

Dephosphorylation studies of SKNSH-SY 5Y cell Tau proteins by endogenous phosphatase activity

C. Soulié^a, J. Lépagnot^b, A. Delacourte^a, M.L. Caillet-Boudin^{a,*}

^aINSERM U 422, Place de Verdun, F-59045 Lille Cedex, France

^bIDRS, 125 Chemin de ronde, F-78290 Croissy sur Seine, France

Received 16 January 1996; revised version received 12 February 1996; accepted 13 February 1996

Abstract

Recent data have shown that the microtubule-associated Tau proteins are phosphorylated but to a lesser extent than PHF-Tau proteins which are the major components of Alzheimer's disease paired helical filaments. These normal Tau proteins are highly sensitive to the endogenous phosphatase activity during post-mortem delay. In order to understand the basic equilibrium between phosphatase and kinase activities, phosphorylation and dephosphorylation mechanisms of Tau proteins were studied in neuroblastoma cells. The present results demonstrate that an endogenous phosphatase activity is present and directed on Tau proteins in the SKNSH-SY 5Y cell extracts. Interestingly, the okadaic acid-induced hyperphosphorylated Tau proteins are more resistant to the phosphatase activity than the control Tau proteins. Our data emphasize the value of this *in vitro* cellular model for the study of biological conditions that control Tau protein phosphorylation levels.

Keywords: Tau protein; SKNSH-SY 5Y cells; Phosphatase; Kinase; PHF-Tau

Pathological Tau proteins (Tau 55, Tau 64, Tau 69; also named PHF-Tau) are the major components of paired helical filaments (PHF) found in degenerating neurons during Alzheimer's disease [4,6,8,9]. For a long time, these proteins were thought to be hyperphosphorylated when compared with Tau proteins in normal adult human brain. In fact, normal Tau proteins found in adult human brain biopsies are more phosphorylated than previously reported and are phosphorylated, but to a lesser extent, on sites that are similar to those found in PHF-Tau proteins [10,16]. Other sites on Tau proteins could be only phosphorylated during Alzheimer's disease [12]. Unlike Tau proteins which are aggregated into PHF, adult human Tau are rapidly dephosphorylated by endogenous phosphatase proteins during post-mortem delay [10]. On the contrary, foetal Tau proteins which are also phosphorylated to a high extent might not be dephosphorylated during protein preparation because of a deficiency in phosphatase activity in the foetal samples [11].

These data suggest that the equilibrium between phosphatase and kinase activities is determinant in PHF-Tau

accumulation. One can ask if the decrease in phosphatase activities [7], and not in phosphatase expression [1,2,13], is sufficient to allow the accumulation of PHF-Tau proteins in Alzheimer's brain neurons. Indeed, aggregation, phosphorylation or other post-transductional modifications can induce conformational changes which could account for PHF-Tau protein resistance to the phosphatase activity.

The study of the influence of Tau phosphorylation level on the endogenous phosphatase activity may be realized using an *in vitro* cellular model. Recently, we showed that Tau proteins synthesized by SKNSH-SY 5Y human neuroblastoma cells are of foetal-type and phosphorylated [5]. Alzheimer-type hyperphosphorylation of these proteins was induced by cell treatment with okadaic acid (OA), a potent inhibitor of phosphatase 1 and 2A proteins [3,5,14,15].

To analyze the Tau protein dephosphorylation by endogenous phosphatase, the SKNSH-SY 5Y cell pellet was washed in PBS buffer, centrifuged, then resuspended in lysis hypotonic buffer and disrupted or not with Dounce homogenizer (12 strokes), at 4°C. Cell lysates were incubated at 37°C for different times. After SDS-PAGE elec-

* Corresponding author. Tel.: +33 20 62 20 73; fax: 33 20 62 20 79.

