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Mitotic-like Tau Phosphorylation by p25-Cdk5 Kinase Complex*

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Among tau phosphorylation sites, some phosphoepitopes referred to as abnormal ones are exclusively found on tau aggregated into filaments in Alzheimer's disease. Recent data suggested that molecular mechanisms similar to those encountered during mitosis may play a role in abnormal tau phosphorylation. In particular, TG-3 phosphoepitope is associated with early stages of neurofibrillary tangles (NFTs). In this study, we reported a suitable cell model consisting of SH-SY5Y cells stably transfected with an inducible p25 expression vector. It allows investigation of tau phosphorylation by p25-Cdk5 kinase complex in a neuronal context and avoiding p25-induced cytotoxicity. Immunoblotting analyses showed that p25-Cdk5 strongly phosphorylates tau protein not only at the AT8 epitope but also at the AT180 epitope and at the Alzheimer's mitotic epitope TG-3. Further biochemical analyses showed that abnormal phosphorylated tau accumulated in cytosol as a microtubule-free form, suggesting its impact on tau biological activity. Since tau abnormal phosphorylation occurred in dividing cells, TG-3 immunoreactivity was also investigated in differentiated neuronal ones, and both TG-3-immunoreactive tau and nucleolin, another early marker for NFT, were also generated. These data suggest that p25-Cdk5 is responsible for the mitotic-like phosphoepitopes present in NFT and argue for a critical role of Cdk5 in neurodegenerative mechanisms.

Tau aggregation is a common feature among neurodegenerative disorders referred to as tauopathies. Mechanisms leading to tau aggregation and neurofibrillary degeneration are poorly understood. However, abnormal phosphorylation seems to be involved in tau conformational changes and aggregation (1, 2). Phosphorylation is a key post-translational modification involved in the regulation of tau function regarding microtubule polymerization and stability. Whereas many phosphorylation sites are common between tau aggregated into filaments (tau-PHF) in AD[1] and normal tau from biopsy-derived material, some phosphoepitopes referred to as abnormal ones are exclusively found on tau-PHF (for a review, see Ref. 3). Our recent data suggest that molecular mechanisms similar to those encountered during mitosis may play a role in the formation of abnormal tau phosphoepitopes (4). Understanding the role of abnormal tau phosphorylation in NFT formation requires identification of kinases leading to these specific phosphoepitopes. A large number of kinases can phosphorylate tau on specific Thr or Ser residues (for a review, see Ref. 3). It was found that many kinases, belonging to the Pro-directed protein kinase family, have an enzymatic activity for one of the 17 Ser/Thr-Pro motifs in full-length tau. Other kinases, however, such as protein kinase A were found to phosphorylate Ser or Thr residues that are not followed by a Pro, whereas GSK-3β can modify both Ser/Thr residues that are or not followed by a Pro (for a review, see Ref. 5). A number of phosphorylation-dependent antibodies were used to monitor tau phosphorylation. For instance, AD2 recognizes phosphorylated residues Ser^{396} and Ser^{404} (6), whereas AT8 and AT180 recognize phospho-Ser^{202}/Ser^{205} and phospho-Thr^{231}, respectively (7, 8). Among abnormal tau phosphoepitopes, TG-3 recognizes phospho-Thr^{231} in a conformation-dependent manner. This phosphoepitope is exclusively found in mitotic cells (9) with only the exception of degenerating neurons of AD and seems to be associated with early stages of the disease (10, 11). The Cdc2 kinase probably generates TG-3 epitope in mitotic cells, but the kinase(s) responsible for this phosphorylation in neurons remain unknown (9, 12, 13). For instance, it was reported that Thr^{231} could be phosphorylated *in vitro* by four kinases (GSK3β, p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase 2) (14). Nevertheless, one of the most evident candidates is Cdk5, the neuronal Cdc2-like kinase. Several data support the idea of Cdk5 involvement in AD (15–17). Cdk5 activity is regulated in neuronal cells by its binding with its activating partner p35, p39, and the p35 proteolytic fragment, p25 protein (for a review, see Ref. 18). p35 proteolysis into p25 occurs in response to diverse insults that probably trigger calpain activation (19–21). p25-Cdk5 complex leads to a deregulated kinase activity that has been linked to tau hyperphosphorylation and neurotoxicity (16, 22, 23). Tau phosphorylation has been well characterized *in vitro* (24), but data on Cdk5-induced tau phosphorylation *in vivo* and in cellular models are lacking. In the present study, biochemical studies have been undertaken to investigate tau phosphorylation by p25-Cdk5 complex. The toxicity linked with p25 expression was avoided by using an appropriate cell model that expresses inducible p25-Cdk5 kinase activity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM nonessential amino acids, and 50 units/ml penicillin/streptomycin (Invitrogen) in a 5% CO_2 humidified incubator at 37 °C. Tau-SY5Y cells were previously described (4, 25). They constitutively

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[1] The abbreviations used are: AD, Alzheimer's desease; NFT, neurofibrillary tangle; NGF, nerve growth factor; Pipes, 1,4-piperazinediethanesulfonic acid.

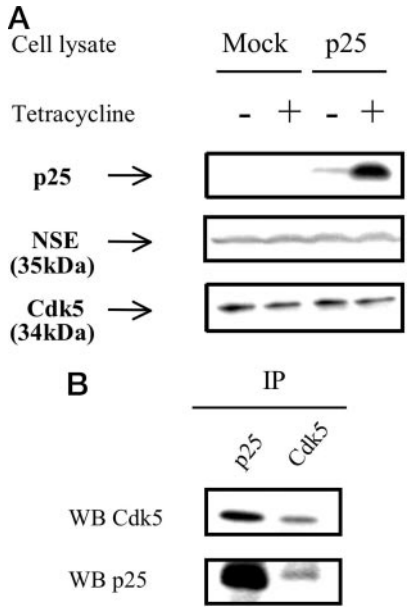

FIG. 1. **Analysis of p25 expression and formation of p25-Cdk5 complex in stably transfected cells, following induction by tetracycline treatment.** *A*, p25 expression. Lysates from mock and p25-inducible cells, treated (+*Tet*) or not (−*Tet*) with tetracycline (1 μg/ml) for 24 h, were immunolabeled with antibodies against p25, Cdk5, and neuronal specific γ-enolase (*NSE*) as control. *B*, cell lysates from p25-overexpressing cells (24-h tetracycline treatment) were subjected to immunoprecipitation (*IP*) by either p25 antibody or Cdk5 antibody. Immunocomplexes were then analyzed by immunoblotting (*WB*) with p25- or Cdk5-directed antibodies.

