Glycogen synthase kinase (GSK) 3-beta directly phosphorylates serine 212 in the regulatory loop and inhibits microtubule affinity regulating kinase (MARK) 2

Thomas Timm*, Kiruthiga Balusamy*, Xiaoyu Li, Jacek Biernat, Eckhard Mandelkow, Eva-Maria Mandelkow#

Max-Planck-Unit for Structural Molecular Biology
Notkestrasse 85, 22607 Hamburg, Germany

* both authors contributed equally

Running title: GSK3β inhibits MARK/Par-1

# Address correspondence to: Eva-Maria Mandelkow; Tel: +49 40 8998 2810;
Email: mandelkow@mpasmb.desy.de

MARK/Par-1, a kinase family with diverse functions, especially in inducing cell polarity, can phosphorylate microtubule-associated proteins in their repeat domain, cause their detachment from microtubules and thereby microtubule destabilization. Because of its role in abnormal phosphorylation of Tau protein in Alzheimer's disease we searched for regulatory kinases. MARK family kinases can be activated by phosphorylation of a conserved threonine (Thr208 in MARK2), and inactivated by phosphorylation of a serine (Ser212), both in the activation loop of the catalytic domain. Activation is achieved by the kinases MARKK/TAO1 or LKB1, but the inactivating kinase was not known. We show here that GSK3β serves the role of the inhibitory kinase. Since GSK3β can also phosphorylate Tau at sites outside the repeat domain, the activation of GSK3β and concomitant inactivation of MARK can shift the pattern of pathological phosphorylation of Tau protein in AD.

Introduction

The kinase MARK/Par-1 (Microtubule associated protein/microtubule affinity regulating kinase / Partitioning defective 1) was initially discovered because of its ability to phosphorylate Tau protein at the KXGS motifs located in the microtubule binding domain (1, 2). Tau can be phosphorylated at various sites by other kinases (e.g. Mitogen-activated protein kinases (MAPK), Glycogen synthase kinase 3β (GSK3β), Cyclin-dependent kinase 5 (Cdk5), cAMP-dependent protein kinase (PKA)), but the phosphorylation at KXGS motifs results in the strongest reduction of Tau binding to microtubules, with the consequence that microtubules, which serve as tracks for axonal transport, are no longer stabilized and fall apart. The detached tau accumulates in the cytosol and gradually polymerizes into paired helical filaments that bundle up into neurofibrillary tangles, forming one of the two pathological aggregates in Alzheimer's disease (reviews 3, 4).

MARK/Par-1 kinases belong to the AMPK/Snf1 subfamily (adenosine monophosphate-activated protein kinase/Sucrose non-fermenting 1) of the Ca2+/calmodulin-dependent kinase II (CaMK) group (5). Homologous genes have been found in eukaryotes ranging from yeast to mammals. In mammals the MARK family consists of four members (MARK1 - 4) with a conserved domain organisation (Fig. 1A). An N-terminal header (N) precedes the catalytic domain (6) which is linked to a putative common docking domain (CD; 7) as found in kinases of the extracellular signal–regulated kinase (ERK) family (8). The UBA and KA1 domains are conserved among the AMPK related protein kinases and MARKs, their functions are poorly understood but may be related to ubiquitin dependent signalling or supramolecular folding with impact on activity (10).

One important function of MARK/Par-1 is the regulation of the microtubule dynamics by altering the affinity of the tau protein and its functional relatives MAP2c and MAP4 towards the microtubules (11, 12, 13).
MARK/Par-1 plays a pivotal role in the establishment and maintenance of cell polarity in different organisms, e.g., asymmetric distribution of P-granules in the C. elegans zygote (14), axis formation in the D. melanogaster embryo (15), asymmetric organization of polarized epithelial cells (16), polarized neurite outgrowth and neuronal polarity in neuroblastoma cells and hippocampal neurons (17, 18, 19). However, other AMPK-related kinases may also be involved in neuronal polarization as recently shown for the SAD-(synapses of amphids defective) kinases (20, 21).

