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Abnormal phosphorylation of Ser409/410 of TDP-43 in FTLD-U and ALS

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Abstract **A monoclonal antibody specific for phosphoserines 409 and 410 of TDP-43 (mAb pS409/410) has been produced. It strongly stained TDP-43-positive inclusions in brain of patients with frontotemporal lobar degeneration and amyotrophic lateral sclerosis, but did not stain nuclei, in which normal TDP-43 is localized. It did not recognize TDP-43 rapidly extracted from brains of rats at various developmental stages, strongly suggesting that phosphorylation of Ser409/410 is an abnormal event. Analysis of postmortem changes of TDP-43 revealed that the amounts of Sarkosyl-insoluble, urea-soluble full-length TDP-43 and a 35 kDa N-terminal fragment increased time-dependently.**

Keywords: Ubiquitin; Inclusions; Tau; α-synuclein; Degradation; Aggregation

1. Introduction

Hyperphosphorylated and ubiquitinated proteins have been identified as the major components of cytoplasmic inclusions in a number of neurodegenerative diseases, such as tau in tauopathies and α-synuclein in α-synucleinopathies [1,2]. Recently, TAR DNA-binding protein of 43 kDa (TDP-43) was identified as the major component of tau-negative and ubiquitin-positive neuronal cytoplasmic inclusions (NCIs), intranuclear inclusions and dystrophic neurites (DNs) in frontotemporal lobar degeneration (FTLD-U), as well as skein-like inclusions and round inclusions in amyotrophic lateral sclerosis (ALS) [3–5]. TDP-43 is a protein belonging to the group of 2 RNA-binding domain (RBD)-Gly RNA-binding proteins, which include the heterogeneous nuclear ribonucleoprotein (hnRNP) family and factors involved in RNA splicing and transport [6,7]. TDP-43 binds hnRNP A/B and hnRNP A1 through its C-terminal region, inhibiting pre-mRNA splicing [8]. More recently, missense mutations in the TDP-43 gene have been identified in familial and sporadic ALS cases [9–13]. These findings strongly suggest that structural and functional dysfunction of TDP-43 lead to aggregation of TDP-43 and neurodegeneration. Dephosphorylation experiments indicated that the TDP-43 deposited in FTLD-U and ALS was hyperphosphorylated [3,4]. We have raised antibodies against 36 candidate sites for phosphorylation and found that several antibodies against phosphopeptides in the C-terminal region of TDP-43 strongly stained TDP-43 lesions in FTLD-U and ALS [14]. Among these antibodies, the antibody to phosphoserine 409 and 410 (pS409/410) showed particularly robust and widespread staining of TDP-43 lesions in FTLD-U and ALS. In addition, in vitro phosphorylation experiments showed that the pS409/410 epitope was generated by incubation of TDP-43 with casein kinase (CK) 1 or CK2, and phosphorylation at these sites increased TDP-43 oligomerization [14].

In order to investigate the phosphorylation of TDP-43 further, we have produced and characterized a monoclonal antibody (mAb pS409/410) directed against phosphoserines 409 and 410 in human TDP-43. We show here that mAb pS409/410 strongly stained all known types of TDP-43 inclusions in FTLD-U and ALS, but did not stain normal TDP-43. This mAb also stained TDP-43 phosphorylated with CK1 or CK2 in vitro. However, it did not react with TDP-43 rapidly extracted from brains of fetal and adult rats with a buffer containing phosphatase inhibitors, though tau phosphorylation was detected. TDP-43 proteins were detected in various tissues with anti-TDP-43 antibody, but not with mAb pS409/410. These results suggest that the phosphorylation of Ser409/410 is an abnormal event that does not occur in normal tissues. We also found that Sarkosyl-insoluble, urea-soluble full-length TDP-43 and a 35 kDa N-terminal fragment increased during postmortem incubation.

