Aberrant phosphorylation of dentatorubral-pallidoluysian atrophy (DRPLA) protein complex in brain tissue

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Dentatorubral-pallidoluysian atrophy (DRPLA) is caused by DRPLA protein which carries expansion of a glutamine repeat. Abnormal high-molecular-mass complex formation by DRPLA protein and its pathological ubiquitination comprise the disease processes in the brains of patients with DRPLA. In this study, DRPLA protein complex was isolated and shown to have pathologically stronger bond formation with DRPLA proteins in DRPLA brain tissue compared with control brain tissue. Immunochemical methods and an enzymic dephosphorylation technique were used to demonstrate that DRPLA protein com-

plex is aberrantly phosphorylated in DRPLA brain tissue. Immunohistochemical studies show that both the ubiquitinated cytoplasmic inclusions and the nuclear membrane are aberrantly phosphorylated in DRPLA-affected neurons. This finding suggests that the nuclear membrane is another pathological focus of DRPLA neurodegeneration.

Key words: DRPLA gene product, glutamine repeat, neuronal inclusion, polyglutamine, ubiquitin.

INTRODUCTION

Dentatorubral-pallidoluysian atrophy (DRPLA) is a hereditary neurodegenerative disorder caused by expansion of a glutamine repeat in DRPLA protein [1–3]. Similar expansions have been found in seven other neurodegenerative disorders: spinobulbar muscular atrophy, Huntington's disease (HD), spinocerebellar ataxia (SCA) 1, Machado-Joseph disease, and SCA 2, SCA 6 and SCA 7, referred to as glutamine-repeat or polyglutamine diseases (here, the diseases are listed in the order in which the genes responsible for them were identified) [4–12]. Neuronal intranuclear inclusions are found in brain tissues of patients with HD and HD transgenic mice [13,14]. The HD gene product was surmised to aggregate in HD neurons. Similar neuronal intranuclear inclusions showing immunoreactivity with the gene product have also been reported in brain tissues of patients with other glutamine-repeat diseases, including DRPLA and the other glutamine-repeat disease transgenic mice [15–19]. Aggregation of the gene product that carries an expanded glutamine repeat seems to be a primary pathological mechanism in glutamine-repeat diseases, although the precise relationship between aggregation and neuronal degeneration is uncertain. Previous studies, using immunoblotting of normal control and DRPLA brain tissue with anti-DRPLA protein antibodies, showed that DRPLA protein forms a high-molecular-mass complex, and that more of the DRPLA protein complex was present in DRPLA brain tissue than in control brain tissue from immunoblotting data obtained under non-reducing conditions [20]. Subsequent immunohistochemical studies of control and DRPLA brain tissue using an anti-DRPLA protein antibody demonstrated that immunoreactivity of DRPLA protein, appearing as cluster formations, corresponds to complex formation by DRPLA protein in immunoblotting [21], and that characteristic neuronal

cytoplasmic inclusions only in DRPLA brain tissue showed that DRPLA protein complex is found in the core of the inclusions, and that this core is surrounded by ubiquitin (UBI)-bonded proteins [22]. These results suggest that DRPLA protein complex is involved in high-molecular-mass complex formation in human brain tissue, and that expansion of the glutamine repeat may affect DRPLA protein complex formation, producing an abnormally large complex in diseased brains.

The aim of the present study is to investigate the possibility that the distinct properties of abnormal DRPLA protein complex result from aberrant post-translational modification of normal DRPLA protein complex. A previous study which analysed DRPLA protein by two-dimensional electrophoresis identified isoelectric variants of DRPLA protein in human brain tissues, and revealed a difference in the pI range of the isoelectric variants in post-translational modifications between wild-type and mutant DRPLA proteins in DRPLA brain tissue [23]. The presence of isoelectric variants of DRPLA protein raised the possibility that DRPLA protein may be phosphorylated in human brain tissues. In addition, because DRPLA protein carries a long serine repeat close to the expanded glutamine repeat in its predicted amino acid sequence [24], perhaps the expanded glutamine repeat influences the phosphorylation of serine residues in DRPLA protein. Post-translational modifications may not appear to specify unique biochemical properties of DRPLA protein itself, but may also be a critical factor in determining the structural integrity of DRPLA protein complex. In the present study, high-molecular-mass DRPLA protein complex was isolated from human control brain tissue and DRPLA brain tissue in SDS/polyacrylamide gels, and phosphorylation of the isolated DRPLA protein complex was examined immunochemically and by an enzymic dephosphorylation technique. Phosphorylation of neuronal proteins may relate to neurodegenerative disorders;

Abbreviations used: DRPLA, dentatorubral-pallidoluysian atrophy; HD, Huntington's disease; SCA1, spinocerebellar ataxia 1; PSR, phosphoserine; PTN, phosphothreonine; UBI, ubiquitin; HNM, human nuclear membrane.

