Integrative Biosciences 11: 39-50, 2007

Integrative Biosciences
http://www.ibiosci.or.kr

Facilitation of SUMO (Small Ubiquitin-like Modifier) Modification at Tau 340-Lys Residue (a Microtubule-associated Protein) through Phosphorylation at 214-Ser Residue

Eun Jeoung Lee[1], Sung Hee Hyun[2], Jaesun Chun[3], Hye Rim Ahn[1] and Sang Sun Kang[1,*]

[1]*School of Science Education, Chungbuk National University, Gaeshin-dong, Heungdok-gu, Chongju, Chungbuk, 361-763, Korea;* [2]*Department of Pre-medicine, Eulji University School of Medicine, Daejeon 301-832, Korea;* [3]*Department of Biology Education, Korea National University of Education, Chongwon, Chungbuk 363-791, Korea*

Abstract: Tau plays a role in numerous neuronal processes, such as vesicle transport, microtubule-plasma membrane interaction and intracellular localization of proteins. SUMO (Small Ubiquitin-like Modifier) modification (SUMOylation) appears to regulate diverse cellular processes including nuclear transport, signal transduction, apoptosis, autophagy, cell cycle control, ubiquitin-dependent degradation, as well as gene transcription. We noticed that putative SUMOylation site is localized at ^{340}K of Tau (^{339}VKSE^{342}) with the consensus sequence information (ΦKxE; where Φ represents L, I, V or F and x is any amino acid). In this report, we demonstrated that ^{340}K of Tau is the SUMOylation site and that a point mutant of Tau S214E (an analog of the phospho ^{214}S Tau) promotes its SUMOylation at ^{340}K and its nuclear or nuclear vicinity localization, by co-immunoprecipitation and confocal microscopy analysis. Further, we demonstrate that the Tau S214E (neither Tau S214A nor Tau K340R) mutant increases its protein stability. However, the SUMOylation at ^{340}K of Tau did not influence cell survival, as determined by FACS analysis. Therefore, our results suggested that the phosphorylation of Tau on ^{214}S residue promotes its SUMOylation on ^{340}K residue and nuclear vicinity localization, and increases its stability, without influencing cell survival.

Key words: Tau, SUMO (Small Ubiquitin-like Modifier), subcellular localization, signal transduction, apoptosis, cell cycle control

Tau is a family of microtubule-associated proteins that are expressed predominantly in neurons and are expressed by alternative splicing of a single gene (reviewed by Maccini et al., 2001; Johnson, and Stoothoff, 2004; Feinstein and Wilson, 2005). Tau, inseveral isoforms, plays a role in numerous neuronal processes, such as vesicle transport, microtubule-plasma membrane interaction and intracellular localization of proteins (Goldsbury et al., 2006; Raynaud and Marcilhac, 2006). The well-known function of Tau is the binding and stabilization of microtubules (Feinstein and Wilson, 2005). Filamentous Tau aggregate is the major component of neurofibillary tangles (NFTs), the most common neuropathological hallmark in several neurodegenerative disorders, including Alzheimer's disease (AD). NFTs consist of highly phosphorylated microtubule-associated Tau that assembles to form fibrila with β-sheet structures within the cell body and dendrites of neurons (Luna-Munoz et al., 2005). Generally, increased Tau phosphorylation tends to negatively regulate Tau-microtubule interactions, suggesting that Tau is one of major proteins for the cell cycle, growth, differentiation, apoptosis, and morphogenesis (Chong et al., 2005). In AD brain, abnormally hyperphosphorylated Tau accumulates and forms paired helical filaments (Takashima, 2006). Since abnormally phosphorylated Tau does not bind to microtubules, abnormal Tau phosphorylation in AD brain is thought to cause a loss of Tau function, microtubule dysfunction, and neuron degradation (Raynaud and Marcilhac, 2006). The role of Tau phosphorylation, however, is still unclear (Butterfield et al., 2005).

Recently, a new covalent modification of protein, especially of transcriptional regulator and nuclear pore protein has been described: SUMO (Small Ubiquitin-like Modifier) modification (SUMOylation) (reviewed by Gill, 2005). This is a covalent modification leading to the attachment of SUMO to specific lysine residues of target proteins, mainly

*To whom correspondence should be addressed.
Tel: 82-43-261-3278; Fax: 82-43-271-0526
E-mail: jin95324@cbu.ac.kr

nuclear proteins (Wilson and Rangasamy, 2001; Maria et al., 2004; Kikuchi et al., 2006). SUMO modification appears to regulate diverse cellular processes, including nuclear transport, signal transduction, apoptosis, autophagy, cell cycle control, ubiquitin-dependent degradation, as well as gene transcription (Mabb et al., 2006, Suico et al., 2006). SUMO represents a class of ubiquitin-like proteins conjugated, like ubiquitin, by a set of enzymes to cellular proteins (Melchior and Hengst, 2002; Gill, 2004). Although mechanistically similar to ubiquitination, SUMOylation does not promote protein degradation and the two processes involve distinct enzymes. Protein SUMOylation involves SAE1/SAE2 heterodimer acting as E1 enzyme in mammals (Aos1/Uba2 in yeast) and Ubc9 acting as E2 SUMO-conjugating enzyme (Boggio et al., 2004). In ubiquitination, E3 ubiquitin ligases, that promote ubiquitin transfer from E2 enzyme to the target lysine, are responsible for substrate specificity. Although target specificity remains unclear in the SUMO modification pathway, proteins of the mammalian PIAS (protein inhibitor of activated STAT) family, Ran Bp (Ran binding protein) 2 and the polycomb PC2 repressor, have recently been shown to function as E3-type SUMO ligases (Pichler et al., 2002; Kagey et al., 2003; Bischof et al., 2006; van Waardenburg et al., 2006). The analysis of many SUMO substrates indicates that SUMO modification occurs at a particular sequence, ΦKxE/D (where Φ represents L, I, V or F and x is any amino acid); thus, specificity of SUMO conjugation might be conferred by recognition of this sequence by the thioester-linked Ubc9-SUMO conjugate (Duprez et al., 1999; Sampson et al., 2001; Yang et al., 2006).

We previously demonstrated that the SGK1 phosporylation site is located at 214-serine residue (^{207}GSRSRTPSLP^{216}) of human Tau 40 (hTau40) that creates one of 14-3-3 binding sites (RSX$_{(1-2)}$ S*XP, S* is a phosphor serine residue), with the SGK1 substrate consensus sequence information (R-X-R-X-X- (S/T)-Φ; Φ indicates a hydrophobic amino acid) (Chun et al, 2004). We also observed that ^{214}S phospho Tau was localized to the nuclear region or nuclear vicinity in COS-1 cell. Thus, we speculated that the subcellular localization of ^{214}S phospho Tau seemed to be related with the differential phosphorylation of Tau for SUMOylation. In addition, we noticed two putative SUMOylation sites in Tau40 (^{340}K in ^{339}VKSE^{342}, and ^{385}K in ^{384}AKTD^{387}) (Dorval and Fraser, 2006). Thus, we initially intended to identify SUMOylation site by confocal microscopy analysis and immunoblotting assay. During this process, we observed that SUMOylation of hTau40 on ^{340}K residue is facilitated by Tau S214E point mutation (the phosphorylation analog on ^{214}S of Tau), and that this mutation increases its stability and nuclear vicinity localization, without any cell viability change. Even though the biological significance of phosphorylation on ^{214}S residue still remains to be characterized, we propose here that the phosphorylation on ^{214}S of Tau facilitates the SUMOylation on its ^{340}K, and that this may be a new mechanism modulating its subcellular localization and stability.

MATERIALS AND METHODS

Cell culture

COS-1 was purchased from ATCC (Manassas, VA, USA). Media and supplements were obtained from GIBCO (Grandisland, NY, USA). The cell line was maintained in Dulbecco's Modified Essential Medium (DMEM) containing 10% heat inactivated (for 30 min at 56°C) fetal bovine serum (FBS), 100U potassium penicillin/ml, 100 μg/ml streptomycin, 2 mM glutamine and 20 mM sodium bicarbonate. The cells were incubated at 5% CO_2, 95% humidity and 37°C chamber. The growth medium was changed every 3 days. SUMO fusion protein was obtained from Calbiochem (Grandisland, NY, USA).