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2. Materials and methods

2.1. Antibodies

Monoclonal antibody (mAb) pS409/410 was raised against a synthetic phosphopeptide, CMDSKS(p)S(p)GWGM (amino acid residues 405–414 in human TDP43), where S(p) represents phosphoserine. The peptide was conjugated at the amino-terminus via a cysteine linkage to thyroglobulin from bovine thyroid, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as a coupling reagent, and the complex was used as the immunogen. BALB/c mice were injected subcutaneously with 50–200 μg of peptide and boosted on days 14 and 21. The spleen cells were removed and the cells were fused with myeloma cells using polyethyleneglycol 4000. Screening of positive clones was performed by immunohistochemistry of Triton-insoluble smears from FTLD-U brains. Ascites were prepared by intraperitoneal injection of cloned hybridoma cells and used in all experiments. Polyclonal antibody (pAb) pS409/410 and anti-TDP43C (405–414) were prepared as described [14]. Anti-TDP-43 antibody and anti-ubiquitin antibody were purchased from Protein Tech and Dako, respectively. Antibodies were diluted as follows: mAb pS409/410 (1:3000–5000), pAb pS409/410 (1:2000–3000), anti-TDP-43 (1:3000–5000), anti-ubiquitin (1:2000) and anti-TDP43C (1:1000–2000).

2.2. Preparation of recombinant TDP-43 and phosphorylation

In a full-length cDNA encoding the 414 amino acids of human TDP-43, mutations were introduced at codons 409 (Ser to Ala: S409A), 410 (Ser to Ala: S410A), or 409 and 410 (Ser to Ala: S409A/S410A) using a site-directed mutagenesis kit (Strategene) according to the manufacturer's instructions. Expression and partial purification of the wild-type (WT) and mutated TDP-43 proteins and phosphorylation of recombinant TDP-43 with CK1 or CK2 were carried as described [14].

2.3. Extraction of TDP-43 and immunoblotting

Brains of embryonic 19-day (E19) and 21-day (E21), and postnatal 1-day-old (1d), 1-week-old (1w), 2-week-old (2w), 4-week-old (4w), and 6-week-old (6w) Wistar rats were homogenized in 10 volumes of extraction buffer (10 mM Tris–HCl, pH 7.5, 1 mM EGTA, 10% sucrose, 0.8 M NaCl) containing phosphatase inhibitors (50 mM β-glycerophosphate, 5 mM NaF, 5 mM $NaVO_3$) and a protease inhibitor cocktail (Calbiochem), and spun at 386000×*g* for 20 min at 4 °C. The resulting supernatants were retained as Tris-soluble fraction and the pellets were homogenized in 10 volumes of buffer containing 1% Triton X-100. The homogenates were left for 30 min at 37 °C. After a 20 min spin at 386000×*g*, the supernatants were retained as Triton-soluble fraction and the pellets were homogenized in buffer containing 1% Sarkosyl. The homogenates were left for 30 min at 37 °C, followed by a 20 min spin at 386000×*g*. The supernatants were retained as Sarkosyl-soluble fraction and the pellets were solubilized in one volume of 50 mM Tris–HCl, pH 7.5, containing 8 M urea, followed by a 20 min spin at 386000×*g*. The supernatant was retained as Sarkosyl-insoluble, urea-soluble fraction. Various tissues of 6-week-old rats were also homogenized as described above, and the extracted proteins were analyzed. Sarkosyl-insoluble fractions from frontal and temporal regions of human control, FTLD-U and ALS brains were prepared as described [14]. The samples before (−) and after (+) the treatment with lambda protein phosphatase (λPPase) were loaded on 10% SDS–PAGE. Immunoblotting was performed as described [14].

2.4. Immunohistochemistry

Human brain tissue was obtained from the Brain Donation Program at Sun Health Research Institute, Sun City AZ and from Aichi Medical University, and Tokyo Metropolitan Matsuzawa Hospital, Japan. Small blocks of brain tissue were dissected at autopsy and frozen rapidly at −70 to −80 °C or fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 2 days. Brain tissue from patients with sporadic FTLD-U, familial FTLD-U with PGRN mutations (mPGRN), sporadic ALS and sporadic FTLD-MND was compared with brain tissue from neurologically normal control subjects. Neuropathological diagnoses of FTLD-U, FTLD-MND and ALS were made in accordance with published guidelines as described [14].

Following cryoprotection in 15% sucrose in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), PFA-fixed tissue blocks were frozen and cut on a microtome at 30 μm thickness. Free floating sections were immunostained with mAb 409/410, an anti-ubiquitin antibody, and a commercially-obtained phosphorylation-independent anti-TDP-43 antibody (ProteinTech), using reported methods [3].