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for example, tau protein was shown to be abnormally phosphorylated in the brain tissues of patients with Alzheimer's disease and hereditary frontotemporal dementia and parkinsonism linked to chromosome 17 [25–28]. The present study demonstrates that abnormal DRPLA protein complex is aberrantly phosphorylated in the diseased DRPLA brain. By gaining insights into abnormal protein-complex formation in DRPLA, one of glutamine-repeat diseases, new evidence is provided of a pathological mechanism for the neurodegeneration observed in glutamine-repeat diseases.

MATERIALS AND METHODS

Sample preparation

Post-mortem brain tissue samples from five DRPLA patients (24–69 years old), whose disease had been diagnosed genetically by PCR analysis and confirmed pathologically, and brain tissue samples from five control subjects (59–79 years old) were examined [3,29,30]. Tissue samples (1 g) from the cerebral frontal cortices were homogenized separately in 5 vol. 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 (Boehringer Mannheim, Mannheim, Germany) and 10 µg/ml pepstatin]. Samples were stored at –80 °C until subjected to gel electrophoresis.

Double-electrophoresis under non-reducing and reducing conditions

Protein concentrations in brain tissue homogenates were measured by bicinchoninic acid protein assay (Pierce). Each sample of brain tissue was mixed with an equal volume of SDS sample buffer (4 % SDS, 160 mM Tris/HCl, pH 6.8, 20 % glycerol) without any reducing agent, and placed in boiling water for 8 min. The first electrophoresis was performed using 0.1 % SDS-containing polyacrylamide gel under non-reducing conditions. The acrylamide concentration of the stacking gels was 5 %, and that of the running gels 7.5 %. Samples (10 µg each) were electrophoresed, and the gels were stained using the standard Coomassie technique to verify bands of protein complex at the top of the stacking gels. Each band was cut out of the SDS/polyacrylamide gel and incubated in reducing buffer (160 mM Tris/HCl, pH 6.8, 20 % 2-mercaptoethanol) for 30 min at 90 °C, before being applied to another SDS/polyacrylamide gel for the second electrophoresis. The gels were then subjected to immunoblotting studies.

Dephosphorylation of DRPLA protein complex in SDS/polyacrylamide gel

After the protein concentrations in the brain tissue homogenates were measured, electrophoresis was run twice under non-reducing conditions. Each sample of brain tissue was mixed with an equal volume of SDS sample buffer without any reducing agent and then placed in boiling water for 8 min. Electrophoresis was performed twice using 0.1 % SDS-containing polyacrylamide gel under non-reducing conditions. Samples (10 µg each) were first electrophoresed in the SDS/polyacrylamide gels, and the gels were stained by the standard Coomassie technique. Each band at the top of the stacking gels was cut out and incubated for 60 min at 37 °C in alkaline phosphatase buffer (160 mM Tris/HCl, pH 9.2, 1 mM pefabloc SC) containing calf intestinal alkaline phosphatase (Sigma) added to a final concentration of 1000 units/ml. For control experiments, gel pieces were incubated in the same buffer without alkaline phosphatase for 60 min at

37 °C. The dephosphorylated and non-dephosphorylated gel pieces were then resolved on another SDS/polyacrylamide gel for the second electrophoresis under non-reducing conditions. Finally, the gels were subjected to immunoblotting.

Immunoblotting

Proteins in the gels were transferred electrophoretically to a PVDF membrane (Immobilon; Millipore), which was then blocked with the Tris buffer solution containing 4 % non-fat milk powder and 1 % crystallized BSA (Sigma) for anti-DRPLA protein polyclonal antibody (C580) and anti-UBI polyclonal antibody (Dako), and with 1 % BSA for anti-phosphoserine (anti-PSR) monoclonal antibody (Sigma) and anti-phosphothreonine (anti-PTN) monoclonal antibody (Sigma), which were used for the localization of proteins containing PSR and PTN. Each Immobilon membrane was left undisturbed for 16 h at 4 °C with four primary antibodies: C580, anti-UBI, anti-PSR and anti-PTN. C580, which was produced in rabbits against synthetic peptides corresponding to the C-terminal end of human DRPLA protein (amino acids 1171–1184), was purified by affinity chromatography as described elsewhere [3,24]. The Immobilon membrane was then incubated for 1 h at room temperature with secondary antibodies. In order to visualize the immunoreaction, an ECL[®] Western-blotting system (Amersham Pharmacia Biotech) was used.