The significant role of MARK/Par-1 in polarity development requires a tight regulation of its activity. Some regulatory aspects have been elucidated (reviewed in 22, 23). The kinases MARKK/TAO1 (thousand and one amino acids) and the serine/threonine kinase LKB1 activate all four MARK isoforms by phosphorylation of a threonine residue (T208 in MARK2) in the activation loop of the catalytic domain (24, 25). This threonine is conserved in many eucaryotic serine/threonine kinases and often regulates their activity. Fig. 1B displays the activation loops of several serine/threonine kinases where phosphorylation sites are highlighted. Another modulator of the activity of MARK is the kinase p21-activated kinase 5 (PAK5): The two kinases interact via their kinase domains so that MARK (but not PAK5) is inhibited (26). The scaffold protein 14-3-3 binds to their kinase domains so that MARK (but not PAK5) is activated (28, 29, 18).

In MARK, the regulatory threonine in the activation loop is followed by a serine (S212 in MARK2) which is phosphorylated at S212 is inactive (24). However the kinase responsible for this regulatory event is a matter of debate. A recent report claimed that GSK3β phosphorylates this site and activates MARK2 (20). By contrast, we report here data showing that GSK3β in fact inhibits MARK by phosphorylating this site. Since both kinases are involved in several interconnected regulatory pathways, this finding has important consequences for interpreting the roles of GSK3β and MARK.

Materials and methods

MARK2 preparation from E. coli and MARKK preparation from Sf9 cells: Recombinant kinases were expressed as fusion proteins with a polyhistidin-tag in E. coli (DE3 pLys) or Sf9 cells using the baculovirus system BaculoGold™ (Pharmingen) respectively. Cells were lysed in buffer A (50 mM Tris-HCl pH 8.5, 100 mM NaCl, 50 mM imidazole, 5 mM CHAPS, 1 mM benzamidine, 1 mM β-mercaptoethanol, 1 mM PMSF) with a French Press. The supernatant was loaded onto a NiNTA™-Column (Qiagen). After washing with buffer A the protein was eluted with a short gradient of 50-500 mM imidazole. The eluted protein was dialysed against buffer B (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM benzamidine, 1 mM DTT, 1 mM PMSF, 50 % glycerol) and stored at −20°C.

GSK3β preparation from E. coli and Sf9 cells: Recombinant GSK3β was expressed as fusion protein with a polyhistidin-tag in E. coli (DE3 pLys) and in Sf9 cells using the baculovirus system BaculoGold™ (Pharmingen). Cells were lysed in buffer C (50 mM sodium phosphate pH 7.9, 100 mM NaCl, 50 mM imidazole, 5 mM CHAPS, 1 mM benzamidine, 1 mM β-mercaptoethanol, 1 mM PMSF) with a French Press. The supernatant was loaded onto a NiNTA™-Column (Qiagen). After washing with buffer C the protein was eluted with a short gradient of 50-500 mM imidazole. Fractions containing GSK3β were pooled and dialysed against buffer D (50 mM sodium phosphate pH 7.9, 100 mM NaCl, 1 mM benzamidine, 1 mM β-mercaptoethanol, 1 mM PMSF). The sample was loaded onto a MonoS™-Column (Pharmacia/GE-Healthcare) and eluted in a gradient of 100-1000 mM NaCl. The eluted protein was dialysed against buffer B (see above) and stored at −20 °C.

