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In situ dephosphorylation of tau by protein phosphatase 2A and 2B in fetal rat primary cultured neurons

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Abstract Using antibodies recognizing the phosphorylation state of specific sites, phosphorylation states of tau were monitored in fetal rat primary cultured neurons. When cultured neurons were treated with okadaic acid (OA) or calyculin A (CalA) at concentrations sufficient to inhibit protein phosphatase 2A (PP2A), phosphorylation of Ser-199/Ser-202 (numbered according to the human tau 441) and Ser-235 increased. On the other hand, treatment with Ca^{2+} ionophore, A23187, induced dephosphorylation of Ser-199/Ser-202, Thr-205, Ser-396 and Ser-404, and this dephosphorylation was repressed by inhibitors of protein phosphatase 2B (PP2B), cyclosporin A and FK506. These results indicate that PP2A and PP2B are differentially involved in dephosphorylation of tau in neurons.

Key words: Protein phosphatase; Tau; Microtubule-associated protein; Primary cultured neuron; cdk5

1. Introduction

Tau is a brain-specific, heat-stable microtubule-associated protein (MAP) with an ability to promote the assembly of tubulin into microtubules [1–3]. Tau is a phosphoprotein and phosphorylation is known to regulate its microtubule polymerization and stabilization abilities [3–6]. In particular, currently phosphorylation of tau has attracted much attention, because of the fact that the highly phosphorylated form of tau is a main component of the paired helical filaments (PHFs) found in Alzheimer's disease brains [7–9]. Although tau protein kinases have been extensively studied [10–12], it has become evident that phosphorylation of tau is also modulated by protein phosphatases. There are several reports on protein phosphatases that dephosphorylate tau in vitro [13–19]. Although there are some contradictions, protein phosphatase 2A (PP2A) and 2B (PP2B) have been shown to dephosphorylate Alzheimer tau or tau phosphorylated by Ca^{2+}-calmodulin kinase II, cAMP-dependent protein kinase, MAP kinase and cdc2 kinase in vitro [13–19].

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Abbreviations: PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; CalA, calyculin A; CysA, cyclosporin A; OA, okadaic acid; cdk5, cyclin-dependent kinase 5; GSK3β, glycogen synthase kinase 3β MAP kinase, mitogen-activated protein kinase; PHF, paired helical filament.

However, the broad substrate specificity of protein phosphatases [20,21] makes it uncertain that these protein phosphatases indeed act on tau in vivo. To resolve this question, we used rat primary cultured neurons and examined which protein phosphatase dephosphorylates which phosphorylation sites of tau. We chose to use cultured neurons because they are amenable to treatments with protein phosphatase inhibitors. Another advantage of using fetal brain neurons was the expression of a single tau species, which differ from adult rat brain in which complicated expression patterns of alternatively spliced isoforms might hinder the observation of phosphorylation-dependent electrophoretic mobility shift [22–24]. Finally, the use of antibodies that can recognize a single site-specific phosphorylation is also a considerable technical improvement over other systems. We report here our findings that PP2A and PP2B are capable of dephosphorylating tau in a site-specific and Ca^{2+}-dependent manner.

2. Materials and methods

2.1. Chemicals and antibodies

Okadaic acid and calyculin A were purchased from Wako Chemicals (Osaka, Japan). Cyclosporin A and FK506 was provided by Drs. Takao Kataoka and Kazuo Nagai (Tokyo Institute of Technology, Tokyo, Japan). Ca^{2+} ionophore, A23187, was purchased from Sigma (St Louis, MO).

Anti-phospho-Thr-205 (PT205), phospho-rat Ser-235 (PS235), phospho-Ser-396 (PS396), and phospho-Ser-404 (PS404) sera were obtained by immunizing rabbits with phosphorylated peptide conjugated with keyhole limpet hemocyanin [25]. Amino acid sequences of these antigen peptides were in the following. PT205, C-PGSPGTpPGSRS; rat PS235, C-RTPPKSpPSASK; PS396, C-EIVYKSpPVVSG; PS404, C-VSGDTSpPRHLS. Here Sp and Tp represent phosphoserine and phosphothreonine, respectively. Antihuman tau antibody was generously provided by Dr. Yasuo Ihara (University of Tokyo, Tokyo, Japan). Anti-Tau1 antibody was purchased from Sigma. Anti-Tau1 recognizes dephosphorylation at Ser-199/Ser-202. Anti-PT205, antirat PS235, anti-PS396 and anti-PS404 recognize phosphorylation at Thr-205, Ser-235, Ser-396 and Ser-404, respectively. Antihuman tau is phosphorylation-independent.