Single-labelling and double-labelling immunohistochemistry

Blocks of tissue from three human control brains and three DRPLA brains were fixed in 10 % formalin and 4 % paraformaldehyde. Sections from the 10 % formalin-fixed blocks were embedded in paraffin, and then immunostained singly with anti-UBI and doubly with anti-UBI and anti-PSR. For single-labelling and double-labelling immunohistochemistry, sections which had been incubated for 30 min in 0.3 % H₂O₂/methanol were then incubated with the first sequence of primary polyclonal antibody against UBI at a dilution of 1:1500 overnight at 4 °C. The sections were then preincubated with secondary biotinylated anti-rabbit antibody, followed by incubation with avidin-biotinylated horseradish peroxidase complex (Vectastain). 3,3'-Diaminobenzidine (Sigma) was used as the first-reaction substrate, and the reaction product appeared as a brown colour. For double-labelling immunohistochemistry, after brown visualization, the sections were washed with a 100 mM Tris/HCl solution, pH 2.2. The sections were reacted with the second sequence of primary monoclonal antibody against PSR overnight at 4 °C, and consecutively stained with secondary anti-mouse antibody (Nichirei Corporation, Tokyo, Japan), using standard indirect peroxidase and streptavidin-biotin techniques (Histofine SAB-POR Kit; Nichirei Corporation). 3,3',5,5'-Tetramethylbenzidine was used as the second reaction substrate, and reaction products were visualized as a blue colour with a True Blue Peroxidase Substrate Kit (KPL, MD, U.S.A.).

To demonstrate that anti-PSR staining clearly shows the structure of neuronal nuclear membrane, frozen sections taken from the 4 % paraformaldehyde-fixed blocks of the control and DRPLA brain tissues [3] were double-immunostained with anti-human nuclear membrane (anti-HNM) antibody (Leinco Technologies, St Louis, MO, U.S.A.) and anti-PSR. Cryostat sections of the 4 % paraformaldehyde-fixed blocks, that had been incubated with 0.3 % H₂O₂/methanol, were reacted with the first sequence of primary anti-HNM at a dilution of 1:20 overnight at 4 °C, and the nuclear membrane appeared brown. The sections were then reacted with the second sequence of primary anti-PSR, and the reaction product appeared blue.

RESULTS

Isolation of DRPLA protein complex in SDS/polyacrylamide gel

The total homogenates of the human control and DRPLA brain tissues were analysed by double-electrophoresis on SDS/polyacrylamide gels under non-reducing and reducing conditions, followed by immunoblotting stained with polyclonal antibody (C580) against the C terminal of DRPLA protein [3]. Coomassie Blue-stained gels showed no difference in the amount of high-molecular-mass complexes at the top of the stacking gels between the control and DRPLA brain tissues (Figure 1A, lanes 1 and 2). On the immunoblot of the DRPLA brain tissue sample, which was once electrophoresed under non-reducing conditions, greater immunoreactivity of DRPLA protein complex was observed at the top of the stacking gels compared with the control brain tissue (Figure 1A, lanes 5 and 6), as previously described [20]. The immunoblot of DRPLA brain tissue, which was double-electrophoresed on an SDS/polyacrylamide gel under non-reducing and reducing conditions and then stained with C580, showed two apparent bands of mutant and wild-type DRPLA proteins with molecular masses of approx. 220 kDa and 200 kDa (Figure 1B, lane 8, arrows). In contrast, the immunoblot of the control brain tissue showed that C580 exhibited little immunoreactivity of a wild-type DRPLA protein band (Figure 1B, lane 7). These findings indicate that a

greater amount of DRPLA protein in the diseased brain tissue is involved in a high-molecular-mass complex at the top of the stacking gels compared with the control brain tissue.

