Glycogen synthase kinase 3 phosphorylates recombinant human tau protein at serine-262 in the presence of heparin (or tubulin)

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Abstract Tau protein, the major component of the aberrant structures termed paired helical filaments (PHFs) present in the brain of Alzheimer's disease patients, is pathologically phosphorylated in sites in and around the tubulin-binding sites. A single protein kinase, glycogen synthase kinase 3 (GSK 3), is able to phosphorylate tau at the flanking regions and, additionally, at the tubulin-binding motifs if heparin or tubulin is present. Serines-262 and -324 have been found to be modified at the tubulin-binding region of tau protein by GSK 3 in the presence of heparin or tubulin.

Key words: Tau phosphorylation; Paired helical filament; Alzheimer's disease

1. Introduction

Microtubule-associated protein tau [1] plays a role in the stabilisation of polymerised microtubules [2–4]. This role is abolished in a neuropathological situation, Alzheimer's disease (AD), in which the capacity for microtubule binding of tau protein decreases [5,6]. Furthermore, tau protein in AD, in a modified form, is the main component of the aberrant structures termed as paired helical filaments (PHFs) present in the brain of the patients [7–12].

Several post-translational modifications have been described for tau protein [11–14]. One of these, phosphorylation, has been suggested to precede PHF formation [15]. Tau phosphorylation has been extensively studied in vitro and in PHF-associated tau [16]. There are three main types of proline-directed protein kinases which could modify tau in vitro. GSK 3 (glycogen synthase-kinase 3) [17,18], cdc-2 like kinases [19–21], and MAP kinases [22]. Proteins related to the first two types that have been found to be associated to microtubules, showing a capacity to phosphorylate tau, are termed as tau kinases I and II [18,23]. The modification of tau by these kinases does not significantly decrease the binding of tau to microtubules, a feature observed for AD tau [5]. However, Biernat et al. [6] have described a modification in Ser-262, which could dramatically decrease the interaction of tau for microtubules. Additionally, Correas et al. [24] described a related serine, present in the second tubulin- binding domain of tau, phosphorylated by protein kinase C. Such phosphorylation also results in a decrease in the interaction of the protein with microtubules.

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Abbreviations: GSK 3, glycogen synthase kinase 3; MAP, microtubule associated protein.

GSK 3 consists of two related proteins, GSK 3α and GSK 3β [24] first described as a modulator of glycogen metabolism, phosphorylating glycogen synthase and mediating insulin regulation of glycogen synthesis [26]. Several studies have described that either GSK 3α or GSK 3β are able to modify tau [17,18,27–30]. Moreover, it has been shown by co-transfection studies that GSK 3β could modify tau in cultured cells at some of the residues that are also modified in AD-tau [31].

2. Materials and methods

2.1. Materials

The peptides KVTSKCGSLGNIHHKPGGG, and KVTAKCGSLGNIHHKPGGG, were purchased from Med Probe A.S. (Oslo, Norway). The peptide TAEEEEDFGEEAEEEA corresponding to tubulin β isoform (mouse β_5) [32] was synthesized on an automatic solid phase peptide synthesizer (type 430A, Applied Biosystems) and purified by reverse-phase HPLC on a NovoPak C18 column. Heparin was obtained from Lab. Farm. Rovi (Barcelona, Spain), S.A. T4 polynucleotide kinase, Klenow enzyme, T4 DNA ligase and restriction enzymes were purchased from Boehringer. T7 DNA polymerase was from Pharmacia. Ampli Taq polymerase was from Perkin Elmer-Cetus. Synthetic oligonucleotides were from Isogen Bioscience (Amsterdam).

2.2. Construction of expression plasmids and purification of recombinant tau isoforms and fragments from E. coli

Human tau cDNA clones (htau 23 and htau 40) were kindly supplied by Dr. M. Goedert (M.R.C. Cambridge, UK). Additionally, several tau fragments were cloned into the expression vector pRK172 [33]. To obtain plasmids pRKT3RC, pRKT2R, pRKT3R and pRKT4R for inducible expression in *E. coli*, mutagenesis was carried out using the polymerase chain reaction (PCR). Oligonucleotides A5 (GCGGATCCATATGCCAGACCTG), which include the initiation codon as well as *Bam*HI and *Nde*I sites for a proper cloning into the vector, was used as 5′ primer. Oligonucleotide A7 (GCGAATTCTTACTCGCGGAAGG), A8 (GCGAATTCTCACAAACCCTGCTTGG) and A10 (CCGGAATTCTAAAGCTTCTCAGATTTTAC), which include a stop codon and an *Eco*RI site downstream, were used as 3′ primers. The fragment generated by PCR amplification on pSGT3RC using primers A5 and A8 (503 bp) was digested with *Nde*I and *Eco*RI and ligated into pRK172 and also digested with *Nde*I and *Eco*RI to obtain pRKT3RC. Primers A5 and A7 were used on pSGT3R and pSGT4R [34] and the amplified fragments (321 bp and 384 bp, respectively) were digested with *Nde*I and *Eco*RI and ligated into *Nde*I- and *Eco*RI-digested pRK172 to obtain pRKT3R and pRKT4R, respectively. Primers A5 and A10 were used to amplify a 205 bp fragment that was used, after digestion with *Nde*I and *Eco*RI and ligation into pRK172 also digested with *Nde*I and *Eco*RI, to obtain pRK2R. All plasmids were analyzed by restriction analysis to test for the 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. Recombinant tau protein was purified [34], including a boiling step to prevent possible contamination of endogenous enzymatic activities.