Kinase assay: Kinase activities were assayed in buffer E (50 mM Tris-HCl pH 8.0, 5 mM MgCl2, 2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 0.5 mM benzamidine) for 120 minutes at 30°C. Final concentration of [32P] ATP (3.7 x 10⁷ MBq/mol) and substrate peptide were 100 µM. As substrate we used a peptide derived from the first repeat of tau protein containing S262 in the KXGS motif (TR1-peptide NVKSKIGSTENLK; 11). Reactions were stopped by addition of half the volume of 30 % (w/v) TCA. After centrifugation the supernatant was applied to phosphocellulose-paperdiscs, washed with phosphoric acid (0.1 M), dried by air and radioactivity was measured in a scintillation counter (Tricarb 1900 CA, Packard). Data show averages of 3 experiments (bars = s.e.m.). Alternatively, reactions were stopped by addition of sample loading buffer and subjected to SDS-PAGE. After staining with Coomassie (RothBlue™) gels were dried and radioactivity was detected with a BAS3000 phospho-imaging system (Raytest). Recombinant
p38/SAPK was expressed in Sf9 cells, ERK1/2 was prepared from porcine brain (52), Cdc2/CyclinB and Cdk5/p35 were obtained from New England Biolabs, and CKI was a generous gift from L.A. Pinna, University of Padova, IT).

**Cell culture:**

**Sf9 (Spodoptera frugiperda) cells** were grown in a 27 °C incubator in monolayer culture with Graces medium supplemented with 10 % FCS and 100 μg/ml penicillin/streptomycin mixture. Confluent monolayers were sub-cultured by scraping the cells and diluting in the ratio of 1:4 in complete medium. For expression of proteins in Sf9 cells the actively growing cells (80 % confluence) were infected with recombinant baculovirus. The MOI (Multiplicity of Infection) was 1-3. The cells were incubated with the virus at 27 °C for 66-72 hours.

**Neuroblastoma N2a/F113** cells stably expressing hTαu40 were grown in a medium containing MEM (Minimal Earle’s medium), 10% FCS, 1% L-glutamine, 1% nonessential amino acids and 600 μg/ml gentamycin 418. The cells were maintained in a humidified atmosphere containing 5 % CO2 at 37 °C. For Western blot analysis, 1.5-3x106 cells/well were grown in a 6 well plate for 24 hours and the cells were transiently transfected with appropriate plasmids using Effectene (Quiagen). 24 hours after transfection, the cells were differentiated with 2 ml of differentiation medium (Minimal Earle’s medium), 10% FCS, 0.1 % L-glutamine, 1% nonessential amino acids and 600 µg/ml gentamycin 418. The cells were maintained in a humidified atmosphere containing 5 % CO2 at 37 °C. For Western blot analysis, 1.5-3x106 cells/well were grown in a 6 well plate for 24 hours and the cells were transiently transfected with appropriate plasmids using Effectene (Quiagen). 24 hours after transfection, the cells were differentiated with 2 ml of differentiation medium (MEM, 0.1 % FCS, 0.1 % nonessential amino acids, 2 µM retinoic acid) for 6 hours. The cells were washed once with 3 ml of PBS. Then 1 ml of PBS was added, the cells were scraped and centrifuged at 14,000 rpm for 10 seconds. The cell pellet was used immediately or frozen in liquid nitrogen and stored at –20 °C.

**CHO wildtype cells** and stably transfected with hTαu40 were grown in HAM's F12 medium with 10% FCS and incubated in a humidified atmosphere containing 5 % CO2 at 37 °C. For stable transfection we added G418 (600 μg/ml, Invitrogen) to the culture medium. Cells were seeded at 70 % confluency in 24-well plates (1.8 cm²) on coverslips and transfected with DOTAP (Roche), followed by immunofluorescence analysis at various time points.

**PC12 cells** (2x10⁴ cells) were grown in a 24 well plate (2 cm²) pre-coated overnight with poly-D-lysine. The cells were incubated at 37°C with 5% CO2. Cells were grown in a medium containing DMEM (Dulbecco's Modified Eagle Medium), 4500 mg/L glucose, 1 % L-glutamine, 10 % FCS, 15 % HS and (100 µg/ml) penicillin/streptomycin. The differentiation of PC12 cells was carried out in differentiation medium (DMEM: F12 = 1:1) containing 0.1 % serum and 100 ng/ml NGF for 24-72 hours.