DRPLA protein complex is aberrantly phosphorylated in DRPLA brain tissue

The total homogenates of control and DRPLA brain tissues were analysed using single-electrophoresis on SDS/polyacrylamide gels under non-reducing conditions. The Coomassie Blue-stained gels showed no difference in the amounts of high-molecular-mass complexes at the top of the stacking gels between the control and DRPLA brain tissues (Figure 2A, lanes 1–4). When the immunoblots of DRPLA brain tissue samples were stained with anti-PSR, apparent immunoreactivity of high-molecular-mass DRPLA protein complex was demonstrated, whereas no immunoreactivity of DRPLA protein complex was observed on the immunoblots of control brain tissue samples (Figure 2A, lanes 5–8). In contrast, when the immunoblots of control and DRPLA brain tissue samples were stained with anti-PTN, no immunoreactive band of high-molecular-mass complex was detected at the top of the stacking gels in any brain tissue samples (Figure 2A, lanes 9–12). Only in diseased brain tissue samples were abnormal DRPLA protein complexes observed containing phosphorylated serine residues.

To verify the findings that DRPLA protein complex is phosphorylated only in DRPLA brain tissue, isolated DRPLA protein complex in an SDS/polyacrylamide gel was examined by an enzymic dephosphorylation technique using alkaline phosphatase. The immunoblot of DRPLA brain tissue, when incubated in buffer not containing alkaline phosphatase, showed that anti-PSR recognized an apparent immunoreactive band of DRPLA protein complex at the top of stacking gels (Figure 2B, lane 2). Conversely, the immunoblot of DRPLA brain tissue treated with alkaline phosphatase showed that the anti-PSR immunoreactivity of the DRPLA protein complex band at the top of the stacking gels was consistently reduced by the alkaline phosphatase treatment and was no longer visible (Figure 2B, lane 1). This result indicates that alkaline phosphatase treatment decreases isolated DRPLA protein complex anti-PSR immunoreactivity, and this decrease is due to dephosphorylation of the complex. In contrast, DRPLA protein complex isolated from control brain tissue did not appear to be phosphorylated, because with or without alkaline phosphatase treatment it showed no anti-PSR immunoreactivity (Figure 2B, lanes 3 and 4). Based on these results, it appears that isolated DRPLA protein complex is aberrantly phosphorylated in DRPLA brain tissue compared with normal brain tissue.

To show that mutant and wild-type DRPLA proteins are aberrantly phosphorylated in DRPLA brain tissue, isolated DRPLA protein complex was studied by double-electrophoresis on SDS/polyacrylamide gels under non-reducing and reducing conditions (Figure 1B, lanes 9 and 10). The immunoblots of DRPLA brain tissue stained with anti-PSR showed no immunoreactivity of mutant and wild-type DRPLA proteins, but showed apparent immunoreactivity of a high-molecular-mass complex at the top of stacking gels (Figure 1B, lane 10). This finding indicates that DRPLA protein is not aberrantly phosphorylated, but DRPLA protein complex contains aberrantly phosphorylated proteins only in DRPLA brain tissue.

UBI-bonded DRPLA protein complex is aberrantly phosphorylated

To assess the role that inappropriate phosphorylation might play in pathological ubiquitination of DRPLA protein complex,

Figure 1 Isolation of DRPLA protein complex

Coomassie Blue (CB)-stained SDS/polyacrylamide gel of a sample of human control brain tissue (lane 1) and a sample of DRPLA brain tissue (lane 2), which was electrophoresed once under non-reducing conditions. Immunoblots of control brain tissue (lanes 3, 5, 7 and 9) and DRPLA brain tissue (lanes 4, 6, 8 and 10) electrophoresed under three different sets of conditions: the immunoblot of samples electrophoresed once under reducing conditions (lanes 3 and 4), the immunoblot of samples electrophoresed once under non-reducing conditions (lanes 5 and 6), and the immunoblot of samples electrophoresed twice under non-reducing and reducing conditions (lanes 7–10). Immunoblots were stained with C580 (lanes 3–8) and with anti-PSR (lanes 9 and 10). ME is 2-mercaptoethanol. Molecular-mass markers are indicated on the left. (A) On the CB-stained gel of the control and DRPLA brain tissue samples, an apparent high-molecular-mass complex is observed at the top of the stacking gel (arrowhead), and there appears to be no difference in the amount between the control and DRPLA brain tissues (lanes 1 and 2). The immunoblot of the DRPLA brain tissue shows greater immunoreactivity of DRPLA protein complex at the top of the stacking gel (arrowhead) compared with the immunoblot of the control brain tissue sample (lanes 5 and 6). (B) The immunoblot of DRPLA brain tissue shows that the high-molecular-mass complex band has migrated into two bands of reduced mutant and wild-type DRPLA proteins (arrows) (lane 8), whereas the immunoblot of the control brain tissue sample shows that C580 detected very little immunoreactivity of DRPLA protein (lane 7). When the immunoblot of DRPLA brain tissue was stained with anti-PSR, a high-molecular-mass complex band was detected at the top of stacking gel (lane 10).

