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Protein kinases involved in the phosphorylation of human tau protein in transfected COS-1 cells

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Abstract

Human tau phosphorylation has been studied in transfected COS-1 cells. Treatment with okadaic acid alters the electrophoretic mobility of human tau protein transiently expressed in transfected cells, due to an increase in the level of phosphorylation. Treatment with okadaic acid also results in an increased phosphorylation of Alzheimer's disease-type phosphoepitopes. Tau phosphorylation within COS-1 cells is partially inhibited by in vivo treatment with DRB, a protein kinase inhibitor. Double treatment of transfected cells with okadaic acid and DRB reveals that phosphorylation of tau protein at the AT8 epitope is achieved by a DRB-resistant protein kinase which is different from that responsible for tau phosphorylation at the SMI-31 epitope, which appears to be sensitive to DRB.

Keywords: Tau protein; Protein kinase; Alzheimer's disease; Phosphorylation; DRB

1. Introduction

Tau protein is a brain microtubule associated protein (MAP) that facilitates tubulin polymerisation in vitro [1,2] and stabilises microtubule assembly in vivo [3–5]. Tau protein is of interest because it has been shown to be the main component of paired helical filaments (PHF), aberrant structures that are present in the brain of Alzheimer's disease (AD) patients [6–15]. In AD, tau protein no longer binds to microtubules [11] in some neurons and it has a higher phosphate content than tau isolated from unaffected controls [6,12,16,17]. Thus, hyperphosphorylation of tau has been correlated with its presence in PHF [18].

Tau phosphorylation has been analysed by using different monoclonal antibodies that recognise either phosphorylated or unphosphorylated forms of the protein. Examples of those antibodies are SMI-31 (recognises serines 396 and 404, in phosphorylated form) [19], AT8 (recognises phosphoserine 202) [20] or tau-1 (recognises serine 202 in unmodified form) [21–23]. Antibodies that recognise phosphoserines react with PHF tau but not control tau. This has therefore been termed Alzheimer-like phosphorylation which could be the consequence of the action of active protein kinases and/or inactive protein phosphatases [24]. Several kinases can modify tau in vitro at the previously indicated serines (for a review see Ref. [25]), but little is known about those acting in vivo.

In order to analyse the in vivo tau phosphorylation, transfections studies have been carried out. Previous studies [26–28] have indicated the suitability of this procedure since there is no interference with endogenous proteins due to the neuronal origin of tau protein, and because tau expressed in these transfected cells is indeed phosphorylated [26,29,30] in an Alzheimer-like state [31,32].

We and others have recently shown that tau protein transiently expressed in COS cells is phosphorylated in some of the epitopes also present in AD-tau [28,31]. In this work we have studied the Alzheimer-like phosphorylation in tau-transfected COS-1 cells looking at the conditions in which tau is phosphorylated, the possible protein kinases involved in that phosphorylation and the functional consequences of that modification.

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2. Materials and methods

2.1. Materials

The protein kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) was purchased from Sigma. The protein phosphatase inhibitor okadaic acid (OkA) was purchased from LC Laboratories (Woburn, MA). Tau monoclonal antibodies 7.51 ([33], a kind gift of Dr. Wischik, MRC, Cambridge, UK); Tau-1 ([34], a kind gift of Dr. Binder, MGC, IL); SMI-31 and SMI-35 (Sternberger Monoclonals, MD) and AT8 (a kind gift of Dr. Dello, Innogenetics, Belgium) were used. Antibodies specific against different protein kinases, including GSK3β (Transduction Laboratories, Lexington, KY), cdk4 (Santa Cruz Biotechnology, CA), casein kinase II, cdc2 and MAP kinase (UBI, Lake Placid, NY), were also used. Alkaline phosphatase was purchased from Boehringer Mannheim and assayed as previously indicated [35]. Synthetic oligonucleotides were from Isogen Bioscience (Amsterdam).

2.2. Cell culture and transfection studies

African green monkey kidney fibroblast (COS-1) cells [36] were grown in Dulbecco's-modified Eagle's medium supplemented with 5% fetal calf serum. Cells were transfected by using lipofectin (Gibco-BRL) essentially as described by Montejo de Garcini et al. [37]. Unless stated, treatment of cells with OkA and DRB was performed in serum-free medium at 1 μM for 2 h and 0.1 mM for 4 h, respectively. OkA and/or DRB were added to the cells at around 60 h post-transfection.