2.2. Cell culture

Cerebral cortical neurons were prepared from 17- or 18-day-old embryonic rat brains. The brains were dissected free of meninges, minced in phosphate-buffered saline, and incubated with 0.125% trypsin and DNase I (Sigma) at 37°C for 15 min. The cortices were dissociated mechanically with a pipette and plated in polyethylenimine-coated tissue culture dishes in Dulbecco's modified Eagle medium and Ham's F-12 (1:1) supplemented with 5% newborn calf serum and 5% horse serum. Cytosine arabinoside (10 μM) was added to culture medium 2 days after plating to inhibit proliferation of nonneuronal cells. The cultures were fed 3× a week and used 10–14 days after plating.

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2.3. Preparation of cell lysate and brain extract

Cultured neurons were homogenized with 5 vols. of 0.1 M MES, pH 2.8, 0.75 M NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA and 0.1 mM EDTA containing 2 mM dithiothreitol, 20 mM NaF, 20 mM β-glycerophosphate and a protease inhibitor, 0.2 mM Pefabloc SC (Merck, Darmstadt, Germany), and immediately boiled for 5 min. After centrifugation at 100,000 × *g* for 30 min, the supernatant fraction was used as the heat-stable cell extract. In some experiments, cultured neurons were directly solubilized in SDS-sample buffer and boiled. Rat brains were homogenized in the same buffer used for cultured neurons and boiled immediately (within 1 min) after decapitation. After centrifugation as described above, the supernatant fraction was used as the heat-stable brain extract.

2.4. SDS-PAGE and immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using an acrylamide concentration of 9.0% [26]. Proteins were transferred to PVDF membrane (Millipore, Bedford, MA) and reacted with antiphosphorylation-dependent antibodies with horse radish peroxidase- or alkaline phosphatase-conjugated antirabbit secondary antibodies [26, 27]. The dilution of antibodies for immunoblots were the following: antihuman tau, 1/1000; anti-Tau1, 1/10,000; anti-PT205, 1/100; antirat PS235, 1/100; anti-PS396, 1/400; anti-PS404, 1/200.

2.5 Protein concentration determination

Protein concentration was determined by the procedure of Bradford [28], using bovine serum albumin as a standard.

3. Results

3.1. Phosphorylation states of tau in fetal rat brains and primary cultured neurons

We examined phosphorylation states of tau in primary cultured neurons and compared with those of tau in fetal brains by immunoblotting with phosphorylation-dependent antibodies. We focused our study on cdk5 (Ser-202, Thr-205, Ser-235 and Ser-404) and GSK3β (Ser-199 and Ser-396) phosphorylation sites because cdk5 and GSK3β have been suggested to be the major tau protein kinases in porcine and bovine brain extracts [26,29,30].

Only a blot of cultured neurons is shown in Fig. 1, because exactly identical results were obtained with the brain extract. Both brain and cultured neuron tau were composed of two species that reacted with the phosphorylation-independent antibody, antihuman tau (Fig. 1, human tau). Treatment with alkaline phosphatase shifted the migration of these two species to a lower single band (see Fig. 5, lane 6: the upper band indicated by arrowhead is *E. coli* alkaline phosphatase reacted with an secondary antibody), indicating that the original two bands observed are isoforms with different phosphorylation states. Both bands reacted with anti-PS396 and anti-PS404 antibodies, these antibodies recognize the phosphorylation of Ser-396 and Ser-404, respectively, (Fig. 1, PS396 and PS404) in almost similar ratio of intensity to those stained with the antihuman tau antibody. On the other hand, antirat PS235 did not react with either of two tau bands (Fig. 1, PS235), although upon longer development of the blots a faint reaction could be seen (data not shown). These two bands were distinguished by anti-PT205 or anti-Tau1 immunoreactivity; the upper band reacted with anti-PT205 antibody while the lower band reacted with anti-Tau1 antibody (Fig. 1, PT205 and Tau1).