Figure 2 Immunoblots stained with antibodies against phosphorylated proteins and enzymic dephosphorylation of DRPLA protein complex

(A) Coomassie Blue (CB)-stained SDS/polyacrylamide gel (lanes 1–4) and immunoblots stained with anti-PSR (lanes 5–8) and anti-PTN (lanes 9–12). Samples of two control brain tissues (lanes 1, 2, 5, 6, 9 and 10) and two DRPLA brain tissues (lanes 3, 4, 7, 8, 11 and 12) were electrophoresed once under non-reducing conditions. On immunoblots of DRPLA brain tissue samples, anti-PSR recognized the high-molecular-mass band of DRPLA protein complex at the top of the stacking gels (arrowhead), whereas it did not detect any immunoreactive band on immunoblots of control brain tissue samples (lanes 5–8). In contrast, immunoblots of neither control nor DRPLA brain tissue samples, when stained with anti-PTN, showed any immunoreactive band at the top of the stacking gels (lanes 9–12). (B) A sample of DRPLA brain tissue (lanes 1 and 2) and a sample of control brain tissue (lanes 3 and 4) were first electrophoresed, followed by incubation with alkaline phosphatase (AP) (lanes 1 and 3) and without AP (lanes 2 and 4). On the immunoblot of DRPLA brain tissue sample treated without AP but incubated with buffer, anti-PSR detected apparent immunoreactivity of DRPLA protein complex at the top of the stacking gels (arrowhead) (lane 2), but on the immunoblot of the AP-treated sample the anti-PSR immunoreactivity of DRPLA protein complex was greatly reduced (lane 1).

DRPLA protein complex was examined on an immunoblot separately stained with anti-UBI and anti-PSR. On the immunoblots of DRPLA brain tissue samples, both anti-UBI and anti-PSR recognized a high-molecular-mass band of DRPLA protein complex at the top of the stacking gels (Figure 3, lanes 7, 8, 11 and 12), whereas they did not recognize any band on the immunoblots of control brain tissue samples (Figure 3, lanes 5,

Figure 3 Aberrant phosphorylation of UBI-bonded DRPLA protein complex

Coomassie Blue (CB)-stained SDS/polyacrylamide gel of two human control brain tissue samples (lanes 1 and 2) and two DRPLA brain tissue samples (lanes 3 and 4), which was electrophoresed once under non-reducing conditions. Also, immunoblots of the control brain tissue samples (lanes 5, 6, 9 and 10) and the DRPLA brain tissue samples (lanes 7, 8, 11 and 12), electrophoresed once on an SDS/polyacrylamide gel under non-reducing conditions, were separately stained with anti-UBI (lanes 5–8) and anti-PSR (lanes 9–12). On the immunoblots of the DRPLA brain tissue samples, both anti-UBI and anti-PSR detected high-molecular-mass DRPLA protein complex at the top of the stacking gel (arrowhead) (lanes 7, 8, 11 and 12). On the immunoblots of the control brain tissue samples, neither anti-UBI nor anti-PSR recognized the DRPLA protein complex (lanes 5, 6, 9 and 10).

6, 9 and 10). This indicates that the high-molecular-mass band of DRPLA protein complex was immunoreactive with the antibodies against both UBI and PSR only in DRPLA brain tissue, suggesting that abnormal UBI-bonded DRPLA protein complex is aberrantly phosphorylated in the brains of DRPLA patients.