3. Results

3.1. Antibody specificities

To investigate the specificity of mAb pS409/410, we performed ELISA assay of synthetic phospho- and non-phosphopeptides. As shown in Fig. 1A, mAb pS409/410 strongly reacted with a pS409/410 phosphopeptide, CMDSKS(p)S(p)GWGM, which was used as the immunogen, but did not react with the peptide containing non-phosphorylated S409/410. It only weakly recognized phosphopeptides pS409 (CMDSKS(p)SGWGM) and pS410 (CMDSKSS(p)GWGM) (Fig. 1A), suggesting that this monoclonal antibody is highly specific for the peptide phosphorylated at both Ser409 and Ser410. This was confirmed by immunoblot analysis of wild-type (WT) and mutated recombinant TDP-43 before and after phosphorylation with CK1. As shown in Fig. 1B, mAb pS409/410 failed to recognize wild-type TDP-43 prior to phosphorylation. However, it did recognize wild-type TDP-43 that had been incubated overnight with CK1. It also weakly recognized phosphorylated S409A and S410A, but failed to stain S409A/S410A (Fig. 1B). Immunoblotting with pAb pS409/410 gave similar results: however, weak staining was detected with non-phosphorylated

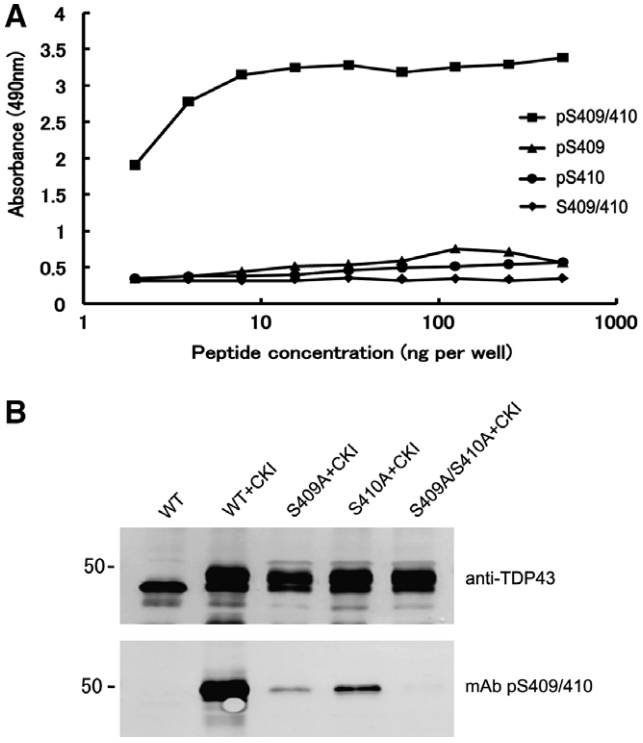

Fig. 1. Characterization of mAb pS409/410A. (A) ELISA assay: a synthetic peptide or phosphopeptides corresponding to residues 405–414 of TDP-43 were probed with mAb pS409/410 antibody. (B) Immunoblot analyses of recombinant TDP-43 phosphorylated in vitro. Wild-type and mutated recombinant human TDP-43 were incubated with or without CKI (10000 units/ml, NEB) in the presence of ATP at 37 °C for 24 h. Immunoblots were stained with anti-TDP-43 (ProteinTech) and mAb pS409/410.

wild-type TDP-43 when a large amount of TDP-43 was loaded (data not shown), suggesting that this antiserum may not be entirely phosphorylation-dependent.

3.2. Immunohistochemical characterization of mAb pS409/410

MAb pS409/410 strongly stained ubiquitin-positive inclusions in both FTLD-U and ALS brains (Fig. 2). It recognized NCIs in the dentate gyrus (Fig. 2A) and DNs in the temporal cortex (Fig. 2B) of sporadic FTLD-U cases, and skein-like inclusions (Fig. 2C), round inclusions (Fig. 2D) and glial inclusions (Fig. 2E) in the spinal cord of ALS cases. These inclusions were unambiguously identified by mAb pS409/410, with no nuclear staining (Fig. 2). In some cases, pAb pS409/410 weakly stained ghost tangles and dot-like structures in the hippocampal region of brains from patients with AD or other related diseases, but mAb pS409/410 did not recognize these structures (data not shown).