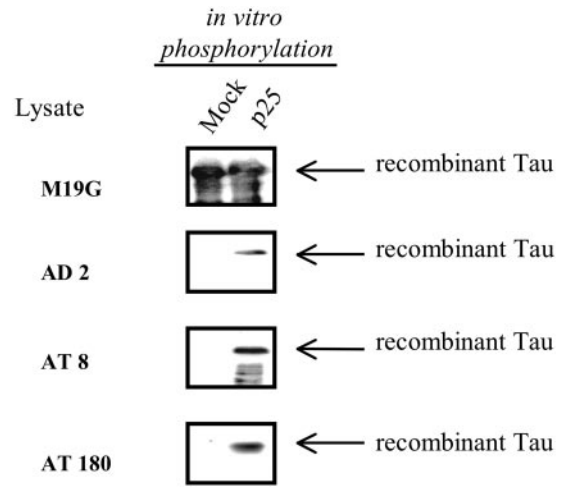

FIG. 2. ***In vitro* tau phosphorylation.** Recombinant tau was subjected to *in vitro* kinase assay by incubation with lysates from either mock or p25-expressing cells (24-h tetracycline treatment) and then analyzed by immunoblotting using phosphorylation-dependent anti-tau antibodies (AD2, AT8, and AT180) and a phosphorylation-independent antibody, M19G.

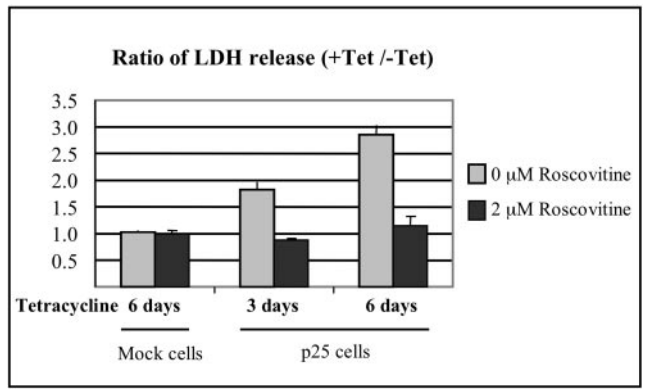

FIG. 3. **Lactate dehydrogenase measurement of p25-induced cytotoxicity.** Histograms represent lactate dehydrogenase release as percentages of maximal lactate dehydrogenase release by detergent lysis. Values of tetracycline-treated cells have been normalized to those of tetracycline-untreated cells. In mock cells, similar data were observed at 3 and 6 days of tetracycline treatment.

express the tau isoform with three microtubule-binding domains (2+3−10−).

Tau-SY5Y and SH-SY5Y cells were used as the basis for a tetracycline-regulated mammalian expression T-Rex system (Invitrogen). p25 cDNA was cloned into pcDNA4/TO vector (Invitrogen). First, stable cell lines that constitutively express tetracycline repressor were generated by transfection of pcDNA6/TR vector (Invitrogen) using ExGen500 (Euromedex, France) according to the manufacturer's instructions. Isolated clones, maintained in medium with 5 μg/ml blasticidin, were then transfected with inducible expression vector alone (mock) or with p25 cDNA. Individual stable clones were generated following Zeocin selection (100 μg/ml), and those that exhibited the weakest basal expression of p25 were selected. For induction of p25 expression, cells were maintained in medium with tetracycline at 1 μg/ml.

NGF-Cell Differentiation

Cells were differentiated for 7 days in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 2 mM L-glutamine, 50 units/ml penicillin/streptomycin, 7 μg/ml progesterone, 1% insulin/transferrin/selenium (Invitrogen), and 10 ng/ml NGF 2.5 S (Sigma). Medium was replenished every 2 days.

Measurement of Cell Toxicity

Cells were seeded in 96-well plates (2000 cells/well). 24 h later, they were replenished with medium either containing or not containing tetracycline and 2 μM roscovitine (Calbiochem) (day 0). Treatments were renewed at day 3, and cytotoxicity assays were conducted at day 6 by measurement of lactate dehydrogenase release according to the manufacturer's instructions (Cyto Tox 96; Promega). For each experiment, measurements were carried out in triplicate.

Antibodies

Anti-tau—The numbering of the tau epitopes is given according to the longest human 441 tau isoform. Phosphorylation-dependent monoclonal antibodies are directed against specific phosphorylated Ser/Thr-Pro sites (for a review, see Ref. 3). They included AD2 (anti-phospho-$Ser^{396-404}$; dilution 1:20,000) (6), AT8 (Innogenetics, Ghent, Belgium) (anti-phospho-Ser^{202} and -Thr^{205}; dilution 0.5 μg/ml) (7), AT180 (Innogenetics) (anti-phospho-Thr^{231}; dilution 0.4 μg/ml) (8), and TG-3 (a gift of P. Davies; a marker of PHF and M phase of eukaryotic cells that recognizes a specific conformation of phospho-Thr^{231}) (26) (dilution 1:20). Phosphorylation-independent antibodies M19G and tau-C-ter are well characterized antisera, directed against the first 19 amino acids (4, 6) and the last 15 amino acids of tau sequence (27), respectively (dilution 1:20,000).

Other Antibodies—Cdk5 monoclonal antibody (J-3; Santa Cruz Biotechnology, TEBU, France) (dilution 1:1000), p35-C-ter polyclonal antibody (C-19; Santa Cruz Biotechnology) (dilution 1:1000), neuronal specific γ-enolase antibody (Santa Cruz Biotechnology) (dilution 1:50,000), nucleolin monoclonal antibody (3G4B2; from Upstate Biotechnology, OZYME, France) (dilution 1:2000), tubulin polyclonal antibody (28) (dilution 1:2000), acetylated tubulin monoclonal antibody (6-11B-1; Sigma) (dilution 1:2000), α-synuclein (gift of Innogenetics) (dilution 1:2000), and poly(ADP-ribose) polymerase polyclonal antibody (Roche Applied Science) (dilution 1:2000).

