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Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau

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The Alzheimer-like state of tau protein includes phosphorylation by a proline-directed Ser/Thr kinase present in normal or pathological human brain. Extending earlier results on MAP kinase, we show here that the proline-directed kinase, GSK3, can induce an Alzheimer-like immune response involving several distinct and phoshorylatable epitopes at Ser–Pro motifs, as well as a gel mobility shift, similar to MAP kinase. Both kinases behave like microtubule-associated proteins in that they co-purify through cycles of assembly and disassembly, and both kinases are directly associated with paired helical filaments.

Phosphorylation; MAP kinase; Protein kinase F_A; Paired helical filaments; Serine-proline motifs

1. INTRODUCTION

Tau protein represents a major component of the paired helical filaments (PHF) of Alzheimer's disease (for reviews see [1–3]). A major goal of Alzheimer research is to find out in what way pathological tau differs from normal tau. One sensitive method is the use of monoclonal antibodies that distinguish between PHF tau and normal tau. We have recently investigated the 'Alzheimer-like state' of tau induced by a certain type of phosphorylation, dominated by phosphorylated Ser–Pro and Thr–Pro motifs [4]. This state can be detected by several independent criteria, such as PHF-specific antibodies with distinct epitopes, and a particular shift in electrophoretic mobility [5,6], and it can be induced by a kinase activity in human brain extract or by purified MAP kinase [7]. These results suggested that MAP kinase could be a key enzyme in the pathological phosphorylation of tau protein.

Building on these observations we branched out in two directions. Firstly, since most of the abnormal phosphorylated motifs were of the Ser–Pro type we tested other proline-directed kinases. We had found earlier that the cell cycle kinase, cdc2, combined with either cyclin A or B, caused only a small degree of phosphorylation and no Alzheimer-like immune response [7]. We then turned to GSK3, a kinase involved in the control of insulin-stimulated signal transduction [8,9], and in this paper we show that the effect of this kinase resembles that of MAP kinase, making it another candidate for the pathological phosphorylation of tau. Secondly, we show that both GSK3 and MAP kinase copurify with cycles of microtubule assembly, making them genuine microtubule-associated proteins, and that both are associated with Alzheimer PHFs. Both isoforms of GSK3 (α, 51 kDa; β, 45 kDa) have similar effects.

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2. MATERIALS AND METHODS

The methods used here have been described in recent publications so only some key points will be repeated. Normal human or bovine brain tau was prepared as described in [10]. Recombinant human tau proteins were derived from the cDNA clones of Goedert et al. [11,12] and expressed in *E. coli* [13], using derivatives of the pET expression vector [14]. Point mutants were made by PCR [5,6]. Phosphorylation by the brain kinase activity was done following [5]. Fig. 1a shows a diagram of the domain structure of htau40 and its main phosphorylation sites.

GSK3 (α and β isoforms) were purified from bovine brain as in Vandenheede et al. [8] with an additional Mono S chromatography step which separates the two isoforms. Most experiments described here were done with immunoprecipitates of GSK3-α on TSK beads, but control experiments with the β isoform showed the same behavior.

Polyclonal anti-peptide antibodies to the α and β isoforms of GSK3 were raised in rabbits and affinity purified on peptide columns [15]. Immunoprecipitates of GSK3 were prepared from PC-12 cytosols in 20 mM Tris-HCl, 1% NP-40, 1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin and 0.2 μg/ml pepstatin. 100 μl of cytosols were incubated with 1 μl α- or β-GSK antibodies (1 mg/ml) or control rabbit antibodies and incubated for 4 h at 4°C; 5 μl of TSK-protein A beads were added and incubated for 1 h, and finally the beads were washed with 10 mg/ml BSA in 20 mM Tris-HCl, 0.5 M LiCl in Tris buffer, and 20 mM HEPES, pH 7.2, with 10 mM $MgCl_2$ and 1 mM DTT. In phosphorylation assays, 2 μl of pellets were incubated with 8 μl of substrate (3 μM) in 40 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA, 0.5 mM DTT and 1 mM PMSF.