Using double-labelling immunohistochemistry, neurons of the dentate nucleus in control brain tissues showed weak, fine granular immunoreactivity appearing as a blue colour in the cytoplasm, indicating that the control neurons have weak immunoreactivity with anti-PSR, but little immunoreactivity with anti-UBI (Figure 4B). In contrast, using the same technique on DRPLA brain tissues, anti-UBI revealed brown-coloured cytoplasmic inclusions in neurons of the dentate nucleus, the site of the most severe lesions in DRPLA brain tissues. Anti-PSR also partially, but strongly, recognized the inclusions, which appeared as a light-black colour (colour combination of brown and blue) due to the immunoreactivity with both antibodies (Figure 4A, arrowheads). In DRPLA brain tissue, approx. 1–2% of the dentate nucleus neurons contained the ubiquitinated cytoplasmic inclusions, and approx. 40% of the cytoplasmic inclusions showed anti-PSR immunoreactivity. This finding indicates that UBI-bonded cytoplasmic inclusions, which were previously shown to contain DRPLA protein [22], are partially phosphorylated in DRPLA-afflicted neurons. Moreover, anti-PSR antibodies labelled the nuclear membrane in the afflicted neurons of DRPLA brain tissues (Figure 4A, arrow), but did not label the nuclear membrane of neurons in control brain tissues (Figure 4B). Anti-PSR antibodies labelled the nuclear membrane in approx. 20% of dentate nucleus neurons in DRPLA brain tissue. This observation indicates that the nuclear membrane is also involved in aberrant phosphorylation in addition to abnormal cytoplasmic inclusions in DRPLA-afflicted neurons. To confirm that the neuronal nuclear membranes definitively showed

Figure 4 Single-labelling and double-labelling immunohistochemistry of ubiquitinated cytoplasmic inclusion

Three photographs of a neuron in the dentate nucleus of DRPLA brain tissue (**A**, **C**, **E**), and two photographs of a neuron in the dentate nucleus of control brain tissue (**B**, **D**). Top two photographs (**A**, **B**), using a double-labelling immunohistochemical method by staining first with anti-UBI (brown) and then with anti-PSR (blue). Middle two photographs (**C**, **D**), using a double-labelling immunohistochemical method by staining first with anti-HNM (brown) and then with anti-PSR (blue). Bottom photograph (**E**), using a single-labelling immunohistochemical method by staining with anti-UBI (brown) with Methyl Green as the counterstain. (**A**) The neuron in the DRPLA brain tissue contains cytoplasmic inclusions which are brown. The inclusions partially show a light-black colour (i.e. combination of brown and blue; arrowheads). Moreover, anti-PSR recognized the nuclear membrane of the DRPLA-affected neuron (arrow). (**B**) The neuron in the control brain tissue shows weak, fine granular immunoreactivity appearing as a blue colour in the cytoplasm. (**C**) The nuclear membrane in the DRPLA-affected neuron appears as a black colour, showing immunoreactivity for both anti-HNM and anti-PSR. (**D**) The nuclear membrane in the control neuron appears as a brown colour, showing immunoreactivity only for anti-HNM. (**E**) Single-labelling immunohistochemical study shows that the cytoplasmic inclusions in the neuron in DRPLA brain tissue appear as a brown colour (arrowhead). Magnification = $\times 690$.

anti-PSR immunoreactivity, double-labelling immunohistochemical studies using anti-HNM and anti-PSR revealed that the neuronal nuclear membrane appeared as a black colour in DRPLA brain tissues (Figure 4C), showing immunoreactivity for both anti-HNM and anti-PSR, whereas the neuronal nuclear membrane appeared as a brown colour in control brain tissues (Figure 4D), showing immunoreactivity only for anti-HNM. The findings indicate that neuronal nuclear membranes definitively showed anti-PSR immunoreactivity only in DRPLA brain tissue.

DISCUSSION

DRPLA protein undergoes greater complex formation in DRPLA brain tissue compared with normal control brain tissue, and

the expanded glutamine repeat enhances complex formation by DRPLA protein [20]. DRPLA protein complex is pathologically ubiquitinated in DRPLA brain tissue, and pathological ubiquitination of DRPLA protein complex is found selectively in affected brain tissue lesions of patients with DRPLA [22]. Furthermore, ubiquitinated DRPLA protein complex is heat-resistant only in DRPLA brain tissue [30]. Ubiquitination is a disease-specific pathological phenomenon that is produced by abnormal DRPLA protein complex formation in DRPLA brain tissue. Thus DRPLA protein complex in DRPLA brain tissue displays biochemical properties which are consistently different from DRPLA protein complex in control brain tissue.