Western Blotting

Cells were harvested in ice-cold radioimmune precipitation assay modified buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) with protease inhibitors (Complete Mini; Roche Applied Science) and a 125 nM concentration of the phosphatase inhibitor okadaic acid (Sigma), sonicated, and stirred 1 h at 4 °C. Cell lysate was recovered in supernatant after centrifugation at 12,000 × *g* at 4 °C for 20 min. Protein concentration was determined by the BCA protein assay kit (Pierce). Samples were mixed with an equal volume of 2× Laemmli buffer and dithiothreitol and heated for 5 min at 100 °C, and then 10–20 μg were loaded onto SDS-PAGE gel. After transfer, membranes were blocked in TNT buffer (Tris-buffered saline, pH 8,

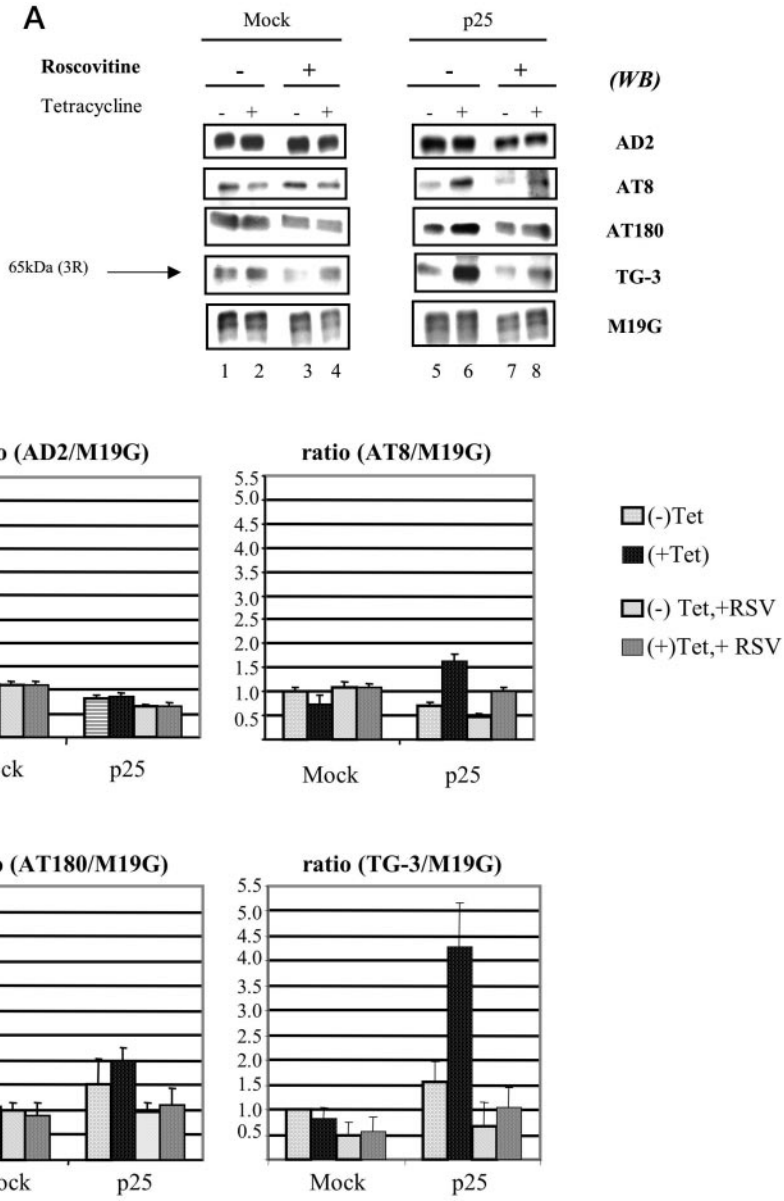

FIG. 4. **Tau phosphorylation in p25 expressing cells.** *A*, immunoblotting analyses. Lysates from mock and p25-inducible cells, treated (+) or not (−) with tetracycline (*Tet*) and roscovitine (*RSV*), were analyzed by phosphorylation-dependent tau antibodies: AD2, AT8, AT180, and TG-3. The total amount of loaded tau proteins is visualized by M19G antibody. *B*, quantification of tau phosphorylation in mock and p25-expressing cells. Ratios of densitometric values of each phospho-tau antibody to those of M19G antibody are presented. These ratios are normalized to those obtained from mock cells with neither tetracycline nor roscovitine treatment ((−)Tet; arbitrary value = 1).

0.05% Tween 20), depending on the antibody without (TG-3 and AT anti-tau antibodies) or with 5% skim milk (the remaining antibodies), and incubated with primary antibody. Horseradish peroxidase-conjugated antibody (Sigma) was used as secondary antibody, and horseradish peroxidase activity was detected with the ECL detection kit (Amersham Biosciences). The IMAGE-MASTER 1D ELITE software (Amersham Biosciences) was used to quantify signals.

Immunoprecipitation

p25-overexpressing cells (24-h tetracycline treatment) were harvested in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) supplemented with protease inhibitors, and cell lysate was prepared as described under "Western Blotting." Protein concentration was determined by the BCA protein assay kit. For the immunoprecipitation experiments, cell lysates (400 μg at 2 mg/ml) were incubated with either anti-Cdk5 or anti-p35-C-ter antibodies (dilution 1/100) overnight at 4 °C and then incubated with 20 μl of protein A/G-agarose beads (Pierce) for 1 h at 4 °C. Immunoprecipitated complexes were washed four times in lysis buffer (centrifugation at 2000 × *g* at 4 °C for 5 min), recovered in Laemmli buffer, boiled for 5 min, and then analyzed by immunoblotting.

In Vitro Kinase Assay

The longest human tau (441 residues) cloned in pET15b was produced in the BL21(DE3) Star *Escherichia coli* strain (Invitrogen). *In vitro* phosphorylation was performed by incubating TALON resin (BD Clontech) saturated with His-tagged tau 441 in the presence of lysates from either mock- or p25-tetracycline-treated cells prepared (as described under "Western Blotting") in lysis buffer: 40 mM Tris-HCl, pH 7.2, 125 nM okadaic acid, 4 mM β-mercaptoethanol, protease inhibitors (EDTA-free Complete Mini; Roche Applied Science); 2 mM $MgCl_2$, and 7.5 mM ATP with gentle shaking at 37 °C for 4 h. Immobilized recombinant tau proteins were then washed and eluted, and their phosphorylation state was analyzed by immunoblotting.

When kinase inhibitors (LiCl (Sigma), PD98059 (Calbiochem), roscovitine (Calbiochem), and SB203580 (Calbiochem)) were used, they were mixed with cell lysates before adding the phosphorylation ATP buffer.

For *in vitro* kinase assay by immunopurified p25-Cdk5 complex, Immunoprecipitation was performed from p25-expressing cells with anti-p35-C-ter antibody. Immunoprecipitated complex was washed in kinase buffer and then used to phosphorylate 100 μg of His-tagged tau 441, as described above. Reaction was stopped by adding Laemmli buffer and boiling for 5 min.