**Cortical Neurons:** Cortex tissue dissected from E18 rat embryos was digested with 0.1% trypsin for 30 minutes. Plating medium was then added and the dissociated cells were gently centrifuged and resuspended in plating medium. The dissociated neurons were plated at a density of 100 neurons/mm² on a 24 well plate (2 cm²) pre-coated overnight with poly-D-lysine. After culturing for 4 hours, the medium was changed to neuronal culture medium (Neurobasal medium with B-27 and L-glutamine) and the cells were grown for 3 to 4 days.

**Immunofluorescence:** Cells were fixed with 3.7% formaldehyde for 15 minutes at RT. Then the cells were washed with PBS (3 times). Permeabilisation was carried out by adding 80% ice cold methanol and incubated for 5 minutes at –20 °C. Cells were washed 3 times with PBS and blocked with 10 % goat serum at 37 °C for 45 minutes. After blocking, the cells were treated with primary antibody (β-actin 1:1000, Sigma; Tubulin 1:250, Sigma; 12E8 1:200, gift of P. Seubert, Elan Pharma; ABP₇T208 1:200, ref 24; GSK3β(pS9) SA-310 1:100, Biomol) for 1 hour or overnight at 37°C and then washed 3 times with PBS. The appropriate secondary antibody was added and the cells were incubated at 37 °C for 1 hour followed by washing 3 times with PBS. Finally the cover slips were mounted for microscopy.

**Western Blotting:** The proteins were separated on SDS gels and electro-transferred to PVDF membranes by semi-dry blotting (1 mA/cm², 1 hour). The membranes were blocked with 5 % nonfat dry milk in 1x TRIS-buffered saline with 0.1 % tween20 (TBST) for 1 hour at RT and then treated with the selected primary antibody (Ab3212 1:500, ref. 24; 12E8 1:1000, Elan Pharmaceuticals; A0024 (K9JA) 1:10000, DAKO; PHF1 1:500; Davies; HA 1:1000, Cell Signaling; β-Actin 1:1000, Sigma; GFP 1:2000, Clontech; MARK1/2 SA4632 1:2000, Eurogentec; GSK3β(pS9) 1:1000, Biomol) in TBST at 37 °C for 1 hour. The membranes were washed 3 times with 1xTBST. The corresponding secondary antibody in TBST was added and the membranes were incubated at 37 °C for 45 minutes followed by washing with 1xTBST (3 times). The substrate reaction was carried out with ECL detection reagents (GE Healthcare) and visualized using the LAS 3000 system (Raytest). Densitometric analysis was performed with the software AIDA V3.42 (Raytest Isotopenmessgeraete GmbH).
Results

GSK3β phosphorylates MARK2 at serine 212 and reduces its activity in vitro.