Antibodies

The monoclonal antibody against the hexahistidine GFP epitope or GFP was purchased from Calbiochem (Emeryville, CA, USA). Antibodies against Tau, actin, Tau 214-Ser, or Tau 422-Ser phospho specific antibody were purchased from Stress Gene (Victoria British Columbia, Canada). Antibodies against ubiquitin or SUMO-1 were purchased from Santa Cruz Biotech. Inc (Santa Cruz, CA, USA).

Site-directed mutagenesis of Tau

To generate Tau S214A mutation (5'-AGC CGC TCC CGC ACC CCG GCC CTT CCA ACC-3', 5'-GGA TGG AAG GGC CGG GGT GCG GCT-3' primers) or K340R (5'-GGA GGT GGC CAG GTG GAA GTA CGA TCT GAG AAG CTT GAC-3', 5'-CTT GAA GTC AAG CTT CTC AGA TCG TAC TTC CAC CTG GCC-3'), Human Tau 40 isoform of 441 amino acids (a gift from Dr. Jung) was used with "Chameleon" double-stranded site-directed mutagenesis kit (Stratagene, CA, USA), according to the manufacturer's instructions. Every mutation was confirmed by DNA sequencing.

Tau expression vector transfection and purification

The mutagenized or wild type human Tau was respectively cloned into EGFP-tagged mammalian expression vector (In Vitrogen) after PCR with forward primer; 5'-ATG GAA GAT CAC GCT GGG ACG-3' and reverse primer; 5'-gag agc tca gta cat gag ctg-3' from wild type (wt) Tau, Tau S214A mutant, or SUMO (K340R, S214E K340R) mutant. For mammalian expression, GFP-Tau constructs were transfected into COS-1 cells by lipofectin transfection method (Gibco-BRL Co). Transfected cells (2×10^7) were

lysed in RIPA lyses buffer. Anti-GFP polyclonal antibody was incubated with 1000 μl of pre-cleaned cell lysate, and precipitated with protein A agarose beads. The beads were washed three times with excess cell lysis buffer. The final pellet was used for immuno assay to detect SUMOylation or ubiquitinization. Western blots were performed to detect the presence of SUMO or ubiquitin with an anti-SUMO-1 or anti-ubiquitin antibody (Maria et al., 2004).

Double Immunofluorescence Microscopy

COS-1 cells were plated at low confluence (~30%) on two-well Lab-Tek Permanox slides (Nalgene Nunc International, Naperville, IL, USA) and transiently transfected with EGFP-Tau wt (hTau40), EGFP-Tau Tau S214A, or SUMO (K340R, S214E K340R) mutant plasmids using the lipofectamine procedure. Cells were starved for 36 h and subsequently treated with 10% calf serum for 15 h. At no time did cell confluency exceed 60%. Cells were fixed, permeabilized, and processed for indirect double immunofluorescence microscopy as described previously with minor modifications. Cells were blocked in normal goat serum (diluted 1 : 30 in PBS), for 15 min and incubated with affinity-purified, anti- antibodies at 1 : 150 dilution, in combination with 1 : 1000 dilution of murine anti- Tau, SUMO-1, or ubiquitin monoclonal antibodies (Santa Cruz Biotech. CA, USA) or anti- pS214, pS422 Tau, GFP polyclonal antibodies (Calbiochem. Inc. La Jolla, USA) for 1-2 h at room temperature on a rocking platform. Washed slides were incubated for 1 h at room temperature with 1 : 150 dilutions of both anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Molecular Probes Inc., Eugene, OR, USA) and Texas red-conjugated goat anti-mouse secondary antibody (Molecular Probes Inc.). Slides were washed and then mounted with Vectashield mounting medium (Vector Laboratories Inc.) and examined using Zeiss Axiophot optics in The Core Facility of Chungbuk National University (Chun et al., 2004).

Expression and purification of recombinant proteins

Wild type (wt) Tau, mutant Tau S214A or SUMO (K340R or S214E K340R) mutant was respectively cloned into a prokaryotic expression vector, pGEX-3T (Phaimacia), after PCR with the same primer set used for generation of the mammalian expression constructs. Each protein tagged by GST was expressed in *Escherichia coli* BL21 and purified with GST-agarose beads according to the manufacturer's instruction. Purified proteins were used for SUMOylation assay substrate protein.

In vitro SUMO-1 conjugation assay

SUMO-1 conjugation assay was performed using the SUMO assay kit purchased from Corgen Inc, according to the manufacturer's recommended protocol. One microgram of purified GST-tagged Tau mutant was mixed with 250 ng of Ubc9 and 125 ng of Aos1/Uba2 with or without 2 μg of SUMO-1, and then incubated for 2 h at 30°C in the presence of 50 mM Tris [pH 7.5], 5 mM $MgCl_2$, with or without (as a negative control) 2 mM ATP in a total volume of 20 μl. Reactions were stopped with SDS-PAGE sample buffer and SUMO conjugates were separated by SDS-PAGE and analyzed by western blotting using mouse monoclonal antibody against SUMO-1 to detect Tau (Maria et al., 2004).

Protein Stability Experiments

COS-1 cells (2.5×10^5 cells per well) in 10 cm plates were transfected with 1.0 μg of expression vector carrying EGFP-Tau wt, EGFP-Tau S214E mutant plasmid. The medium was replaced with medium containing 200 μg/ml cycloheximide 36 h after transfection. Cell lysates were harvested at 0, 8, 16, and 24 h and analyzed by immunoprecipitation and Western blotting using anti-GFP antibodies in triplicate. (Vega et al., 2004.)

FACS

Tau (wt), S214E, S214A, S214E K340R, or EGFP vectors were transfected and the rate of apoptosiswas measured by FACS. EC were added to whole blood or to MNC or were first stained with E1/1 2.3, quantified, and added immediately prior to FACS. MNC with or without EC were stained with E1/1 2.3 on ice for 30 min, washed twice with ice-cold Hanks' balanced salt solution and 5% human pooled serum, and subsequently labelled with FITC-conjugated rabbit anti-mouse immunoglobulin G on ice for 30 min. Cells were washed twice and collected in Hanks' balanced salt solution and 5% human pooled serum. FACS was performed on a Coulter Epics Elite equipped with a gated amplifier and upgraded with enhanced system performance at The Core Facility of Chungbuk National University (Leung et al., 1992).

RESULTS

Subcellular localization of 214-serine phosphorylated Tau in COS-1 cell

In our previous report, we demonstrated that SGK1 phosphorylates the ^{214}S residue of Tau (^{207}GSRSRTPSLP^{216}) (Chun et al., 2004). This phosphorylation site is located in the middle of Tau protein (Fig. 1). To determine the biological function of the ^{214}S residue phosphorylation site by SGK1, we examined sub-cellular localization of endogenous Tau and ^{214}S phospho Tau in COS-1 cells by flourescence microscopy. Using a Tau specific antibody, we detected Tau wild type in the cytoplasm in a fibrous form (Fig. 2A left). On the dother hand, using a specific antibody against ^{214}S phospho Tau, we observed it mainly in the nuclear region