3.3. Immunoreactivity of TDP-43 deposited in FTLD-U and ALS brains and TDP-43 extracted rapidly from rat tissues

MAb pS409/410 strongly stained 45 kDa TDP-43 band, ~25 kDa fragments and smearing substances in the Sarkosyl-insoluble fractions of FTLD-U and ALS brains. Lambda protein phosphatase (λPPase) treatment of Sarkosyl-insoluble TDP-43 resulted in loss of mAb pS409/410 immunoreactivity (Fig. 3A). The patterns of the bands of ~25 kDa fragments detected with mAb 409/410 were different between sporadic cases

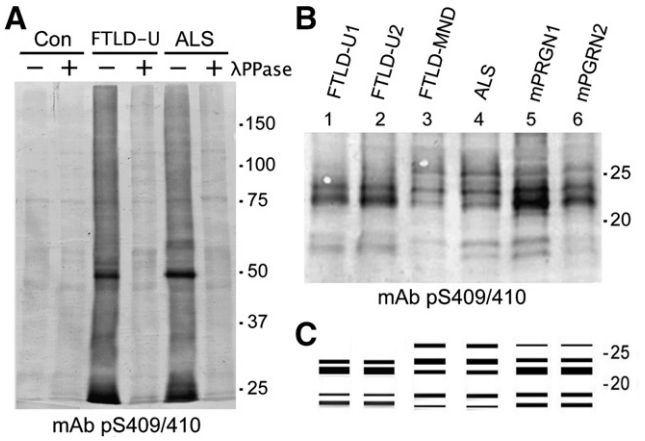

Fig. 3. (A) Immunoblot analyses of the Sarkosyl-insoluble, urea-soluble fractions from human control, FTLD-U and ALS brains with anti-TDP-43 (ProteinTech) and mAb pS409/410 antibodies before (−) and after (+) treatment with lambda protein phosphatase (λPPase). MAb pS409/410 specifically labels the ~45 kDa full-length TDP-43, as well as ~25 kDa fragments and the smearing substances, in FTLD-U and ALS. The labeling is abolished after dephosphorylation. Normal 43 kDa TDP-43 in control and diseased brains is not stained by mAb pS409/410. (B) Immunoblots of the Sarkosyl-insoluble, urea-soluble fractions from sporadic FTLD-U, FTLD-MND, ALS and mPGRN cases with the mAb pS409/410. The samples were loaded on 15% polyacrylamide gel. (C) Schematic diagram of the band pattern of the C-terminal fragments of phosphorylated TDP-43.

