Phosphorylation of tau protein by recombinant GSK-3β: pronounced phosphorylation at select Ser/Thr-Pro motifs but no phosphorylation at Ser262 in the repeat domain

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Abstract Glycogen synthase kinase-3β (GSK-3β) has been described as a proline-directed kinase which phosphorylates tau protein at several sites that are elevated in Alzheimer paired helical filaments. However, it has been claimed that GSK-3β can also phosphorylate the non-proline-directed KXGS motifs in the presence of heparin, including Ser262 in the repeat domain of tau, which could induce the detachment of tau from microtubules. We have analyzed the activity of recombinant GSK-3β and of GSK-3β preparations purified from tissue, using two-dimensional phosphopeptide mapping, immunoblotting with phosphorylation-sensitive antibodies, and phosphopeptide sequencing. The most prominent phosphorylation sites on tau are Ser396 and Ser404 (PHF-1 epitope), Ser46 and Thr50 in the first insert, followed by a less efficient phosphorylation of other Alzheimer phosphoepitopes (antibodies AT-8, AT-270, etc). We also show that the non-proline-directed activity at KXGS motifs is not due to GSK-3β itself, but to kinase contaminations in common GSK-3β preparations from tissues which are activated upon addition of heparin.

Key words: Glycogen synthase kinase; Alzheimer's disease; Tau protein phosphorylation

1. Introduction

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase, originally named after its ability to phosphorylate and inactivate glycogen synthase in the pathway of insulin-dependent glycogen synthesis. It is now known to be involved in a variety of signalling pathways affecting cell fate determination, oncogenesis, and metabolism (for reviews, see [35,53]), notably the wingless signalling pathway in which GSK-3β phosphorylates β-catenin, causing its ubiquitination and degradation. The kinase exists as two main isoforms, GSK-3α, the dominant form in most tissues, and GSK-3β, which is enriched in brain. Both forms occur in the cytoplasm and nucleus, but partial (10%) mitochondrial localization seems to be restricted to GSK-3β [20]. The phosphorylation of glycogen synthase by GSK-3β represents a well-known paradigm of sequential phosphorylation where a priming phosphorylation by CK-II is followed by four further phosphorylation events by GSK-3β in the direction towards the N-terminus, each separated by three intervening residues (motif $S_{P2}XXXS_{P1}$ [36]). This principle of a priming phosphorylation is also realized in other GSK-3 substrates, e.g. phosphatase-1 G-subunit, inhibitor-2 of PP-1, transcription factor CREB and eIF2B [53]. In addition, GSK-3 phosphorylates proline-directed motifs in a variety of proteins without requiring a priming phosphorylation, e.g. transcription factors c-Jun, c-myc, L-myc, and the microtubule-associated protein tau [9,18,28,55]. Because of this preference, GSK-3β is often described as a proline-directed kinase, although there are exceptions to the rule.

GSK-3β has recently received attention in Alzheimer's disease (AD) research for several reasons: the Wnt signalling pathway is implicated in interaction and processing of presenilin 1 (PS1) whose missense mutations are linked to familiar AD. PS1 interacts with β-catenin, GSK-3β, and tau protein, and these interactions are modified in the familial AD mutations of PS1 [48,58,60]. Aβ-induced neurotoxicity is thought to be mediated by GSK-3β [47]. GSK-3β can phosphorylate tau protein (the subunit of Alzheimer neurofibrillary tangles) in a fashion reminiscent of AD tau [18,28]. The epitope of antibody AT-100 that is highly specific for Alzheimer tau is generated by the sequential action of GSK-3β and PKA [59]. Finally, the phosphorylation of mitochondrial pyruvate dehydrogenase by GSK-3β could contribute to the downregulation of acetylcholine synthesis in AD [20].

The activity of GSK-3β is regulated antagonistically by serine and tyrosine phosphorylation. Phosphorylation of Tyr216 in the regulatory loop of GSK-3β is essential for activity; in cells, this tyrosine is constitutively phosphorylated, probably by autophosphorylation, and thus GSK-3β can be regarded as a dual-specificity kinase [32,52,57]. GSK-3β can be inhibited by phosphorylation of Ser9 [45]. The regulatory inhibition can be achieved by different upstream kinases and reversed by PP-2a. It has been observed in three signal transduction pathways: EGF signalling through the MAP kinase pathway, insulin signalling through the PI3 kinase pathway and wingless signalling mediated by a PKC-like protein kinase [10,53].

In the case of tau protein, the enhanced phosphorylation at Ser-Pro or Thr-Pro is a striking feature in AD brains. A number of phosphorylation-dependent antibodies against AD-tau exist whose epitopes require a single or two closely spaced Ser/Thr-Pro motifs; examples are PHF-1, AT-8, AT-270, AT-180, SMI-31, TP-3 [15] (Fig. 1). These antibodies are used to diagnose the stage of AD and other tauopathies, and the direct analysis of phosphorylation sites in tau from AD brains indeed shows that many sites are in Ser/Thr-Pro motifs [31]. This has directed attention to the kinases responsible for this type of phosphorylation, i.e. the proline-directed kinases. They include enzymes such as MAP kinase and its relatives

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; GSK-3, glycogen synthase kinase-3; NFT, neurofibrillary tangle; PHF, paired helical filament; PS1, presenilin 1

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(ERK, JNK, p38 stress-activated kinase [11,16]), cell cycle kinases such as cdc2 or its neuronal relative cdk5 [1,33,50], or GSK-3β [18,19,23,28,30,38,42,56]. The activity of such kinases towards tau was studied in various cell models after transfection of tau and/or the kinases and monitoring phosphorylation with the diagnostic antibodies. These experiments showed that GSK-3β is particularly interesting since it phosphorylates tau well in a cellular context [26,44,8].