MARK isolated from porcine brain is in part phosphorylated at both T208 and S212 (numbering according to MARK2; see Fig. 1) in the activation loop (11). Phosphorylation of the first threonine (T208) by MARK leads to the activation of all four MARK isoforms (24). This activation is possible only if the subsequent serine (S212) is present: changing S212 to Ala or Glu abolishes the kinase activity completely, despite the activating phosphorylation of T208 (24). This suggests that the phosphorylation of this residue might also lead to inactivation of MARK. Since the S212 in the activation loop is followed by a proline, we tested several proline-directed kinases as candidates. As seen in Fig. 2, different kinases such as the stress- or mitogen-activated protein kinases p38/SAPK, ERK1, ERK2, the cyclin dependent kinases Cdc2, Cdk5, and GSK3β were tested. Casein kinase I (CKI) was also tested because the serine in the activation loop of MARK resembles the substrate recognition site of CKI (http://phospho.elm.eu.org/). Only GSK3β exhibited an effect on MARK by reducing its activity (Fig. 2A). This led us to focus on GSK3β which is also of great interest in the context of AD because it phosphorylates tau efficiently at Ser/Thr-Pro motifs that are elevated in MARK2T208A and MARK2 S212A mutants. Similar to the wild type kinase, MARK2T208A is reduced in its activity by GSK3β (Fig. 2C, compare lane 1 with 2 and 3 with 4). The mutation of the S212 to Ala renders the kinase inactive (see also 24), hence there is no effect of GSK3β (lanes 5 and 6). The autoradiography in Fig. 2D shows that the inactivation of MARK by GSK3β is accompanied by its phosphorylation. Only the wild type and the T208A mutant are phosphorylated, but not the S212A mutant (lanes 2 and 4, compare with 6). We conclude that GSK3β phosphorylates MARK2 on S212 to inactivate the kinase. This is further confirmed by a western blot of equivalent samples shown in Fig. 2E (lanes 1, 2, 3). An antibody specific for phosphorylated S212 of MARK2 (AB[5212], rabbit antibody raised against a peptide from the activation loop phosphorylated at S212; see 24) gives a signal in the cases of wildtype and T208A mutants phosphorylated by GSK3β (lanes 2, 3), but not with the S212A mutant (lane 4). Lane 1 confirms that the antibody reacts with MARK phosphorylated at S212. This lane shows MARK, a partially purified kinase from porcine brain identified as a mix of MARK1 and 2 (11). Note that the apparent molecular weight is significantly higher than expected for MARK1 or MARK2 (110 kDa vs. 88 or 81 kDa), probably due to post-translational modifications, especially phosphorylation. This enzyme is easily degraded by proteases, as evidenced by lower Mr fragments (2, 11). Furthermore, GSK3β inhibits MARK2 even when it is activated by MARKK or by the activating mutation T208E (Fig. 2F). The activity of MARK2, activated by MARKK (compare lane 1 with 2) is reduced upon addition of GSK3 (lane 3). As expected, the inactive mutant S212A is neither activated by MARKK (lanes 4, 5), nor inhibited by GSK3β (lane 6). The active mutant T208E (lane 7) is markedly reduced in its activity by incubation with GSK3β (lane 8). These results clearly indicate that GSK3β inhibits MARK2 by phosphorylating S212. The same results were obtained with GSK3β expressed and purified from Sf9-cells.

MARK2 wildtype and mutants phosphorylate tau differentially.

The activity of MARK2wt and mutants was checked in cellular conditions by transfecting the kinases into N2a/F113 cells stably expressing htau40 (human Tau, longest isoform in CNS). After 24 hours the cells were differentiated with retinoic acid for 6 hours. The results were monitored on western blots with the pan Tau antibody K9JA and the pS262-specific Tau antibody 12E8. As expected, wildtype MARK2 exhibited high activity towards Tau at S262, compared with untransfected cells (Fig. 3A, lanes 1, 2). The non-
activatable MARK2T208A mutant had only low activity (lane 3) and the S212A mutant as well as the T208A/S212A mutant resulted in no 12E8 reactivity (lanes 4, 5). The level of MARK2wt and mutants and the level of tau are similar in all transfections (shown by the loading control of β-actin staining). These data on cells correlate closely with the in vitro data, arguing that S212 must be present for the activity of MARK2 (i.e. it cannot be changed by mutation or phosphorylation), and that the phosphorylation of T208 is needed for activation.

**Co-expression of active GSK3βS9A with MARK2wt or active MARK2T208E in cells reduces the phosphorylation of tau at S262.**

In order to investigate the effect of GSK3β on MARK in cells we transfected N2a/F113 cells with either MARK2wt or the constitutively active mutant MARK2T208E alone or together with constitutively active GSK3βS9A. After 24 hours of transfection, the cells were differentiated with retinoic acid for 6 hours. The activity of MARK was monitored by the phosphorylation of Tau at S262 using the 12E8 antibody in western blots. Comparable amounts of kinases in single and double transfections are depicted by HA-antibody for MARK2wt and with GFP-antibody for MARK2T208E and GSK3βS9A which are both fused to ECFP. As seen in Fig. 3B, the expression of MARK2wt and constitutively active MARK2T208E increased the phosphorylation of tau at S262 compared to untransfected cells (compare lanes 2, 3 with lane 1). But upon co-transfection with active GSK3βS9A this phosphorylation strongly decreased (lanes 4, 5). Although we see a reduction in the expression level of MARK2T208E when co-transfected with active GSK3βS9A (lane 5) the phosphorylation of Tau at S262 is reduced to a much greater extent. These results demonstrate that GSK3β inhibits MARK2 not only in vitro but also in cells.