2.3. Protein preparation

GSK 3 was purified from bovine brain essentially as described by Woodgett [25], by chromatography on DEAE-cellulose, phosphocellulose and heparin-Sepharose. In some cases the further step in column

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filtration on Sephacryl was replaced by a mono S-2 chromatography on a FPLC system (Pharmacia). A specific activity of 0.15 μmol of phosphate incorporated/min/mg of substrate (recombinant tau protein) was calculated for the GSK 3 preparation.

Tubulin depleted of MAPs was isolated from bovine brain by phosphocellulose chromatography [1]. Tubulin lacking its C-terminal region was obtained by subtilisin digestion [35]. The proteins were characterized by gel electrophoresis, and in the case of labelled proteins, by autoradiography as previously described [28].

2.4. Enzymatic assays

GSK 3 assays were carried out as described by Stambolic and Woodgett [36] in a buffer containing 50 μM [γ-^{32}P]ATP, 25 mM Tris pH 7.5, 1 mM DTT, 10 mM $MgCl_2$ and 1 mM EGTA.

The interaction of tau fragments, containing three tubulin-binding motifs in phosphorylated or unmodified form, with microtubules was carried out as described by Medina et al. [34].

2.5. Protein sequencing

Tryptic peptides of tau protein were fractionated by HPLC, using a NovoPack column, in the conditions described by Nieto et al. [5], and these peptides containing phosphate label were blotted on immobilon paper (Millipore) and sequenced with an Applied-Biosystem gas phase sequenator (model 470A) equipped with an on-line phenylthiohydantoin amino acid analyser (model 120A).

3. Results

3.1. Recombinant tau is phosphorylated by GSK 3

Fig. 1 shows that the whole molecule of tau (isoform tau 31) is a substrate for GSK 3. It has been indicated that heparin could increase the phosphorylation of tau by kinases like PKA [37] and also by GSK 3 [18]. Thus, we have tested the incorporation of phosphate in tau, by GSK 3, after adding heparin. In the presence of heparin, an 8-fold increase was observed in the incorporation of phosphate into tau 31 per mol of protein. This increase was found to be dependent on both protein and heparin concentration.

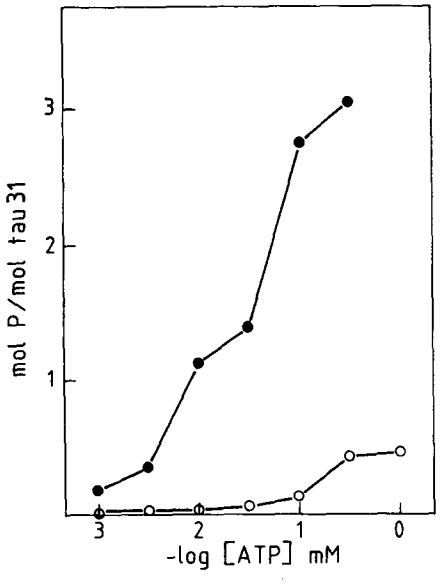

Fig. 1. Determination of phosphate incorporated into tau 31 by GSK 3. 10 μl aliquots of purified tau 31 (0.35 mg/ml) were incubated with GSK 3 at different concentrations of ^{32}P-labelled ATP for 20 min at 37°C, in the absence (○) or in the presence of 0.1 mg/ml of heparin (●). After incubation, each aliquot was run on SDS-gel electrophoresis, and the region containing tau protein was excised from the gel. The radioactivity associated with tau was measured by Cerenkov radiation, taken it into account to calculate the phosphate incorporated into the protein.

