 92% homologous to that of the human gene, consisting of 1180 amino acids, and because the sites recognized with the two antibodies are the same in the sequences of the human and rat DRPLA proteins [23], we identified the approximately 195kDa band as rat DRPLA protein. No p100 which was present on the immunoblots of the human control and DRPLA brain tissue samples with C580, was detected on the immunoblots of the rat brain tissue samples. Whether p100 is the product of endoproteolytic cleavage or is a degradation product of the DRPLA protein is still not clear, but using an immunohistochemical method, we identified the actual localization of the rat DRPLA protein.

Immunohistochemical staining of the rat brain tissues showed that the immunoreactivity of C580 is located primarily in the neuronal cytoplasm in rat brain tissues, confirming that DRPLA proteins have a predominantly neuron-specific distribution (Fig. 3B).

DISCUSSION

We found that the DRPLA protein forms a high molecular weight complex with disulfide bondings on immunoblots of NEM-untreated human brain tissue sam-

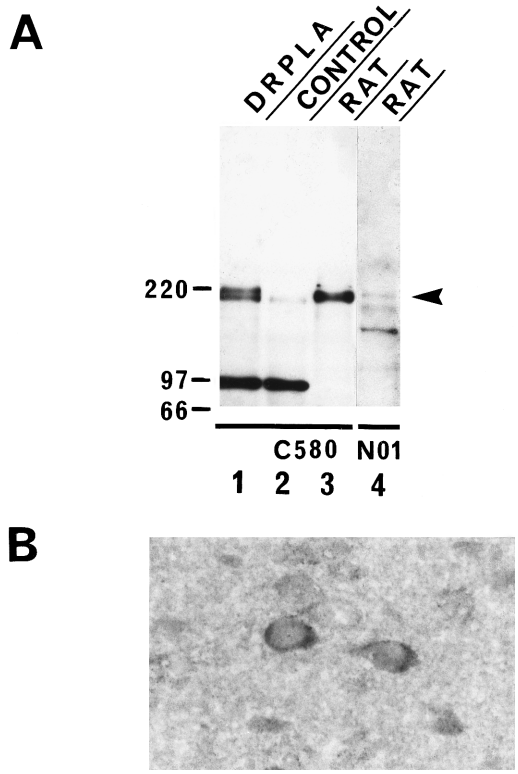

FIG. 3. (A) Immunoblots of the total homogenates of the cerebral cortices of a DRPLA patient (lane 1), human control (lane 2), and of rat brain tissues (lanes 3, 4) (10 μg of protein per lane). Stained with C580 (lanes 1-3) and with N01 (lane 4) under reducing conditions. Immunoblots of the rat brain tissue samples, obtained under reducing conditions and stained with C580, showed that the rat DRPLA protein migrated in a single band with an apparent molecular mass of 195kDa similar to that of the human wild-type DRPLA protein (arrow). There was no p100 immunoreactivity. (B) Immunohistochemistry of rat brain tissues stained with C580. The antibody is located in the cytoplasms of the neurons in the rat cerebral cortex. Magnification, ×280.

ples under non-reducing conditions. To determine the effect of the expanded glutamine repeat on the complex formation of the DRPLA protein in the diseased brains, we compared disulfide bond formation of the DRPLA proteins in human control brains with that in the diseased brains, and showed that more of the disulfide-bond complex of the DRPLA protein was present in DRPLA brains than in the control brains based on immunoblot data obtained under non-reducing conditions. As there is more DRPLA protein complex in human diseased brains than in the control brains, the expanded glutamine repeat may affect DRPLA protein complex formation, producing greater complex in the diseased brains. The development of neuronal intranuclear inclusions in HD transgenic mice with expanded CAG repeats is suspected to have a role in developing the HD phenotype [24]. These intranuclear inclusions were found to be abnormal accumulations of the HD gene product that localizes in the cytoplasms of neurons in rat and human brains [25-28]. The dense accumulation of the huntingtin also has been reported in the cytoplasms of neurons in HD transgenic mice. Intranuclear inclusions have been reported in MJD and DRPLA brains [29, 30], and neuronal intranuclear inclusions and dystrophic neurites in HD brains [31]. Abnormal aggregation of the truncated gene product was shown to occur in glutamine repeat disease. Our findings suggest that untruncated DRPLA protein may undergo abnormal complex formation due to the protein interaction produced by the expanded glutamine repeat in DRPLA brains. Moreover, it is interesting that the untruncated wild-type DRPLA protein in DRPLA brains is different in complex formation from that in human control brains, but similar to the mutant DRPLA protein. This result is consistent with the previous findings of the DRPLA protein using the 2-dimensional electrophoretic analysis, which showed the isoelectric variants of the wild-type protein in DRPLA brains were different from those of the wild-type protein in human control brains, but similar to those of the mutant protein [32]. As Perutz et al hypothesized that glutamine repeats may function as polar zipper and acquire excessively high affinities for each other when they become too long [18], we speculate that the mutant protein with expanded glutamine repeat may interact with the wild-type protein and affect the complex formation of the wild-type protein in DRPLA brains.

We have characterized the DRPLA protein as a disulfide bonding complex and showed that these proteins may form a large molecular protein complex under specific conditions. Human DRPLA protein carries three cysteine residues in its sequence that are downstream from the glutamine repeat on the C-terminal half [21]. Because the immunoblots of the NEM-treated human brain samples showed the non-reduced form and those of the NEM-untreated samples showed the complex form, we speculate that the cysteines are involved in forming the disulfide bonds of the DRPLA proteins and have a role in forming the large molecular protein complex. The immunohistochemical study with the antibodies against the C-terminus of the DRPLA protein showed that the DRPLA protein is localized in the cytoplasms of rat neurons. Recently, full-length DRPLA cDNA was found to express DRPLA protein in the cytoplasms of COS-7 cells [30]. The DRPLA protein therefore is a cytoplasmic protein. Formation of disulfide bonds between cysteine residues often serves to stabilize the structure of extracellular proteins [33], and such bonds commonly are present in exported proteins in Escherichia coli as in eukaryotes [34-37], but they rarely are present in cytoplasmic proteins [37]. The absence from the cytoplasm of proteins with disulfide bonds generally is attributed to the reducing environment [34] although the endoplasmic reticulum maintains an oxidative environment under the reducing en-

vironment of the cytoplasm [38]. Restriction of the cellular compartment is one way to form cytoplasmic disulfide bonds [35, 37]. In order to rule out the possibility that the DRPLA protein is an exported protein, we examined whether cultured HeLa cells secrete DRPLA protein into the medium and found very little of it on the immunoblots, indicative that the DRPLA protein is not an exported protein (data not shown). These findings suggest that the disulfide bond formation by the DRPLA protein, one of the cytoplasmic proteins in the neurons, may be preserved by restriction of the cellular compartment. Disulfide bonds are important to structural folding and the stability of many proteins [39, 40], but little is known about the vivo mechanisms of their formation. The findings presented here suggest that the rare cytoplasmic disulfide bonds are important to the folding and stability of the DRPLA protein.

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