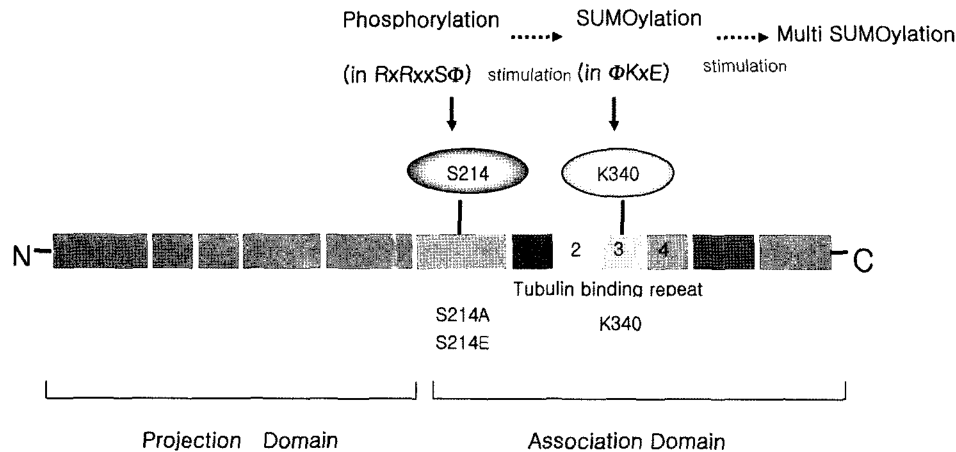

Fig. 1. Summary of Tau domains and mutants used in SUMOylation study. Two major domains (Projection and Association domain) are in Humain Tau protein 40. There are four tubulin binding domains in the Association domain (Friedhoff et al., 2000). The SUMOylation site (^{340}K in ^{339}VKSE^{342}) and SGK1 phosphorylation site (^{214}S in ^{207}GSRSRTPSL^{215}) are indicated above. Tau mutants (K340R, S214A, and S214E K340R), indicated below, were constructed by site directed mutagenesis. Tau mutants (K340R, S214A, and S214E K340R) were also inserted into EGFP fusion expression vector. The phosphorylation on ^{214}S in ^{207}GSRSRTPSL^{215} seems to stimulate the SUMOylation on ^{340}K in ^{339}VKSE^{342}, resulting in multi SUMOyaltion on other K residues.

(Fig. 2A middle). To compare the specificity of ^{214}S phosphorylation with another phosphorylation in Tau, we performed the flourescence microscopy with a specific antibody against ^{422}S phosphor Tau (Fig. 2A right). The ^{422}S phosphor Tau was found mainly in the cytoplasm. Thus, these observations suggested that the phosphorylation on ^{214}S residue of Tau by SGK1 bestows a mechanism to specifically localize to the nucleus or the nuclear vicinity in COS-1 cells.

To further define our above observation for the ^{214}S phospho Tau localization specificity, we constructed Tau S214E (as an analog of ^{214}S phosphorylated Tau) and Tau S214A (as an analog of ^{214}S unphosphorylated Tau) by site directed mutagenesis, and cloned them into an EGFP expression vector (Fig. 1). By flourescence microscopic analysis, we observed that EGFP Tau S214E was mainly detected at the nuclear vicinity (Fig. 2B middle), whereas EGFP Tau S214A was in the cytoplasm (Fig. 2B right). EGFP Tau wild type (wt) was observed in the cytoplasm in fibrous forms (Fig. 2B left) which was similar with the result of endogenous Tau (Fig. 2A left). We speculated that the difference in subcellular localization between the endogenous ^{214}S phospho Tau (Fig. 2A) and EGFP Tau S214E (Fig. 2B) was due to the EGFP tagging in EGFP Tau S214E. The EGFP moiety inhibits EGFP Tau S214E nuclear translocation, making it remain at the nuclear vicinity (Figs 2B).

To eliminate the cell line specificity, we repeated the above experiment with NIH3T3 cells, and observed the same phenomena as with COS-1 cell (data not shown). Thus, these results also suggested that because both endogenous ^{214}S phospho Tau and its analog (S214E) were localized to the nucleus or nuclear vicinity, the phosphorylation at ^{214}S residue of Tau is related with its subcellular localization.

Next, we speculated that the nuclear region or nuclear vicinity localization of the endogenous ^{214}S phosphor Tau and EGFP Tau S214E was related with SUMOylation, because the attachment of SUMO to specific lysine residues of target proteins mainly occurs to nuclear proteins (Duprez et al., 1999; Wilson and Rangasamy, 2001). To address this question, we performed confocal microscopy using anti-Tau or anti-SUMO-1 antibodies. As shown in Fig. 2C, the endogenous ^{214}S phospho Tau (green) was merged (yellow) with SUMO-1 (red) in the nuclear region. Thus, the endogenous ^{214}S phospho Tau seemed to be transported into the nuclear region (Fig. 2A middle) and SUMOylated. Thus, these results suggested that the phosphorylation on ^{214}S residue of Tau by SGK1 regulates not only its subcellular localization but also its SUMOylation.

Tau SUMOylation *in vitro*

Because two putative SUMOylation consensus sequences (Φ KxE), are bound at ^{340}K and ^{385}K in the tubulin binding domain of Tau (Fig. 1), it was possible that Tau is a SUMO modified protein (Dorval and Fraser, 2006; Yang et al., 2006). Because ^{340}K in (^{339}VKSE^{342}) of Tau seems to be more similar with the SUMOylation consensus motif, we chose this site and constructed Tau mutants (K340R, S214A, and S214E K340R) which are indicated in Fig. 1, by site directed mutagenesis and inserted them into EGFP fusion expression vector or GST fusion expression vector.

To determine that Tau SUMOylation occurred at the ^{340}K residue in ^{339}VKSE^{342}, we purified the GST-fusion proteins (Tau WT and Tau K304R mutant) from *E. coli*, and we

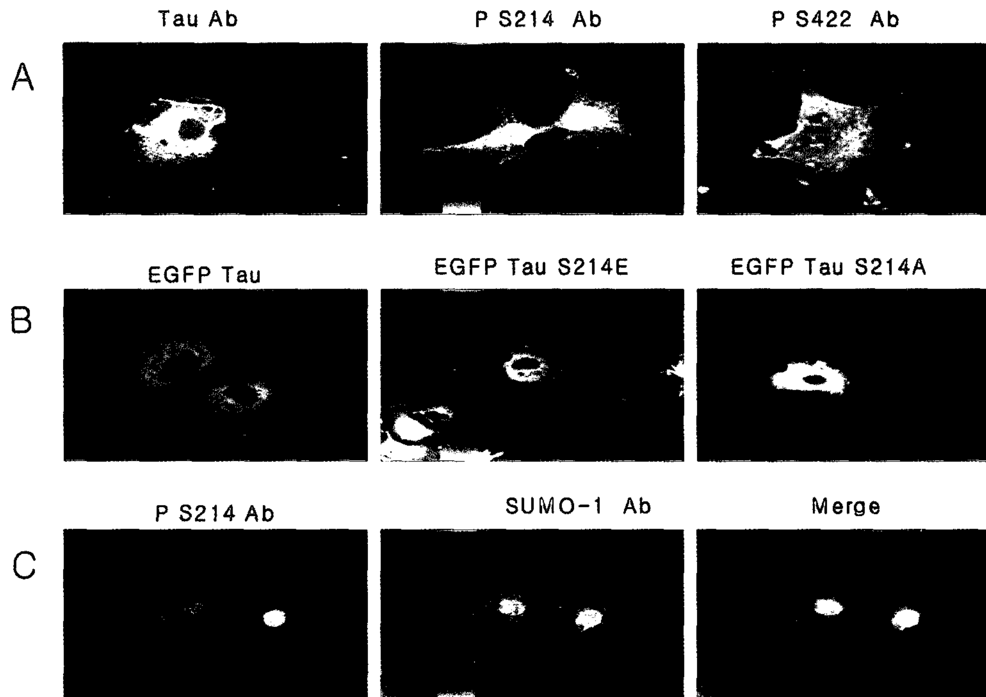

Fig. 2. Nuclear or nuclear vicinity localization of 214 Ser phospho Tau. Tau, a microtubule-associated protein, was detected along with microtubule fiber (A, left). The 214-serine phospho Tau was found at the centriole/microtubule organizing centriole and the nuclear vicinity, but not with the microtubule (A, middle), whereas the 422-serine phospho Tau was found in both cytoplasmic and nuclear regions (A, right). With EGFP Tau wt, Tau protein was detected along with microtubule fiber (B, left). We observed that EGFP Tau S214E (analog to 214-serine phosphor Tau) was found at the nuclear vicinity (B, middle), whereas Tau S214A (analog to 214-serine unphosphor Tau) was evenly distributed in the cytoplasm (B, right). Confocal microscopic analysis (C) of the endogenous 214-serine phospho Tau (green color) was performed to determine whether it merged with the SUMO-1 (red color).The phosphorylated Tau on ^{214}S residue was merged (yellow) with the SUMO-1 (red color) in the nucleus.

used them as substrate proteins in the SUMOylation assay as described in Materials and Methods. As shown in Fig. 3A, SUMOylation of Tau WT was detected as several high molecular weight protein bands (right lane), whereas this was not observed with the K340R mutant (middle lane). For a negative control, Tau WT was used without ATP (left lane). To monitor the protein amount to be used in the experiment, western blotting was performed with a Tau monoclonal antibody (Fig. 3B). Thus, these results suggested that 340K residue in ^{339}VKSE^{342} of Tau is the SUMOylation site. During our study, other researchers had already identified that SUOMylation site is the 340K in (^{339}VKSE^{342}) of Tau (Dorval and Fraser, 2006).