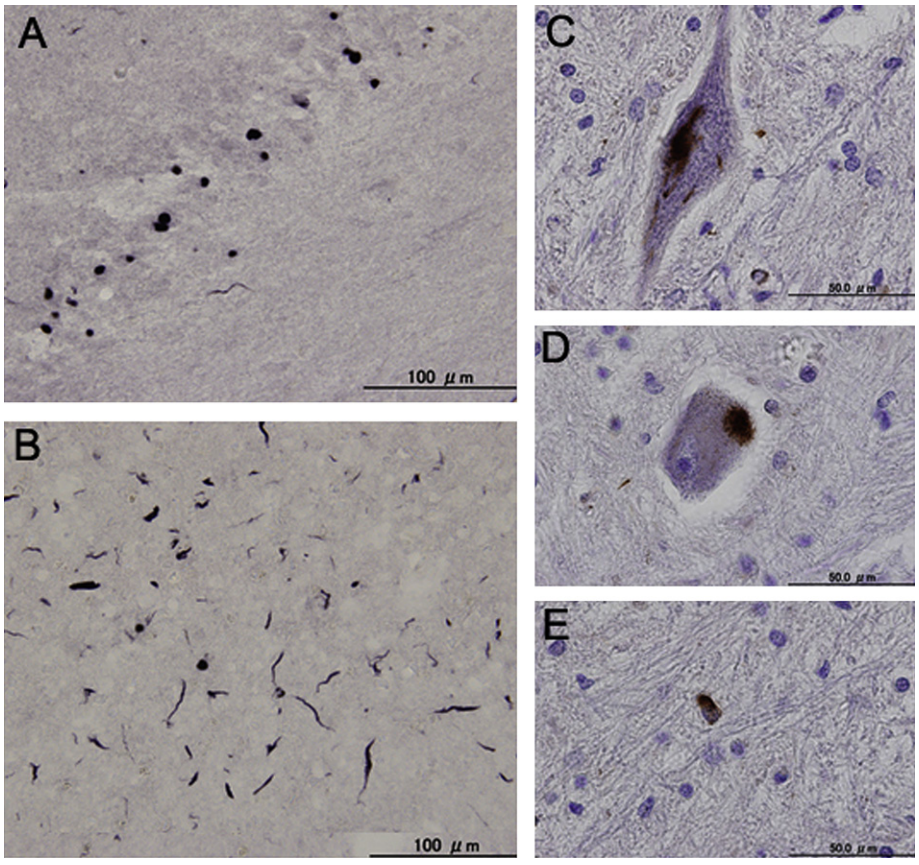

Fig. 2. Immunohistochemistry of TDP-43 lesions. Thirty-micrometer-thick free floating sections of the dentate gyrus of hippocampus (A) and temporal cortex (B) from FTLD-U and of spinal cords from ALS (C–E) were immunostained with mAb pS409/410. TDP-43-positive NCIs in dentate gyrus and DNs in cortex are specifically stained. Note the absence of nuclear staining in A–E. Bars 100 μm (A,B) and 50 μm (C–E).

of FTLD-U, ALS and familial cases of FTLD-U with progranulin mutation (Fig. 3B), as previously observed with pAb pS409/410 [14]. Sporadic FTLD-U cases showed two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa, while FTLD-MND and ALS cases showed three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa (Fig. 3B and C). The band pattern of mPGRN cases was intermediate between those of FTLD-U, FTLD-MND and ALS cases (Fig. 3B and C).

We next examined whether the phosphorylation of Ser409/410 can be detected in TDP-43 rapidly extracted from fetal or adult rats with a buffer containing phosphatase inhibitors, because phosphorylation seen at many sites of tau and at Ser129 of α-synuclein under pathological conditions is also present in a significant fraction of normal tau [15] or α-synuclein [16], and these sites are rapidly dephosphorylated postmortem [17,18].

Anti-TDP-43 antibody strongly labeled the TDP-43 band at 43 kDa in Tris-soluble fractions of brains from fetal to 6-week-old adult rats (Fig. 4A). In contrast, mAb pS409/410 failed to detect any of the TDP-43 bands in these fractions (Fig. 4A), though the antibody strongly stained recombinant TDP-43 phosphorylated with CK1. On the other hand, an antibody specific for phosphorylated tau at Ser396 (pS396) strongly recognized phosphorylated tau bands in fetal and adult rat brains (Fig. 4B). Similar results were obtained with pAb pS409/410 (data not shown). These findings indicate that phosphorylation of Ser409/410 does not occur in rat brain during normal development. Immunoblot analysis of extracts from various tissues revealed that TDP-43 is broadly expressed in various tissues (Fig. 4C). The 43 kDa band was strongly immunostained in cerebellum, brain stem, spinal cord, liver, lung, spleen and ovary, but only weakly in heart and kidney (Fig. 4C). In addition to the 43 kDa band, some low-molecular bands at 30–35 kDa in most of the tissues and a doublet at ~30 kDa in kidney were detected with anti-TDP-43 (Fig. 4C). In contrast, no apparent immunoreactivity was observed with mAb pS409/410, which was confirmed to strongly immunostain recombinant TDP-43 that had been phosphorylated with CK1.

3.4. Changes of TDP-43 during postmortem incubation

Rapid postmortem dephosphorylation occurs in tau and α-synuclein, and the Cdk5 activator p35 is degraded to p25 during postmortem incubation [19]. Therefore, we investigated