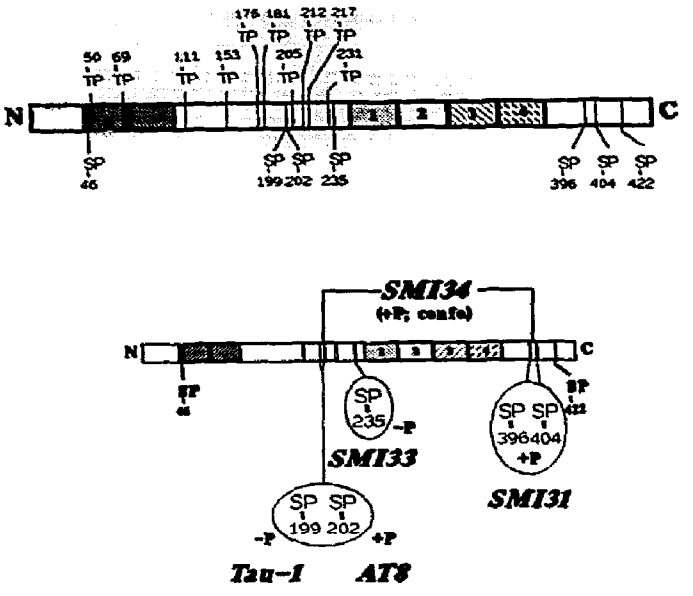

Fig. 1. (a) Bar diagram of htau40, showing the location of all Ser–Pro and Thr–Pro motifs. The numbering follows that of htau40 [12]. The two hatched boxes near the N-terminus (29 residues each) are inserts that may be absent in smaller tau isoforms (e.g. both absent in htau23). The four boxes in the C-terminal half indicate the microtubule-binding repeats. Repeat 2 may be absent (e.g. in htau23, htau37, htau39). MAP kinase can phosphorylate all Ser–Pro or Thr–Pro motifs [7]. GSK3 phosphorylates mainly Ser-199, -202, -235, -396, -404, all followed by Pro. (b) Bar diagram of htau40 with epitopes of antibodies sensitive to the Alzheimer-like state of tau (see [5,6]. +P or −P means phosphorylation or not. TAU1 requires dephosphorylated Ser-199 and -202, the PHF-specific antibody, AT8, requires both phosphorylated. SMI33 requires dephosphorylated Ser-235, SMI31 requires phosphorylated Ser-396 and -404. SMI34 is conformation sensitive and requires phosphorylated Ser–Pro motifs on either side of the repeat region.

Antibodies against tau were obtained from Dr. L. Binder (TAU1, [16]), Innogenetics SA (AT8, [17]), and Sternberger Monoclonals Inc. (SMI 31, 33, 34 [18]). We had previously determined their epitopes [5,6]; they are diagrammed in Fig. 1b. TAU1 and SMI33 react with normal (or recombinant unphosphorylated) tau; AT8, SMI31 and SMI34 react with Alzheimer PHFs, or with tau phosphorylated by MAP kinase or the brain extract.

3. RESULTS

3.1. *Time–course of phosphorylation and antibody response induced by GSK3*

The Alzheimer-like state of tau protein can be assessed by a combination of several criteria; a gel shift, phosphorylation, and reactivity with antibodies that distinguish between PHF-tau and normal tau. The Alzheimer-like gel shift takes place in three main stages which can be induced by a kinase activity in brain extract [5], purified MAP kinase, but not by the cell cycle kinase cdc2 [7]. The extract activity and MAP kinase phosphorylate mainly Ser–Pro or Thr–Pro motifs [4]; in the case of activated MAP kinase this reaches ≈10 Pi per htau23 molecule, approaching the total number of 14 Ser–Pro and Thr–Pro motifs, while the extract kinase activity incorporates only ≈6 and affects some non-Ser–Pro sites as well. In both cases, there is a characteristic complementarity between PHF antibodies (which recognize phosphorylated epitopes) and non-PHF antibodies (against unphosphorylated epitopes); the transition to the PHF-like state takes place during the second stage of phosphorylation.

