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Histone H3 is aberrantly phosphorylated in glutamine-repeat diseases

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Abstract

Double-labeling immunohistochemical studies staining with anti-ubiquitin and anti-phosphoserine antibodies and application of an enzymatic dephosphorylation technique reveal neuronal inclusions and affected nuclei to be aberrantly phosphorylated in brain tissues with patients with glutamine-repeat diseases. Regional distribution of the phosphorylated nuclei in neurons correlates with the pathology. To identify the target nuclear protein, transient expression of Huntington's disease exon 1 gene containing an expanded glutamine repeat was generated in a cell culture and nuclear inclusions were isolated with a fluorescence-activated cell sorting system. Immunoblotting studies of the aggregated nuclear proteins using anti-phosphoserine antibody demonstrate the protein of the aberrant phosphorylation as histone H3. The immunoblots of control and diseased brain tissues demonstrate that the phosphorylation of histone H3 is commonly increased in the diseased brains. Aberrant phosphorylation of histone H3 is surmised to be a shared pathological process in glutamine-repeat diseases.

Keywords: Glutamine-repeat disease; Polyglutamine disease; Histone H3; Phosphorylation; Huntington's disease; Spinocerebellar ataxia 1; Dentatorubral-pallidoluysian atrophy; Machado–Joseph disease; Nuclear inclusion; Phosphoserine

Glutamine-repeat diseases are a group of hereditary neurodegenerative disorders caused by expansion of a glutamine repeat in responsible gene products. This group is also referred to as polyglutamine disease and includes spinobulbar muscular atrophy, Huntington's disease (HD), spinocerebellar ataxia 1 (SCA1), dentatorubral-pallidoluysian atrophy (DRPLA), Machado–Joseph disease (MJD), SCA2, SCA6, and SCA7 [1–11]. Neuronal intranuclear inclusions are found in brain tissues of patients with HD and HD transgenic mice [12,13]. The HD gene product is surmised to aggregate in HD neurons. Similar neuronal intranuclear and cytoplasmic inclusions showing immunoreactivity with the gene product have also been reported in brain tissues of patients with other glutamine-repeat diseases and transgenic mice [14–19]. Aggregation of the gene products that carry 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 unclear.

Our previous studies of DRPLA demonstrated that several disease processes arising from abnormal protein complex formation involving the DRPLA gene product (DRPLA protein) occur in brain tissues of patients with DRPLA based on immunoblotting data obtained by electrophoresis under non-reducing conditions [20–25]. The first process is large complex formation due to abnormally strong bonding between DRPLA protein molecules [21,22]. The second is pathological ubiquitination of the DRPLA protein complex [23,24]. Immunoblotting studies using anti-phosphoserine antibody (anti-PSR) and enzymatic dephosphorylation of isolated DRPLA protein complexes recently enabled us to

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demonstrate that DRPLA protein complexes are aberrantly phosphorylated in DRPLA brain tissues as the third disease process [25]. Moreover, double-labeling immunohistochemical studies using anti-ubiquitin antibody (UBI) and anti-PSR demonstrated that the aberrant phosphorylation is within ubiquitinated inclusions and the nucleus of DRPLA-affected neurons, suggesting that the nucleus is another pathological focus of DRPLA neurodegeneration [25]. Because the target of the aberrant phosphorylation was not the DRPLA protein but another protein forming the complexes with DRPLA protein [25], there is a possibility that the aberrant phosphorylation is not simply a pathological process in DRPLA, but a process shared in common by DRPLA and the other glutamine-repeat diseases.

Materials and methods

Double-labeling immunohistochemistry. Postmortem brain tissues from three HD (39–64 years old), four DRPLA (37–69 years old), one SCA1 (28 years old), and one MJD (32 years old) patients, whose diseases had been diagnosed genetically by PCR analysis and confirmed pathologically, and brain tissue samples from five control subjects (59–79 years old) were examined [20,26]. Blocks of brain tissue were fixed in 10% formalin, sections of which were embedded in paraffin. Sections of the cerebral cortex, cerebellar cortex, dentate nucleus, caudate nucleus, and pons from the diseased brain tissues were selected for examination. The sections were stained with hematoxylin and eosin, and observed by a light microscopy (Table 1). The sections were immunostained doubly with anti-ubiquitin polyclonal antibody (anti-UBI; DAKO) and anti-phosphoserine monoclonal antibody (anti-PSR; Sigma), as previously described (Table 2) [25]. The sections were incubated with the first sequence of primary anti-UBI. 3,3′-Diaminobenzidine (DAB; Sigma) was used as the first reaction substrate and the reaction product appeared as a brown color. After visualization, the sections were washed with a 100 mM Tris–HCl solution, pH 2.2. The sections were then reacted with the second sequence of primary anti-PSR and the reaction product was visualized as a blue color with a True Blue Peroxidase Substrate Kit (KPL). To confirm that ubiquitinated inclusions and nuclear structures in neurons were phosphorylated, diseased and control brain tissue sections were dephosphorylated and then double-immunostained with anti-UBI and anti-PSR antibodies. Before the sections were incubated with the primary anti-UBI, they were incubated for 18 h at 37 °C in alkaline phosphatase buffer (50 mM Tris–HCl, pH 9.0, 150 mM NaCl) containing calf intestinal alkaline phosphatase (Sigma) added to a final concentration of 1000 U/ml. As a control, sections were similarly incubated in the same buffer without alkaline phosphatase.