The functional analysis of tau phosphorylation is still a matter of debate. A common assumption holds that phosphorylation of tau decreases its affinity for microtubules and thereby makes microtubules more dynamic, up to the point of their breakdown in Alzheimer's degenerating neurons. However, in this regard the effect of phosphorylated Ser/Thr-Pro motifs is relatively weak [2,49]. It is greatly surpassed by phosphorylation at other non-Pro-directed motifs, such as Ser262 located in the first KXGS motifs of the repeat domain [2,12], or Ser214 in the flanking domain upstream of the repeats [6,22]. These sites are phosphorylated by non-proline-directed kinases such as PKA (mostly at Ser214) and MARK (Ser262 and other KXGS motifs) [12,25,37,54,59]. Cell models with inducible phosphorylation at Ser262 by MARK can lead to the breakdown of the microtubule network and cell degeneration [13]. This is reminiscent of the situation in degenerating neurons in AD where microtubules are disrupted, tau shows elevated phosphorylation and is no longer competent to bind to microtubules [5,31]. In the light of the strong effect of phospho-Ser262 on the tau-microtubule interaction it was interesting that GSK-3β was claimed to be competent to phosphorylate this site, even though it is not part of a Ser-Pro motif [29,42]. Conceptually this could provide a basis for the inhibitory effects of GSK-3β on the microtubule cytoskeleton; on the other hand, a direct role of Ser/Thr-Pro motifs was deduced from mutagenesis experiments [19,51], although this, too, remains a matter of debate [54].

In order to resolve the discrepancies in the literature we have investigated the phosphorylation of tau by several types of GSK-3β preparation, recombinant GSK-3β expressed in *Escherichia coli*, commercial GSK-3β purified from rabbit muscle, both in the absence and in the presence of heparin which acts as a kinase enhancer. The results show that recombinant GSK-3β phosphorylates the regions flanking the repeats and the N-terminal domain on tau, it has a strong preference for proline-directed motifs and does not phosphorylate Ser262, with or without heparin. However, GSK-3β preparations from rabbit muscle tissue phosphorylate proline- and non-proline-directed motifs, including Ser262, especially in the presence of heparin. This is explained by impurities in the tissue-derived kinase which copurify through several steps of purification (such as MARK or PKA). The proline-directed activity of tissue-derived GSK-3β can be blocked by LiCl which is a highly specific inhibitor of GSK-3β [46], but LiCl does not affect the activity of the kinase impurities, proving that the two kinds of activities arise from different enzymes.

2. Materials and methods

2.1. Proteins

Recombinant human tau proteins were expressed in *E. coli* using variants of the pET vector and purified by making use of its heat stability and by Mono S fast protein liquid chromatography [2]. GSK-3β was expressed in active form in *E. coli* following [52]; the original human cDNA clone was generously provided by J.R. Woodgett [45]. GSK-3β purified from rabbit muscle was purchased from UBI (Upstate Biotechnology, New York, USA).

2.2. Phosphorylation reactions

Phosphorylation reactions were carried out in 40 mM HEPES (pH 7.2) containing 10 μM tau protein, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM EGTA, 0.2 mM PMSF and 1 mM [γ-^{32}P]ATP (100–200 Ci/mol) in the presence or absence of 50 μg/ml heparin. For inhibition of GSK-3β 50 mM LiCl was added. Phosphorylation was assayed in SDS gels [2] or on phosphocellulose paper discs (Life Technologies, Inc.). The specific activity of rGSK-3β was measured against phospho-CREB peptide at 37°C [52]. Autophosphorylation of GSK-3β was done for 30 min at 37°C in phosphorylation buffer with 1 μM [γ-^{32}P]ATP (2 Ci/μmol).

2.3. Monoclonal antibodies

Antibodies AT8, AT180 and AT270 were obtained from Innogenetics (Ghent, Belgium); monoclonal antibody 12E8 was from Athena Neurosciences (South San Francisco, CA, USA [39]); monoclonal antibody PHF-1 was a gift from P. Davies (Albert Einstein College, Bronx, NY, USA).

2.4. Immunoblots

For immunoblot analysis, the proteins were transferred to PVDF membranes (Millipore, Eschborn, Germany). Residual membrane binding sites were blocked with 5% non-fat dry milk in Tris-buffered saline following incubation with primary antibodies. The antibodies

Table 1
Phosphopeptides and phosphorylation sites of tau protein induced by phosphorylation with GSK-3β

Spot	Remark	Sequence			Mass [D]		Ser	Thr
					calc.	obs.	-P	-P
S46/T50	a,d	24	DQGGYT...	67	4749	4730	+	+
S46/T50	a,d	25	KDQGGYT...	67	4877	4864	+	+
T50	b	45	ESPLQTPTEDGSEEPGSETSDAK	67				+
T69	b	68	STPTAEDVTAPLVDEGAPGK	87				+
T153	c	151	IATPR	155				+
T181	b	175	TPPSSGEP...	190				+
S199	b	195	SGYSSPGSPGTPGSR	209			+	
S202/T205	d,e	195	SGYSSPGSP...	209			+	+
T212	b	210	SRTPSLPTPPTR	221	1390	1388		+
T212/T217	b	210	SRTPSLPTPPTR	221	1470	1470		+
S235	b	231	TPPKSPSSAK	240			+	
S396/S404	a,d	386	TDHGAE...	406	2375	2377	+	
S404	c,f	396	SPVVSGDTSPR	406	1182	1184	+	
T403/S404	b	396	SPVVGSDGTSPR	406			+	+

Remarks: (a) Doubly phosphorylated peptides as determined by MALDI. (b) Site was determined for MAPK [22]; another Ser or Thr might be phosphorylated. (c) Sequence and phosphorylated residue were completely determined. (d) The first amino acids were determined as indicated. (e) Partly phosphorylated at S198 and S199. (f) Containing a small fraction of S400.