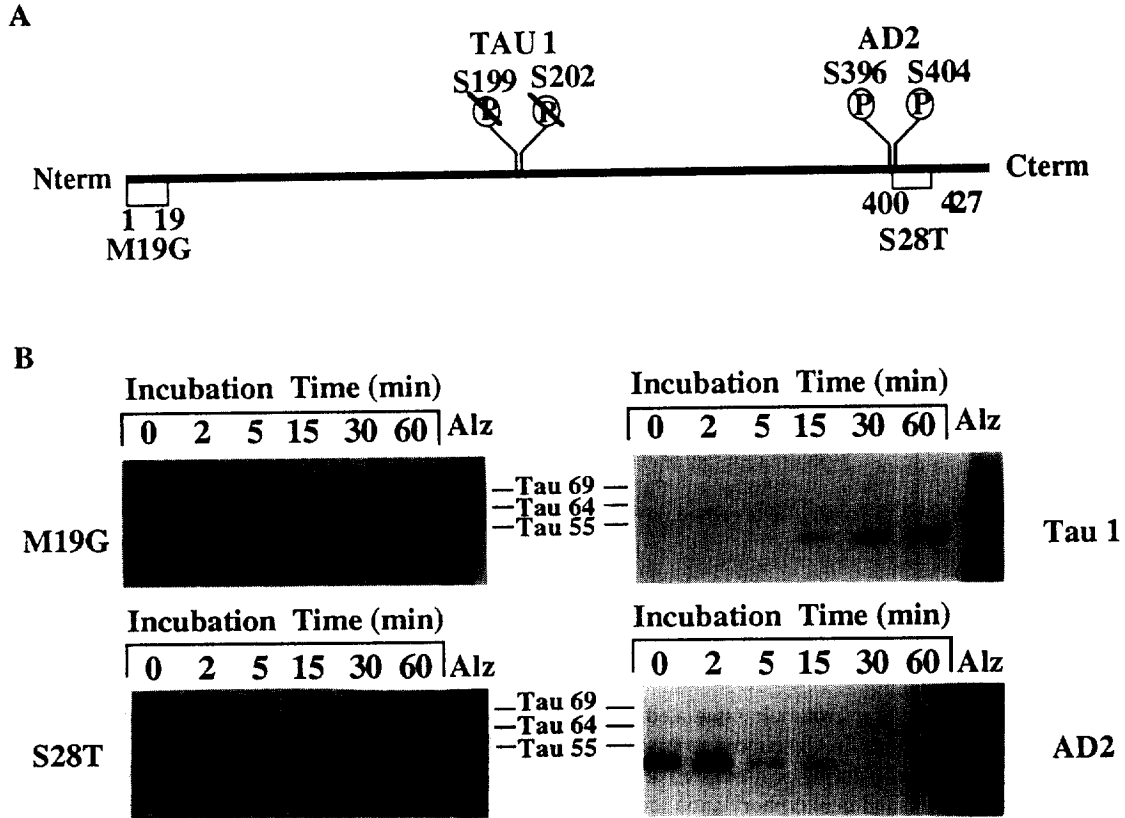
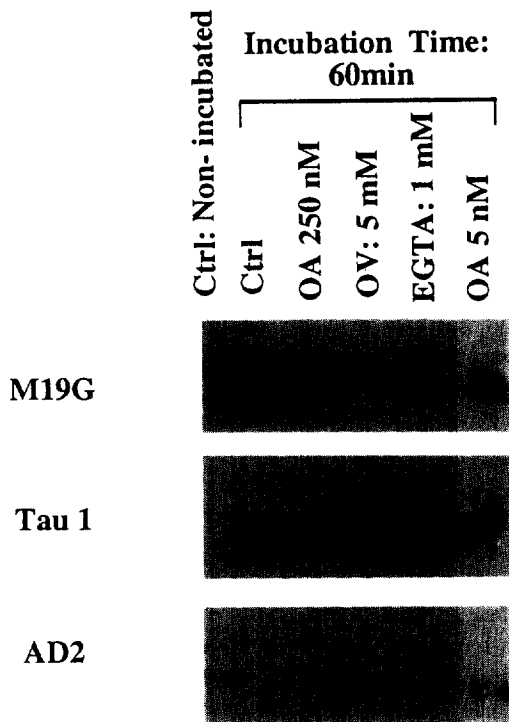


Fig. 1. (A) Schematic illustration of the 441 amino acid Tau isoform with location of the epitopes recognized by the anti-Tau antibodies. The numbers correspond to the amino acid numbers. When epitopes are phosphorylation-dependent, the presence (P) or the absence (R) of phosphate group required for antibodies binding is indicated. (B) Cell lysates incubation for different times at 37°C (see times above the figures). Tau modifications were analyzed by Western blotting: M19G and S28T showed that MW decreased. The increase in Tau 1 immunoreactivity and the simultaneous decrease in immunodetection using AD2 antibody indicated that Tau dephosphorylation occurred during incubation. PHF-Tau triplet (Tau 55, Tau 64, Tau 69) of an Alzheimer brain control (Alz) is indicated as positive control.