Cell Fractionation into Cytosol and Microtubule Fractions

An equivalent number of mock and p25-expressing cells was recovered in equal volumes of warmed lysis buffer (80 mM Pipes, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 30% glycerol, 0.1% Triton X-100) with protease inhibitors and okadaic acid. After ultracentrifugation (100,000 × *g* at 21 °C) for 30 min, supernatants were collected as cytosolic fractions. The remaining pellets were resuspended by sonication in ice-cold modified radioimmune precipitation assay buffer with protease inhibitors

and okadaic acid in a volume equal to that of cytosolic fractions and then centrifuged at 12,000 × *g* (4 °C) for 20 min. Supernatants were recovered as microtubule fractions. Samples were then mixed to an equal volume of 2× Laemmli buffer and boiled at 100 °C for 5 min; equivalent volumes were then loaded on SDS-PAGE and analyzed by immunoblotting.

Detergent Tau Solubilization

For separation of soluble and insoluble tau proteins, an equivalent number of mock and p25-expressing cells was recovered in equal volumes of ice-cold saline buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, protease inhibitors, okadaic acid) with 1% SDS and then ultracentrifuged (100,000 × *g* at 4 °C) for 30 min. Supernatants were recovered as the soluble fractions and mixed (v/v) with 2× Laemmli buffer. The remaining pellets were solubilized in 70% formic acid, dried, and resuspended in Laemmli buffer (insoluble fractions). Samples were then subjected to immunoblotting analyses (soluble and insoluble fractions were loaded with the proportion of 1:6, with reference to cell number).

For differential detergent tau solubility, an equivalent number of cells was recovered in equal volumes of ice-cold saline buffer and centrifuged as above to collect supernatants referred to as S1 fractions. The remaining pellets were successively homogenized by sonication in equivalent volumes of subsequent lysis buffers containing increased concentrations of Triton X-100 (0.01, 0.025, 0.05, 0.1, 0.25, and 1%), centrifuged as above to collect supernatants referred to as fractions (S2, S3, S4, S5, S6, and S7, respectively). Samples were mixed with an equal volume of 2× Laemmli buffer; equivalent volumes were then loaded on SDS-PAGE and analyzed by immunoblotting.

RESULTS

Characterization of Stable Tau-SY5Y Cells Inducibly Expressing p25 Protein—Stable transfection of SH-SY5Y with the p25-inducible expression vector was unsuccessful, probably because of the toxicity of the low basal level expression of p25 protein (see "Discussion"). However, viable stable cell lines were obtained using SH-SY5Y cells that constitutively express tau protein (tau-SY5Y) (25). As shown in Fig. 1*A*, p25 was not found in mock tau-SY5Y cells. Conversely, p25 noninduced cells displayed a low basal expression of transgene protein, whereas tetracycline treatment induced a high p25 expression without affecting the endogenous level of its catalytic subunit Cdk5. Furthermore, co-immunoprecipitation analysis has been performed to ascertain the formation of p25-Cdk5 complex in p25-overexpressing cells (Fig. 1*B*).

To investigate kinase activity in lysates from p25-overexpressing cells, we used recombinant tau protein as substrate. Western blotting with specific phosphorylation-dependent antibodies (Fig. 2) showed that lysate from p25-overexpressing cells but not that from mock ones allowed tau phosphorylation at the reported Cdk5 kinase sites *in vitro* including AD2, AT8, and AT180 epitopes (24). Thus, tetracycline treatment of p25-inducible cells allowed a substantial expression of p25 protein and its direct association with Cdk5 to allow kinase activity.

Tau Phosphorylation in p25-expressing Cells—P25 overexpression has been reported to be toxic in mammalian cells (16). Thus, prior to tau phosphorylation analyses, we monitored the effect of time course p25-induced overexpression on cell death. Measurement of lactate dehydrogenase release showed that the toxic effect of p25 on tau-SY5Y cells is detectable from at least 3 days up of tetracycline treatment (Fig. 3). Moreover, p25 toxicity is attenuated by roscovitine, known as a preferential inhibitor of Cdk5 activity (Fig. 3). No evidence of apoptosis was observed at 24 h following p25 induction, since poly(ADP-ribose) polymerase proteolysis and carboxyl-terminal tau cleavage were not observed by Western blotting (data not shown).

To investigate tau phophorylation in p25-inducible cells independently of any p25 toxic effect, experiments have been performed from 24-h tetracycline-induced p25 overexpression. Immunoblots were carried out using four phosphorylation-dependent anti-tau antibodies (AT8, AD2, AT180, and TG-3) and the phosphorylation-independent antibody M19G, used to normalize for the amount of tau proteins (Fig. 4*A*). Tau phosphorylation was quantified as a ratio of each phosphorylation-dependent antibody immunoreactivity to that of M19G (Fig. 4*B*). This ratio was compared between tetracycline-treated (+*Tet*) and -untreated (−*Tet*) cells.

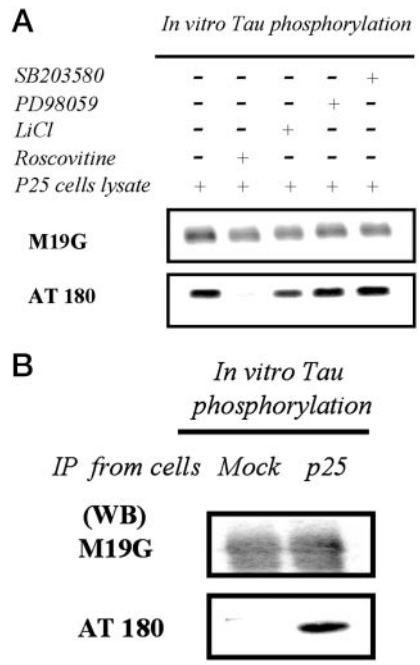

FIG. 5. **Direct tau phosphorylation at Thr^{231} by p25-Cdk5 kinase.** Recombinant tau was subjected to *in vitro* kinase assay by incubation with the following. *A*, crude extract from p25-expressing cells (24 h of tetracycline), alone and in the presence of kinase inhibitors: roscovitine (10 μM), LiCl (20 mM), PD98059 (50 μM), and SB203580 (10 μM). *B*, immunopurified p25-Cdk5 complex from mock cells (control) or p25-expressing cells. Tau phosphorylation was analyzed by immunoblotting (*WB*) with AT180. The anti-tau M19G was used as a control antibody.