**Co-expression of GSK3β with MARK2 stabilizes the microtubule network.**

In CHOwt cells the activity of MARK2 leads to drastic morphological changes (13). Since CHO cells do not have Tau, MARK phosphorylates the related MAP4-like protein at its KXGS sites in the repeat domain and abolishes its microtubule stabilizing function. As a result the microtubules break down, the cells shrink and finally die. CHO cells were transfected with constitutively active MARK2 (EYFP-MARK2T208E). After 16 hours, cells were fixed and stained for microtubules with antibody YL1/2. Untransfected cells have an extended shape with a clear microtubule network. As expected, transfection of active EYFP-MARK2T208E alone leads to loss of microtubules and shrinkage in 55% of the cells (Fig. 4, upper row, arrows). In contrast, co-expression of both active MARK2 and active GSK3β retains the microtubule network in 60% of the cells (Fig. 4, lower row) indicating that MARK2 is inhibited by GSK3β in these cells.

**GSK3β inhibits MARK in PC12 cells.**

In contrast to the detrimental effect of MARK on microtubules in CHO cells, overexpression in PC12 cells results in neurite outgrowth. Active MARK (ECFP-MARK2T208E) or active GSK3β (mRFP-GSK3βS9A) or both were transfected into PC12 cells and the cells were differentiated with NGF for 48 hours. In transfected cells ECFP-MARK2T208E is located at the plasma membrane, similar to the endogenous protein (24). These cells exhibit strong 12E8 staining and the formation of neurite outgrowth after addition of NGF (Fig. 5, upper row) showing that the exogenous MARK is not toxic in these cells. In contrast, transfection of active mRFP-GSK3βS9A leads to a lower level of phosphorylated S262-Tau than in untransfected cells, and after NGF treatment no neurite outgrowth is observed (middle row), indicating that GSK3β inhibits the endogenous MARK. Moreover, upon co-transfection of active GSK3βS9A and active MARK2T208E the activity of MARK2 is inhibited and as a result the cells do not form neurites (lower row).

**Endogenous inactive GSK3β and active MARK co-localize in the neurite tips of PC12 cells and in the growth cones of rat cortical neurons.**

To further investigate the functional relationship between GSK3β and MARK we examined the endogenous activities of both kinases in differentiated PC12 cells and in stage three cortical neurons (34). To monitor the activation state of MARK we probed the cells with an antibody against phospho-threonine 208 in the activation loop of MARK, labeling the activated enzyme (ABpT208, ref. 24). Furthermore we probed the cells with an antibody against phospho-serine 262 of Tau as an indicator for MARK activity. Both signals co-localize strongly in the tips of the cell processes and at the membranes (Fig. 6 and 7, A, B and C). At the same time, inactive GSK3β (detected by an antibody against phospho-serine 9) is also predominantly found at the tips, co-localizing with active MARK (Fig. 6 and 7, A, B and C). The tips also show a high level of co-localization of actin and active MARK, consistent with our previous observations (24) (Fig. 6 and 7, A, B and C). These...
results indicate a zone of a highly dynamic actin and microtubule cytoskeletons where active MARK and inactive GSK3β are involved in the growth of an axon.