In our search for other proline-directed kinases we have repeated these experiments with GSK3 (alias phosphatase activating factor F_A [8]). Fig. 2a shows a time–course of phosphorylation of htau40 with GSK3, and the corresponding autoradiogram and immunoblots. In most respects the behavior is similar to that obtained with the brain kinase activity or with purified MAP kinase (compare Figs. 2 and 3 of [6], and Fig. 7 of [7]). Phosphorylation induces a gel shift in three main stages; it incorporates ≈4 Pi, it induces the reactivity of antibodies AT8, SMI34, and SMI31, but reduces the reactivity of TAU1 and SMI33.

3.2. *Phosphorylation sites of GSK3 on tau*

The main phosphorylation sites can be determined from antibody epitopes and point mutants (Fig. 2). The epitopes of AT8, TAU1, and the SMI antibodies and their phosphorylation dependence has been determined previously (Fig. 1b, and see [5,6]). TAU1 requires that both Ser-199 and Ser-202 are unphosphorylated, AT8 requires them both phosphorylated. Thus when only one of the two serines is phosphorylated these antibodies do not react. This means that Ser-199 and Ser-202 both become phosphorylated during stage 2 (Fig. 2, panels 3,4). Similarly, antibody SMI31 requires the phosphorylation of both Ser-396 and Ser-404, which means that both serines become phosphorylated rapidly during stage 1 (Fig. 2, panel 6). SMI33 reacts only when Ser-235 is unphosphorylated so the gradual loss of reactivity means that this residue becomes phosphorylated only slowly (panel 7). Together these residues would account for 5 Pi, but only ≈4 Pi are observed by autoradiography, indicating that not all of these serines are phosphorylated at 100%. There are some subtle differences in the time–course of the immune response, compared to MAP kinase. For example, the SMI31 reactivity sets in early and precedes that of AT8 and SMI34, while the reactivity of SMI33 persists for a longer time, indicating that the mode of action of GSK3 is not identical to that of MAP kinase.

Additional information can be obtained by point mutations. As shown previously, the initial strong mobility shift induced by the kinase activity from brain extracts and by MAP kinase is due to the phosphorylation of Ser-404 (Fig. 6 of [4]). The same is true for GSK3, as illustrated in Fig. 3 (lanes 1–3). When Ser-404 is mutated into Ala, the initial rapid shift disappears, and initial phosphorylation is reduced to a low level (Fig. 3, compare lanes 2 and 3).