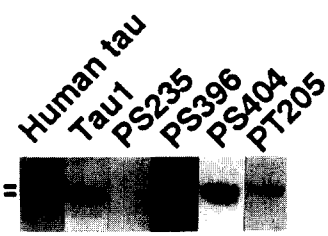

Fig. 1. Western blot analysis of phosphorylation states of tau in fetal rat primary cultured neurons. Heat-stable extract of cultured neurons were separated on 9% SDS-PAGE, transferred to PVDF membrane, and reacted with antihuman tau (Human tau), anti-Tau1 (Tau1), antirat PS235 (PS235), anti-PS396 (PS396), anti-PS404 (PS404) or anti-PT205 (PT205).

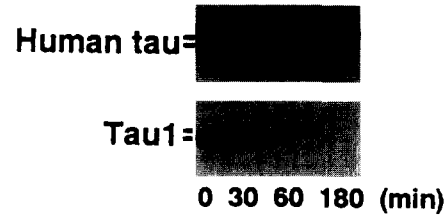

Fig. 2. Effect of okadaic acid on phosphorylation at Tau1 site of tau in primary cultured neurons. Fetal rat primary cultured neurons treated with 100 nM OA for 0, 30, 60 and 180 min were homogenized, boiled and their heat-stable supernatants were blotted with antihuman tau and anti-Tau1 antibodies.

3.2. Involvement of PP2A in dephosphorylation of Ser-235 and a portion of Ser-199/Ser-202 in rat primary cultured neurons

As shown in Fig. 1, a portion of tau (the lower band) was dephosphorylated at the anti-Tau1 site (Ser-199/Ser-202) in contrast to the major tau band, which was phosphorylated. Ser-235, which is a favorable phosphorylation site for cdk5 in vitro, was largely dephosphorylated (Fig. 1). To determine which protein phosphatase was involved in dephosphorylation of these sites, cultured neurons were treated with okadaic acid (OA). 100 nM OA shifted the electrophoretic mobility of tau upward, abolished the reactivity with anti-Tau1 antibody (Fig. 2), and generated the antirat PS235 epitope (Fig. 5, lane 2 of PS235), indicating that increased phosphorylation of Ser-199/Ser-202 and Ser-235 had occured. The same result was obtained with 100 nM calyculin A (CalA) treatment (data not shown). CalA is an OA class protein phosphatase inhibitor.

To determine whether PP1 or PP2A is the phosphatase responsible for dephosphorylation at these sites, cultured neurons were treated with various concentrations of OA or CalA. PP1 and PP2A are distinguished by the sensitivity to OA or CalA. PP2A is inhibited by similar concentrations of OA and CalA, whereas PP1 is about 1000 × more sensitive to CalA than OA. The electrophoretic mobility shift and the disappearance of the anti-Tau1 reactivity occurred at 10 nM OA (Fig. 3), which inhibited the in vitro dephosphorylation of the anti-Tau1 site in fetal tau by purified PP2A. When cultured neurons were treated with 10 nM CalA, similar changes, the electrophoretic mobility shift (data not shown) and the disappearance of the anti-Tau1 reactivity (Fig. 3), were obtained. These results suggested the involvement of PP2A in the electrophoretic mobility shift and the disappearance of the reaction with the anti-Tau1 antibody (Ser-199/Ser-202).