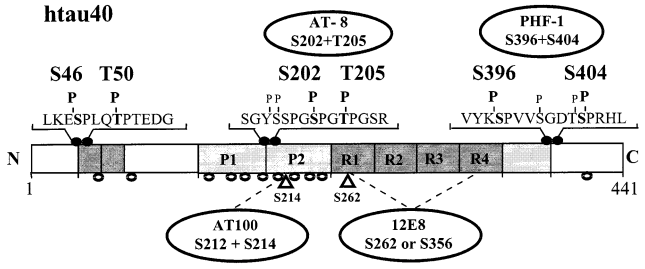

Fig. 1. Diagram of major phosphorylation sites and epitopes of antibodies on tau protein. The bar shows htau40, the longest tau isoform in the human CNS (441 residues). It contains four repeats in the C-terminal half which represent the core of the microtubule binding domain, flanked by proline-rich basic regions. The two inserts near the N-terminus and repeat R2 may be absent in other isoforms due to alternative splicing. Construct K18 contains only the four repeats and no Ser/Thr-Pro motifs, K12 has three repeats and the downstream flanking region (see Fig. 3). Below the bar all 17 Ser/Thr-Pro motifs are indicated (open circles), as well as Ser214 and Ser 262 (open triangles) which have a strong influence on tau-microtubule binding. The sites phosphorylated by GSK-3β are shown above the bar (most prominent sites in bold, Ser396, Ser404, Ser46, Ser50, Ser202, Thr205). Epitopes of phosphorylation-sensing antibodies are indicated (AT-8, PHF-1, 12E8, AT-100).

were detected with a peroxidase-conjugated antibody and visualized using enhanced chemoluminescence (ECL) according to manufacturer's instructions (Amersham-Buchler, Braunschweig, Germany).

2.5. *Phosphopeptide mapping by thin layer electrophoresis/chromatography*

Following in vitro phosphorylation, kinase proteins were removed by boiling of the samples in 0.5 M NaCl, 5 mM dithiothreitol and centrifugation. Tau remains in the supernatant and was precipitated by 15% trichloroacetic acid on ice. Cysteine residues were modified by performic acid. The protein was digested overnight with trypsin (Promega, sequencing grade) in the presence of 0.1 mM $CaCl_2$, using two additions of the enzyme in a ratio of 1:10–1:20 (w/w). Two-dimensional phosphopeptide mapping on thin layer cellulose plates (Macherey and Nagel, Düren, Germany) was performed according to [4]. First dimension electrophoresis was carried out at pH 1.9 in formic acid (88%)/glacial acetic acid/water (50:156:1794), second dimension chromatography at pH 3.5 in *n*-butyl alcohol/pyridine/glacial acetic acid/water (150:100:30:120). The phosphorylation pattern was scanned with a Bas2000 Bio-Imaging Analyzer (Fuji, Tokyo, Japan) and analyzed with TINA 2.0 software (Raytest, Straubenhardt, Germany). Determination of phosphorylation sites was done by MALDI, sequencing and running samples together with samples of identified phosphorylation patterns, as described previously [22] (Table 1).

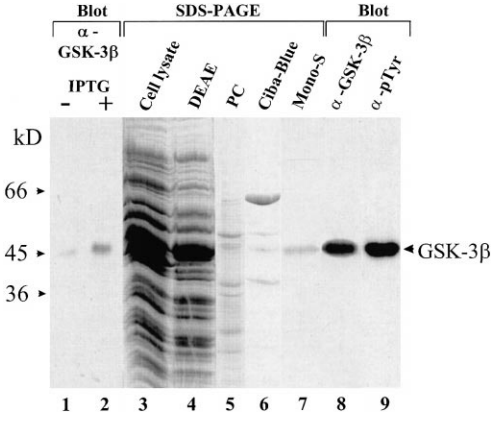

Fig. 2. Preparation of recombinant GSK-3β. Lanes 1, 2, immunoblot of *E. coli* cell lysate with an antibody against GSK-3β before and after induction with IPTG. Lanes 3, 4, SDS gel of cell lysate before and after DEAE chromatography; lane 5, after phosphocellulose chromatography; lane 6, after Cibacron blue chromatography; lane 7, after Mono-S chromatography; lanes 8, 9, immunoblots with antibodies against GSK-3β and phosphotyrosine (indicates activated kinase).

3. Results

Fig. 2 illustrates the expression and purification of GSK-3β from *E. coli*. After the final purification the protein has a concentration of 100 μg/ml and a specific activity of 450 nmol/min/mg (measured against phospho-CREB peptide at 37°C). Like other kinases, GSK-3β requires an activating phosphorylation at a Tyr in its regulatory loop [52]. This phosphorylation can be achieved by autophosphorylation of the enzyme, as seen from the reaction with a phosphotyrosine antibody (Fig. 2, lane 9). We tested the ability of this enzyme to phosphorylate different tau constructs, including htau40 (the longest isoform in humane CNS) and constructs comprising only the repeat domain where the KXGS motifs are lo-

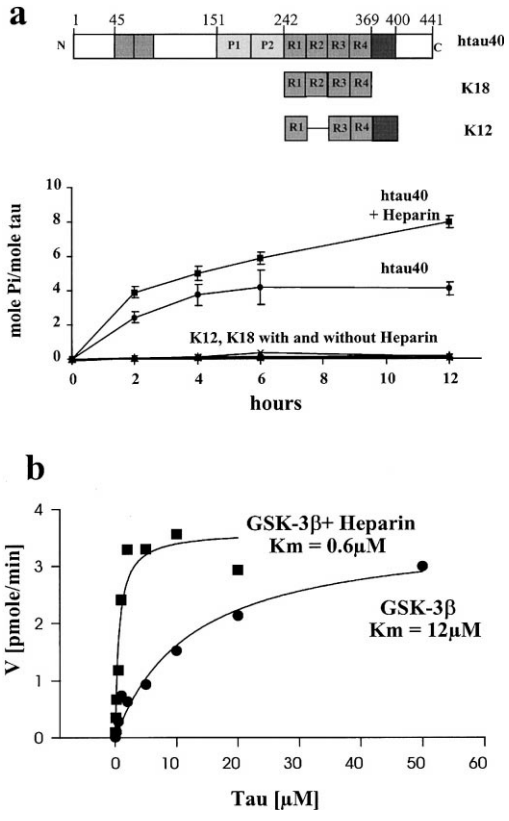

Fig. 3. a: Time course of incorporation of total phosphate into htau40 or constructs K18 and K12 containing the repeat domain. Bar diagrams of constructs are shown on top. Bottom curves: K12 (triangles) and K18 (×) are not significantly phosphorylated by rGSK-3β in the absence or presence of heparin, indicating that the repeat domain does not contain major phosphorylation sites. Middle curve (circles): htau40 can be phosphorylated to about 4 Pi/molecule in the absence of heparin. Top curve (squares): heparin roughly doubles the rate of phosphate incorporation, up to 8 Pi/molecule. b: Michaelis-Menten kinetics of phosphorylation of htau40 by GSK-3β without and with heparin (bottom curve = circles, top curve = squares). The ATP concentration was held constant at 100 μM and the reaction was performed for 15 min at 37°C with 0.2 μM of GSK-3β. Note that heparin (50 μg/ml) does not affect the V_{max} (about 3 pmol phosphate per min), but strongly reduces the K_M (from 12 μM to 0.6 μM), indicating an enhanced affinity of the kinase to the substrate tau.