Discussion

The microtubule associated protein Tau stabilizes microtubules in axons as tracks for axonal transport. In degenerating neurons, tau becomes missorted, hyperphosphorylated and detaches from microtubules, which leads to the breakdown of the microtubules and the collapse of energy supply and transport in the axons of neurons. In addition, the hyperphosphorylated tau aggregates into paired helical filaments (PHFs), which deposit as neurofibrillary tangles (NFTs) in the neurons. Binding of tau protein to microtubules is primarily regulated by phosphorylation of the KXGS motifs located in the repeats of the microtubule binding domain of tau. MARK has been identified as a kinase family phosphorylating the structural microtubule-associated proteins (MAPs) tau, MAP2 and MAP4 at the KXGS motifs, thereby reducing their affinity to microtubules and disrupting microtubule stability (11). This type of phosphorylation is enhanced in the early stages of Alzheimer’s disease (35). Furthermore, it was proposed that in a *Drosophila* model the activation of MARK primes tau for the phosphorylation by other kinases like GSK3β and Cdk5, which in turn triggers the aggregation of tau into PHFs, a hallmark of AD neurofibrillary pathology (36).

MARK and its homologues PAR-1 are involved in generating and maintaining cell polarity during development in *Drosophila* and *C.elegans* (37). In mammalian cells MARK/PAR-1 is important for establishment of neuronal polarity in neuroblastoma cells and hippocampal neurons (17, 18, 19). Other important functions are linked to cell cycle regulation (38), Wnt signaling (39), and exocytosis (10). As MARK/PAR-1 is involved in the regulation of many essential pathways, it is important to understand the control of its activity.

MARK/Par-1 kinases are activated by MARKK/TAO-1 or LKB1 by phosphorylation of a conserved threonine in the activation loop, T208 in MARK2 (see Fig. 1B; 24, 40). Unlike other kinases, a fraction of MARK2 purified from brain is additionally found to be phosphorylated at a second site S212, which is adjacent to the T208 (11). Site directed mutagenesis of this residue to the phosphoserine-mimicking Glu suggested that phosphorylation might be inhibitory (24). X-ray analysis of the catalytic domains of MARK1 and MARK2 (41, 7) confirm the important function of this particular serine in stabilizing the activation loop. Fig. 8 displays the structural model of MARK2. It shows that S212 forms hydrogen bonds to the catalytic Asp (D175) and a nearby Lys (K177), as proposed for PKA (Fig. 8B, residues involved in PKA are T201, D166 and K168; 42). Change of S212 to Ala or Glu disrupts this stabilizing interaction, and the same is achieved when S212 is phosphorylated (Fig. 8C). The data presented here demonstrate that GSK3β is able to phosphorylate MARK2 at S212 *in vitro* and that this indeed results in an inactive kinase, irrespectively of the phosphorylation status of the activating residue T208. Since the activation loops of the four MARK isoforms are similar, these results hold for all isoforms.

To confirm the inhibition of MARK by GSK3β we expressed combinations of these kinases in several cell lines. Wild type and constitutively active mutants of MARK and GSK3β were expressed in CHO, N2a/F113 and PC12 cells. In all cases the co-expression of GSK3β with MARK resulted in the inhibition of MARK activity and the preservation of the microtubule network.

Many substrates of GSK3β have to be primed by pre-phosphorylation at a site four residues downstream of the phosphorylation site. The classic case is glycogen synthase (primed by casein kinase II), others are Tau (primed by Cdk5/p35), β-catenin (by casein kinase I), or CREB (cAMP-response element-binding protein, by PKA) (reviews 43, 44). In this respect, MARKs are not typical substrates for GSK3β because no priming phosphorylation is necessary. The activating phosphorylation on T208 by MARKK is N-terminal to the GSK3β target site and has no influence on the phosphorylation of S212 by GSK3β.

Our conclusions are opposite to those of Kosuga et al. (30) who claimed that MARK could be activated by phosphorylation of S212 by GSK3β. Our explanation of the discrepancy is that these authors have only tested point mutations of MARK in cells without checking their actual kinase activities. For instance, they observed that overexpression of the MARKS212A mutant had no effect on the phosphorylation of Tau at S262 and concluded that S212 of MARK was the residue responsible for activation by phosphorylation, without noticing that the S212A mutant is not active as such because it cannot stabilize the activation loop by H-bonds (see above).