2.3. Protein preparation

Microtubule protein was isolated as described by Shelanski et al. [38]. Taxol-stabilised microtubules were purified by the method of Vallee [39]. Brain tubulin depleted of MAPs was isolated from bovine brain by phosphocellulose chromatography [1]. Cytoplasmic extracts of transfected cells were obtained by several freezing-thawing cycles in buffer A (100 mM Pipes, pH 6.5; 0.5 mM $MgCl_2$; 2 mM EGTA) containing protease inhibitors (1 mM PMSF; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 10 μg/ml pepstatin). After incubation in a boiling water bath for 5 min, the heat-stable supernatant was stored frozen until use.

2.4. Western blot analysis

Protein characterisation by gel electrophoresis and Western blot [40] was carried out using tau-specific monoclonal antibodies (see above) and goat anti-mouse peroxidase conjugated antibody (Dako). The peroxidase reaction was developed using 4-chloro-1-naftol and hydrogen peroxide as substrates.

2.5. Assay for the interaction of tau protein with microtubules

To calculate the dissociation constant (K_d) for the interaction of tau with microtubules, constant amounts of tau protein from transfected COS-1 cells were mixed with increasing amounts of taxol-polymerised microtubules following the method described by Butner and Kirschner [41]. K_d corresponds to the concentration of tubulin at which 50% of tau protein is associated with the assembled polymers.

2.6. Expression and purification of recombinant tau from E. coli

Human tau cDNA clone htau37 was kindly supplied by M. Goedert (MRC, Cambridge, UK). Recombinant tau proteins were expressed in *E. coli* and purified as described elsewhere [28].

2.7. Construction of expression plasmids

Construction of plasmids pSGT30, pSGT40, pSGT32, pSGT42, pSGT3RC and pSGT4RC for transient expression in COS-1 cells has been already described [28,37]. To obtain plasmids pRKT32N and pRKT3RC for inducible expression in *E. coli*, mutagenesis was carried out using the polymerase chain reaction (PCR). Oligonucleotides A5 (GCGGATCCATATGCCAGAC CTG) and A6 (GCGGATCCATATGGCTGAGCCC), which include the initiation codon as well as *Bam* HI and *Nde* I sites for a proper cloning into the vector, were used as 5′ primers. Oligonucleotides A7 (GCGAATTCTTACTCGCGGAAGG) and A8 (GCGAATTCTCACAAACCCTGCTTGG), which include a stop codon and an *Eco* RI site downstream, were used as 3′ primers. The fragment generated by PCR amplification on pSGT32N using primers A6 and A7 (1058 bp) was digested with *Nde* I and *Eco* RI and ligated into *Nde* I- and *Eco* RI-digested pRK172 [42], thus obtaining pRKT32N. In a similar way, the fragment generated by PCR amplifications on pSGT3RC or pSGT4RC using primers A5 and A8 (503 bp) were digested with NdeI and EcoRI and ligated into pRK172 also digested with NdeI and EcoRI to obtain pRKT3RC or pRKT4RC, respectively. All plasmids were analysed by restriction analysis to test for proper orientation and correct size of the inserts. Finally, when cDNAs obtained by PCR amplification were tested by sequencing, no changes regarding the template sequences were observed. An schematic diagram of all cDNA constructs used throughout this study is shown in Fig. 1.

2.8. Chromatography

Fractionation of tau protein kinase activities present in COS-1 cells was achieved by fast performance liquid

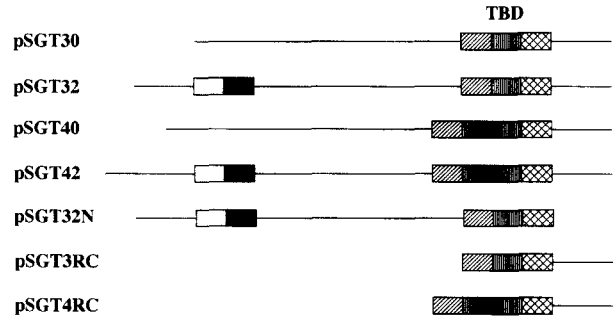

Fig. 1. Human tau cDNA constructs used throughout this study. Their construction and expression of these constructs are described in Section 2. Extra exons at the amino region and the tubulin binding domain (TBD) are shaded.