As expected, tau proteins were found phosphorylated in mock cells and −Tet p25 cells. However, p25 overexpression in +Tet cells induced tau phosphorylation at the AT8 epitope, the major phosphorylation site of Cdk5 reported *in vivo* (29, 30), whereas no significant increase in AD2 immunoreactivity was observed. Interestingly, a robust increase in tau phosphorylation was observed at the AT180 epitope and more strongly at the TG-3 epitope, suggesting that phospho-Thr^{231} could be a major phosphorylation site of p25-Cdk5 *in vivo*. No significant increase in phosphorylation was detected in p25-overexpressing cells following roscovitine treatment (+*RSV*), suggesting a specific action of the complex p25-Cdk5. However, the direct tau phosphorylation by p25-Cdk5 has to be clearly established, since this kinase complex may also activate other kinases. In fact, Thr^{231} is a residue that can be phosphorylated by a large number of kinases other than Cdk, including c-Jun N-terminal kinase, p38, extracellular signal-regulated kinase 2, and GSK3β (14).

Direct Phosphorylation of Thr^{231} by the Complex p25-Cdk5—To investigate whether p25-Cdk5 directly phosphorylates Thr^{231}, we first performed an *in vitro* tau phosphorylation using the p25-overexpressing cell lysate in the presence of well recognized kinase inhibitors including PD98059 (MEK1 inhibitor), SB203580 (p38 inhibitor), LiCl (GSK3β inhibitor), and roscovitine (Cdk inhibitor) (31). Tau phosphorylation at Thr^{231} was monitored by Western blotting (Fig. 5*A*). Whereas LiCl slightly decreased phosphorylation at Thr^{231}, roscovitine completely abolished the signal. Other inhibitors did not have any effect on Thr^{231} phosphorylation. Altogether, these data

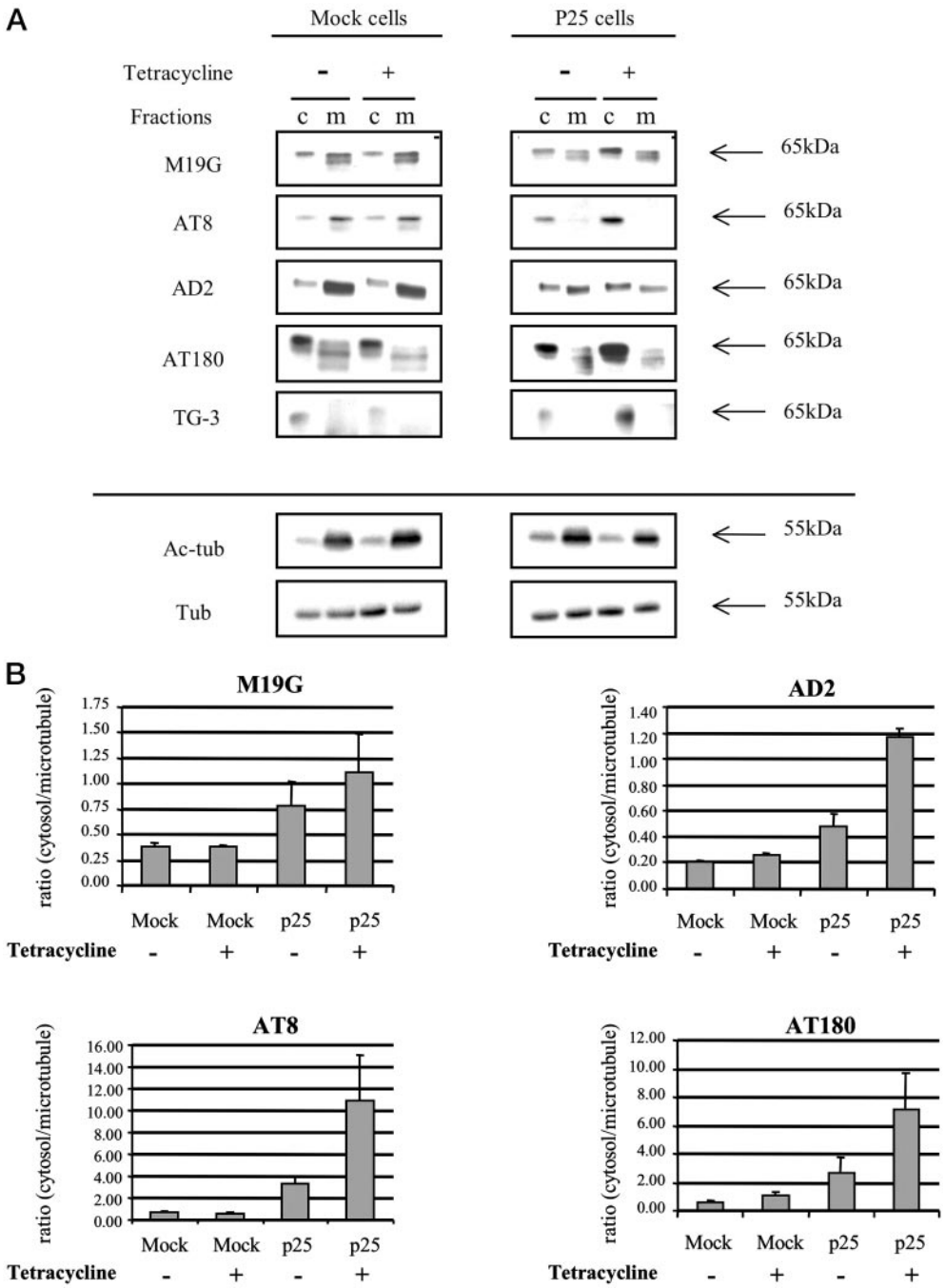

FIG. 6. **Analysis of phospho-tau distribution in cytosol and microtubule fractions.** *A*, representative immunoblots of cytosol (*c*) and microtubule (*m*) fractions, from mock and p25-inducible cells, untreated (−) or treated (+) with tetracycline. Tau phosphorylation was analyzed with phosphorylation-dependent and -independent antibodies. Levels of loaded proteins were evaluated with α-tubulin (*Tub*) antibody that showed no variation among samples. As expected, immunoreactivity of acetylated α-tubulin is mostly localized in the microtubule fraction, as showed by a specific antibody (*Ac-tub*). *B*, densitometric analysis and schematic representation of the ratio of cytosolic to microtubule immunoreactivities.

strongly suggested that *in vivo* tau phosphorylation at Thr^{231} resulted from a direct effect of p25-Cdk5 kinase. To definitely confirm this direct phosphorylation, the p25-Cdk5 was immunoprecipitated, using the anti-p25 antibody from the lysate. An *in vitro* tau phosphorylation was performed using the immunoprecipitated complex. Phosphorylation at Thr^{231} was visualized using the AT180 antibody, indicating that the complex p25-Cdk5 can directly phosphorylate tau at Thr^{231} (Fig. 5*B*).