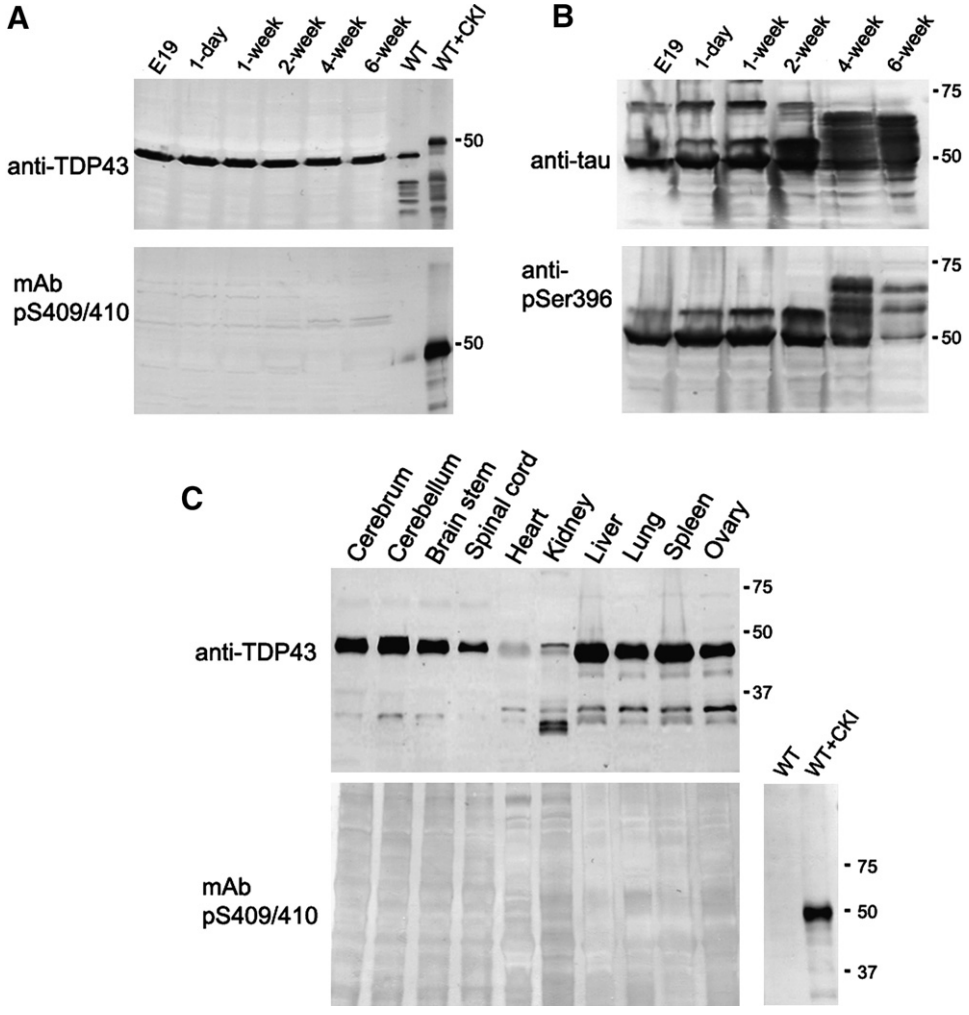

Fig. 4. (A) Immunoblot analyses of TDP-43 differentially extracted with Tris–HCl, Triton-X100, Sarkosyl and 8 M urea, from rat brains at various developmental stages, with anti-TDP43 antibody (ProteinTech), mAb pS409/410, an anti-tau antibody (T46: Zymed) and an anti-pS396 tau antibody (Calbiochem). (B) Immunoblots of Tris-soluble extracts from various tissues of 6-week-old rats with anti-TDP-43 and mAb pS409/410.

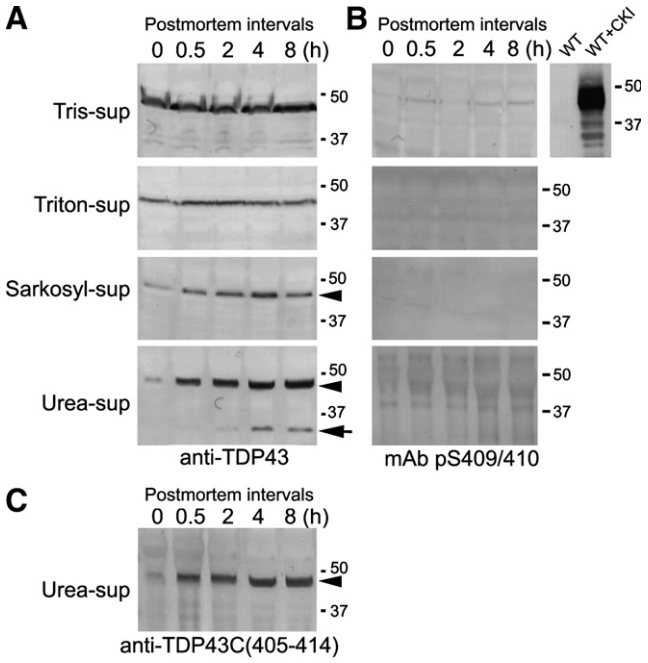

Fig. 5. Immunoblot analyses of brain extracts from rats after different postmortem intervals with anti-TDP43 antibody (ProteinTech) (A), mAb pS409/410 (B) and anti-TDP43C (405–414) (C).