3.3. Involvement of PP2B in dephosphorylation of tau at Ser-396, Ser-404 and putative involvement at Ser-199/Ser-202 and Thr-205 in Ca^{2+} ionophore-treated cultured neurons

PP2B has also been reported to dephosphorylate the anti-

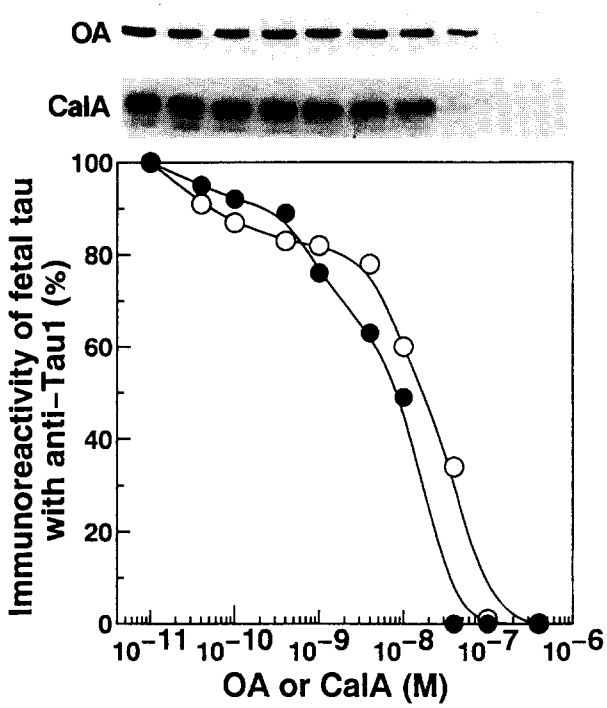

Fig. 3. Disappearance of anti-Tau1 immunoreactivity dependent on concentrations of okadaic acid (OA) and calyculin A (CalA). Fetal rat primary cultured neurons were treated with various concentrations of OA or CalA for 60 min. Cell lysates were probed on Western blot with anti-Tau1 antibody (upper panels) and quantitation with densitometry was done for the immunoreactive band in lysate of cells treated with OA (○) or CalA (●) (lower panel).

Tau1 site in vitro [17,18]. To test if this is true in neurons, rat primary cultured neurons were treated with inhibitors of PP2B, cyclosporin A (CysA) or FK506. These immunosuppressants inhibit PP2B through their respective intracellular binding proteins, cyclophilin and FKBP [31]. 1 μM CysA or 100 nM FK506 did not show any effect on either the band pattern or the reaction with anti-Tau1 antibody during treatments as long as 24 h (data not shown), suggesting that PP2B is not the protein phosphatase that maintains part of the Ser-199/Ser-202 sites in a dephosphorylated state in quiescent cultured neurons. This results is in good agreement with a recently published paper in which rat brain slice cultures were studied [32].

To investigate whether PP2B is capable of dephosphorylating tau when neurons are activated, cultured neurons were treated with Ca^{2+} ionophore (A23187). When 5 μM A23187 was added to the culture medium, tau increased its electrophoretic mobility and showed with enhanced reactivity to the anti-Tau1 antibody. The band indicated by the arrow showed the same mobility as tau that had been dephosphorylated by alkaline phosphatase (see Fig. 5, lanes 6 and 7). A downward shift on SDS-PAGE suggests that net action of PP2B is prevailing over action of Ca^{2+}-calmodulin-dependent protein kinase on tau in Ca^{2+} ionophore-treated neurons. Because proteolytic fragments appeared over 60 min incubation, most likely due to Ca^{2+}-activated protease, the incubation time was limited to 30 min to minimize proteolysis. This increased mobility shift was shown to be due to dephosphorylation by PP2B rather than due to protein degradation because this shift was suppressed by either CysA or FK506 (Fig. 4).

Phosphorylation states of Thr-205, Ser-235, Ser-396 and Ser-404 after treatment with A23187 and CysA are shown in Fig. 5 (lanes 3 and 4). Thr-205 was readily dephosphorylated by A23187 treatment. Addition of CysA did not inhibit this dephosphorylation, suggesting either that Thr-205, as well as Ser-199/Ser-202 (indicated by the increased reaction with anti-Tau1 antibody even in the presence of CysA or FK506 as shown in Fig. 4), may be very sensitive to PP2B or that other phosphatases could be activated under this condition. Ser-235 remained in the dephosphorylated state (Fig. 5, PS235). Anti-PS396 and anti-PS404 did not react with the fastest migrating tau band (arrow in lane 7), which showed the same electrophoretic mobility as dephosphorylated tau. Compared with the reaction of tau with anti-PS396 and anti-PS404 in A23187-treated neurons (Fig. 5, lane 3), the increased reaction in the presence of CysA (Fig. 5, lane 4) indicated that Ser-396 and Ser-404 are dephosphorylated by PP2B under conditions in which intracellular Ca^{2+} concentration is elevated.