Biochemical Characterization of Free and Microtubule-bound Tau in p25-inducible Cells—To examine the effect of increasing tau phosphorylation on its microtubule association, mock and p25-inducible cells were fractionated into a cytosol fraction (which contains free tau) and a microtubule fraction (which contains microtubule-associated tau), and they were analyzed by immunoblotting (Fig. 6*A*). Immunoreactivity of each tau antibody was quantified, and ratios of cytosol *versus* microtubule fractions are shown in Fig. 6*B*.

M19G antibody labeling revealed that the majority of tau proteins associated with microtubules in mock cells. Tau level in the cytosol fraction, proportionally to that in microtubule fraction, increased significantly in p25-noninduced cells, and to a greater extent in p25-induced cells, suggesting that p25 overexpression altered tau interaction with microtubules.

Analysis of phosphorylated tau distribution showed that AT8 and AD2 immunoreactivities in mock cells are proportional to tau levels and were detected in both cytosol and microtubule

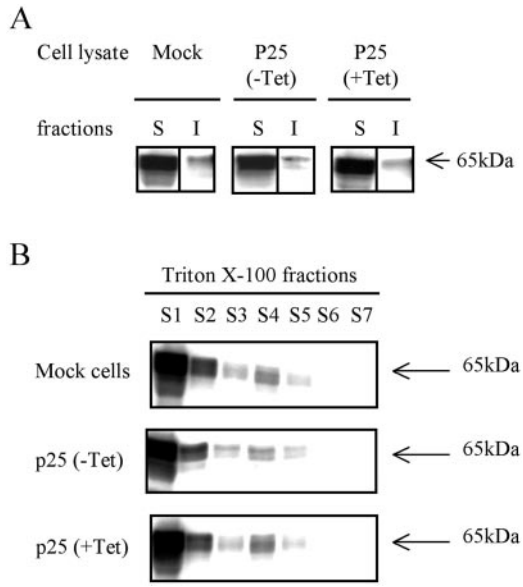

FIG. 7. *A*, analysis of tau aggregation. SDS-soluble (*S*) and acid formic fractions (*I*) were immunoblotted by using phosphorylation-independent antibody M19G. *B*, analysis of detergent tau solubility. Fractions recovered in salt buffer (S1) and in Triton X-100 (0.01% (S2), 0.025% (S3), 0.05% (S4), 0.1% (S5), 0.25% (S6), and 1% (S7)) were immunoblotted with M19G antibody. Representative immunoblots from mock and p25 tetracycline-untreated (−*Tet*) and -treated (+*Tet*) cells are shown.

fractions. In agreement with previous data, phosphorylation at these sites did not seem to affect tau biological function regarding microtubule binding (32, 33). However, AT180 epitope displayed intensive immunoreactivity in cytosol *versus* microtubule fractions, although the levels of tau were lower, and TG-3 labeling was exclusively detected in the cytosol fraction. Hence, tau phosphorylation at AT180 and TG-3 epitopes is probably a critical factor affecting tau binding to microtubules.

In p25-overexpressing cells, the ratio of cytosol to microtubule fractions of AD2-, AT8-, and AT180-reactive tau displayed a substantial increase compared with those in mock and p25-noninduced cells. Furthermore, TG-3-immunoreactive tau proteins were not found in microtubules despite their increased level in p25-induced cells (Fig. 6*B*).

Detergent Solubility of Tau in p25-expressing Cells—In an attempt to determine whether pathological tau phosphorylation by p25-Cdk5 complex triggers any change in tau conformation, we performed biochemical studies to investigate detergent tau solubility from p25-overexpressing tau-SY5Y. Cell lysates were separated into SDS (1%)-soluble and -insoluble (formic acid) fractions and then analyzed by immunoblotting with M19G antibody. Results showed that most tau proteins are recovered in SDS-soluble fraction (*S*) and that only a weak proportion of tau proteins was recovered in the insoluble fraction (*I*) (Fig. 7*A*). The presence of insoluble tau aggregates is probably due to the high level of tau proteins, since the concentration of tau proteins can be a critical factor for tau conformational changes (34). Indeed, tau proteins displayed the same solubility in mock, p25-noninduced, and p25-induced cells, suggesting that tau phosphorylation in p25-expressing cells did not modulate tau aggregation. We then looked into the presence of subtle differences in tau solubility by using solubilization buffer with increasing concentrations of Triton X-100 (ranging from 0.01 to 1% (v/v)). In all analyzed cell lines, tau proteins were mostly extractable in salt buffer (S1 fractions) with little proportions with intermediate solubility that are recovered in fractions S2–S5, corresponding to solubilization in 0.01, 0.025, 0.05, and 0.1% of Triton X-100, respectively (Fig. 7*B*). Hence, in these conditions, we did not see any variation in tau aggregation.

Mitotic Epitopes in Neuronal Cells Overexpressing p25 Protein—TG-3 epitope is exclusively found in mitotic cells (9). To ascertain that tau phosphorylation by p25-Cdk5 complex in tau-SY5Y cells was not linked to their proliferation state, the effect of p25 expression has been analyzed in differentiated neuronal cells. After differentiation by 7 days of NGF treatment, cells displayed morphological criteria of neuronal cells, and biochemical analysis by immunoblotting revealed the expression of α-synuclein, a well defined marker for neuronal differentiation (35); that expression was undetectable in undifferentiated tau-SY5Y cells (data not shown). Our analysis also showed an increase in the amount of endogenous 60-kDa tau, associated with its phosphorylation at some sites such as AD2 epitope (data not shown), as reported for differentiated SH-SY5Y cells (33). It is interesting to note that p25 overexpression in these cells induced profound changes in cell morphology (Fig. 8). Neurites retracted after 48 h. After 72 h, rounded cells were observed, and only a few short neurites were seen, suggesting that there was a severe reorganization of the microtubule network involved in neurite sprouting and stability as previously described (16). Hence, analyses of tau phosphorylation have been undertaken at 24 h of tetracycline treatment.

p25 overexpression in differentiated tau-SY5Y cells induced a marked tau phosphorylation at AT180 and TG-3 epitopes (Fig. 9*A*, *lane 4*). Conversely, only a faint tau immunoreactivity at these epitopes was observed in differentiated mock cells and noninduced p25 cells (Fig. 9*A*, *lanes 1-3*). Analysis of tau phosphorylation on other epitopes like AD2 showed no effect of p25-induced expression. Therefore, these data suggest that p25-Cdk5 complex in neuronal cells strongly phosphorylates tau at AT180 and at the mitotic TG-3 epitope.