It has been reported that SUMO-1 (but not SUMO-2 and -3) monomerically conjugates the ε amino group of K residue in SUMO-1 acceptor consensus sequences (Φ KxE) (Sampson et al., 2001; Wilson and Rangasamy, 2001; Gill, 2004). As shown in Fig. 3 (right lane), however, SUMOylated Tau WT was detected as several high molecular weight protein bands (~200 kD). Multiple SUMOylation on several other K residues of Tau seemed to have occurred. Interstingly, however, SUMOylation did not occur on Tau K340R mutant (Fig. 3), even though ^{340}K is only one of the two potential sites (^{340}K and ^{385}K) as SUMO-1 acceptor consensus sequences (Φ KxE/D). Thus it seemed that ^{340}K site is both the UBC9 (E2) binding and the primary site for SUMOylation (Dorval and Fraser, 2006; Yang et al., 2006). Consequently, the SUMOylation on ^{340}K site triggers to stimulate each SUMOylation reactions on several other K residues in Tau (multiple SUMOylation). Even though SUMO forms a homo or hetero dimer in the cell, SUMOylation by the other SUMO isotypes (SUMO-2, 3 and 4) is excluded in the experiment, because hTau40 contains many K residues (total 42 sites) and the multiple SUMOylation by SUMO-1 on hTau40 is possible on these K residues. However, it is presently unclear why the SUMOylation of Tau wt occurs as several high molecular weight protein bands in *in vitro* SUMOylation assay with SUMO-1 antibody.

Confocal microscopic analysis of Tau SUMOylation

To determine further whether Tau undergoes SUMOylation at 340K residue, we analyzed COS-1 cells that were transiently transfected with constructs encoding Tau mutants in Fig. 1. The transfected EGFP-Tau wt, K340R, S214E or Tau S214E K340R (all constructs were shown as green color) was detected by fluorescence microscopy and the SUMO-1 position was shown in red (Fig. 4). We

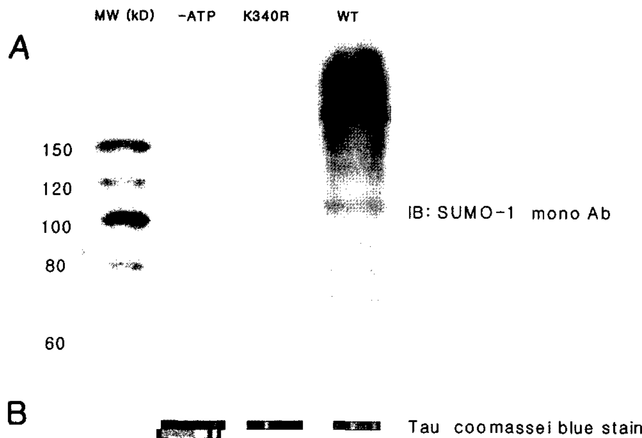

Fig. 3. Tau SUMOylation at 340K residue in 339VKSE342. The SUMOylation site (^{340}K in ^{339}VKSE^{342}) was noticed in its tubulin binding domains, as shown in Fig.1. A) The purified GST-Tau WT or GST-Tau K349R fusion protein was used as the substrate protein in the SUMOylation assay as prescribed in Materials and Methods. SUMOylation of Tau WT was detected as a high molecular weight protein band (right lane), whereas that of K340R mutant in ^{339}VKSE^{342} was totally inhibited (middle lane). For a negative control, Tau WT was used without ATP (left lane). B) To monitor Tau protein amount, Tau protein used in (A) was stained with coommassi green.

observed that SUMO-1 modification proteins were mainly detected in the nucleus (Fig. 4 middle lane). In the case of EGFP-Tau wt, which was observed in the cytoplasm as fibrous form (similar with Fig. 2A), it was slightly merged (yellow) with SUMO-1 at the nuclear vicinity (Fig. 4 the first row). However, even though EGFP-Tau S214A was evenly detected in the cytoplasm, it was not merged (yellow) with SUMO-1 at the nuclear vicinity (Fig. 4 the second row).

Surprisingly, EGFP-Tau S214E was mainly observed at the nuclear vicinity (consistent with Fig. 2A) and merged well (yellow color) with SUMO-1 (Fig. 4 the third row). To confirm that the ^{340}K residue is the SUMOylation site, we used Täu S214E K340R in which SUMOylation site was eliminated (Fig. 4 the fouth row). Even though EGFP-Tau S214E K340R was detected around the nucleus (similar with EGFP-Tau S214E confocal microscopy results in Fig. 4, third row), it was not merged with SUMO-1 (yellow). Thus, these results confirmed that ^{340}K residue in ^{339}VKSE^{342} of Tau is SUMOylation site and that the phosphorylation on ^{214}S promotes SUMOylation on ^{340}K.

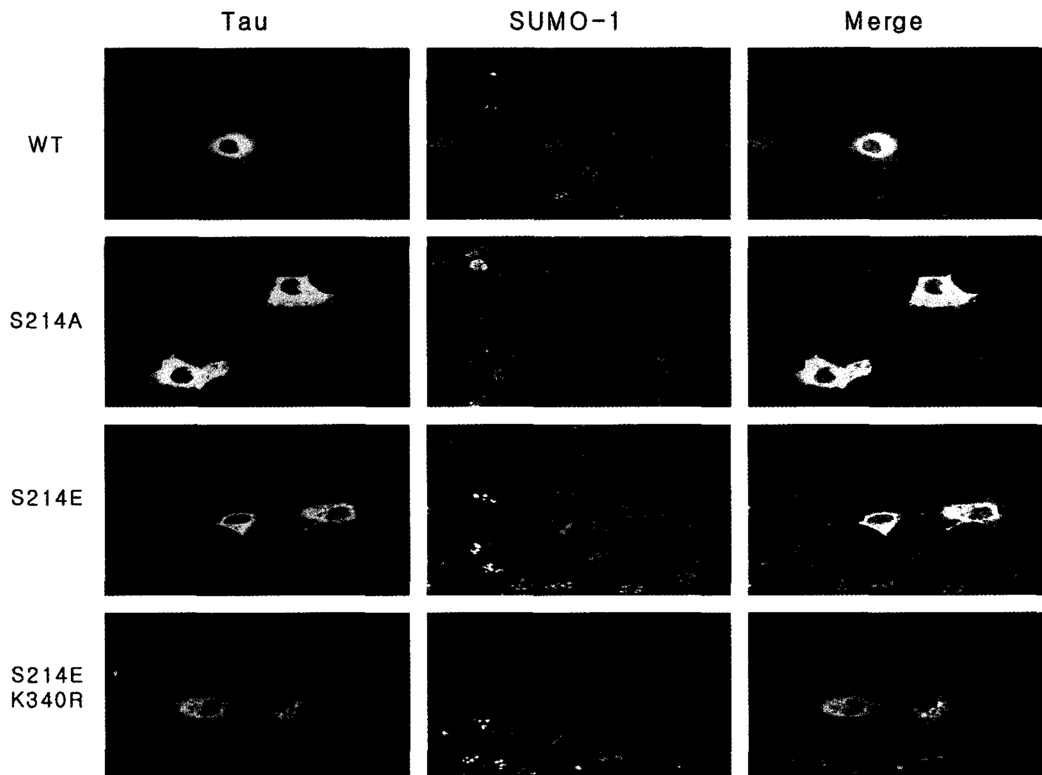

Fig. 4. Observation of Tau wt, S214A, S214E or Tau S214E K340R SUMOylation by Confocal microscopic analysis. The transfected EGFP-Tau wt, S214A, S214E or Tau S214E K340R (all constructs were shown as green color) was detected by fluorescence microscopy. For the SUMO-1 position, the SUMO-1 specific antibody was used (red color). SUMO-1 modified proteins were mainly detected in the nuclear region (middle row). EGFP-Tau S214E was mainly localized in the nuclear vicinity, whereas EGFP-Tau S214A was evenly detected in the cytoplasm. EGFP-Tau S214E was also merged well (yellow color) with SUMO-1 around the nucleus (C lane). Tau S214E K340R, in which SUMOylation site was eliminated, was not merged with SUMO-1, but was mainly localized in the nuclear vicinity, similar with S214E localization (D lane).