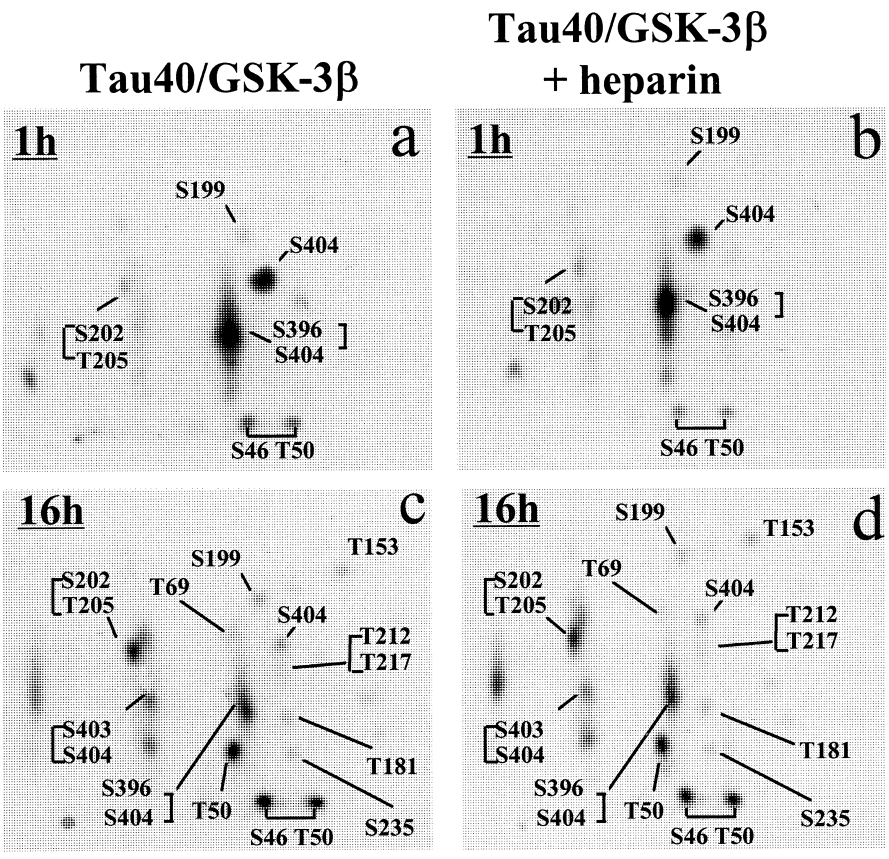

Fig. 4. Phosphopeptide maps of tau protein phosphorylated by recombinant GSK-3β in the absence or presence of heparin. Left, phosphorylation of tau with GSK-3β for 1 h and 16 h leads to incorporation of 1 and 4 mol phosphate/mol tau. Right, phosphorylation for 1 h and 16 h in the presence of 50 μg/ml heparin leads to 2 and 7 mol phosphate/mol tau. The phosphorylation sites which were identified by MALDI, sequencing and running samples together with samples of identified phosphorylation patterns [22] are marked. After 1 h incubation, tau protein is mainly phosphorylated at Ser396/Ser404, Ser404 and Ser46/Thr50, whereas extended phosphorylation leads to more than a dozen spots (16 h). Note that phosphorylation specificity is not altered significantly after the addition of heparin.

cated, including Ser262 in the first repeat. As seen in Fig. 3a, rGSK-3β efficiently phosphorylates htau40 up to four phosphates per molecule. The efficiency is increased two-fold by including heparin in the reaction mixture. However, the constructs of the repeat domain show almost no phosphorylation, indicating that the non-proline-directed activity of rGSK-3β is minimal, irrespective of heparin. In order to characterize the nature of the heparin effect we determined how the phosphorylation rates depended on the concentration of tau. The Michaelis-Menten plot (Fig. 3b) shows that heparin increases the affinity between enzyme and substrate (K_M value drops 20-fold) but leaves the maximal velocity unchanged, arguing that the mechanism and specificity of the phosphorylation reaction are not altered by the anionic cofactor.

The nature of the phosphorylation sites was determined by two-dimensional phosphopeptide mapping. htau40 was phosphorylated to various extents by GSK-3β without or with heparin, phosphopeptides were obtained by tryptic cleavage and separated in two dimensions (Fig. 4). The identification of the spots was done by phosphopeptide sequencing, mass spectrometry, and by reference to defined phosphopeptides (for details on tau phosphopeptides obtained in this way see [22,59]). The most prominent phosphorylation sites observed at early times are seen in Fig. 4a: Ser396 and Ser404 in the flanking region behind the repeats, followed by Ser46 and Thr50 in the first insert in the N-terminal domain, followed by Ser202 and Thr205 in the flanking region upstream of the repeats. At later times other sites became noticeable as well, e.g. Thr212, Ser235, or Thr181. All the significant sites were in Ser/Thr-Pro motifs, and most of them were located in epitopes of phosphorylation-sensitive antibodies (see below). Specifically, no phosphorylation was detected at Ser262 or other KXGS motifs. Heparin increased the rate of phosphorylation, but the phosphopeptide pattern was indistinguishable, show-

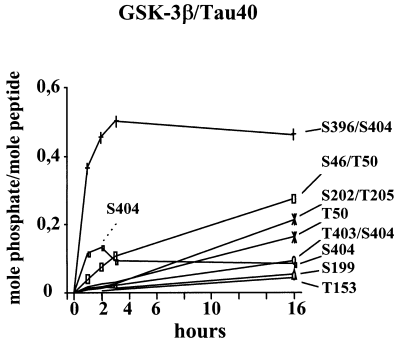

Fig. 5. Time course of phosphorylation at selected sites. The highest phosphorylation occurs in the peptide carrying both phospho-Ser396 and Ser404, followed by the doubly phosphorylated peptides S46/T50 and S202/T205. Note the transient maximum in the singly phosphorylated peptide S404 which is probably caused by sequential phosphorylation (first S404, then S396).