4. Discussion

It has become evident that phosphorylation of tau is modulated not only by protein kinases but also protein phosphatases. PP1, PP2A and PP2B have all been reported to dephosphorylate tau in vitro [13–19]. Because of the broad substrate specificity of protein phosphatases [20,21], however, many phosphorylation sites could be artificially dephosphorylated by multiple protein phosphatases in vitro. An important question is whether these protein phosphatases are, in fact, involved in dephosphorylation of tau in vivo. In this paper, using rat fetal primary cultured neurons, we demonstrated that phosphorylation of tau can be regulated by PP2A and PP2B in site-specific and Ca^{2+}-dependent manners. Because the phosphorylation states of tau in cultured neurons were the same as those in brain, we believe that the same protein phosphatases are acting on tau in both brain and cultured neurons.

A portion of anti-Tau1 sites (Ser-199/Ser-202) was dephosphorylated by PP2A in cultured neurons. Heterogeneous phosphorylation at the anti-Tau1 site might reflect its localization in neurons as has been shown in rat brains, in which the dephosphorylated form is found in axon and the phosphorylated form in the cell body and dendrite [33]. If so,

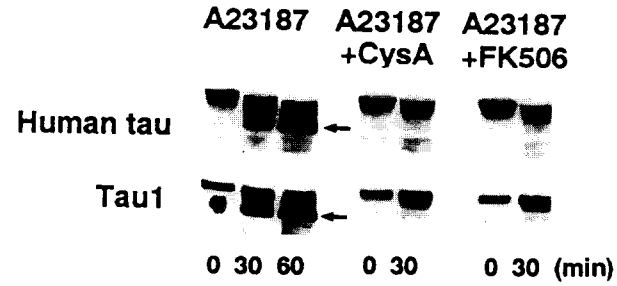

Fig. 4. Ca^{2+} ionophore-induced tau dephosphorylation and its inhibition by cyclosporin A (CysA) and FK506. Ca^{2+} ionophore, A23187 (5 μM), was added to culture medium of fetal rat primary cultured neurons. After incubation for 0, 30 and 60 min, cell lysate was prepared and subjected for Western blot with antihuman tau and anti-Tau1 antibodies. 1 μM CysA or 0.1 μM FK506 was added to the culture medium at the same time of A23187 addition. Cell lysates prepared at 0 and 30 min incubation were subjected to Western blotting with antihuman and anti-Tau1 antibodies. Arrows indicate the position of tau dephosphorylated with *E. coli* alkaline phosphatase.