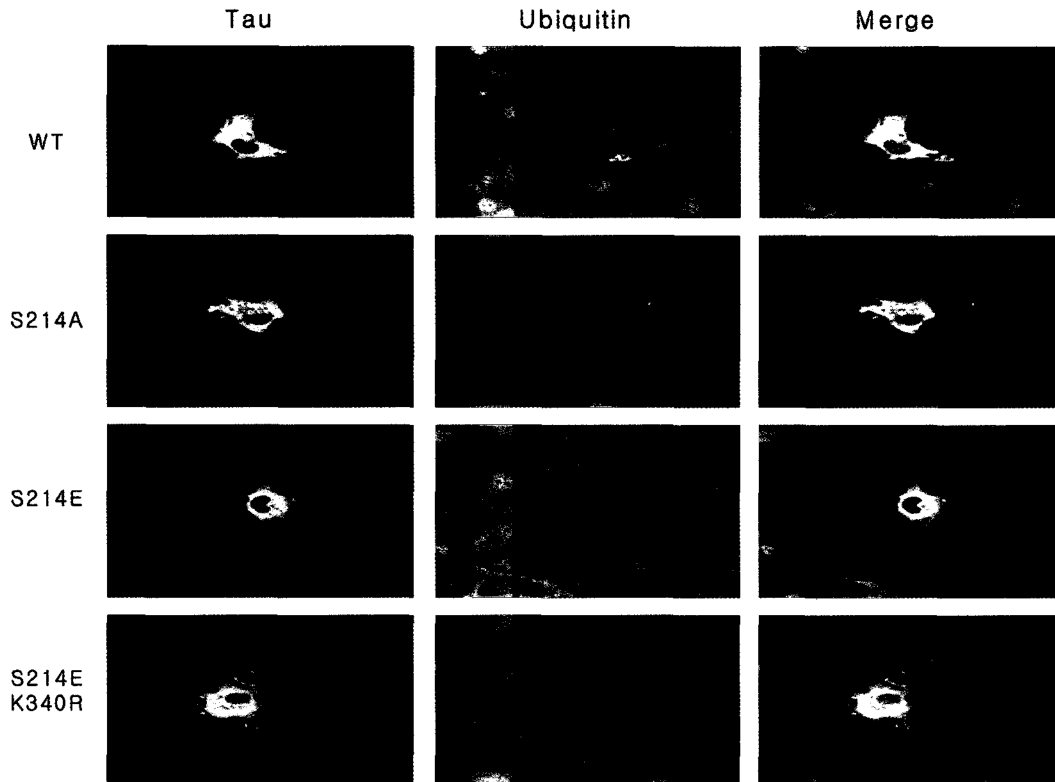

Fig. 5. Confocal microscopic analysis of Tau wt, S214A, S214E or Tau S214E K340R Ubiquitinization. Confocal microscopic analysis of transfected EGFP-Tau wt, S214A, S214E or Tau S214E K340R (all constructs were shown as green color) was performed to determine whether it merged with ubiquitin (red color). EGFP-Tau wt, S214A, Tau S214E, or Tau S214E K340R did not merge (yellow) with ubiquitin.

Confocal microscopic analysis of Tau ubiquitinization

SUMO represents a class of ubiquitin-like proteins conjugated, like Ubiquitin (Kikuchi et al., 2006; Boggio et al., 2004). Tau is also known to be ubiquitinated depending on its phosphorylation state (Feinstein and Wilson, 2005; Iqbal and Grundke-Iqbal, 1991). Thus, we speculated that the phosphorylation on ^{214}S residue promotes not only its SUMOylation but also its ubiquitinization. To determine whether phosphorylation on ^{214}S Tau residue only facilitates its SUMOylation, we analyzed ubiquitination (as red color) of transfected EGFP-Tau wt, K340R, S214E or Tau S214E K340R (all constructs were shown as green color) by fluorescence microscopy (Fig. 5). Similar with SUMOylation results in Fig. 4, we also observed ubiquitinated protein which was mainly detected in the nuclear (middle lane). The subcellular localization result of each EGFP-Tau protein in Fig. 5 was also observed similar with that in Fig. 4. However, EGFP-Tau wt, K340R, S214E or Tau S214E K340R (green color) was not merged with ubiquitin (as red color). Although it was mainly observed around the nuclear vicinity, EGFP-Tau S214E was not merged with ubiquitin (Fig. 5 third row). Thus, the results suggested that the phosphorylation on ^{214}S Tau residue (S214E) does specifically stimulate its SUMOylation (Fig. 4), but not ubiquitination (Fig. 5).

Tau SUMOylation in COS-1 cell

To confirm again the observation in Fig. 4 that Tau S214E is more effectively SUMOylated, immunological analysis method was applied. Each EGFP-Tau (WT, S214A, S214E, and S214E K340R) expression vector was transfected into COS-1 cells and immunoprecipitated with GFP polyclonal antibody. The immunoprecipitant was immunoblotted with SUMO-1 monoclonal antibody (Fig. 6A) or GFP monoclonal antibody (Fig. 6B), or ubiquitin monoclonal antibody (Fig. 6C), as prescribed in Materials and Methods. Untransfected COS-1 cell lyate was used as a negative control (right lane). As shown Fig. 6A, SUMOylation of Tau was detected as several high molecular weight bands. The relative optical density (OD) was indicated below.

Consistent with the results in Fig. 3 and 4, SUMOylation of S214E mutant seemed to be seven times more effective than that of S214A (as an analog of 214-serine un phosphor Tau) or wilde type Tau (Fig. 6A middle lane). The SUMOylation of Tau S214E K340R mutant in ^{339}VKSE^{342} was totally inhibited (Fig. 6A left lane). To eliminate the cell line specificity, we performed the experiment with NIH 3T3 cell and observed the same results (data not shown). Together with the above confocal results (Figs 2 and 4), these results confirmed again that Tau SUMOylation occurred at 340K residue and S214E, an analog of the