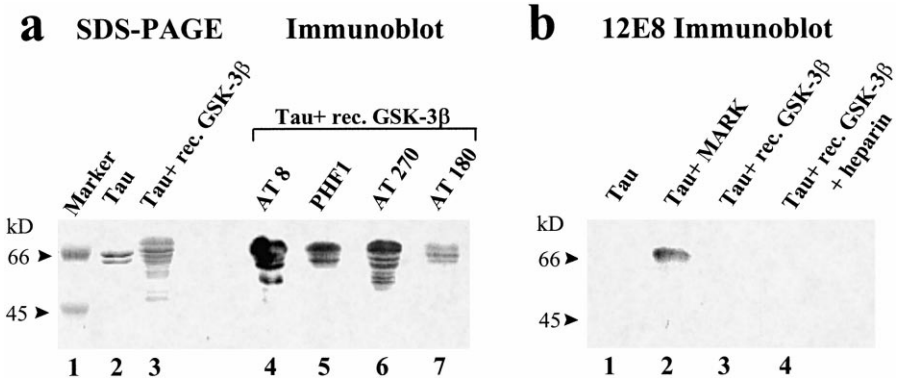

Fig. 6. Analysis of phosphoepitopes of tau after phosphorylation with recombinant GSK-3β for 4 h. a: Lane 1, SDS gel of marker proteins; 2, unphosphorylated control of htau40; 3, phosphorylated htau40, showing several bands shifted to higher M_r because of multiple phosphorylation sites. Lanes 4–7, immunoblots with antibodies AT-8, PHF-1, AT-270, AT-180 (for epitopes see Fig. 1). b: Immunoreactivity with antibody 12E8. Lane 1, recombinant htau40 (unphosphorylated control); 2, htau40 phosphorylated with MARK, resulting in phosphorylation at 12E8 epitope (Ser262, Ser356); 3 and 4, htau40 phosphorylated with GSK-3β without and with heparin, showing no 12E8 reaction.

ing that the same types of sites were phosphorylated with or without heparin, and with the same relative efficiencies. The relative intensities of the phosphorylation reaction are confirmed by the time courses of phosphorylation at selected sites shown in Fig. 5. The transient maximum of the singly phosphorylated peptide at S404 suggests a sequential phosphorylation in the N-terminal direction (first S404, then S396), somewhat reminiscent of the sequential action of GSK-3β on glycogen synthase. A similar situation holds for the pair Thr50 (first) and Ser45 (second, data not shown).

Several of the phosphorylation sites detected by phosphopeptide mapping are located in epitopes of phosphorylation-dependent antibodies against Alzheimer tau, and therefore one would expect a reaction with these antibodies. This is confirmed by the SDS gels and immunoblots shown in Fig. 6a. After phosphorylation by GSK-3β, the M_r of tau tends to shift upwards, depending on the combination of phosphorylation sites (e.g. phospho-Ser404 induces a noticeable shift). Antibodies AT-8, PHF-1, AT-270, AT-180 and others react with the phosphorylated tau. We note, however, that the intensity of the immunostaining does not necessarily reflect the degree of phosphorylation since this depends on the affinity of the antibodies. For example, the blots show the strongest staining with AT-8 and AT-270, even though the corresponding sites (Ser202/Thr205 and Thr181) are minor ones in the phosphopeptide maps (Fig. 4c,d).

In contrast to the Ser/Thr-Pro motifs, the KXGS motifs of the repeats are not phosphorylated by GSK-3β, as judged by phosphopeptide mapping (Fig. 4), and this is confirmed by the lack of reactivity with antibody 12E8 (sensitive to phosphorylated Ser262 and Ser356, Fig. 6b, lanes 3, 4). As a control we phosphorylated these motifs with MARK, resulting in a clear immunoreaction with 12E8 (Fig. 6b, lane 2). We therefore conclude that GSK-3β does not phosphorylate Ser262 or other KXGS motifs, irrespective of whether heparin is present or not.

There is a discrepancy between the results shown here and some reports claiming that GSK-3β phosphorylates not only Ser/Thr-Pro motifs but other sites as well, notably Ser262 in the first KXGS motif [29,42]. We knew from previous experience [12] that protein kinases tend to copurify during preparation from tissue, presumably because of the similarity of the kinase catalytic domains. Since earlier results on tau phosphorylation by GSK-3β reported in the literature were mostly obtained with the tissue-derived enzyme we performed the experiments with commercial GSK-3β (UBI) prepared from rabbit muscle. The protein concentration of this enzyme preparation is so low that it cannot be demonstrated by SDS-PAGE, but the purity can be assessed by allowing the sample to autophosphorylate in the presence of [γ-^{32}P]ATP. The autoradiography reveals a wide range of bands which may be phosphorylated either by GSK-3β (M_r 46 kDa) or possibly by other contaminating kinases (Fig. 7, lane 1). By contrast, recombinant GSK-3β revealed only one pure band (Fig. 7, lane 2). Next we phosphorylated tau with the commercial GSK-3β prepared from muscle tissue and probed the phosphorylation sites with antibodies PHF-1 (for the pair of Ser-Pro motifs Ser396/Ser404) and 12E8 (for Ser262 or Ser356 in