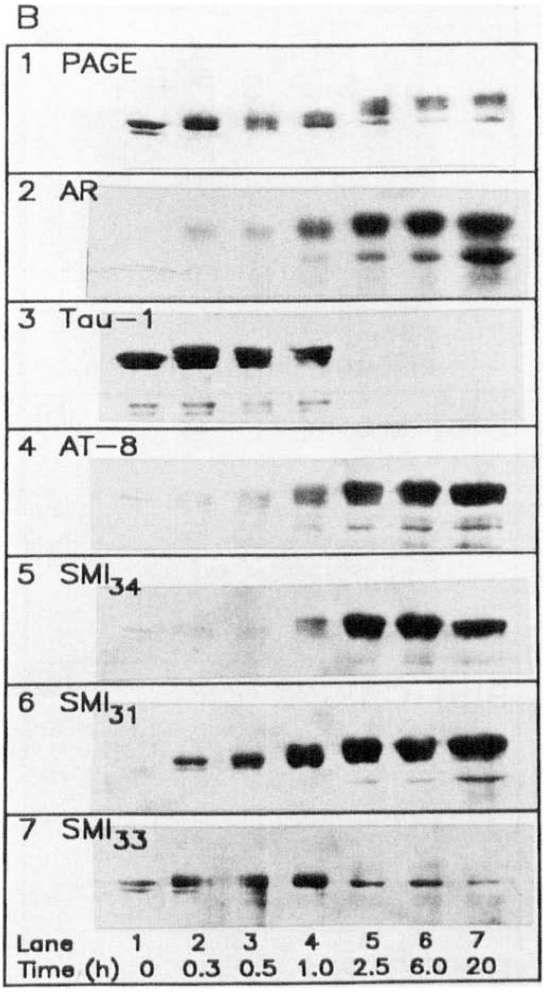

Fig. 2. Time-course of phosphorylation of htau40 by GSK3 and immune response. (Panel 1) SDS-PAGE of htau40 after incubation with the kinase between 0 and 20 h at 37°C. The minor lower band in lane 1 is a fragment. Note the progressive shift to higher M_r values, similar to the effects of brain extract and MAP kinase. (Panel 2) Autoradiography. (Panel 3) Immunoblot with the antibody TAU1 whose reactivity is lost after ≈2 h (following the phosphorylation of Ser-199 and Ser-202). (Panel 4) Immunoblot with the PHF-specific antibody AT8 whose reactivity requires the phosphorylation of Ser-199 and Ser-202. (Panel 5) Immunoblot with antibody SMI34 (conformation sensitive and against phosphorylated Ser). Panel 6) Blot with SMI31 (epitope includes phosphorylated Ser-396 and Ser-404). (Panel 7) Blot with antibody SMI33 which requires a dephosphorylated Ser-235. There are some differences with respect to phosphorylation by MAP kinase or the brain extract. The SMI33 staining persists for a long period, suggesting that Ser-235 is only slowly phosphorylated by GSK3. The staining of SMI31 appears very quickly, before that of AT8 or SMI34, showing that Ser-396 and Ser-404 are among the earliest targets of GSK3.

Another conclusion from the immunoblots is that GSK3 strongly prefers Ser–Pro motifs, in contrast to MAP kinase which also affects Thr–Pro. This can be concluded because the ≈4 Pi incorporated are needed to account for the phosphorylated epitopes. To test this we made construct AP11, a derivative of htau23 in which all 6 Ser– Pro are replaced with Ala–Pro (Fig. 4,

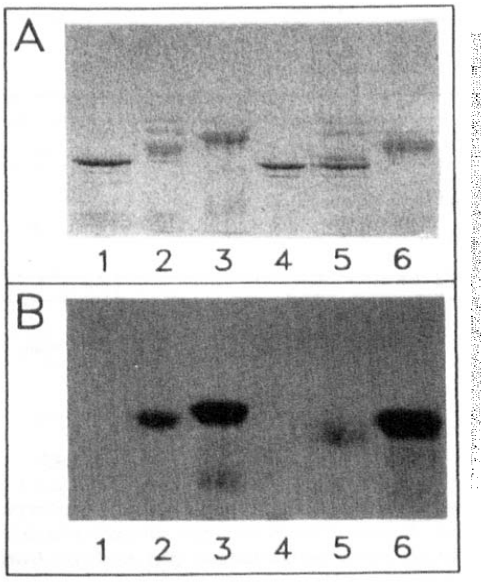

Fig. 3. Mobility shift of htau23 vs. mutant htau23/A404 upon phosphorylation with GSK3. A, SDS gel B, autoradiogram. Lanes 1–3, htau23 unphosphorylated and phosphorylated for 2 or 20 h. Note the pronounced shift and the clear incorporation of phosphate. Lanes 4–6, mutant Ser-404→Ala, unphosphorylated and phosphorylated for 2 and 20 h. The shift after 2 h is much smaller and the degree of phosphorylation much lower. This shows that the first strong shift and phosphorylation is at Ser-404, similar to MAP kinase and the brain extract kinase activity.