trophoresis and transfer, Tau protein modifications were analyzed by Western blotting using both phosphorylation-dependent (Tau 1 and AD2) and phosphorylation-independent (M19G and S28T) antibodies as described in [5] (Fig. 1A). When using M19G and S28T, a decrease in Tau proteins molecular weight (MW) was observed (Fig. 1B). This decrease was induced by protein dephosphorylation as demonstrated by phosphorylation-dependent antibody reactivity: Tau 1 immunoreactivity (specific of Tau proteins when Ser 199 and Ser 202 are not phosphorylated) increased whereas AD2 immunoreactivity (specific of phosphorylated Ser 396 and Ser 404 residues of Tau proteins) progressively disappeared during the 30 min of incubation (Fig. 1B). The immunoreactivity of these antibodies indicated that the dephosphorylation of Tau proteins occurred at various sites.

Endogenous phosphatase activity did not depend on cell differentiation as the same results were obtained on undifferentiated or NGF-differentiated cell extracts (data not shown).

Fig. 2. Inhibition of Tau protein dephosphorylation was tested by adding the following compounds in the incubation buffer: OA (5 or 250 nM), OV (5 mM) or EGTA (1 mM). Control blotting (Ctrl) was obtained from non-treated lysates incubated or non-incubated at 37°C.