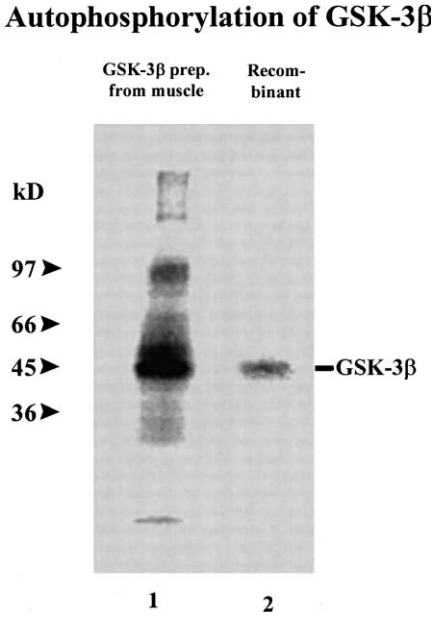

Fig. 7. Purity of commercial and recombinant GSK-3β. Lane 1, Autophosphorylation of 'GSK-3β' fraction derived from muscle tissue and incubated with [γ-^{32}P]ATP; lane 2, autophosphorylation of recombinant GSK-3β. The tissue-derived enzyme fraction contains a wide range of impurities, the recombinant enzyme shows a single homogeneous band.

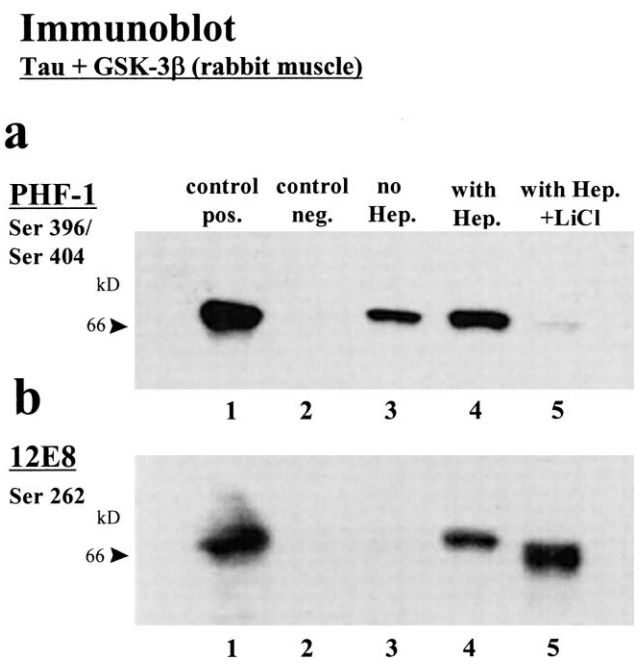

Fig. 8. Tau phosphorylated by the tissue-derived GSK-3β fraction and blotted with antibodies PHF-1 and 12E8. Each lane contains 250 ng of htau40. The kinase fraction can phosphorylate the PHF-1 epitope (Ser395/Ser404) without or with heparin (lanes 3, 4), but this activity is strongly suppressed by the specific GSK-3β inhibitor LiCl (lane 5). The epitope of 12E8 is also induced by GSK-3β, but becomes noticeable only in the presence of heparin (lane 4). However, this activity cannot be suppressed by 50 mM LiCl (lane 5), showing that this is not due to GSK-3β but to another contaminating kinase, e.g. MARK or PKA.

the KXGS motifs of repeats 1 or 4). In addition we used heparin to enhance the activity of GSK-3β, and LiCl to inhibit it. Fig. 8a shows that the PHF-1 epitope becomes phosphorylated without heparin, it is enhanced by heparin, and almost completely blocked by LiCl, showing that this epitope is indeed a target of GSK-3β. By contrast, the 12E8 epitope is not phosphorylated without heparin, phosphorylation becomes visible with heparin, but LiCl cannot inhibit it. This proves that the phosphorylation is due to a contaminating kinase whose activity requires enhancement by heparin but cannot be blocked by LiCl. Both MARK and PKA would qualify as the contaminating kinase because they phosphorylate the 12E8 epitope, are enhanced by heparin, and are not sensitive to LiCl. As a consequence, it is not likely that GSK-3β regulates microtubule stability in the cell via targeting of Ser262 in tau.

4. Discussion

The phosphorylation of tau protein at Ser262 has been the subject of several investigations because phosphorylation at that site is particularly potent in dissociating tau from microtubules, both in vitro and in cell models [2,13,49]. Since Ser262 of tau shows enhanced phosphorylation in AD [31], and since tau from AD brains has lost its microtubule-binding capacity [5], it is possible that an upregulation of a Ser262 kinase could cause neuronal degeneration in AD, leading to the loss of microtubules and breakdown of axonal traffic. Purification and cloning of a Ser262 kinase lead to the identification of MARK as the most potent kinase of Ser262 and related KXGS motifs in tau [12,13]. Other kinases were reported to have some activity towards Ser262 as well; they included PKA [12,25,37,54,59], CaMK-II [25,41], phosphorylase kinase [34], GSK-3α [56] and GSK-3β [29,42]. The activity of MARK, PKA or CaMK-II might appear understandable in view of the similar consensus target sequences (which include a positive charge three or four residues upstream of the phosphorylated serine, such as the lysine in the KXGS motif [21]), but the activity of GSK-3β seems counterintuitive since GSK-3β is a kinase which requires a subsequent proline or a priming kinase activity which creates the -S-X-X-X-Sp- motif. On the other hand, GSK-3β is particularly interesting in the context of AD research because it phosphorylates tau efficiently at Ser/Thr-Pro motifs that are elevated in AD [18,19,23,26,28,30,40,42,59]. It is involved in the wingless signalling pathway which may be perturbed in AD due to mutations in the PS1 gene which are related to familial AD [48,58,60]. Thus, GSK-3β may link different reaction pathways that are important for the neurodegeneration in AD. Because of this it would be interesting to know whether GSK-3β would qualify as a Ser262 kinase capable of detaching tau from microtubules, thus regulating microtubule dynamics and intracellular transport [13,14]. We show here that GSK-3β is not a Ser262 kinase. This would be consistent with GSK-3β transfection experiments in COS or CHO cells showing phosphorylation at Ser/Thr-Pro motifs but not at Ser262 [19,27].