middle). AP11 is phosphorylated only to a minimal extent, <0.1 Pi per molecule, confirming that the Thr–Pro motifs remain largely unphosphorylated. The same result is obtained with construct AP17 (all 6 Ser–Pro and 8 Thr–Pro replaced by Ala–Pro, Fig. 4, top). Another construct, K18, containing only the 4 repeats (Fig. 4, bottom), is also not phosphorylated, indicating that no major sites are within the microtubule binding region. Thus, GSK3 and MAP kinase are similar in that they are both proline directed, but MAP kinase is more active with respect to Thr–Pro motifs.

3.3. *GSK3 and MAP kinase are associated with microtubules and with PHFs*

Considering that tau is a microtubule-associated protein one might expect that kinases that phosphorylate tau might be localized in the vicinity of microtubules. An example is the regulatory subunit of PKA which is bound to MAP2 [19]. We therefore tested whether MAP kinase or GSK3 were MAPs according to the usual criterion of co-purification through repeated cycles of assembly and disassembly. This was indeed the case. Fig. 5b shows that both the p42 and p44 isoforms of MAP kinase co-purified with porcine brain microtubules. Fig. 5c,d demonstrates the same for the case of GSK3 α and β. Interestingly, the microtubule-associated MAP kinase was not in an activated state since it

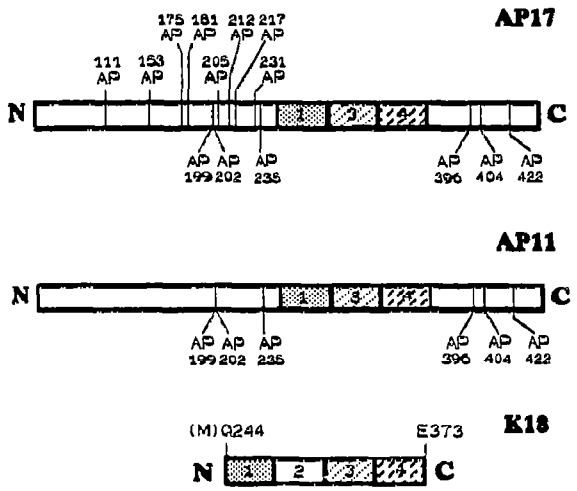

Fig. 4. Diagrams of tau constructs. Top, AP17, a derivative of htau23 with all Ser–Pro or Thr–Pro motifs altered into Ala–Pro. Middle, AP11, only Ser–Pro motifs changed into Ala–Pro. Bottom, K18, only 4 repeats of tau (derived from htau40).

was not phosphorylated on Tyr (as judged by immunoblotting, not shown).

Considering this result we asked whether the kinases were also associated with Alzheimer PHFs. The immunoblots of Fig. 6a demonstrate that GSK3 is present in normal and in Alzheimer brain in roughly equivalent amounts; the presence of MAP kinase was already shown earlier [7]. Moreover, the kinases co-purify directly with PHFs isolated by two different procedures, following [20] (Fig. 6b, lane 1) and [21] (lane 2).

The fact that GSK3 is associated with microtubules and PHFs and phosphorylates tau would suggest that the kinase might be able to affect the interaction between tau and microtubules. This would be in agreement with a common notion about the pathological effects of tau phosphorylation. However, to our surprise there was no influence on the binding. Fig. 7 shows the binding of htau23 to microtubules without phosphorylation, with phosphorylation by GSK3, and by the kinase activity of the brain extract. In the latter case there is a strong reduction in affinity (as reported earlier, [4]), but the effect of GSK3 itself is minimal.