We further investigated whether p25-Cdk5 phosphorylates another TG-3 antigen, nucleolin. TG-3-immunoreactive nucleolin is exclusively detected in mitotic cells and, as for TG-3-labeled tau, is a marker for early NFT (10–13). Immunoblot analysis of lysates from native cells (Fig. 9*B*, *panel A*) revealed an increase in nucleolin TG-3 immunoreactivity in p25-overexpressing tau-SY5Y cells and, to a lesser extent, in noninduced p25 cells compared with mock cells (*panel A*, *lanes 4*, *3*, and *1* and *2*, respectively). This increase is markedly observed in differentiated cells (Fig. 9*B*, *panel B*) that exhibited strong nucleolin phosphorylation at the TG-3 epitope following p25 overexpression (*lane 4*) compared with noninduced p25 and mock cells that showed undetectable levels of TG-3-reactive nucleolin (*lanes 1–3*).

Taken together, these results strongly indicate that p25-Cdk5 complex phosphorylates both tau and nucleolin and generates mitotic TG-3 epitope in differentiated neuronal cells.

DISCUSSION

Abnormal tau phosphorylation is likely to be a critical mechanism involved in tau aggregation and neurofibrillary degeneration in Alzheimer's disease (1, 2). Furthermore, each abnormal phosphorylation site may have its own impact on tau aggregation (1, 36). Indeed, correlation between specific epitopes of phosphorylated tau and the severity of neuropathological stage in Alzheimer's disease has been reported (11, 37). As with that correlation, TG-3-reactive tau is detected in early stages of preneurofibrillary tangles (10, 11) and may be critical to the initiation of early conformational changes leading to tau aggregation. However, the significance of such phosphorylation in tau aggregation remains to be established. Understanding the role of abnormal tau phosphorylation in NFT formation and neurofibrillary degeneration requires the identification of kinases leading to these specific epitopes and relies on the devel-

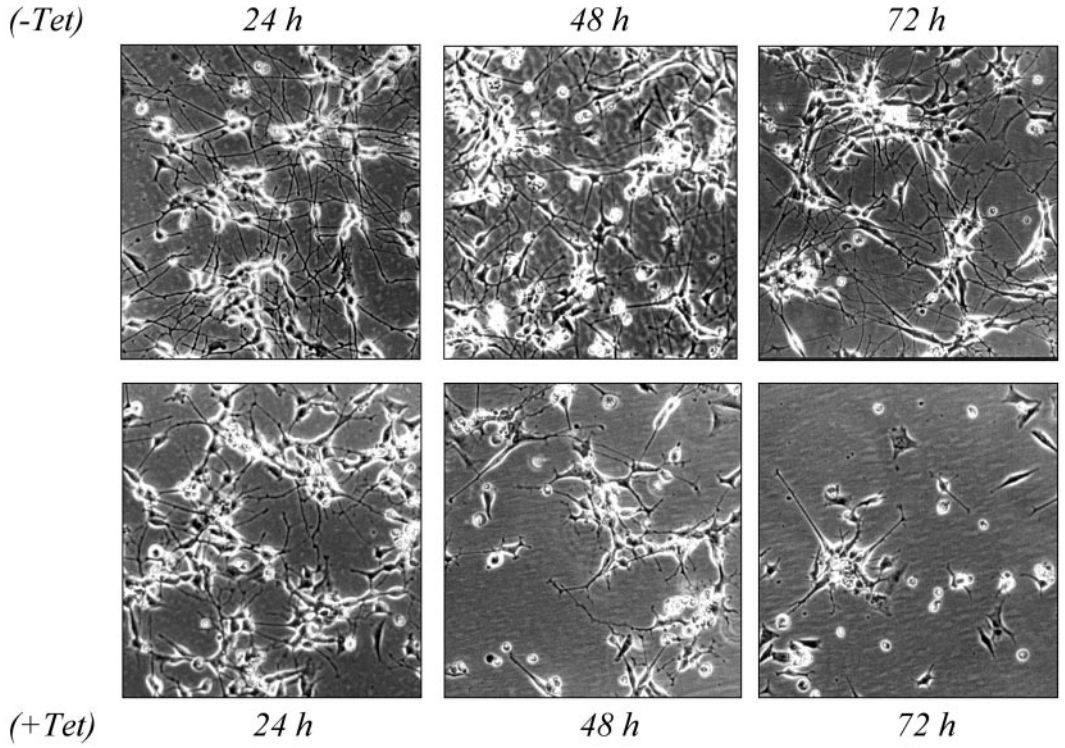

FIG. 8. **Cell morphology of p25 cells by phase-contrast microscopy.** Cells were differentiated by 7 days of NGF treatment, and then p25 expression was induced by tetracycline during the indicated times (24, 48, and 72 h) in differentiation medium. Noninduced p25 cells maintained in differentiation medium were used as controls.

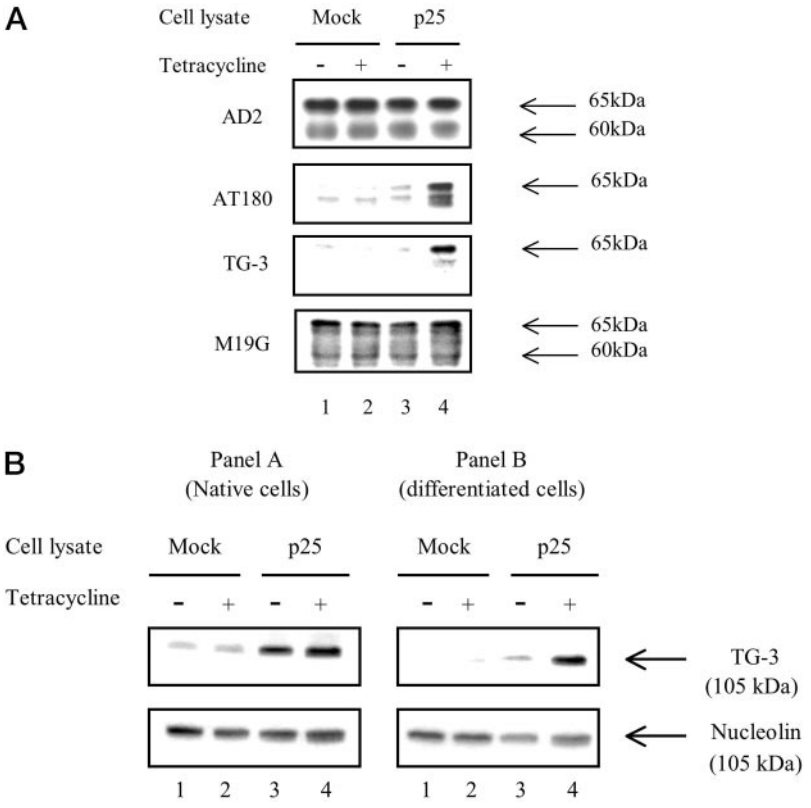

FIG. 9. **Mitotic-like phosphorylation in neuronal cells.** Lysates from differentiated (7 days of NGF) mock and p25-inducible cells, treated (+) or not (−) with tetracycline, were analyzed for the following. *A*, tau phosphorylation, using phospho-dependent antibodies (AD2, AT180, and TG-3) and phospho-independent antibody M19G. *B*, nucleolin phosphorylation, using phospho-dependent antibody TG-3, followed, after stripping, by a phospho-independent antibody to visualize the amount of loaded nucleolin.

opment of appropriate cellular and animal models.