Isolation of nuclear inclusions containing HD gene product with an expanded glutamine repeat in cell culture, and immunoblotting and immunocytochemistry. Transient expression of HD exon 1 fragment containing 74 glutamine repeats and green fluorescent protein (GFP) fusion protein was generated in Neuro 2A mouse neuroblastoma (N2A) cell culture [27]. The HD exon 1 fragment was cloned into pEGFD-N1 expression vector (Clontech). Nuclear and cytoplasmic inclusions that contained GFP fusion proteins were isolated with a fluorescence-activated cell sorting (FACS) system [27]. FACS-purified nuclear and cytoplasmic inclusions were treated with concentrated formic acid at 37 °C for 30 min, resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (SDS–PAGE) using 5–20% gradient gel (Atto), and transferred electrophoretically to a polyvinylidene difluoride (Immobilon) membrane (Millipore). The appropriate bands on Coomassie brilliant blue (CBB)-stained membranes were cut and their proteins were analyzed with an amino acid sequence analyzer (Applied Biosystems, Model 491).

For immunoblotting studies, the Immobilon membrane was blocked with a Tris-buffer solution containing 4% nonfat milk powder and 1% bovine serum albumin (crystallized, Sigma) when staining by anti-histone H3 polyclonal antibody (anti-H3; Upstate Biotechnology) and anti-GFP antibody (anti-GFP; DAKO), and with 1% bovine serum albumin when staining by anti-phosphoserine monoclonal antibody (anti-PSR; Sigma). The membrane was left undisturbed for 16 h at 4 °C with the primary antibodies: anti-H3, anti-GFP, and anti-PSR. Then the Immobilon membrane was incubated for 1 h at room temperature with secondary antibodies. In order to visualize the immunoreaction, an enhanced chemiluminescence (ECL) Western blotting system (Amersham Pharmacia Biotech) was used. For immunocytochemical studies, the N2A cells were fixed with 10% formaldehyde at 24 h after transfection. The cells were incubated with anti-PSR primary antibody at 4 °C for 16 h and then incubated with 1:100 diluted Alexa 546-linked anti-mouse secondary antibody (Molecular Probes). They were mounted with Mounting medium (Vectastain) and observed by fluorescence microscopy (Karl Zeiss).

Table 1
Neuropathological findings in glutamine-repeat diseases

Disease	Cerebral cortex	Caudate nucleus	Cerebellar purkinje cell	Dentate nucleus	Pontine nuclues
HD	+	++	–	–	–
DRPLA	–	–	–	++	–
SCA1	–	–	++	–	++
MJD	–	–	+	++	++

–, unaffected; +, affected with neuronal loss and gliosis (+, mild; ++, severe).

Table 2
Aberrant phosphorylation of neuronal nuclear structures

Disease	Cerebral cortex	Caudate nucleus	Cerebellar purkinje cell	Dentate nucleus	Pontine nucleus
HD	+	+	–	–	–
DRPLA	–	–	–	+	–
SCA1	–	–	+	–	+
MJD	–	–	+	+	+

+, phosphorylated; –, not phosphorylated.

Sample preparation of human brain tissues and immunoblotting. Postmortem brain tissue samples from the three HD, four DRPLA, and one SCA1 patients and the control brain tissue samples were examined. Tissue samples (1 g) of the frontal cerebral cortex were homogenized separately in 10 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). Samples were stored at −80 °C until subjected to gel electrophoresis. Protein concentrations in brain tissue homogenates were measured by bicinchoninic acid (BCA) 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, and 10% of 2-mercaptoethanol) and placed in boiling water for 8 min. The acrylamide concentration of the stacking gels was 5% and that of the running gels 5–20% (Atto) and 15%. Samples (10 μg each) were electrophoresed in the gels and proteins in the gels were transferred electrophoretically to an Immobilon membrane, which was then