4. DISCUSSION

One goal of current Alzheimer research is to define the modes of phosphorylation of tau protein and how this relates to the 'abnormal' phosphorylation of tau in PHFs [22]. Tau can be phosphorylated by several common kinases, such as PKA, PKC, CaMK, or CK II, in vitro [13,23], but in such cases the problem is to assess whether these modifications occur in vivo as well, and whether they are normal or pathological. Conversely, several phosphorylation sites have been identified in PHFs [24,25], but the kinases responsible are not

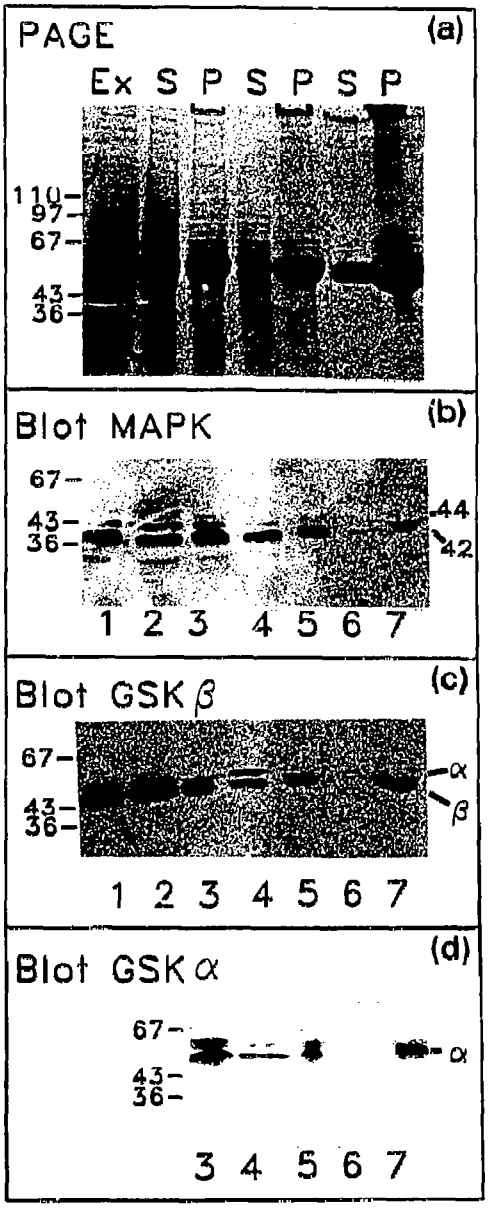

Fig. 5. Co-polymerization of MAP kinase and GSK3 with porcine brain microtubules. (a) SDS gel of microtubule purification stages. Ex, brain extract, supernatant after first cold spin; S, supernatant of first hot spin (tubulin and MAPs not assembled into microtubules after warming to 37°C); P, pellet of redissolved microtubules. The other lanes (S,P) show two further cycles of assembly and disassembly by temperature shifts (last pellet of microtubule protein was concentrated). (b) Blot with anti-MAP kinase, showing mainly the p42 isoform and some of the p44 isoform. (c) Blot with anti-GSK3β; note that this antibody shows some cross-reactivity with GSK3α. (d) Blot with anti-GSK3α. The blots show that both kinases and their isoforms co-purify with the cycles of microtubule assembly.

known. We have taken a hybrid approach in that we define the Alzheimer-like state of tau by a set of several independent criteria, search for kinases that induce this state, and identify the phosphorylation sites. The criteria include (i) the reaction with several antibodies that