chromatography (FPLC) following basically the method described by Ahn et al. [43]. COS-1 cells were homogenised in a buffer containing 50 mM glycerophosphate, pH 7.3; 1 mM EGTA; 0.1 mM Na_3VO_4; 1 mM DTT; 1 μg/ml leupeptin and 10 μg/ml aprotinin. COS-1 cell homogenate was fractionated through a Mono Q HR5/5 column for anion exchange by using the FPLC system (Pharmacia-LKB Biotechnology). After loading the sample and a brief wash, protein was eluted with a 0–0.4 M NaCl gradient in the same buffer at 0.5 ml/min. 1-ml fractions were collected, the optical density at 280 nm was determined and fractions were used to test for protein kinase activity using bacterially expressed tau protein as substrate.

2.9. Protein kinase assays

30 μl from the fractions eluted from the FPLC column were assayed for protein kinase activity on tau protein in a buffer containing 20 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 1 μM okadaic acid; 1 μg/ml leupeptin; 10 μg/ml aprotinin; 2 μg tau protein from *E. coli*; 50 μM ATP and 10 μCi [γ-^{32}P]ATP. Reactions were performed at 37°C for 2 h and stopped by adding electrophoresis loading buffer and boiling for 3 min. Phosphorylation was analysed by autoradiography after running the phosphorylated samples on a 10% acrylamide gel electrophoresis. Those fractions containing protein kinase activities able to phosphorylate tau protein were then assayed in the same buffer, with increased cold ATP at 1 mM and incubated for 6 h. Phosphorylation of tau protein was followed by Western blot analysis using monoclonal antibodies specifically recognising phosphorylated epitopes. For in vitro inhibition experiments, DRB was used at 50 μM.

3. Results

3.1. Tau phosphorylation in transfected COS-1 cells

3.1.1. Treatment with okadaic acid alters the electrophoretic mobility of tau protein

Expression of the different human tau isoforms in COS-1 cells results in the appearance of more than one electrophoretic species [28]. Since it has been suggested that the presence of more than one isoform could be the consequence of phosphorylation events [28–30], transfected cells were incubated with okadaic acid (OkA) to further analyse that modification.

Okadaic acid, a polyether fatty acid isolated from a marine sponge, is a very potent specific inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (reviewed in Ref. [44]). As OkA is hydrophobic, it can enter cells, and is therefore of use for studying tau phosphorylation in different systems such as human brain slices [45], LA-N-5 [46] and SKNSH-SY 5Y [47] neuroblastoma cells and neuronal primary cultures [48].

COS-1 cells were transfected with plasmids pSGT32 or pSGT3RC (see Fig. 1) and after treatment with the phosphatase inhibitor a mobility shift was observed for T32, appearing as a major band with a lower electrophoretic mobility. In the case of T3RC, all tau protein appears to migrate as a band with slower electrophoretic mobility (data not shown). Further in vitro treatment with alkaline phosphatase of those tau isoforms expressed in COS-1 cells resulted in an increase in electrophoretic mobility (not shown), strongly suggesting that the electrophoretic mobility shift induced by okadaic acid is indeed due to an increase in the level of tau phosphorylation.

3.1.2. Okadaic acid induces an increase in the AD-type phosphorylation

To test if the observed increase in the level of tau phosphorylation occurs in those sites reported for the Alzheimer-like state, tau isolated from transfected COS-1

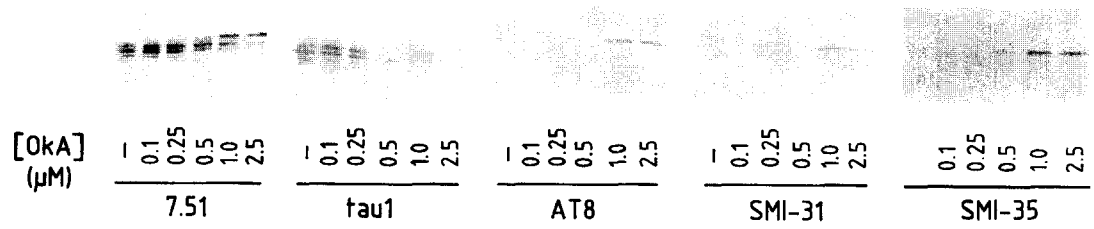

Fig. 2. Appearance of AD-type phosphorylated epitopes after OkA treatment. COS-1 cells were transfected with pSGT32 and treated with increasing amounts of okadaic acid. Cell extracts were run on a 10–20% acrylamide electrophoresis gel, electroblotted and developed with monoclonal antibodies 7.51, tau-1, AT8, SMI-31 and SMI-35.