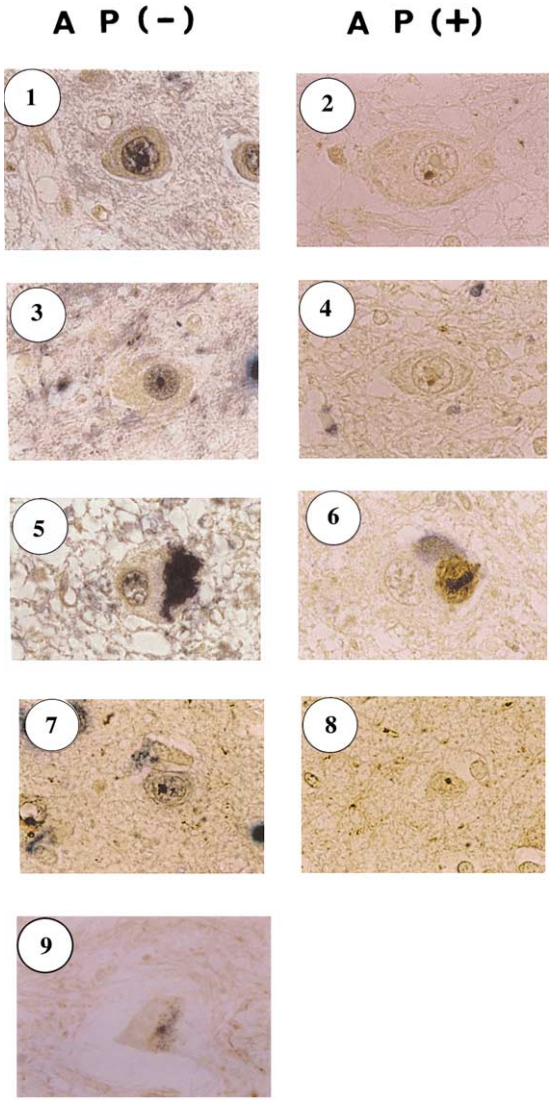

Fig. 1. Brain tissues of patients with SCA1 (1, 2), MJD (3, 4), DRPLA (5, 6), and HD (7, 8) were examined using a double-labeling immunohistochemical method by staining first with anti-UBI polyclonal antibody (yielding a brown color) and then with anti-PSR monoclonal antibody (yielding a blue color). Sections of the SCA1 pontine nucleus (2), MJD pontine nucleus (4), DRPLA dentate nucleus (6), and HD caudate nucleus (8) were dephosphorylated with alkaline phophatase (AP). The non-dephosphorylated sections of the SCA1 (1), MJD (3), DRPLA (5), HD (7), and human control (9) brain tissues and the phosphorylated sections (2, 4, 6, and 8) were double-immunostained. The non-dephosphorylated sections of the diseased brain tissues show that the neurons contain intranuclear (SCA1, MJD, HD, and DRPLA) and cytoplasmic (DRPLA) inclusions which stain black (i.e., combination of brown and blue), indicating the presence of immunoreactivity for both anti-PSR and anti-UBI antibodies. Anti-PSR also stained the nuclear membrane and matrix of the affected neurons in addition to the inclusions. The dephosphorylated sections show an apparent decrease in the anti-PSR immunoreactivity of the ubiquitinated inclusions, and disappearance of anti-PSR immunoreactivity of the nuclear membrane and matrix in the affected neurons, which appear as a brown color. Magnification: 460×.