Abnormal DRPLA protein complex in the diseased brain has these unique properties which are not shared with normal

DRPLA protein complex. These properties demonstrate that abnormal DRPLA protein complex may play a pathological role in DRPLA neurodegeneration. The present study demonstrates that abnormal DRPLA protein complex isolated from DRPLA brain tissue includes a quantitatively greater amount of DRPLA protein than normal DRPLA protein complex. This is consistent with previous observations on immunoblots of DRPLA brain tissue stained with C580 after electrophoresis was performed once under non-reducing conditions. Previous study demonstrated that C580 reacted with a single band of high-molecular-mass DRPLA protein complex on immunoblots of DRPLA brain tissue, whereas this antibody reacted with two bands on immunoblots of control brain tissue, i.e. a reduced band of wild-type DRPLA protein and a band of high-molecular-mass DRPLA protein complex [22]. The present and previous studies showed that an abnormally large amount of DRPLA protein is involved in the complex and that the DRPLA protein complex in diseased brain tissue is formed with pathologically stronger bonding than in normal DRPLA protein complex in normal brain tissue. Because DRPLA protein complex shows pathologically strong bond formation only in DRPLA brain tissue, DRPLA protein complex may trigger abnormal aggregation in DRPLA brains. Thus abnormal aggregation produced by abnormal DRPLA protein complex formation plays a role in the pathology of DRPLA. In the present study, moreover, abnormal DRPLA protein complex in DRPLA brain tissue displayed another unique biochemical property that is different from normal DRPLA protein complex in control brain tissue. That unique property is aberrant phosphorylation. Aberrant phosphorylation is shown to be selectively present in DRPLA protein complex of diseased brain tissue and specifically within ubiquitinated cytoplasmic inclusions in DRPLA-affected neurons. Thus three disease processes arising from abnormal DRPLA protein complex are present in patients with DRPLA: first, a large complex is formed due to abnormally strong bonding between DRPLA protein molecules; secondly, pathological ubiquitination of DRPLA protein complex occurs, followed (thirdly) by aberrant phosphorylation of the DRPLA protein complex. These three disease processes may contribute to DRPLA neurodegeneration.

Immunochemical studies using immunoblotting with anti-PSR antibody and enzymic dephosphorylation of DRPLA protein complex have demonstrated that DRPLA protein complex undergoes aberrant phosphorylation in diseased brain tissue. Protein phosphorylation and dephosphorylation are basic mechanisms for the modification of protein function in eukaryotic cells. Recently, abnormally phosphorylated tau proteins were reported to aggregate in neurons in a number of neurodegenerative diseases (referred to as tauopathies), including Alzheimer's disease and frontotemporal dementia and parkinsonism linked to chromosome 17 [25–28]. Aberrant phosphorylation plays an important role in the pathological mechanism of neurodegenerative diseases. The findings of the present study suggest that aberrant phosphorylation of DRPLA protein complex plays a pathological role in DRPLA neurodegeneration. However, the target of the aberrant phosphorylation is not DRPLA protein, but is rather the other protein or proteins which the DRPLA protein complex contains. Many questions remain concerning the functional significance of aberrant phosphorylation of the DRPLA protein complex in the diseased brain. Aberrant phosphorylation may actively modulate the surface properties of the DRPLA protein complex and contribute to functional interactions of the complex. Immunohistochemical studies demonstrated that both the nuclear membrane and the ubiquitinated cytoplasmic inclusions are aberrantly

phosphorylated in DRPLA-affected neurons. This finding suggests that the nuclear membrane is another pathological focus of DRPLA neurodegeneration, and that aberrant phosphorylation may induce abnormal interaction between the UBI-bonded cytoplasmic inclusions and the nuclear membrane in DRPLA-affected neurons. Previously, by expressing a truncated DRPLA cDNA with expanded CAG repeats in COS-7 cells, truncated DRPLA protein was reported to be localized in perinuclear cytoplasmic and intranuclear inclusions [17]. This perinuclear localization of DRPLA protein was also observed in an immunohistochemical study of DRPLA brains stained with C580 (results not shown). In HD and SCA1, nuclear localization of their mutant gene products, huntingtin and ataxin-1, was shown to be essential for initiating pathogenesis in transgenic models [13,19,31]. Thus these experiments suggest that the gene product changes its location from the cytoplasm to the nucleus, inducing cell death in diseased neurons in glutamine-repeat diseases. In the present study, aberrant phosphorylation of the DRPLA protein complex may play a role in changing the localization from the cytoplasm to the nucleus, leading to DRPLA neurodegeneration.

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