cells and treated with OkA was tested for their reaction with tau-specific monoclonal antibodies tau-1, AT8, SMI-31 and SMI-35. Higher concentrations of OkA may be needed in the medium of cultured cells because the intracellular concentrations of PP1 and PP2A often lie in the range of 0.1–1.0 μM [44,49]. For this reason we wanted to study the effect of increasing amounts of OkA on tau phosphorylation in transfected COS-1 cells.

Fig. 2 indicates that OkA induces an increase in the slow-electrophoretic migrating form, detected with an antibody, 7.51, which recognises tau protein [33] independently of its phosphorylation state. In addition, treatment of cells with increasing concentrations of OkA, results in a decrease in the interaction of tau protein with the antibody tau-1 (which recognises an epitope containing serine 202 in a dephosphorylated form), whereas the reaction with antibody AT8 (which recognises serine 202 when is modified) or with antibody SMI-31 (which recognises an epitope including phosphoserines 396 and 404) increase in those conditions.

As expected, appearance of AD-type phosphorylated epitopes is dose-dependent, with AT8 and SMI-31 reactions appearing around 0.5 μM OkA. Reaction with another monoclonal, SMI-35 (which recognises undefined phosphorylated epitopes on tau protein), was also observed to increase upon OkA treatment of the transfected cells (data not shown). Also as expected, reaction with these antibodies was abolished by in vitro treatment with alkaline phosphatase, with the exception of tau-1, which was increased.

3.1.3. *Tau phosphorylation is partially inhibited by treatment of cells with DRB*

As a complement to the previous experiments using OkA, we interfered with the phosphorylation process by inhibiting protein kinases involved in the observed tau phosphorylation. A ribofuranosyl-benzimidazole derivative was used as a possible protein kinase inhibitor. 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB), which has the ability to pass through the cell membrane [52], is a powerful inhibitor of casein kinase-1 (CK1) and -2 (CK2) [50,51].

Fig. 3 shows the results obtained for COS-1 cells transfected with different human tau isoforms and fragments and then treated with DRB. Treatment with DRB induces a change in the relative intensity of the two bands corresponding to tau protein, increasing the amount of tau protein with a higher mobility, both in the whole tau isoforms and the N-terminal truncated tau fragments. An increase in the amount of DRB (up to 1 mM) or in the time of treatment (up to 8 h) does not further affect tau mobility. This result strongly suggests that DRB is only partially able to inhibit tau phosphorylation within COS-1 cells, as determined by the induced electrophoretic mobility shift.

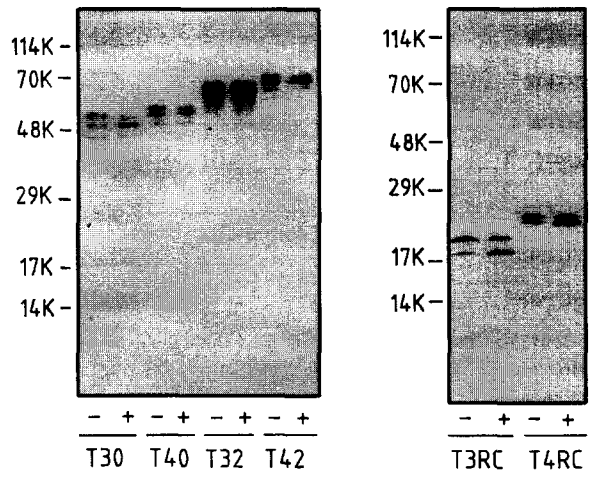

Fig. 3. Treatment of transfected COS-1 cells with DRB. COS-1 cells were transfected with either pSGT30, pSGT40, pSGT32, pSGT42, pSGT3RC or pSGT4RC and then treated (+) or untreated (−) with DRB as indicated in Section 2. Cell extracts were run on a 10–20% acrylamide electrophoresis gel, electroblotted and developed with monoclonal antibody 7.51.