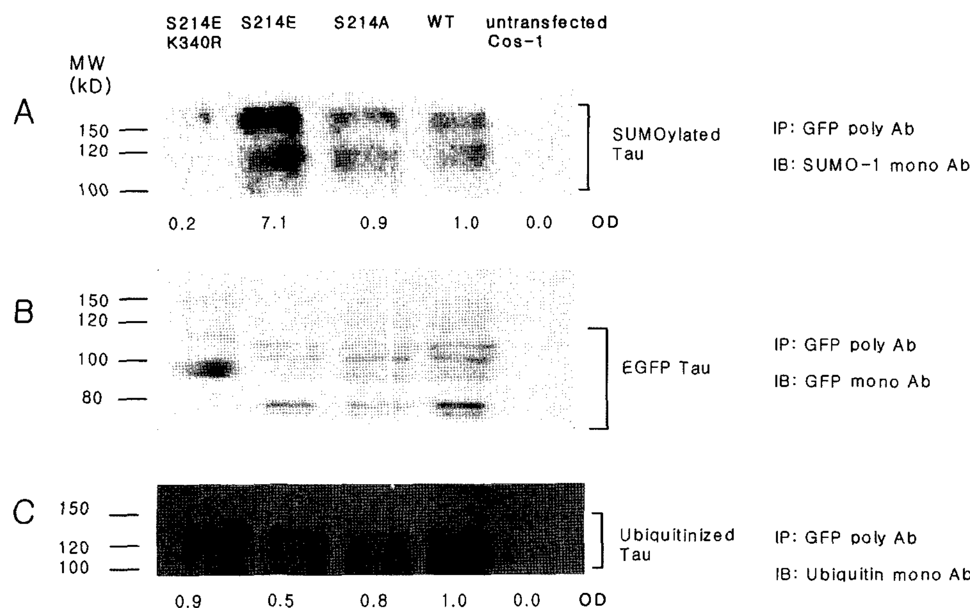

Fig. 6. Tau S214E, an analog of the phosphorylated Tau at 214S, promotes Tau SUMOylation at 340K residue. To confirm again the Fig. 4 observation which Tau S214E is more effectively SUMOylated, each EGFP-Tau (WT, S214A, S214E, and K340R) expression vector was transfected into COS-1 cells, and immunoprecipitated with GFP polyclonal antibody. The immunoprecipitant was immunoblotted with SUMO-1 monoclonal antibody (A) or GFP monoclonal antibody (B) as described in Materials and Methods. Untransfected COS-1 cell lysate was used as a negative control. SUMOylation of Tau was detected as several high molecular weight bands (A). The relative optical density (OD) was destermined by image analysis of the dried SDS-PAGE gel with the Fuji Image Quant software. The SUMOylation of S214E mutant in ^{207}GSRSRTPSL^{215} (as an analog of 214-serine phosphor Tau) seems to be seven time more effective than that of S214A (as an analog of 214-serine un phospho Tau) or wild type Tau (A middle lane). The SUMOylation of Tau K340R mutant in ^{339}VKSE^{342} was totally inhibited (A left lane). To monitor the amount of Tau protein, the western blot sheet was reprobed with GFP monoclonal antibody (B). Because Tau is a highly post translationally modified protein, several Tau protein bands are shown. Ubiquitinization of EGFP-Tau wt, S214A, S214E or Tau S214E K340R was analyzed with a ubiquitin monoclonal antibody (C). The relative optical density (OD) was indicated below. EGFP-Tau S214E was less ubiquitinized than EGFP-Tau wt, S214A, or Tau S214E K340R. These are the results of one of three repeat experiments.

phosphorylated Tau at 214S, promotes Tau SUMOylation (Fig. 6).

The SUMOylation of EGFP Tau S214E K340R mutant was not observed (Fig. 6A left lane), thus supporting again that ^{340}K residue of Tau is the primary SUMOylation site that triggers multiple SUMOylation on several K residues in Tau, similar with the observations in Fig. 3. It seems that the multiple SUMOylation with SUMO-1 occur on several K residues among total of 42 sites, because SUMO-1 can not be poly-SUMOylated (Melchior and Hengst, 2002; Zhang, et al., 2002).

Next, to confirm correlation between the phosphorylation on ^{214}S Tau residue (S214E) and the SUMOylation, *ubiquitination* of EGFP-Tau wt, K340R, S214E or Tau S214E K340R was analyzed with a ubiquitin monoclonal antibody (Fig 6C) (Shimura et al., 2004). Comparing with its SUMOylation result in Fig. 6A, we observed that EGFP-Tau S214E was less ubiquitinated than EGFP-Tau wt, S214A, or Tau S214E K340R (Fig. 6C). The relative optical density (OD) data (Fig. 6C) suggested that the ubiquitination of EGFP-Tau S214E seems to be reversibly related with the SUMOylation of EGFP-Tau S214E (Fig. 6A). Therefore, these results suggested that the phosphorylation on ^{214}S Tau residue (S214E) does inhibit its ubiquitination, in contrast to its SUMOylation (Fig. 6A). Taken together, this supported again that the phosphorylation on ^{214}S Tau residue (S214E) facilitates only its SUMOylation (Fig. 2 C, Fig. 4 and 6A), and not its ubiquitinization (Fig. 5 and 6C).

Tau protein stability

To compare Tau protein stability, we performed pulse-chase experiments as described in Materials and Method. Each EGFP-Tau (WT and S214E) expression vector was transfected into COS-1 cells, and immunoprecipitated with GFP polyclonal antibody, after cyclohexamide treatment (Fig. 7 A). Tau proteins were chased for the indicated time periods, immunoprecipitated with a polyclonal anti-GFP antibody, and subjected to SDS-PAGE followed by western blot with Tau antibody. To control the protein amount, we monitored actin in each sample by (Fig. 7A). Quantification of the pulse-chase experiment was achieved by image analysis of the dried SDS-PAGE gel with the Fuji Image Quant software. As shown in Fig. 7, protein stability of EGFP-Tau S214E was twice higher than that of EGFP-Tau WT. The stability of EGFP-Tau S214A or S214E K340R was almost the same as that of EGFP-Tau WT (data not

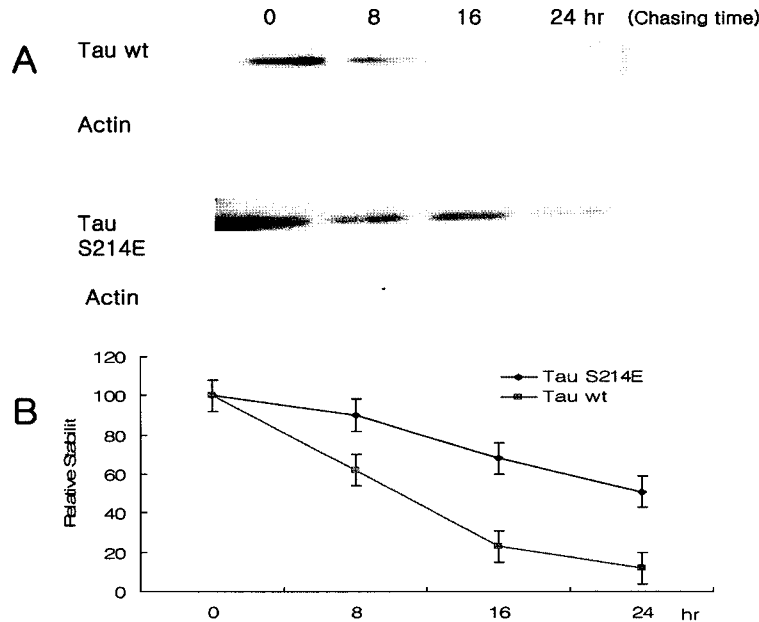

Fig. 7. Comparison of Tau protein stability by pulse-chase experiments. Tau and Tau-S214E were transfected into COS-1 cells and cells were treated with cyclohexamide. Tau proteins were chased for the indicated time periods. GFP-Tau proteins were immunoprecipitated with a polyclonal anti-GFP antibody and subjected to SDS-PAGE followed by western blot with a monoclonal Tau antibody (A). To monitor the protein amount, the equal amount of cell lysate was subjected to western blot with an actin antibody. These are results of one of three repeat experiments. Quantification of the pulse-chase experiment was done by image analysis of the dried SDS-PAGE gel with the Fuji Image Quant software (B).

shown). Thus, these results suggested that the phosphorylation on ^{214}S of Tau seems to increase its protein stability twice than that of unphosphorylated Tau (Fig. 7), through the stimulation of multiple SUMOylation on its several K residues (Fig. 1, 4 and 6).

Cell viability change

We measured cell viability with FACS analysis to determine whether Tau SUMOylation and its phosphorylation on ^{214}S influence the cell viability. As shown in Table 1, no Tau construct showed any the cell survival rate change. Even though EGFP-Tau S214E was dominantly localized in the nuclear vicinity area and SUMOylated on ^{340}K, it did not promote the cell survival, compared to the other Tau constructs. Thus it seems to be that both SUMOylation on ^{340}K and phosphorylation on ^{214}S of Tau did not affect cell viability (Table 1).

In summary, our results suggested that the phosphorylation of Tau on Ser^{214} residue (S214E) facilitates the SUMOylation on its ^{340}K residue, with its nuclear vicinity localization. The phosphorylation on Tau ^{214}S (S214E) also increases its protein stability without influencing cell survival.