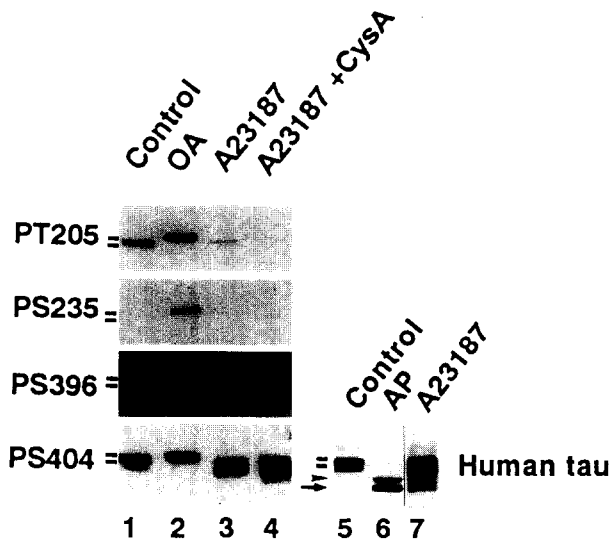

Fig. 5. Effect of okadaic acid (OA), A23187 and A23187 plus cyclosporin A (CysA) on phosphorylation of tau at Thr-205, Ser-235, Ser-396 and Ser-404. Heat-stable extracts were prepared from primary cultured neurons (control, lane 1), those treated with 100 nM OA for 30 min (lane 2), those treated with 5 μM A23187 for 30 min (lane 3) and those treated with 5 μM A23187 and 1 μM CysA for 30 min (lane 3). After separation on 9% SDS-PAGE and transfer to PVDF membrane, tau was probed with anti-PT205, antirat PS235, anti-PS396, and anti-PS404, respectively. As reference, blots with antihuman tau antibody are shown in lane 5 (control), lane 6 (tau dephosphorylated with *E. coli* alkaline phosphatase) and lane 7 (tau prepared from cultured neurons treated with A23187 for 30 min). Arrow indicates the dephosphorylated tau and arrowhead indicates *E. coli* alkaline phosphatase.

PP2A, which is active on tau, should also colocalize with anti-Tau1 reactive tau molecules. Recently, a pool of PP2A has been shown to colocalize with microtubules in mammalian cultured cells [34]. It is possible that a subset of microtubules with tau dephosphorylated at its anti-Tau1 site associates with PP2A in neurons as well as in cultured fibroblasts.

Ser-235 of Alzheimer tau is reported to be dephosphorylated by PP2B but not PP2A in vitro [18]. This is apparently contradictory to our present result that PP2A is a likely protein phosphatase responsible for keeping Ser-235 in the unphosphorylated state in cultured primary neurons. Normal tau could have different conformation from Alzheimer tau, and in this conformation, it could become accessible to PP2A. In our study, because Ser-235 was dephosphorylated before the treatment with Ca^{2+} ionophore, we could not determined whether PP2B showed potential to dephosphorylate Ser-235. As we have reported, there are OA-sensitive and -insensitive Ser-235 phosphatases in bovine brain extracts [26], therefore, Ser-235 can be dephosphorylated by several protein phosphatases in vitro.

Ser-396 and Ser-404 have been reported to be dephosphorylated by PP2A in vitro [16–19]. This seemed not to be the case in fetal rat cultured neurons, although we can not rule out the possibility that, as in the case of the anti-Tau1 site, there is a small fraction of tau whose Ser-396 and Ser-404 are dephosphorylated by PP2A. Such tau, if present, would be detected by antibodies recognizing the unphosphorylated state of Ser-396 or Ser-404.

In contrast to PP2A, PP2B appeared to dephosphorylate most tau phosphorylation sites (Ser-199/Ser-202, Thr-205, Ser-396 and Ser-404) when cultured neurons were treated with Ca^{2+} ionophore (Figs. 4, 5). However, there seemed to be some variation in sensitivity to Ca^{2+} treatment, depending on phosphorylation sites. Ser-199/Ser-202 and Thr-205 were dephosphorylated easily even in the presence of PP2B inhibitor. This may indicate the possibility that other protein phosphatases are activated by Ca^{2+} ionophore treatment or that PP2B not completely inhibited by CysA dephosphorylates them. On the other hand, Ca^{2+} ionophore-dependent dephosphorylation of Ser-396 and Ser-404 was clearly shown to be mediated by PP2B, because it was suppressed by CysA or FK506.