3.1.4. *Double treatment of cells with okadaic acid and DRB*

The phosphorylation state of a protein within the cell is the result of a balance between the protein kinase and protein phosphatase activities acting on it. As shown above, DRB can partially inhibit tau phosphorylation within transfected COS-1 cells. In order to know how DRB affects the AD-type tau phosphorylation, double treatment of transfected cells with OkA and DRB was performed and tau phosphorylation was studied using AT8 and SMI-31 antibodies.

The reactivity of tau protein expressed in COS-1 cells with monoclonal antibodies AT8, SMI-31 and SMI-35 in the absence of OkA treatment is very weak, if any, as previously reported [28,31]. The observed reactivity after OkA treatment of transfected cells can be reversed by the presence of DRB, particularly in the case of SMI-31 antibody, when reactivity almost disappears (data not shown). This difference in sensitivity to DRB treatment result seems to suggest that the protein kinase activity responsible for phosphorylation at both sites is different.

3.2. *Interaction of phosphorylated tau with microtubules*

To analyse the effect of OkA-induced tau hyperphosphorylation on its ability to bind microtubules, an assay was carried out to measure the interaction of tau with taxol-stabilised microtubules. The interaction of COS-1 expressed tau, after treatment with OkA, was measured by taking a constant amount of protein from COS-1 cell extracts transfected with tau (T32) and mixed with increasing amounts of taxol-stabilised microtubules from bovine brain tubulin, following the procedure of Butner and

Kirschner [41]. We took into account that the microtubule concentration, in which half of the tau protein was associated to the assembled polymer, corresponds to the dissociation constant (K_d) for the interaction of tau with microtubules.

A K_d of 0.7 ± 0.2 μM was determined for tau protein obtained from COS-1 cells in the absence of OkA, whereas a K_d of 0.9 ± 0.3 μM was found for tau from OkA-treated COS-1 cells. This result indicates that, in the conditions in which serines 202, 396 and 404 are modified, no great differences in the interaction with microtubules, with respect to tau with unmodified serines, can be observed

3.3. Tau protein kinase activities in COS-1 cells

3.3.1. FPLC fractionation of tau protein kinase activities from COS-1 cells

In order to identify the protein kinases present in COS-1 cells which are able to phosphorylate tau protein, the procedure of Ahn et al. [43] was followed. A COS-1 cell homogenate was loaded on a mono Q fast protein liquid chromatography (FPLC) column, eluted with a gradient of NaCl and the resulting fractions were tested for protein kinase activity using recombinant tau protein as substrate. Fig. 4A shows the fractionation profile determined by optical density at 280 nm.

The different fractions obtained by FPLC were assayed for protein kinase activity in the presence of recombinant tau protein or its absence (as a control measuring endogenous phosphorylation of the column fractions). Fig. 4B shows those fractions in which a protein kinase activity able to phosphorylate tau protein. Since tau protein cannot be quantitatively precipitated by the addition of cold trichloroacetic acid, phosphorylation level was determined by gel electrophoresis followed by counting radioactivity associated with tau protein bands.

3.3.2. Identification of protein kinase activities from COS-1 cells

To identify which protein kinase activity is present in each FPLC column fraction, a dot blot analysis using antibodies specifically recognising different protein kinases was carried out, including cdc2, cdk4, MAP kinase (ERK1 and ERK2), GSK 3 (β isoform) and CK2. As shown in Fig. 4C, GSK3β is present in the fraction not retained in the column (F, flowthrough), whereas cdc2 appears in fraction 12, MAP kinase seems to be present mainly in fraction 16 and and CK2 is present in fraction 29 (not shown in the figure). Less clear is the position of cdk4 because the reaction was very weak.

The presence of these protein kinases in the indicated fractions was confirmed by running a sample of each column fraction on a electrophoresis gel and performing a Western blot analysis with the specific antibodies as shown in Fig. 5A. It must be noted that the different protein kinases-enriched fractions after chromatography are obviously not homogenous and the presence of other protein kinases for which we have not tested cannot be ruled out.