the changes of phosphorylation and degradation of TDP-43 postmortem. One-year-old rats were lethally anethetized and the brains were excised 0, 0.5, 2, 4 or 8 h later, then homogenized differentially. The extracted fractions were analyzed by immunoblotting (Fig. 5). As shown in Fig. 5B, pS409/410 immunoreactivity was not detected in soluble or insoluble fractions of the brains, nor was it generated during postmortem incubation, indicating that phosphorylation of Ser409/410 does not occur even in aged rats. Interestingly, the levels of Sarkosyl-insoluble and urea-soluble TDP-43 of 43 kDa as well as Sarkosyl-soluble TDP-43 (Fig. 5A, arrowheads) and the levels of urea-soluble 35 kDa fragment (Fig. 5A, arrow) gradually increased during postmortem incubation at room temperature, although no apparent reduction was detected in TS-soluble TDP-43. The postmortem increase of Sarkosyl-insoluble and urea-soluble TDP-43 was also observed with antiserum directed against the C-terminus of TDP-43 (405–414) (Fig. 5C).

4. Discussion

Abnormal phosphorylation of TDP-43 has been suggested in the initial reports that identified TDP-43 as the major component of ubiquitin-positive inclusions in FTLD-U and ALS [3,4]. In the present study, we have produced a monoclonal antibody that distinguishes the disease-associated phosphorylation of TDP-43. By means of ELISA assay of phosphopeptides and a combination of site-directed mutagenesis and in vitro phosphorylation, we have shown that mAb pS409/410 is highly specific for TDP-43 phosphorylated at both Ser409 and Ser410. MAb pS409/410 strongly stained TDP-43 lesions in FTLD-U brains and ALS brains/spinal cords, as well as abnormal TDP-43 bands and smears on immunoblots, in agreement with previous observations using pAb pS409/410 [14]. In contrast, mAb pS409/410 did not recognize normal human TDP-43 of 43 kDa or TDP-43 rapidly extracted from brains of fetal to 1-year-old adult rats. It also did not react with TDP-43 expressed in other various tissues. These results indicate that phosphorylation of TDP-43 at Ser409/410 is a pathological event. This is different from the cases of tau or α-synuclein, where phosphorylation at most of the sites can be detected in normal fetal or adult brain. It remains unknown whether the abnormal phosphorylation of TDP-43 at Ser409/410 precedes assembly of TDP-43, or whether it is a reaction associated with aggregation or conformational change of TDP-43. However, it is clear that the phosphorylation is a specific biological marker for detection of abnormal TDP-43 deposits in FTLD-U and ALS brains/spinal cords.

In the course of characterization of mAb pS409/410, we found that the levels of Sarkosyl-insoluble and urea-soluble TDP-43 increased during postmortem incubation. A similar increase of Sarkosyl-insoluble TDP-43 was observed during the incubation of recombinant human TDP-43 at 37 °C for 24 h (data not shown). These results suggest that the sarkosyl-insoluble, urea-soluble TDP-43 of 43 kDa and the 35 kDa fragment detected in brains of human controls and patients with FTLD-U or ALS may have been artifacts generated postmortem. Zhang et al. reported that the amounts of the 35 and 25 kDa fragments were increased in cells treated with staurosporin or PGRN siRNA and in brains of patients of FTLD-U [20], whereas Shankaran found that these fragments of TDP-43 were generated independently of PGRN knock-down in cell lines and zebrafish [21]. Our results show that a 35 kDa N-terminal fragment of TDP-43 is generated during postmortem incubation and recovered in insoluble fractions, but this is irrelevant to the pathology of TDP-43 proteinopathy.

As had been found with pAb pS409/410, mAb pS409/410 revealed that the 18–26 kDa C-terminal fragments of TDP-43 are the major species of TDP-43 deposited in patients with FTLD-U and ALS, and the patterns of the fragments are closely related to the neuropathological subtypes of the inclusions. Although further studies are needed to clarify the molecular mechanisms of the fragmentation and aggregation of TDP-43, the results presented here suggest that aggregation of TDP-43 is implicated in FTLD-U and ALS, just as aggregation and phosphorylation of tau or α-synuclein may be involved in other neurodegenerative diseases. MAb pS409/410 may be a useful probe for detection of abnormal TDP-43 in tissues of patients and for evaluation of cellular or animal models of TDP-43 proteinopathy. Antibodies specific for the abnormal phosphorylation sites of TDP-43 may be useful for antibody therapy, or the antigens may be suitable for immunization against FTLD-U and ALS.

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