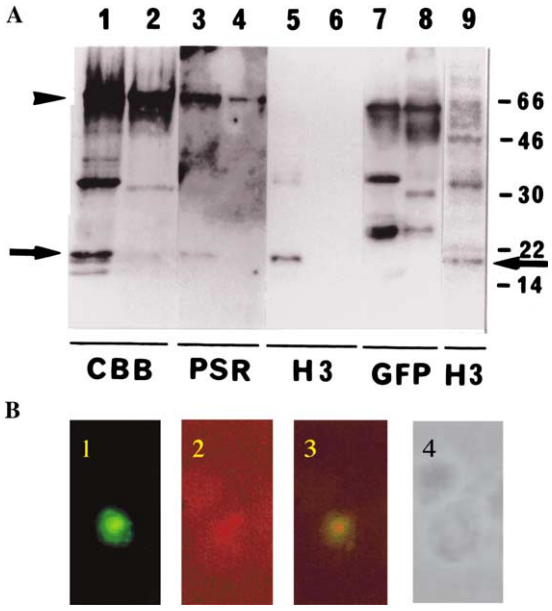

Fig. 2. Inclusions obtained from the expression study of HD exon 1 fragments containing 74 CAG repeats in N2A cells were isolated into nuclear particles and cytoplasmic particles by FACS [27]. (A) Samples of the nuclear (lanes 1, 3, 5, and 7) and cytoplasmic inclusions (lanes 2, 4, 6, and 8) were treated with formic acid. Then the samples of the inclusions and a sample of human control brain tissue (lane 9) were subjected to SDS–PAGE and immunoblotting studies. The Immobilon membrane was stained with CBB (lanes 1, 2) and the other membrane was immunostained with three antibodies; anti-PSR (lanes 3, 4), anti-H3 (lanes 5, 6, and 9), and anti-GFP (lanes 7, 8). On the immunoblots of the nuclear inclusions, anti-PSR detected two immunoreactive bands with apparent molecular weights of 65 kDa (arrowhead) and 17 kDa (arrow), but only a 65-kDa band on the immunoblots of the cytoplasmic inclusions. The band with an approximate molecular weight of 65 kDa showed the same electrophoretic mobility as that of HD exon 1-GFP fusion protein on the immunoblots stained with anti-GFP. The band with an apparent molecular weight of 17 kDa, which was recognized on the CBB-stained membrane, was identified as histone-H3 by amino acid sequence analysis [27]. Anti-H3 confirmed that the 17 kDa band is identical to histone-H3 on the immunoblots. (B) Immunocytochemical study of the inclusions in the N2A cells, expressed HD exon 1 fragments containing an expanded glutamine repeat and GFP. The N2A cells were immunostained with anti-PSR (2) and observed by phase contrast microscopy (4). Two fluorescent images of GFP and anti-PSR are merged into an image (3). The nuclear inclusions, labeled with GFP (1), showed strong immunoreactivity with anti-PSR. Magnification: 720×.

blocked and left undisturbed for 16 h at 4 °C with the primary antibodies, i.e., anti-H3, anti-PSR, and anti-histone H2B polyclonal antibodies (anti-H2B; Chemicon). Then the Immobilon membrane was incubated for 1 h at room temperature with secondary antibodies and the immunoreaction was visualized by ECL.

Results

Using double-labeling immunohistochemical staining with anti-UBI and anti-PSR antibodies, neurons in the control brain tissues showed weak, fine granular immunoreactivity appearing as a blue color in the cytoplasm (Fig. 1). In contrast, the neurons in the affected lesions of diseased brain tissues showed intranuclear inclusions that appeared as a black color (color combination of brown and blue) due to immunoreactivity with both anti-UBI and anti-PSR antibodies (Fig. 1). Enzymatic dephosphorylation of the diseased brain tissue sections decreased the anti-PSR immunoreactivity of the neuronal inclusions, but the anti-UBI immunoreactivity remained, appearing as a brown color (Fig. 1). These findings indicate that ubiquitin-bonded intranuclear inclusions contained phosphorylated serine residues which were aberrantly phosphorylated in the affected neurons. The immunohistochemical staining also revealed anti-PSR immunoreactivity of the nuclear membrane and matrix in the affected neurons, in addition to the immunoreactivity of the inclusions (Fig. 1). Enzymatic dephosphorylation eradicated the anti-PSR immunoreactivity of the nuclear membrane and matrix (Fig. 1). These immunohistochemical findings suggest that the nucleus is a focus of glutamine-repeat disease neurodegeneration. When the regional distribution of the neurons displaying aberrant phosphorylation of the nucleus was assessed in the glutamine-repeat disease brain tissues, it clearly correlated with the distribution of the lesions showing neuropathological changes of neuronal loss and gliosis (Tables 1 and 2). These findings suggest that the aberrant phosphorylation of the neuronal nucleus may be a shared pathological process in glutamine-repeat disease neurodegeneration.

To identify the target nuclear protein which undergoes aberrant phosphorylation and interacts with the disease gene products, we used the Neuro 2A mouse neuroblastoma (N2A) cell culture, which expresses HD exon 1 fragments containing 74 glutamine repeats [27]. The expression study demonstrated that the purified aggregated proteins involved the N-terminal fragment of HD gene product, ubiquitin, and other nuclear proteins. Immunoblotting studies of the aggregated nuclear proteins using anti-PSR showed a band with an approximate molecular weight of 17 kDa (Fig. 2A), which was found to be histone H3 by amino acid sequence analysis [27]. Immunoblots stained with anti-histone H3 antibody (anti-H3) confirmed that the band with an approximate molecular weight of 17 kDa was identical to histone H3 (Fig. 2A). In addition, an immunocytochemical study of the N2A cells demonstrated that nuclear inclusions that contained GFP and the N-terminus of the HD gene product showed an apparent increase in anti-PSR immunoreactivity (Fig. 2B). Thus, the expression study indicates that the nuclear protein which aggregates with the HD gene product to form nuclear inclusions is histone H3, and that histone H3 contains aberrantly phosphorylated serine residues in the nuclear inclusions of the N2A cells which expressed HD exon 1 with the expanded glutamine repeat.