GSK-3β phosphorylates predominantly Ser/Thr-Pro motifs in tau protein which occur in closely spaced pairs, in the order S396/S404, S46/T50, and S202/T205, followed by others. The first and the third pair are epitopes of antibodies PHF-1 and AT-8 which explains the reaction of these antibodies (Fig. 6a). Note, however, that the antibody reaction does not provide an accurate measure of the relative levels of phosphorylation. The same is true for other antibodies directed against phosphorylated Ser-Pro motifs which show a clear reaction, even though the phosphorylation at their epitopes is only a minute fraction of the total (e.g. AT-270 or AT-180, Fig. 6a). Not shown in this study is the reaction with AT-100, one of the most specific antibodies against AD tau. Its epitope requires the sequential phosphorylation of Thr212 (a Thr-Pro motif) by GSK-3β, followed by the phosphorylation of Ser214 by PKA [59]. Phospho-Thr212 is visible in the phosphopeptide maps (Fig. 4), but since PKA was not present the epitope of AT-100 was not generated. Summarizing the observed sites, we conclude that none of them qualifies for a major influence on the binding of tau to microtubules [2].

Another important conclusion is that the phosphorylation of Ser262 by GSK-3β, reported in other studies, is an artefact due to contaminations in kinase preparations from tissue. This activity is not observed with recombinant GSK-3β, and it cannot be suppressed by GSK-3β inhibitors such as LiCl. The contaminating activity was only observed upon stimulation with heparin, presumably because of the low concentration of the contaminant.

It is interesting to compare the specificity of GSK-3β towards tau with that of the other major proline-directed kinases discussed in the field, MAP kinase and cdc2 or cdk5. Several groups have reported phosphorylation sites induced by these kinases (reviewed in [15]), but in most cases they used antibody reactions or mass spectrometry. This makes it difficult to determine the relative occupancies of sites because

the signals are not linear (compare the strong signal with antibody AT-8, Fig. 6, with the low extent of phosphorylation at Ser202/Thr205, Fig. 4a,b; or the strong signal with antibody AT-270, Fig. 6, with almost no phosphorylation at Thr181, Fig. 4c,d). The problem of quantitation can be largely overcome by phosphopeptide mapping. Thus, MAP kinase phosphorylates most Ser/Thr-Pro sites rather efficiently (Thr153, Ser235 and Thr50 being most prominent [22]). The kinases cdc2 or cdk5 show a strong preference for Ser235, Ser404, and Ser202/Thr205 [22,49]. Therefore, even though GSK-3β, MAP kinase, and cdk5 are almost exclusively proline-directed with respect to tau, there is a clear difference in their phosphorylation profile.

The cellular effects of GSK-3β on the cytoskeleton are only partly understood at present. The kinase phosphorylates both neurofilament subunits [17] and microtubules via their MAPs; in the latter case there is a debate on whether GSK-3β reduces the interaction of tau with microtubules and ability of microtubules to form bundles in cells (see [51,54]). We found that the phosphorylation of GSK-3β target motifs on tau are somewhat inhibitory for process formation in transfected Sf9 cells, but the phosphorylation of targets of PKA or MARK has much more pronounced effects on microtubule dynamics in mitosis and cell process formation [3,13]. Finally, one should note that tau has several distinct functions which include not only microtubule stabilization, but also anchoring of enzymes (e.g. PP-2a or PP-1 [24,43]) and membrane attachment [7], and the regulation of intracellular transport by motor proteins [14]. It remains to be shown how these functions are controlled by different kinases and their target sites on tau.