In the present study, we used an inducible expression system that allowed investigation of tau phosphorylation by the p25-Cdk5 kinase complex in proliferating neuroblastoma tau-SY5Y cells and in differentiated neuronal ones, independently of any effect of p25-induced toxicity. Our results showed that p25-Cdk5 complex is linked to tau phosphorylation at the pathologic epitope TG-3 in proliferating as well as in neuronal cells. Besides TG-3-labeled tau, p25-Cdk5 also generated TG-3-reactive nucleolin in postmitotic differentiated cells. Several studies, mostly based on analysis of mitotic epitopes, support the idea of a reactivation of mitotic mechanisms in AD (9, 10, 12,

13). The present data strongly suggest that p25-Cdk5 may be responsible for the generation of mitotic phosphoepitopes, reported as markers of early stages preceding neurofibrillary tangle formation, thus arguing for a pathological role of Cdk5 activity in AD. Further studies are needed to determine whether this kinase is also involved in the reported expression of cell cycle markers in AD (13, 38–41).

It is interesting to note that elevated Cdk5 immunoreactivity is found in neurons with early neurofibrillary tangles (17), again suggesting its involvement in early events linked to AD neurodegeneration. Besides these observations, several studies established an increased Cdk5 activity in AD brains (15, 16), possibly in relation to abnormal cleavage of p35 into p25 (16, 42). Furthermore, mammalian cells and animal models showed that p25-Cdk5 but not p35/Cdk5 induced tau phosphorylation and cytoskeletal disruption (16, 29, 43, 44). However, tau phosphorylation by p25-Cdk5 is not yet well characterized *in vivo*. Although several Cdk5 phosphorylation sites on tau have been determined by *in vitro* studies (24), only the AT8 epitope was shown to be phosphorylated by Cdk5 *in vivo* (16, 29, 30). In the present study, we demonstrated that, in addition to AT8 epitope, p25 expression led to a substantial and robust tau phosphorylation at AT180 and TG-3 epitopes. Analyses of the tau phosphorylation pattern in proliferating tau-SY5Y cells and in differentiated neuronal cells strongly suggest that these two epitopes can be major phosphorylation sites of p25-Cdk5 *in vivo*.

AT180 and TG-3 epitopes are probably critical in determining tau biological activity in terms of inhibiting its binding to microtubules. Tau phosphorylation at Thr^{231}/Ser^{235} inhibits its binding to microtubules (32, 45, 46). Consistent with these data, our analysis of fractionated free and microtubule-bound tau showed that AT180 and TG-3 immunoreactivities are very poorly associated with microtubule fraction (Fig. 6). The elevated phosphorylation at these sites following 24 h of p25-induced expression is linked to an accumulation of phosphorylated tau in the cytosol fraction with a relatively lower remaining tau level in the microtubule fraction. Interestingly, alterations of tau phosphorylation and its distribution preceded the deleterious effect of p25 on cells that occurred at 3 days expression and up (Fig. 3). These observations led us to hypothesize that an accumulation of soluble phosphorylated tau and/or inhibition of tau activity is likely to be involved in p25-mediated cytotoxicity. Such a mechanism may explain the inability to establish viable stable p25-inducible SH-SY5Y that only expresses endogenous tau. Indeed, in SH-SY5Y overexpressing tau, although the basal level of p25 phosphorylates tau, there still remains enough hypophosphorylated tau to assume the stabilization of microtubules required for preserving the cytoskeleton function and thus cell viability.

Besides its critical role in tau biological activity, TG-3 epitope is also relevant in AD, since it labels pre-NFT stages and thus probably induces early conformational changes leading to tau aggregation (10, 11). Analysis of tau aggregation in p25-overexpressing cells that displayed strong TG-3 immunoreactivity showed no perceptible change, compared with p25-noninduced and mock cells. Our data were in agreement with those from p25 transgenic mice studies that showed cytoskeletal abnormalities with no neurofibrillary tangle formation (29, 43). Interestingly, during the revision of this manuscript, studies with the double transgenic mice overexpressing p25 and the tau mutant P301L demonstrated that the complex p25-Cdk5 potentiates neurofibrillary degeneration formation (47).

Altogether, these data did not *rule out* the possibility that tau phosphorylation at TG-3 epitope is a prerequisite in promoting neurofibrillary tangle formation. Indeed, additional events or factors could be required to induce tau conformational changes. For instance, it has been recently reported that a cooperative effect of several kinases is able to induce tau aggregation (2). Hence, additional phosphorylation of tau at other critical sites could be required for its conformational changes. Moreover, other molecules than kinases could be involved in such conformational changes (*i.e.* peptidyl-prolyl *cis-trans*-isomerases). In this context, Pin1 protein represents a good candidate, since it seems to be involved in AD, and more interestingly Pin1 binds tau protein on phospho-Thr^{231} (48–52), characterized in the present study as a major phosphorylation site by p25-Cdk5 kinase.

In conclusion, this report described the characterization of a suitable cell model for investigating tau phosphorylation by p25-Cdk5 kinase in a neuronal context, with the advantage of avoiding p25-induced cytotoxicity. Our data strongly suggest that *in neuronal cells* p25-Cdk5 mainly phosphorylates tau at AT180 and TG-3 epitopes, which are also associated with the microtubule-free fraction. This cell model, which reproduces markers of early stages of AD, constitutes a useful tool to identify additional molecular events that lead to tau aggregation and to clarify whether mitotic tau phosphorylation is a prerequisite in NFT formation or only represents a molecular marker of AD without any significant role in triggering tau aggregation.

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Mitotic-like Tau Phosphorylation by p25-Cdk5 Kinase Complex

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