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References

[1] Cleveland, D.W., Hwo, S-Y. and Kirschner, M.W. (1977) J. Mol. Biol. 116, 207–225.
[2] Hirokawa, N., Shiomura, Y. and Okabe, S. (1988) J. Cell Biol. 107, 1449–1461.
[3] Lee, G. (1990) Cell Motil. Cytoskel. 15, 199–203.
[4] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K. and Ihara, Y. (1995) J. Biol. Chem. 270, 823–829.
[5] Chapin, S.J. and Bulinski, J.C. (1992) Cell Motil. Cytoskel. 23, 236–243.
[6] Drechsel, D.N., Hyman, A.A., Cobb, M.H. and Kirschner, M.W. (1992) Mol. Biol. Cell 3, 1141–1154.
[7] Lee, V.M.-Y. and Trojanowski, J.Q. (1992) Curr. Opin. Neurobiol. 2, 653–656.
[8] Mandelkow, E.-M. and Mandelkow, E. (1993) Trends Biochem. Sci. 18, 480–483.
[9] Goedert, M. (1993) Trends Neurosci. 16, 460–465.
[10] Drewes, G., Lichtenberg-Kraaag, K., Boring, F., Mandelkow, E.-M., Biernat, J., Goris, J., Doree, M. and Mandelkow, E. (1992) EMBO J. 11, 2131–2138.
[11] Ishiguro, K., Takamatsu, M., Tomizawa, K., Omori, A., Takahashi, M., Arioka, M., Uchida, T. and Imahori, K. (1992) J. Biol. Chem. 267, 10897–10901.
[12] Paudel, H.K., Lew, J., Ali, Z. and Wang, J.H. (1993) J. Biol. Chem. 268, 23512–23518.
[13] Yamamoto, H., Saitoh, Y., Yasugawa, S. and Miyamoto, E. (1990) J. Neurochem. 55, 683–690.
[14] Goedert, M., Cohen, E.S., Jakes, R. and Cohen, P. (1992) FEBS Lett. 312, 95–99.
[15] Gong, C.-X., Singh, T.J., Grundke-Iqbal, I. and Iqbal, K. (1994) J. Neurochem. 62, 803–806.
[16] Matsuo, E.S., Shin, R.-W., Billingsley, M., de Voorde, A.V., O'Connor, M., Troyanowski, J.Q. and Lee, V.M.-Y. (1994) Neuron 13, 989–1002.
[17] Mawal-Dewan, M., Henley, J., de Voorde, A.V., Trojanowski, J.Q. and Lee, V.M.-Y. (1994) J. Biol. Chem. 269, 30981–30987.
[18] Wang, J.-Z., Gong, C.-X., Zaidi, T., Grundke-Iqbal, I., Iqbal, K. (1995) J. Biol. Chem. 270, 4854–4860.
[19] Ono, T., Yamamoto, H., Tashima, K., Nakashima, H., Okumura, E., Yamada, K., Hisanaga, S., Kishimoto, T., Miyakawa, T., and Miyamoto, E. (1995) Neurochem. Int. 26, 205–215.
[20] Cohen,P. and Cohen, P.T.W. (1989) J. Biol. Chem. 264, 21435–21438.
[21] Shenolikar, S. (1994) Annu. Rev. Cell Biol. 10, 55–86.

[22] Goedert, M., Spillantini, M.G., Cairns, N.J. and Crowther, R.A. (1992) Neuron 8, 159–168.
[23] Goedert, M., Spillantini, M.G., Potier, M.C., Ulrich, J. and Crowther, R.A. (1989) EMBO J. 8, 393–399.
[24] Kosik, K.S., Orecchio, L.D., Bakalis, S. and Neve, R.L. (1989) Neuron 2, 1389–1397.
[25] Tsukamoto, M., Kato, R., Ishiguro, K., Uchida, T. and Sato, K. (1991) Tetrahed. Lett. 32, 7083–7086.
[26] Hosoi, T., Uchiyama, M., Okumura, E., Saito, T., Ishiguro, K., Uchida, T., Okuyama, A., Kishimoto, T. and Hisanaga, S. (1995) J. Biochem. 117, 741–749.
[27] Kobayashi, S., Ishiguro, K., Omori, A., Takamatsu, M., Arioka, M., Imahori, K. and Uchida, T. (1993) FEBS Lett. 335, 171–175.
[28] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[29] Ishiguro, K., Omori, A., Sato, K., Tomizawa, K., Imahori, K. and Uchida, T. (1991) Neurosci. Lett. 128, 195–198.
[30] Ishiguro, K., Omori, A., Takamatsu, M., Sato, K., Arioka, M., Uchida, T. and Imahori, K. (1992) Neurosci. Lett. 148, 202–206.
[31] Liu, J., Farmer, J.D., Lane, W.S., Friendman, J., Weissman, I. and Schreiber, S.L. (1991) Cell 66, 807–815.
[32] Fleming, L.M. and Johnson, G.V.W. (1995) Biochem. J. 309, 41–47.
[33] Papasozomenos, A.C. and Binder, L.I. (1987) Cell Motil. Cytoskel. 8, 210–226.
[34] Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S. and Mumby, M.C. (1995) J. Cell Biol. 128, 1131–1144.