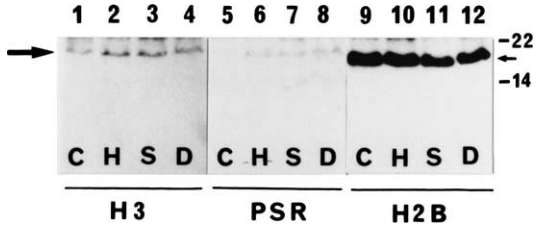

Fig. 3. Immunoblots of human control brain tissues and the diseased brain tissues of the patients with HD, SCA1, and DRPLA. Samples of the brain tissues of human control (C; lanes 1, 5, and 9), HD (H; lanes 2, 6, and 10), SCA1 (S; lanes 3, 7, and 11), and DRPLA (D; lanes 4, 8, and 12) patients were resolved by SDS–PAGE using 15% polyacrylamide gels, and subjected to immunoblotting. The Immobilon membrane was immunostained with anti-H3 (lanes 1–4), anti-PSR (lanes 5–8), and anti-H2B (lanes 9–12). The immunoblots revealed that anti-PSR showed stronger immunoreactivity for histone H3 (large arrow) in the diseased brain tissues than in the control brain tissues, whereas anti-H3 and anti-H2B (small arrow) showed similar immunoreactivity in the control and the diseased brain tissues.

To assess the phosphorylation of histone H3 in the brain tissues of HD and other glutamine-repeat diseases, control human brain tissues and diseased brain tissues of HD, DRPLA, and SCA1 patients were examined in an immunoblotting study using anti-PSR and anti-H3. The immunoblots stained with anti-H3 demonstrated that the immunoreactivity of histone H3 showed little difference among the control and the diseased brain tissues (Fig. 3, 1–4), but the immunoblots stained with anti-PSR antibody showed increased immunoreactivity of histone H3 with an apparent molecular weight of 17 kD in the diseased brain tissues of HD, DRPLA, and SCA1 compared with that in the control brain tissues (Fig. 3, 5–8). These immunoblotting data indicate that the abnormal increase in anti-PSR immunoreactivity is selectively found in the diseased brain tissues, although there is a possibility that the amounts of histone H3 may be different among the brain tissues.

Discussion

The N2A expression study shows that the anti-PSR immunoreactivity of the 17 kDa protein is that of histone H3. Histone H3 is aberrantly phosphorylated in the

diseased brain tissues. These data from the expression experiment and the immunoblotting study of the diseased brain tissues are both consistent with the observations in the double-labeling immunohistochemical study. The anti-PSR immunoreactivity of nuclear structures in the immunohistochemical studies is due to an ectopic increase in anti-PSR immunoreactivity of histone H3 in the glutamine-repeat disease brains. Because the aberrant phosphorylation correlated with the glutamine-repeat disease pathology, the aberrant phosphorylation of histone H3 is a shared pathological process in glutamine-repeat disease neurodegeneration. Histone proteins, assembled with DNA to form nucleosomes, are the basic building blocks of chromatin [28,29]. Histone H3 phosphorylation plays an important role in transcriptional activation, although how histone H3 phosphorylation affects gene expression is not known. One possibility is that histone H3 phosphorylation may serve as a recognition site for recruitment of transcriptional factors [29]. The transcriptional cofactor CBP (CREB-binding protein), which specifically binds to phosphorylated CREB (cyclic adenosine monophosphate-responsive element-binding protein), is speculated to interact directly with phosphorylated histone H3 [29]. When histone H3 is aberrantly phosphorylated in the nuclei of glutamine-repeat disease neurons, abnormal interaction between CBP and histone H3 may induce unnecessary bindings to the transcriptional factors and generate ectopic gene expression, thereby causing abnormal protein accumulation in glutamine-repeat diseases. Recently, binding of expanded glutamine repeats to CBP and $TAF_{II}130$, transcriptional cofactors is reported to interfere with CREB-dependent transcriptional activation [30,31]. Although we are not certain whether expanded glutamine repeats inactivate CREB-dependent transcription and cause neurodegeneration, the present study elucidates that the gene activation system is the target of neurodegeneration in glutamine-repeat diseases.

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