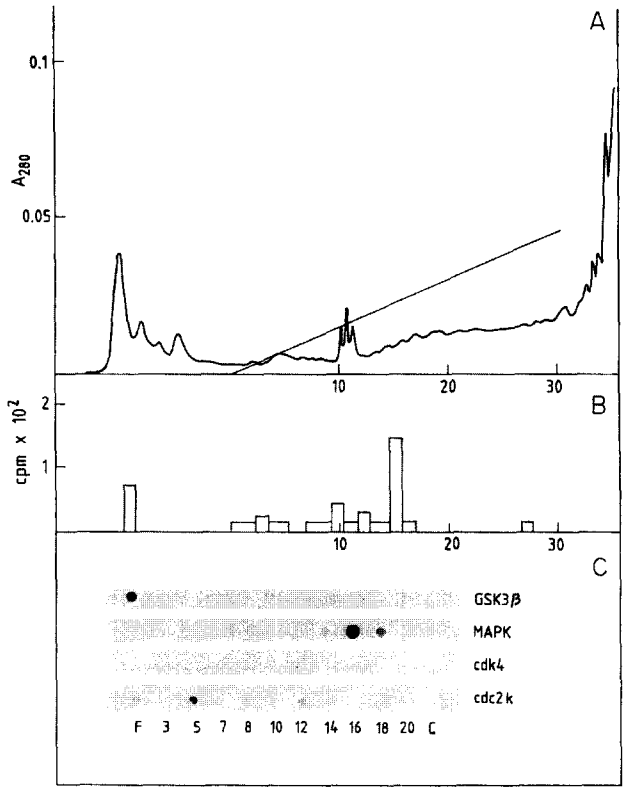

Fig. 4. FPLC fractionation of tau protein kinase activities from COS-1 cells. A COS-1 cell homogenate was loaded on a mono-Q FPLC column and eluted with a NaCl gradient as described in Section 2. A. Fractionation profile determined by optical density at 280 nm. B. In vitro tau phosphorylation by protein kinase activities present in mono-Q column fractions. C. Dot blot analysis of column fractions using specific antibodies against different protein kinases.

A further analysis was carried out to test whether the modification by any of these protein kinases would in turn alter the electrophoretic mobility of tau protein. As shown in Fig. 5B, an electrophoretic mobility shift is more evident when tau protein is modified by the protein kinase activity present in fraction F than the one present in fraction 16. Since a possibility still remains that GSK3β may not be the only protein kinase activity present in fraction F, a further purification step was followed by performing an heparin-sepharose chromatography [53]. GSK3 (β isoform) purified after this step was also shown to modify tau protein in such a way that its electrophoretic mobility decreased (Moreno et al., unpubl. results).

In vitro tau phosphorylation experiments in the presence of this inhibitor were carried out in order to identify the protein kinase activities from COS-1 cells which are inhibited by in vivo DRB treatment. Our preliminary results indicate that the protein kinase activities present in both MAP kinase- and GSK 3-enriched fractions are, at least partially inhibited by DRB when assayed in vitro on recombinant tau protein (data not shown).

3.3.3. In vitro phosphorylation of AD-type epitopes by protein kinase activities from COS-1 cells

We were also interested in whether some of these protein kinase activities were able to modify tau protein at the epitopes recognised by monoclonal antibodies AT8 and SMI-31. As shown in Fig. 5C and D, fraction 16 (MAP kinase-enriched fraction)-kinase modifies those serines present in the epitopes recognised by both antibodies, whereas fraction F (GSK 3β-enriched fraction)-kinase mainly modifies the region recognised by antibody SMI-31.

To complement the previous analyses, we tested for the ability of the protein kinase activities present in the different mono-Q column fractions to modify the amino-terminal region, the carboxy-terminal region of tau protein, or both. To do this, tau fragments T32N and T3RC expressed in *E. coli* were used as substrates. We have used either fractions with the maximum activity or the adjacent ones. In the case of Fig. 6 we have used fraction 14, which according to Fig. 4C have both cdc2 and MAP kinase activities, but not cdk4, which can be weakly detected on fraction 12. We use then fraction 10 trying to use a more cdc2 kinase-enriched fraction, with no presence of the previous two activities.