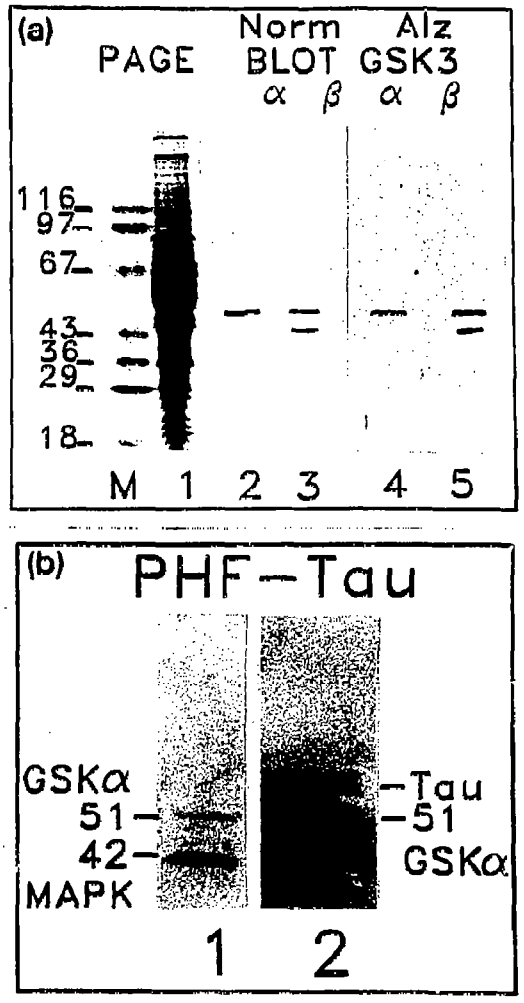

Fig. 6. (a) Identification of GSK3α and β in normal and Alzheimer brain extracts. M, markers; lane 1, SDS gel of normal brain extract; lane 2, immunoblot with anti-GSKα; lane 3, immunoblot with anti-GSK3β (with some cross-reactivity to α); lanes 4 and 5, same blots with Alzheimer brain extracts. (b) Immunoblot of PHF tau prepared according to [20] in lane 1 and [21] in lane 2. The blot of lane 1 was first developed with anti-MAP kinase, then stripped and developed with anti-GSKα. The blot of lane 2 was first developed with Alz50 antibody (against tau), then stripped and developed with anti-GSKα. Similar results are obtained with anti-GSKβ (not shown).

discriminate between PHF tau and normal tau and are directed towards distinct epitopes, (ii) the phosphorylation dependence of these antibodies, (iii) an electrophoretic mobility shift of a particular magnitude. This approach allowed us to prepare a brain kinase activity inducing the required characteristics in tau. In the course of the work we defined the epitopes of several antibodies, and in each case they were sensitive to phosphorylation [5,6]. Sequencing showed that the main sites were of the Ser–Pro or Thr–Pro type [4], and this lead the way towards the identification of MAP kinase as one key enzyme which was capable of inducing the Alzheimer-like state of tau [7].

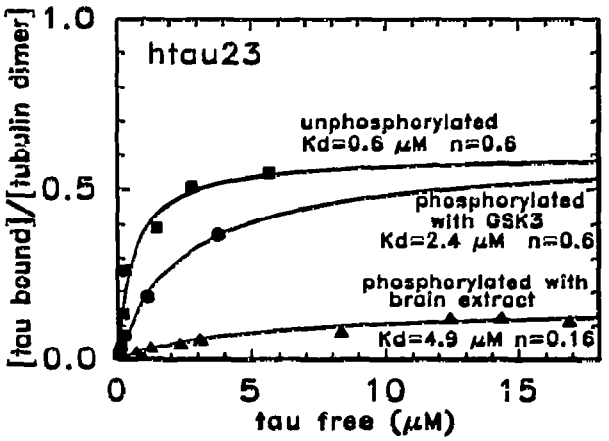

Fig. 7. Binding curves of htau23 to microtubules (made from 10 μM tubulin in the presence of 20 μM taxol). (■) htau23 unphosphorylated. (●) htau23 phosphorylated with GSK3, showing a stoichiometry comparable to the unmodified tau protein (saturating 0.6 per tubulin dimer). (▲) Control of htau23 phosphorylated with the brain kinase activity, showing a pronounced decrease in stoichiometry (see [4]). The solid lines show the best fits assuming independent binding sites.