DISCUSSION

Tau is a microtubule-associated protein, and its main function in neurons is to bind and stabilize microtubules (Maccini et al., 2001; Johnson and Stoothoff, 2004.). In our effort to understand the functional significance of Tau ^{214}S phosphorylation, we tested whether the Tau phosphorylation affected its subcellular localization or stability. In addition, we examined whether Tau is a direct substrate of SUMO-1 posttranslational modification in intact cells and under cell-free conditions, since Tau has two consensus SUMO-1 attachment motifs at ^{340}K and ^{385}K in human 441 amino acid lengths Tau 40.

To address that Tau SUMOylation occurs at the ^{340}K residue in ^{339}VKSE^{342}, we performed SUMOylation assay with the GST-fusion proteins (Tau WT and Tau K304R mutant) from *E. coli* as substrate proteins (Fig. 3), confocal microscopic analysis (Fig. 4), and co-immunoprecipitation assay (Fig. 6). With these observations, we demonstrated that the ^{340}K residue in ^{339}VKSE^{342} of Tau is the SUMOylation site (Fig. 1). Thus, identification of this lysine residue as a target for Tau SUMOylation will help determine whether it plays a role in normal tubulin assembly and whether it may be involved in Alzheimer disease progression (Luna-Munoz et al., 2005). Because lysine serves as the attachment site for several modifications including ubiquitination, acetylating and methylamine, it is also reasonable to speculate that Tau SUMOylation plays a role by antagonizing other post-translational modifications. Importantly, Tau can also be acetylated in vitro and in vivo at the same lysines as those required for SUMO modification (Feinstein and

Wilson, 2005; Luna-Munoz et al., 2005). Although the molecular mechanism underlying Tau SUMOylation and its regulation remain unknown, our study has demonstrated the importance of SUMO modifications as a novel posttranslational step for the regulation of Tau function. Further studies are essential to better elucidate the complexity of the molecular mechanism involved in Tau mediated microtubule assembly. In particular, it will be important to determine exactly when and where Tau SUMOylation occurs, how it is regulated and how it contributes to the physiological role of Tau.

Because Tau is phosphorylated at multiple sites by several protein kinases, the differential phosphorylation of Tau by specific kinases may regulate its biological functions (Maccini et al., 2001; Takashima, 2006). To date, at least 29 phosphorylation sites have been identified in PHF-tau. It is believed that several protein kinases are involved in the phosphorylation of tau, and no single kinase can phosphorylate all of these sites. Thus, it is presently unclear which site is more effective in regulating microtubule-Tau interaction, microtubule dynamics, and SUMOylation. It has been reported that ^{214}S is also phosphorylated in PHF-Tau (Friedhoff et al., 2000; Johnson, and Stoothoff, 2004). However, it is unknown whether or not the phosphorylation on ^{214}S is sufficient to block Tau-microtubule interaction, causing SUMOylation on ^{340}K and microtubule instability in PHF-Tau. The major protein kinases responsible for the Tau hyperphosphorylation and PHF-Tau are glycogen synthase kinase-3 (GSK-3β and cell cycle dependent protein kinase 5 (Maccini et al., 2001; Johnson and Stoothoff, 2004; Takashima, 2006). It has been reported that the phosphorylation on ^{214}S of Tau occurs by SGK1, PKA, or Akt kinase (Chun et al., 2004; Ksiezak-Reding et al., 2004). These three protein kinases are known to involve in the cell survival. Further, Akt kinase phosphorylates ^{9}S residue of GSK-3β and inhibits its kinase activity (Takashima, 2006). Therefore, the phosphorylation on ^{214}S residue of Tau seems to be one of the cell survival effectors of Akt or SGK1 kinase through not noly the promotion of SUMOylation but also the inhibition of ubiquitinization (Fig. 6), resulting in the increase of its protein stability (Fig. 7). However, our FACS results suggested that the phosphorylation on ^{214}S residue of Tau itself was not enough to enhance the cell survival (Table 1). We speculated that because Tau is one of the cell survival downstream effectors of these kinases, the phosphorylation of ^{214}S residue of Tau itself did not effect cell survival.

We showed here the significance of Tau ^{214}S phosphorylation for its subcellular localization and SUMOylation, by confocal microscopy and immunological analysis (Fig. 3-6). The ^{214}S residue phosphorylation site by SGK1 is located in the middle of Tau protein (Fig. 1). Tau is a microtubule-associated protein, and its main function in neurons is to bind and stabilize microtubules. Phosphorylation of Tau reduces Tau's affinity for microtubules (Johnson and Stoothoff, 2004, Feinstein and Wilso, 2005). Thus, it seems that the phosphorylated Tau becomes more monomeric and more accessible to SUMOylation. Therefore, the phosphorylation of Tau seems to regulate not only the microtubule-Tau interaction, but also its SUMOylation. In this case, the type of phosphorylation that most potently detaches Tau from microtubules *in vitro* (at KXGS motifs in the repeat domain) is necessary for cell process outgrowth, rather than inhibitory (Fig. 1). Therefore, it seems that the phosphorylated forms of Tau protein are more SUMOylated, resulting in more dissociation from microtubule and more hyperSUMOylation on Tau, together with hyperphosphoylation. This is a simple explanation as to why the ^{214}S residue phosphorylation promotes Tau SUMOylation. In this case, we can not explain the under-ubiquitinization of Tau S214E mutant (Fig. 5), because the dissociation from microtubule also increases the chance to be ubiquitizated. Thus, it seems that Tau ^{214}S residue phosphorylation stimulates its SUMOylation, suggesting a differential role of each phosphorylation. We also observed enhancement of Tau ^{340}K SUMOylation by the phosphorylation of Tau ^{214}S (Fig 4 and 5). However, we do not know whether other phosphorylation sites in Tau are responsible for SUMOylation in a phosphorylation-dependent manner in the cell. Generally, phosphorylation of Tau, which is also known to dissociate it from the microtubule, seems to enhance its SUMOylation (Luna-Munoz et al., 2005). The phosphorylated Tau is more easily accessible to the nuclear region where the enzymes for SUMOylation are concentrated (Maria et al., 2004). Recently, several researchers reported that, in a subset of SUMO substrates which contains the consensus KxExxSP, the phosphorylation of the SP motif within this consensus sequence plays an important role in promoting SUMOylation of several substrates including MEF2A (Gregoire et al., 2006; Hietakangas et al., 2006; Shalizi et al., 2006). Interestingly, ^{340}K of Tau is noted in

Table1. FACS results with Tau mutants

Tau construct	Rate of apoptosis (%) by FACS
Tau (wt)	23 ± 3
S214A	23 ± 3
S214E	23 ± 3
S214E/K340R	23 ± 3
EGFP (vector)	23 ± 3
Mean value of 5 repeats	

Tau (wt), S214E, S214A, S214E K340R, or EGFP vector was transfected and the rate of apoptosis was measured by FACS. Tau S214E, which was dominantly localized into the nuclear area SUMOylated, did not promote cell survival, compared to the other Tau construct. Thus, cell survival was not promoted by Tau S214E and K340 SUMOyaltion. For detail, see Materials and Methods.

^{339}VKSEKL**D**FK**D**^{348}where the phosphorylated S residue corresponds to an acidic D residue. It was also repoted that Ubc9 prefers to SUMOylate its substrate proteins which have a consensus sequence with the acidic tail Φ**K**xE/DxxE/DxxE/D, where Φ represents L, I, V or F and x is any amino acid. Thus, ^{340}K of Tau in ^{339}**VKSEKLDFKD**^{348} seems to meet the requirement for the negatively charged amino acid dependent SUMOylation motif (Yang et al., 2006). Therefore, the stimulation of Tau SUMOylation through its ^{214}S phosphorylation seems to be one of alternative stimulation mechanisms.

We demonstrated here that the SUMOylation on ^{340}K of Tau is stimulated by its ^{214}S phosphorylation and controls its subcellular localization along with SUMO. Since Tau is known as a cytoplasmic protein, it is unclear yet, for Tau SUMOylation, what are E1 enzyme, E2 SUMO-conjugating enzyme, or E3 SUMO ligases and how they operate for it. Thus, more studies will be required to elucidate the physiological significance of SUMOylation on ^{340}K. We are pursuing now to identify which phosphorylation sites are more responsible to influence on ^{340}K residue SUMOylation. In this study, we used the established cell line such as COS-1 and 3T3 cell. To generalize our observation, further study should be performed in primary neural cell or in animals.