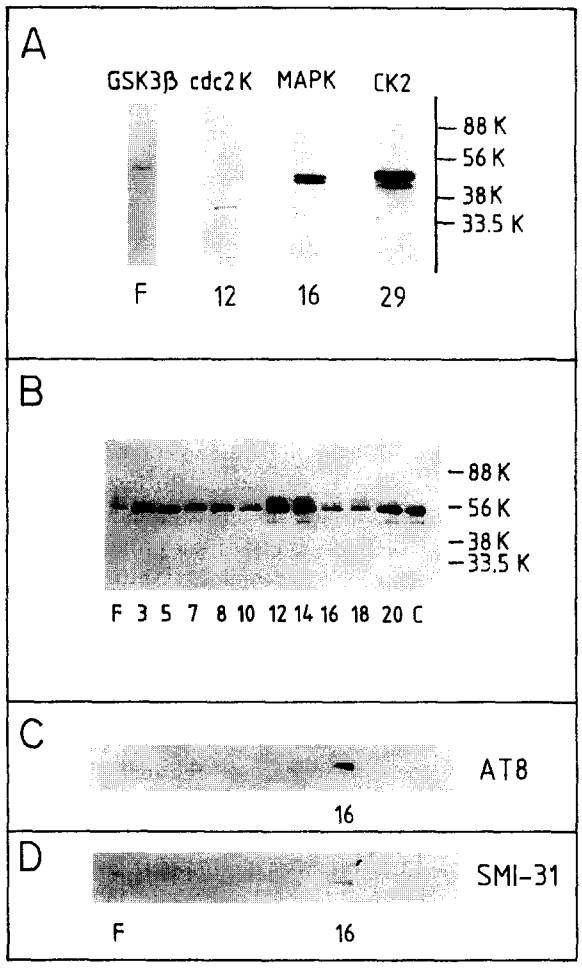

Fig. 5. Identification of protein kinase activities from COS-1 cells. A. Western blot of column fractions F, 12, 16 and 29 using specific antibodies against different protein kinases. B, C and D. Western blots of the in vitro tau phosphorylation by protein kinase activities present in the mono-Q column fractions using tau-specific monoclonal antibodies 7.51 (B), AT8 (C) or SMI-31 (D).

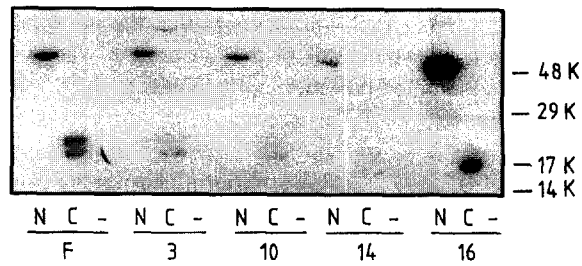

Fig. 6. In vitro tau phosphorylation by protein kinase activities from COS-1 cells. Column fractions F, 3, 10, 14 and 16 were used to phosphorylate in vitro tau fragments T32N (N) and T3RC (C) in the presence of [^{32}P]ATP. A negative control (−) without adding tau protein is also shown. After reaction, samples were run on a electrophoresis gel and exposed to X-ray film. The autoradiograph is shown.

As shown in Fig. 6, all fractions tested were able to modify both tau fragments, with a significant mobility shift observed for T3RC, specially in the case of fraction F. These data also show that both bands of the doublet are phosphorylated, suggesting that phosphorylation at certain residues is required for inducing a mobility shift, whereas phosphorylation at different residues than those does induce it.

4. Discussion

The phosphorylation state of a protein within a cell depends on the relative activities of protein kinases and protein phosphatases acting on it. We have studied tau phosphorylation within transfected COS-1 cells by in vivo interference with these phosphorylation/dephosphorylation processes. Tau phosphorylation status can be followed by changes in the electrophoretic mobility of the protein and also by using monoclonal antibodies specifically recognising phospho-epitopes.

Tau phosphorylation has been studied in a number of different mammalian cell lines, including CHO [32], 3T3 [30] and COS [28,31] cells. Transfected tau in CHO and 3T3 cells displays some AD-type phosphoepitopes, as indicated by its reactivity with some monoclonal antibodies such as AT8 and SMI-31. However, when expressed in COS cells, a very weak reactivity with these antibodies can be detected [28,31].

We have presented here evidence that, after treatment of transfected cells with OkA, tau protein expressed in COS-1 cells acquires some of the features of tau from PHF, as determined by its slower electrophoretic mobility, hyperphosphorylation and the presence of AD-type phosphorylated epitopes.

The observed differences in the phosphorylation state of tau protein between the different mammalian cells lines are

likely to be due to different tau protein kinase/phosphatase activity ratios in those cell lines. An explanation could be that the apparent higher turnover rate of phosphate observed in COS cells (as suggested by the low reactivity with antibodies AT8 and SMI-31 in the absence of OkA treatment) may be due to a higher phosphatase activity in these cells.