We also tested other kinases of the same family to see if they had a similar effect as MAP kinase. Kinases of this family include the two or more MAP kinase isoforms (p42 or p44 MAP kinases, ERK1, ERK2, ERK3, for review see [26]), as well as KSS1, cdc2, GSK3α and β, and others (for review see [27]). We had already shown that cdc2 kinase had no measurable effect on antibody binding and gel shift. However, it has now turned out that GSK3 affects both properties. GSK3 was less efficient than MAP kinase in phosphorylating tau. The common feature of both kinases is that they phosphorylate Ser–Pro motifs; MAP kinase affects Thr–Pro motifs as well, in contrast to GSK3. This is possibly explained by the purification and the concentration of the enzyme. However, since the antibodies and the gel shift assay are mainly sensitive to the phosphorylation status of Ser–Pro motifs, the effects seen by SDS-PAGE, immunoblots and autoradiography are very similar with both enzymes (Fig. 2), with only minor differences that relate mainly to the kinetics of phosphorylation. The GSK3 preparation used in these experiments was obtained from bovine brain (or as immunoprecipitates from the cytosol of PC12 cells), and one could therefore expect to find it in human brain as well. This was indeed the case (Figs. 5 and 6).

Since the physiological function of tau appears to be regulated by phosphorylation one would expect a role not only for kinases but also for phosphatases. Several authors have shown that tau can be phosphorylated by several kinases (e.g. PKA, CaM kinase) whose effects can be reversed by several phosphatases; this is achieved most efficiently by PP-2a (for review see [28]). This phosphatase is also capable of counteracting MAP kinase and GSK-3 (Drewes et al., unpublished).

One surprising aspect is that MAP kinase and GSK3 are both microtubule-associated proteins (MAPs) as defined by the classical criterion of co-purification through cycles of microtubule assembly; moreover they are both associated with PHFs. It has been known for a long time that some kinases cycle together with microtubule protein. Since microtubule protein contains tubulin and various MAPs, the kinases could be associated with either one of these components. Both possibilities seem to occur. Some kinases are bound directly to tubulin and phosphorylate either tubulin-bound GDP (NDP kinase, [29]) or tubulin itself (the proto-oncogene c-*mos*, [30]). Others are bound to certain MAPs; the classical example is that of the R_{II} subunit of PKA attached to MAP2 [19]. The second case would seem to be the most interesting one in the context of Alzheimer pathology since a MAP-associated kinase might also become PHF associated. Recently, Ishiguro et al. [31] described a tubulin-activated kinase that phosphorylated tau, and Vincent and Davies [32] described a PHF-associated kinase. Finally, there are kinases associated with other cytoskeletal elements (e.g. neurofilaments) which can phosphorylate tau as well [33]. Since the phosphorylation sites of these kinase preparations are not well defined it is difficult at this moment to relate them directly to our findings.

In conclusion, GSK3 and MAP kinase must now be counted among the microtubule-associated and PHF-associated kinases. This is unexpected in view of their potentially pathological role, but it illustrates that the distinction between the 'normal' and 'pathological' state may be very small. GSK3 induces the Alzheimer-like characteristics in tau protein in terms of several independent criteria, similar to the ones described for MAP kinase [7]. As the name implies, GSK3 phosphorylates glycogen synthase and thereby inactivates this enzyme, and it phosphorylates other proteins as well, for example the nuclear proto-oncogenes, c-*jun* and c-*myb* (for review see [34]). One of the earliest functions discovered was the activation of protein phosphatase-1, hence the alternative name of activating factor, F_A [8]. Its two isoforms α and β have been cloned and are highly homologous [9]. An interesting feature is that GSK3 often operates by a stepwise mechanism that depends on a priming phosphorylation by another kinase (for review see [35]). In the case of recombinant tau this mechanism is unlikely, but we cannot exclude it for the phosphorylation in vivo. In any case, the fact that both MAP kinase and GSK3 play an important role in cellular signal transduction strengthens the view that the disregulation of some signal may eventually be responsible for the pathological degeneration of tau protein.

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