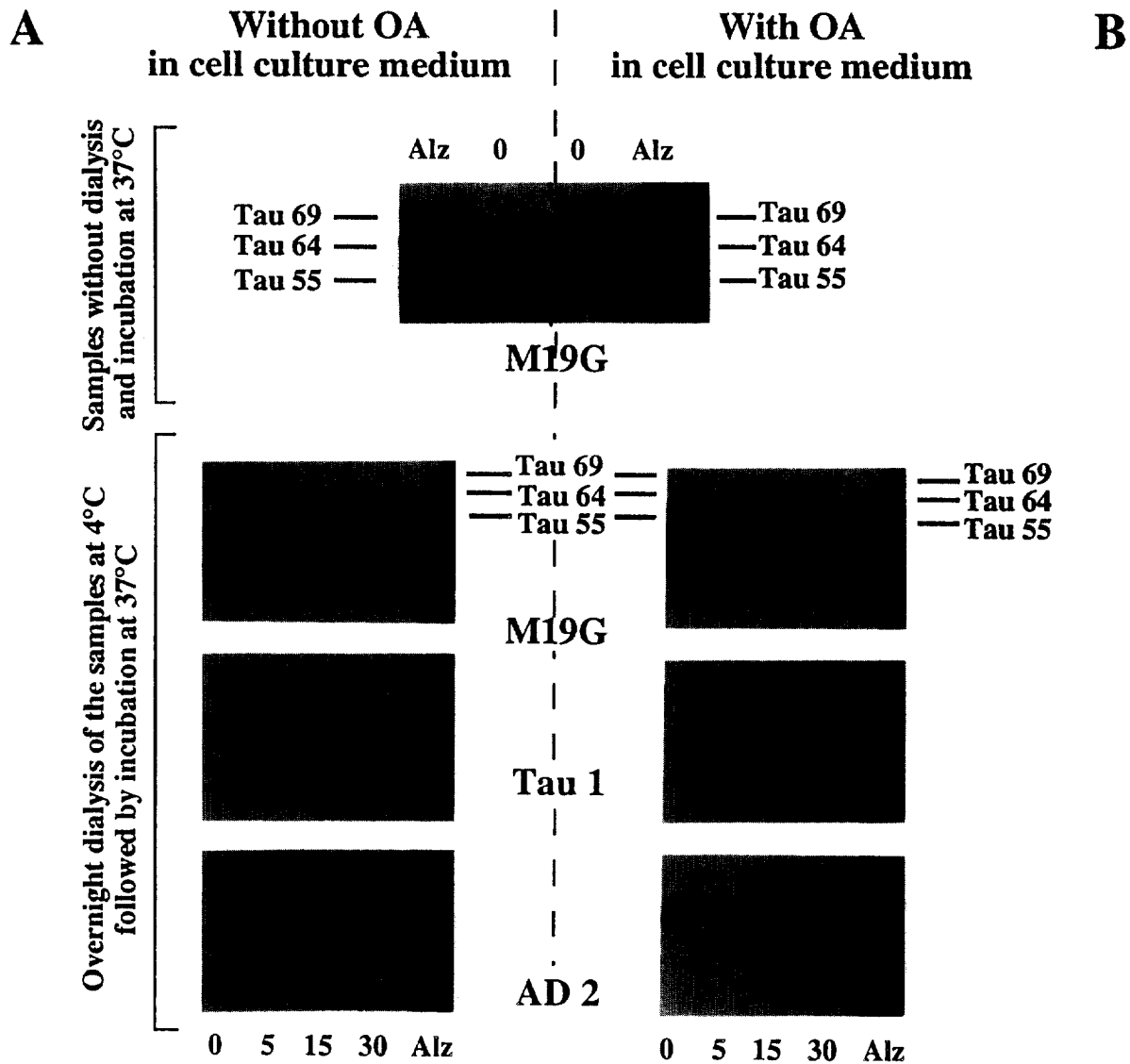


Fig. 3. Effect of endogenous phosphatase activity on normal or hyperphosphorylated Tau proteins. SY 5Y cells were treated (B) or not (A) by adding 250 nM OA in culture medium buffer for 6 h. The cell lysates were prepared, dialyzed overnight or not and then incubated at 37°C for different times (0, 5, 15, 30 min as indicated). The antibody used for Western blotting detection is mentioned. Note the electrophoretic migration change of Tau proteins after OA cell treatment when lysates were not dialyzed and incubated.

The effects of various inhibitors of this endogenous phosphatase activity have been studied by adding them to the incubation buffer (Fig. 2). Adding 250 nM OA (Phosphatase 1 and 2A inhibitor) was necessary for complete inhibition of the enzyme activity. Orthovanadate (OV), which inhibits several tyrosine protein phosphatases, inhibited the phosphatase activity. 5 nM OA and 1 mM EGTA (Ca^{2+} complexing agent) only inhibited partially the phosphatase activity since some discrete bands of lower molecular weight were detected with the M19G serum under the major band. Furthermore, with 5 nM OA, Tau 1 immunoreactivity increased but to a lesser extent than in control lysates. Thus, phosphatase 1 and 2A seemed to be implicated in Tau dephosphoryla-

tion mechanism but other phosphatases, such as tyrosine phosphatases, could also be involved.

Since OA treatment of SY 5Y cells induced a shift from foetal-type to Alzheimer-type Tau proteins [5,14, 15], the dephosphorylation process of these OA-modified Tau proteins was studied. Before lysate incubation at 37°C, overnight dialysis (with three or four buffer changes) was performed to eliminate the intracellular OA amount in the OA-treated cell lysate. A similar dialysis of control untreated cell extract was also performed. Although dialysis was performed at 4°C, the dephosphorylation process of Tau proteins in untreated cell extract appeared during the dialysis procedure as demonstrated by the low MW and the weak Tau detection with AD2.

After incubation at 37°C, the change in Tau-1 immunoreactivity and the disappearance of AD2 immunoreactivity showed that phosphatase proteins were still active and the dephosphorylation process occurred. In the OA-treated cell lysate, Tau proteins were not modified during the dialysis and the incubation at 37°C was not efficient enough to induce Tau 1 epitope detection or to dephosphorylate AD2 site (Fig. 3). In these conditions, a longer incubation time was necessary for obtaining a dephosphorylation process in OA-modified Tau proteins.

In conclusion, the present results demonstrated that endogenous phosphatase activity, similar to the activity which controls the phosphorylation level of Tau proteins in neurons and which is activated during a post-mortem delay, was present in the SY 5Y cell extracts. The foetal-type cellular Tau proteins were sensitive to this phosphatase activity. Furthermore, the OA-induced hyperphosphorylated Tau proteins were more resistant to the phosphatase activity than the control Tau proteins. This might be due to conformational changes occurring in case of phosphorylation at some specific sites. This OA-induced modification is complementary to those previously reported [5,14,15]. Note that in our cellular cell model of Tau protein phosphorylation, we observed a light but significant molecular weight change of Tau proteins after OA treatment, even in the absence of phosphatase activity (Fig. 3). Interestingly, a similar change was described for PHF-Tau proteins when compared to biopsy Tau [16].

The presence of both phosphatase activity and OA-induced Tau modifications suggest the use of SKNSH-SY 5Y cells as a suitable model for the study of the mechanisms implicated in the control of phosphorylation level of Tau proteins.

This work was supported by the Institut National de la Santé et de la Recherche Médicale and the Institut de Recherches Servier. C.S. is a recipient of a grant from Servier. We thanks Dr. J.C. Beauvillain, M.C. Chartier-Harlin and L. Buée for helpful discussions. AD2 was developed through a collaboration between UMR-9921 from Montpellier University (Prof. B. Pau, Dr. C. Mourtou-Gilles), Sanofi/Diagnostic Pasteur and INSERM.