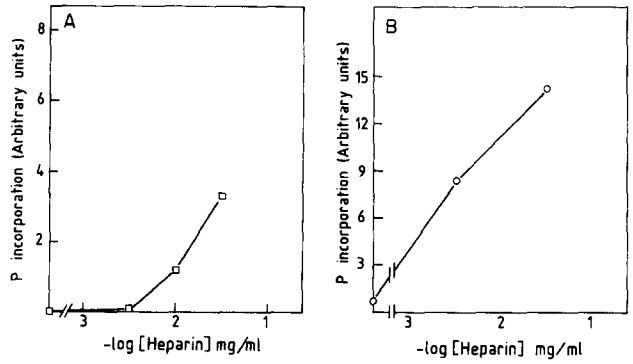

Fig. 2. Effect of heparin on the phosphorylation of tau fragments 3RC and 3R. The enzyme was assayed in the presence of the indicated increasing heparin concentrations using tau 3RC at 0.2 mg/ml (A) or tau 3R at the same concentration (B). Phosphate incorporation was measured as indicated in Fig. 1. In ordinate is indicated the comparison of the incorporation of phosphate into protein determined by gel electrophoresis, and autoradiography.

3.2. Localization of tau residues modified by GSK 3

Tau fragment lacking the amino terminal region (tau 3RC) is also a suitable substrate for GSK 3 (Fig. 2). Additionally, the modification of tau 3RC fragment also increases in the presence of heparin (Fig. 2). Since the tau fragment tested (3RC) contains both the tubulin-binding and carboxy-terminal regions, we tested whether one or both of these regions could be modified by GSK 3. Fig. 2 indicates that both of them are phosphorylated by GSK 3, since not only fragment 3RC, but also fragment 3R (containing the three tubulin-binding motifs), could be phosphorylated. However, the presence of heparin appears to be essential for the modification of 3R, since no phosphorylation was detected in its absence. Since different, but related, tubulin binding motifs can be found in tau isoforms, it is possible that GSK 3 could modify serine residues present in those related motifs. To test this, different tau frag-

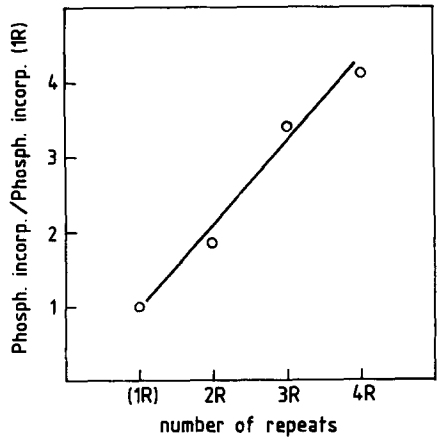

Fig. 3. Phosphorylation of tubulin binding motifs present in tau protein by GSK 3 in the presence of heparin. The enzyme was assayed in the presence of 0.1 mg/ml heparin and the different tau fragments containing four, three, two and a part of a single tubulin-binding repeat. The amount of radioactivity incorporated into the different tau fragments was determined as indicated in legend of Fig. 1.

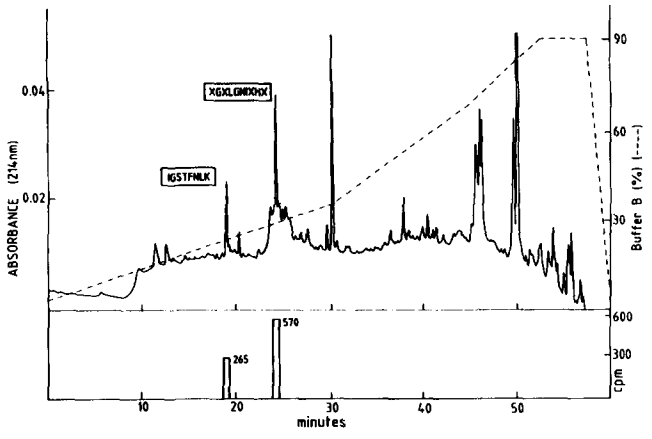

Fig. 4. GSK 3 phosphorylation sites on tau tubulin binding domain of tau protein. Tau 2R was phosphorylated with GSK 3 in the presence of heparin and ^{32}P-ATP. Tryptic peptides of phosphorylated tau were fractionated by HPLC and the peptides containing phosphate label were blotted onto Immobilon paper and sequenced as described under section 2. Peptide sequences obtained are indicated as inserts.

ments containing four, three, two and a part of a single tubulin-binding repeat were incubated in the presence of GSK 3 and the relative phosphate incorporation was measured. Fig. 3 indicates that the incorporation of phosphate into the different tau fragments is related to the number of repeats present in each fragment. The result suggests that serines present in related sequences of the motifs could all be phosphorylated. These serines could be related to the presence of residue 324 at the third repeat (of a four-repeat tau protein), since a similar value was obtained when the incorporation of phosphate into peptide IR (KVTSKCGSLGNIHHKPGGG) was compared to peptide (KVTAKCGSLGNIHHKPGGG), after incubation with GSK 3 and heparin.