However, as mentioned, tau protein from transfected COS-1 cells appears to be in both unphosphorylated or phosphorylated forms, both states being differentiated by changes in the electrophoretic mobility of the protein. The constitutive presence of phosphoisoforms of tau could be due to the absence of a phosphatase activity, such phosphatase activity could be PP2B, that should have a very low level in COS-1 cells. However, preliminary experiments suggest that the constitutive tau phosphoisoforms cannot be dephosphorylated in vitro by that phosphatase.

Another possibility could be that the appearance of phosphorylated tau is the result of a low level of PP2A activity that could actively work on phosphorylated tau [54,55]. This phosphatase could be downregulated in transfected COS-1 cells, since the enzymatic activity is cell cycle regulated [56] and transfected cells are out of cell cycle.

OkA is a potent inhibitor of both PP1 and PP2A. Under normal in vitro assay conditions, PP2A is completely inhibited by 1 nM OkA, while PP1 is unaffected at this concentration, its IC_{50} being 10–15 nM [40]. However, the use of this inhibitor to discriminate between the effects of two target enzymes is not as powerful in living cells as it is in cell extracts. Inhibition constants in cells may be influenced by a number of factors, including intracellular concentrations of either competing target enzymes or effectors that may compete at the inhibitor binding site, cell permeability, half-life of compounds, etc. Our results show that we cannot distinguish between PP1 vs. PP2A effects in living cells by using OkA. However, based on previous in vitro experiments, a possible effect of PP1 on the dephosphorylation of certain residues of tau protein, such as those recognised by SMI-31 antibody, could be excluded [55,60].

In order to characterise the protein kinase activities involved in the modification of tau protein in COS-1 cells, we tried to interfere with the phosphorylation process by using DRB as a protein kinase inhibitor. Our results strongly suggest that treatment of transfected cells with DRB partially inhibits tau phosphorylation. This inhibitor has been described as specific for CK1 and CK2 [51,52] and also for an undefined protein kinase activity from HeLa cells which is able to modify the synthetic peptide $K(YSPTSPS)_4$ [57]. Sequence of this peptide seems to be suitable for modification by proline-directed protein kinases. We have tried to identify the different protein kinase activities present in COS-1 cells upon fractionation by FPLC and to assay their ability to phosphorylate tau in vitro.

We have been able to identify MAP kinase-, GSK 3, cdc 2-, cdk4- and CK2-enriched fractions from a COS-1 cell extract. As expected, protein kinase activities present in these fractions were able to phosphorylate tau protein in vitro both at the N- and C-terminal regions of the protein. However, we cannot rule out the involvement of other protein kinases for which we have not tested.

Any of the previous protein kinases could be responsible for the in vivo modification of tau in COS-1 cells. Although co-transfection experiments using tau and GSK 3 or MAP kinase cDNAs have demonstrated that tau protein is a substrate for GSK 3α and β (but not for MAP kinase) in living cells [31,61], the nature of the protein kinase activities phosphorylating tau protein in vivo still remains to be elucidated.

Our results also indicate that both MAP kinase- and GSK 3-enriched fractions are at least partially sensitive to in vitro DRB treatment, thus suggesting that these kinases may be involved in tau phosphorylation within COS-1 cells. Interestingly, MAP kinase in non-neural cells is believed to down regulate GSK 3 through phosphorylation [58,59]. Thus, depending on the metabolic state of the cell, one or other protein kinase may be active.

MAP kinase activity in non-neural cells is higher in proliferating than non-proliferating cells [59], so that in transfected cells it should have a decreased activity. If this is the case, then probably GSK 3 is the protein kinase acting mainly on tau in transfected cells. This possibility is supported by the fact that many of the features of tau-kinase in COS-1 cells are shown by GSK 3, such as electrophoretic mobility shift or the modification of the C-terminal residues (at the SMI-31 epitope) [28]. In addition to this, we have recently shown that GSK 3 β may phosphorylate tau protein in vitro at the tubulin binding domain, in residues which are important for binding to microtubules and also phosphorylated in PHF-tau [62]. Thus, GSK 3 should be considered as a possible candidate for AD-type tau phosphorylation in COS-1 cells.

On the other hand, although both MAP kinase and GSK3 can modify tau in an Alzheimer-like state, phosphorylated and unmodified tau show similar capacities to bind microtubules. The probable action of the previous kinases could be complemented by other(s) kinase(s) in order to have features of Alzheimer-like phosphorylation.

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