- [1] Billingsley, M.L., Ellis, C., Kincaid, R.L., Martin, J., Schmidt, M.L., Lee, V.M.Y. and Trojanowski, J.Q., Calcineurin immunoreactivity in Alzheimer's disease, *Exp. Neurol.*, 126 (1994) 178–184.
- [2] Brion, J.P., Couck, A.M. and Conreur, J.L., Calcineurin (phosphatase 2B) is present in neurons containing neurofibrillary

tangles and in a subset of senile plaques in Alzheimer's disease, *Neurodegeneration*, 4 (1995) 13–21.

- [3] Cohen, P., Holmes, C.F.B. and Tsukitani, Y., Okadaic acid: a new probe for the study of cellular regulation (review), *Trends Biochem. Sci.*, 15 (1990) 98–103.
- [4] Delacourte, A., Flament, S., Dibe, E.M., Hublau, P., Sablonnière, B., Hemon, B., Scherrer, V. and Défossez, A., Pathological proteins Tau 64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease: demonstration with a panel of antibodies against Tau proteins, *Acta Neuropathol.*, 80 (1990) 111–117.
- [5] Dupont-Wallois, L., Sautière, P.E., Cocquerelle, C., Bailleul, B., Delacourte, A. and Caillet-Boudin, M.L., Shift from fetal-type to Alzheimer-type phosphorylated Tau proteins in SKNSH-SY 5Y cells treated with okadaic acid, *FEBS Lett.*, 357 (1995) 197–201.
- [6] Flament, S. and Delacourte, A., Abnormal Tau species are produced during Alzheimer's disease neurodegenerating process, *FEBS Lett.*, 247 (1989) 213–216.
- [7] Gong, C.X., Shaik, S., Wang, J., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K., Phosphatase activity toward abnormally phosphorylated τ : decrease in Alzheimer disease brain, *J. Neurochem.*, 65 (1995) 732–738.
- [8] Greenberg, S.G. and Davies, P.A., Preparation of Alzheimer paired helical filaments that displays distinct Tau-proteins by polyacrylamide gel electrophoresis, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 5827–5831.
- [9] Lee, V.M., Balin, B.J., Otvos, Jr., L. and Trojanowski, J.Q., A 68: a major subunit of paired helical filaments and derivatized forms of normal tau, *Science*, 251 (1991) 675–678.
- [10] Matsuo, E.S., Shin, R.W., Billingsley, M.L., Van de Voorde, A., O'Connor, M., Trojanowski, J.Q., and Lee, V.M.Y., Biopsy-derived adult brain Tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau, *Neuron*, 13 (1994) 989–1002.
- [11] Mawal-Dewan, M., Henley, J., Van de Voorde, A., Trojanowski, J.Q. and Lee, V.M.Y., The phosphorylation state of Tau in the developing rat brain is regulated by protein phosphatases, *J. Biol. Chem.*, 269 (1994) 30981–30987.
- [12] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K. and Ihara, Y., Proline-directed and non-proline-directed phosphorylation of PHF-tau, *J. Biol. Chem.*, 270 (1995) 823–829.
- [13] Pei, J.J., Sersen, E., Iqbal, K. and Grundke-Iqbal, I., Expression of protein phosphatases (PP-1, PP-2A, PP-2B, and PTP-1B) and protein kinases (MAP kinase and P34 cdc2) in the hippocampus of patients with Alzheimer disease and normal aged individuals, *Brain Res.*, 655 (1994) 70–76.
- [14] Sautière, P.E., Caillet-Boudin, M.L., Watzet, A., Buée-Scherrer, V. and Delacourte, A., Alzheimer-type Tau epitope detection after okadaic acid treatment of neuroblastoma cells, *C. R. Acad. Sci. Paris*, 316 (1993) 533–535.
- [15] Sautière, P.E., Caillet-Boudin, M.L., Watzet, A. and Delacourte, A., Detection of Alzheimer-type Tau proteins in okadaic acid-treated SKNSH-SY 5Y neuroblastoma cells, *Neurodegeneration*, 3 (1994) 53–60.
- [16] Sergeant, N., Bussièrre, T., Vermersch, P., Lejeune, J.P. and Delacourte, A., Isoelectric point differentiates PHF-Tau from biopsy-derived human brain Tau proteins, *NeuroReport*, 6 (1995) 2217–2220.