In conclusion, by point mutagenesis analyses, we showed that the SUMOylated lysine residue is ^{340}K of Tau, which is located in the tubulin binding repeat domain (Fig. 1), and that the phosphorylation on ^{214}S residue of Tau promotes its SUMOylation on ^{340}K residue, together with its nuclear vicinity localization and protein stability, indicating the differential phosphorylation of Tau by the specific protein kinase.

ACKNOWLEDGMENTS

This work was supported by Korea Research Foundation Grant (KRF-2005-003-C00142) and KOSEF Grant (R01-2006-000-10167-0) to SSK. We appreciate The Core Facility of Chungbuk National University

REFERENCES

Bischof O, Schwamborn K, Martin N, Werner A, Sustmann C, Grosschedl R, Dejean A (2006) The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis. *Mol. Cell.* 22: 783-794

Boggio R, Colombo R, Hay RT, Draetta GF, Chiocca S (2004) A mechanism for inhibiting the SUMO pathway. *Mol. Cell.*16: 549-61

Butterfield DA, Abdul HM, Opii W, Newman SF, Joshi G, Ansari MA, Sultana R (2006) Pin1 in Alzheimer's disease. *J. Neurochem.*98: 1697-1706

Chong ZZ, Li F, Maiese K (2005) Stress in the brain: novel cellular mechanisms of injury linked to Alzheimer's disease. *Brain Res Brain Res Rev.* 49: 1-21

Chun J, Kwon T, Lee EJ, Kim CH, Han YS, Hong SK, Hyun S, Kang SS (2004) 14-3-3 Protein mediates phosphorylation of microtubule-associated protein tau by serum- and glucocorticoid-induced protein kinase 1. *Mol.Cells* 18: 360-368

Dorval V, Fraser PE (2006) Small ubiquitin-like modifier (SUMO) modification of natively unfolded proteins tau and alpha-synuclein. *J. Biol. Chem.* 281: 9919-9924.

Duprez E, Saurin AJ, Desterro JM, Lallemand-Breitenbach V, Howe K, Boddy MN, Solomon E, de The H, Hay RT, Freemont PS (1999) SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications for nuclear localisation. *J. Cell Sci.* 112 : 381-393

Feinstein SC, Wilson L (2005) Inability of tau to properly regulate neuronal microtubule dynamics: a loss-of-function mechanism by which tau might mediate neuronal cell death. *Biochim Biophys Acta.* 1739: 268-279

Vega FM, Sevilla A, Lazo PA (2004) p53 Stabilization and Accumulation Induced by Human Vaccinia-Related Kinase 1. *Mol. Cell. Biol.* 24: 10366-10380

Friedhoff P, von Bergen M, Mandelkow E M, Mandelkow E (2000) Structure of tau protein and assembly into paired helical filaments. *Biochim. Biophys. Acta.*1502: 122-132

Gill G (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev.* 18: 2046-2059

Goldsbury C, Mocanu MM, Thies E, Kaether C, Haass C, Keller P, Biernat J, Mandelkow E, Mandelkow EM (2006)Inhibition of APP trafficking by tau protein does not increase the generation of amyloid-beta peptides. *Traffic* 7: 873-888.

Gregoire S, Tremblay AM, Xiao L , Yang Q, Ma K, Nie J, Mao Z, Wu Z, Giguere V, Yang XJ (2006) Control of MEF2 transcriptional activity by coordinated phosphorylation and SUMOylation *J Biol Chem* 281: 44234433

Hietakangas V, Anckar J , Blomster HA, Fujimoto M, Palvimo JJ, Nakai A, Sistonen L (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc. Nat.l Acad. Sci. USA* 103: 4550

Iqbal K, Grundke-Iqbal I (1991) Ubiquitination and abnormal phosphorylation of paired helical filaments in Alzheimer's disease. *Mol. Neurobiol.* 5: 399-410

Johnson G.V, Stoothoff WH (2004) Tau phosphorylation in neuronal cell function and dysfunction. *J Cell Sci.* 117: 5721-5729

Kagey MH, Melhuish TA, Wotton D (2003) The polycomb protein Pc2 is a SUMO E3. *Cell* 113: 127-137

Kikuchi A, Kishida S, Yamamoto H (2006) Regulation of Wnt signaling by protein-protein interaction and post-translational modifications. *Exp. Mo.l Med.* 38: 1-10.

Ksiezak-Reding H, Pyo HK, Feinstein B, Pasinetti GM (2003) Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. *Biochim. Biophys. Acta.* 1639: 159-168

Leung MF, Sokoloski JA, Sartorelli AC (1992) Changes in microtubules, microtubule-associated proteins, and intermediate filaments during the differentiation of HL-60 leukemia cells. *Cancer Res.* 52: 949-954.

Luna-Munoz J, Garcia-Sierra F, Falcon V, Menendez I, Chavez-Macias L, Mena R (2005) Regional conformational change involving phosphorylation of tau protein at the Thr231, precedes the structural change detected by Alz-50 antibody in Alzheimer's disease. *J. Alzheimers Dis.* 8: 29-41

Mabb AM, Wuerzberger-Davis SM, Miyamoto S (2006) PIASy

mediates NEMO SUMOylation and NF-kappaB activation in response to genotoxic stress. *Nat. Cell Biol.* 8: 986-993

Maccioni RB, Otth C, Concha II, Muoz JP (2001) The protein kinase Cdk5: Structural aspects, roles in neurogenesis and involvement in Alzheimer's pathology. *Eur. J. Biochem.* 268: 1518-1527

Vassileva MT, Matunis MJ (2004) SUMO Modification of Heterogeneous Nuclear Ribonucleoproteins. *Mol. Cell. Biol.* 24: 3623-3632

Melchior F, Hengst L (2002) SUMO-1 and p53. *Cell Cycle* 1: 245-249

Pichler A, Gast A, Seeler JS, Dejean A, Melchior F (2002) The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* 108: 109-120

Raynaud F, Marcilhac A (2006) Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease. *FEBS J.* 273: 3437-3443

Raynaud F, Marcilhac A (2006)Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease. *FEBS J.* 273: 3437-3443

Sampson DA, Wang M, Matunis MJ (2001) The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J. Biol. Chem.* 276: 21664-21669

Shalizi A, Gaudilliere B, Yuan Z, Stegmuller J, Shirogane T, Ge Q, Tan Y, Schulman B, Harper JW, Bonni A (2006) A calcium-regulated MEF2 SUMOylation switch controls postsynaptic differentiation. *Science* 311: 10121017

Shimura H, Schwartz D, Gygi SP, Kosik, KS (2004) CHIP-Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival, *J. Biol. Chem.* 279: 4869-4876

Suico MA, Nakamura H, Lu Z, Saitoh H, Shuto T, Nakao M, Kai H (2006) SUMO down-regulates the activity of Elf4/myeloid Elf-1-like factor. *Biochem. Biophys. Res. Commun.* 348: 880-888

Takashima A (2006) GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J. Alzheimers Dis.* 9: 309-317

van Waardenburg RC, Duda DM, Lancaster CS, Schulman BA, Bjornsti MA (2006) Distinct functional domains of Ubc9 dictate cell survival and resistance to genotoxic stress. *Mol. Cell Biol.* 26: 4958-4969

Wilson VG, Rangasamy D (2001) Intracellular targeting of proteins by sumoylation. *Exp. Cell Res.* 271: 57-65

Yang SH, Galanis A, Witty J, Sharrocks AD (2006) An extended consensus motif enhances the specificity of substrate modification by SUMO. *EMBO J.* 1-11 (Epub ahead of print)

Zhang, H., H. Saitoh, and M. J. Matunis (2002) Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. *Mol. Cell. Biol.* 22: 6498-6508.

[Received April 3, 2007; accepted June 15, 2007]