3.3. Serine-262 is modified by GSK 3 in the presence of heparin

In order to identify the residues modified by GSK 3, the tau fragment containing the first two repeats was incubated with the kinase in the presence of heparin and afterwards proteolyzed with trypsin. Those tryptic fragments containing phosphate label were sequenced. Fig. 4 indicates the chromatography pattern of tryptic peptide fractionation and the sequence of those peptides showing a higher phosphorylation level. In one case a phosphopeptide contains serine-262, present in the first tubulin-binding motif and in the other case the phosphopeptide contains serine-324, which is present in a related sequence to that of serine-262, in the third tubulin-binding motif of tau protein.

3.4. The phosphorylation of tau binding motifs reduces the interaction of the modified protein for microtubules

To test whether the phosphorylation of tubulin-binding motifs of the tau protein by GSK 3, in the presence of heparin, modifies the interaction of the protein for microtubules, tau fragment 3R, containing only the tubulin-binding motifs that can interact with microtubules [38], was incubated with GSK 3 plus heparin. After incubation, heparin was removed by DEAE-chromatography, and both modified and unmodified proteins were incubated in the presence of increasing concentrations of assembled microtubules to study their binding affinities for them. A three fold increase in the K_d (17 μM) of the modified protein compared to that of the unmodified tau fragment (K_d, 6 μM) was observed.

3.5. Tubulin-binding motifs present in tau protein are phosphorylated by GSK 3 in the presence of tubulin

The carboxy terminal region of tubulin contains a cluster of acidic residues involved in the binding of microtubule-associated proteins [35,39], which could be considered as a polyanion. GSK 3 was incubated with tau fragment 3R in the presence of increasing concentrations of heparin or tubulin in order to test whether tubulin could, through the presence of the negative charges, facilitate the phosphorylation of tau by GSK 3, at its tubulin-binding repeats. Fig. 5 shows that both, heparin and tubulin, act as promoters of GSK 3 phosphorylation, but not tubulin lacking its acidic carboxy terminal region. However, a peptide containing the last residues of a tubulin β isoform (mouse β_5 [32]) is able to favour the phosphorylation of tau by GSK 3.

4. Discussion

In the present work it has been shown that tau protein in the presence of heparin (or tubulin) could be phosphorylated at serine-262, a residue that is phosphorylated in vivo in the brain of AD patients [12]. Such phosphorylation also results in a decrease in the interaction of tau protein for microtubules [6]. Despite multiple phosphorylation through the whole tau molecule by MAP kinase or GSK 3 [17,18,22,27–30], PHF cores are formed by the tubulin binding domains of tau protein, lacking the flanking regions [8]. Serine-262 is located within the first tubulin binding domain, thus becoming phosphorylation on this residue more interesting, since it is present in truncated tau from PHF cores [40].

In relation to the phosphorylation at the tubulin-binding motifs of tau, it has been suggested that some kinases could phosphorylate at serine-262 [6] or at serine-324 [24,41]. In the

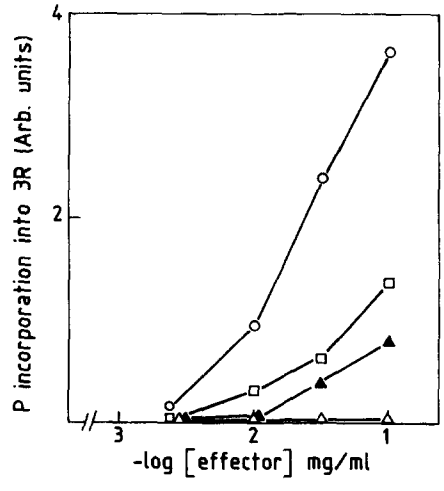

Fig. 5. Phosphorylation of tubulin-binding motifs present in tau protein by GSK 3 was stimulated by heparin and tubulin. The enzyme was assayed in the presence of increasing amounts of heparin (circles), tubulin (squares) and subtilisin-digested tubulin lacking the terminal region (triangles) or the peptide corresponding to the C-terminal region of mouse β_5 isoform (filled triangles) at the indicated concentrations, using tau 3R at 0.2 mg/ml. Phosphate incorporation was measured as indicated in the legend of Fig. 2.

present work it has been shown that GSK 3 could also modify the previous serines. Thus, a single kinase GSK 3 could phosphorylate at both tubulin-binding and flanking regions.

The action of heparin on GSK 3 activity on tau appears to be similar to that of tubulin. It could be due to the anionic character of heparin and the C-terminal region of tubulin subunits, a region involved in the binding of microtubule-associated proteins [35], including tau protein [39]. Thus, a tubulin-GSK 3-tau complex could be formed, resulting in the modification of tau, at the serine-262, of the tubulin-binding motifs.

As indicated in this work and previous works [6], the result of this modification could be a decrease in the interaction of the tau protein with microtubules. As a consequence of the lower microtubule association of tau, and the reported observation indicating that modified protein could have a slower turnover [42], the formation of tau aggregates, even that of PHF's, could be favoured.

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