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References

[1] Baumann, K., Mandelkow, E.M., Biernat, J., Piwnica-Worms, H. and Mandelkow, E. (1993) FEBS Lett. 336, 417–424.
[2] Biernat, J., Gustke, M., Drewes, G., Mandelkow, E.M. and Mandelkow, E. (1993) Neuron 11, 153–163.
[3] Biernat, J. and Mandelkow, E.M. (1999) Mol. Biol. Cell 10, 727–740.
[4] Boyle, W.J., van der Geer, P. and Hunter, T. (1991) Methods Enzymol. 201, 110–149.
[5] Bramblett, G.T., Trojanowski, J.Q. and Lee, V.M. (1992) Lab. Invest. 66, 212–222.
[6] Brandt, R., Lee, G., Teplow, D.B., Shalloway, D. and Abdel-Ghany, M. (1994) J. Biol. Chem. 269, 11776–11782.
[7] Brandt, R., Leger, J. and Lee, G. (1995) J. Cell Biol. 131, 1327–1340.
[8] Brownlees, J., Irving, N.G., Brion, J.P., Gibb, B.J., Wagner, U., Woodgett, J. and Miller, C.C. (1997) NeuroReport 8, 3251–3255.
[9] Chen, G., Huang, L.D., Jiang, Y.M. and Manji, H.K. (1999) J. Neurochem. 72, 1327–1330.
[10] Cohen, P., Alessi, D.R. and Cross, D.A. (1997) FEBS Lett. 410, 3–10.
[11] Drewes, G., Lichtenberg-Kraag, B., Doering, F., Mandelkow, E.M., Biernat, J., Goris, J., Doree, M. and Mandelkow, E. (1992) EMBO J. 11, 2131–2138.
[12] Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H.E., Mandelkow, E.M. and Mandelkow, E. (1995) J. Biol. Chem. 270, 7679–7688.
[13] Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E.M. and Mandelkow, E. (1997) Cell 89, 297–308.
[14] Ebneth, A., Godemann, R., Stamer, K., Illenberger, S., Trinczek, B. and Mandelkow, E. (1998) J. Cell Biol. 143, 777–794.
[15] Friedhoff, P. and Mandelkow, E. (1999) in Guidebook to the Cytoskeletal and Motor Proteins (Kreis, Th. and Vale, R., Eds.), in press, Oxford University Press, Oxford.
[16] Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A. and Cohen, P. (1997) FEBS Lett. 409, 57–62.
[17] Guidato, S., Tsai, L.H., Woodgett, J. and Miller, C.C. (1996) J. Neurochem. 66, 1698–1706.
[18] Hanger, D.P., Hughes, K., Woodgett, J.R., Brion, J.P. and Anderton, B.H. (1992) Neurosci. Lett. 147, 58–62.
[19] Hong, M., Chen, D.C., Klein, P.S. and Lee, V.M. (1997) J. Biol. Chem. 272, 25326–25332.
[20] Hoshi, M. et al. (1996) Proc. Natl. Acad. Sci. USA 93, 2719–2723.
[21] Hunter, T. (1991) Methods Enzymol. 200, 3–37.
[22] Illenberger, S. et al. (1998) Mol. Biol. Cell 9, 1495–1512.
[23] Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T. and Imahori, K. (1993) FEBS Lett. 325, 167–172.
[24] Liao, H., Ki, Y., Brautigan, D.L. and Gundersen, G.G. (1998) J. Biol. Chem. 273, 21901–21908.
[25] Litersky, J.M., Johnson, G.V., Jakes, R., Goedert, M., Lee, M. and Seubert, P. (1996) Biochem. J. 316, 655–660.
[26] Lovestone, S. et al. (1994) Curr. Biol. 4, 1077–1086.
[27] Lovestone, S., Hartley, C.L., Pearce, J. and Anderton, B.H. (1996) Neuroscience 73, 1145–1157.
[28] Mandelkow, E.M., Drewes, G., Biernat, J., Gustke, N., Van Lint, J., Vandenheede, J.R. and Mandelkow, E. (1992) FEBS Lett. 314, 315–321.
[29] Moreno, F.J., Medina, M., Perez, M., Degarcini, E.M. and Avila, J. (1995) FEBS Lett. 372, 65–68.
[30] Moreno, F.J., Munoz-Montano, J.R. and Avila, J. (1996) Mol. Cell. Biochem. 165, 47–54.
[31] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K. and Ihara, Y. (1995) J. Biol. Chem. 270, 823–829.
[32] Murai, H., Okazaki, M. and Kikuchi, A. (1996) FEBS Lett. 392, 153–160.
[33] Paudel, H.K., Lew, J., Ali, Z. and Wang, J.H. (1993) J. Biol. Chem. 268, 23512–23518.
[34] Paudel, H.K. (1997) J. Biol. Chem. 272, 1777–1785.
[35] Plyte, S.E., Hughes, K., Nikolakaki, E., Pulverer, B.J. and Woodgett, J.R. (1992) Biochim. Biophys. Acta 1114, 147–162.
[36] Roach, P.J. (1991) J. Biol. Chem. 266, 14139–14142.
[37] Schneider, A., Biernat, J., von Bergen, M. and Mandelkow, E. (1999) Biochemistry 38, 3549–3558.
[38] Sengupta, A., Wu, Q.L., Grundke-Iqbal, I., Iqbal, K. and Singh, T.J. (1997) Mol. Cell. Biochem. 167, 99–105.
[39] Seubert, P. et al. (1995) J. Biol. Chem. 270, 18917–18922.
[40] Singh, T.J., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1995) FEBS Lett. 358, 4–8.
[41] Singh, T.J., Wang, J.Z., Novak, M., Kontzekova, E., Grundke-Iqbal, I. and Iqbal, K. (1996) FEBS Lett. 387, 145–148.
[42] Song, J.S. and Yang, S.D. (1995) J. Protein Chem. 14, 95–105.
[43] Sontag, E., Nunbhakdi-Craig, V., Lee, G., Bloom, G.S. and Mumby, M.C. (1996) Neuron 17, 1201–1207.
[44] Sperber, B.R., Leight, S., Goedert, M. and Lee, V.M.Y. (1995) Neurosci. Lett. 197, 149–153.
[45] Stambolic, V. and Woodgett, J.R. (1994) Biochem. J. 303, 701–704.
[46] Stambolic, V., Ruel, L. and Woodgett, J.R. (1996) Curr. Biol. 6, 1664–1668.
[47] Takashima, A. et al. (1995) Neurosci. Lett. 198, 83–86.
[48] Takashima, A. et al. (1998) Proc. Natl. Acad. Sci. USA 95, 9637–9641.
[49] Trinczek, B., Biernat, J., Baumann, K., Mandelkow, E.M. and Mandelkow, E. (1995) Mol. Biol. Cell 6, 1887–1902.
[50] Vulliet, R., Halloran, S.M., Braun, R.K., Smith, A.J. and Lee, G. (1992) J. Biol. Chem. 267, 22570–22574.
[51] Wagner, U., Utton, M., Gallo, J.M. and Miller, C.C.J. (1996) J. Cell Sci. 109, 1537–1543.
[52] Wang, Q.M., Fiol, C.J., DePaoli-Roach, A.A. and Roach, P.J. (1994) J. Biol. Chem. 269, 14566–14574.

[53] Welsh, G.I., Wilson, C. and Proud, C.G. (1996) Trends Cell Biol. 6, 274–279.
[54] Xie, H.Q., Litersky, J.M., Hartigan, J.A., Jope, R.S. and Johnson, G.V.W. (1998) Brain Res. 798, 173–183.
[55] Yang, S.D., Song, J.S., Yu, J.S. and Shiah, S.G. (1993) J. Neurochem. 61, 1742–1747.
[56] Yang, S.D., Yu, J.S., Shiah, S.G. and Huang, J.J. (1994) J. Neurochem. 63, 1416–1425.
[57] Yang, C.C., Hsu, C.P., Sheu, J.C., Mai, X.Y. and Yang, S.D. (1998) J. Protein Chem. 17, 329–335.
[58] Zhang, Z.H. et al. (1998) Nature 395, 698–702.
[59] Zheng-Fischhofer, Q., Biernat, J., Mandelkow, E.M., Illenberger, S., Godemann, R. and Mandelkow, E. (1998) Eur. J. Biochem. 252, 542–552.
[60] Zhou, J., Liyanage, U., Medina, M., Ho, C., Simmons, A.D., Lovett, M. and Kosik, K.S. (1997) NeuroReport 8, 1489–1494.