However, our data are in line with results from other investigators: Jiang et al. (45) showed that GSK3β...
activity is differentially distributed in the axon versus the
dendrites. A constitutively active GSK3β mutant
inhibited axon formation, whereas reduction of GSK3β
activity by pharmacological or peptide inhibitors or
siRNAs resulted in multiple axons. A pool of inactive
S9-phosphorylated GSK3β is localized to the tips of
axons where the highest dynamics of microtubules is
needed and therefore MARK is expected to be active.
We confirmed this by showing that endogenous MARK
is active and endogenous GSK3β is inactive at the tips of
neurites in NGF-differentiated PC12 cells and in the
growth cones of differentiating rat cortical neurons.
Activation of GSK3β at the leading edge of neuronal
growth cones by semaphorin 3A inhibits growth cone
advance and is followed by growth cone collapse (46),
consistent with MARK inhibition. Furthermore, active
GSK3β impairs neuronal polarisation by phosphorylating
and inhibiting CRMP-2 (collapsing response mediator
protein 2), which binds to tubulin dimers and promotes
MT assembly when unphosphorylated (47). Conversely,
in neurospheres, the inhibition of GSK3β results in
increased differentiation of neuronal precursors into
dopaminergic neurons (48). Partial or complete knock-
down of GSK3β by shRNA in cultured neurons and
tissue slices (49) showed that inactivation of GSK3β at
neurite tips leads to axon elongation, whereas reduction
of GSK3β throughout the neuron causes axon branching,
and strong suppression of GSK3β results in termination
of axon growth.

Luo and colleagues have shown that reduction of
MARK2 activity by siRNA or by the Par3/Par6/aPKC
complex leads to a loss of polarity and multiple axons in
hippocampal neurons (18). Conversely, the elevation of
MARK activity promotes axons outgrowth, and the same
holds for the related SAD kinase (17, 20). This supports
the model that for neurite outgrowth MARK has to be
largely inactive in the shafts of axons where the cells
need stable microtubules. On the other hand, MARK has
to be very active in the growth cone where highly
dynamic microtubules are required for growth and
retraction of the axon tip. Another recent report revealed
an additional pathway signalling from LKB1 via SAD-
kinases to pS262-Tau in cortical neurons (21). To date it
is unclear if GSK3β is also acting on SAD in an
inhibitory manner, but it is interesting to note that in
Xenopus the kinase LKB1 regulates Wnt-signalling by
inhibition of GSK3β (50). Kojima and co-workers
showed evidence that phosphorylation of Tau at S262 by
MARK2 not only causes suppression of tubulin
polymerization but also proteasome-mediated
degradation of Tau (51). This indicates a higher degree
of complexity in the signalling pathways which has yet
to be elucidated.
References


**Footnotes:**

**Acknowledgements:** We are grateful to Dr. P. Davies (Albert Einstein College, Bronx, USA) and Dr. P. Seubert (Elan Pharmaceuticals, South San Francisco, USA) for providing the antibodies PHF-1 and 12E8. We thank Edda Thies and Kerstin Skokann for help with cell culture procedures. This research was supported in part by grants from DFG.

**Keywords:** Kinase regulation / microtubules / tau / cell polarity / Alzheimer's disease

**Running title:** GSK3β inhibits MARK/Par-1

**Abbreviations:** AD = Alzheimer's disease; GSK3β = glycogen synthase kinase 3β; MAP = microtubule-associated protein; MARK = MAP/microtubule affinity regulating kinase; MARKK = MARK activating kinase; Par-1 = partitioning-defective mutant 1 kinase.
Figure legends:

**Fig. 1: Diagram of MARK2.**
(A) Kinases of the MARK family comprise five basic domains, shown here for MARK2. The amino-terminal header sequence (N) is followed by the catalytic domain. Adjacent are the common docking site (CD) and the ubiquitin-associated domain (UBA). A spacer domain connects it with the tail domain which contains the kinase-associated domain (KA1). MARK can be activated by phosphorylation of the conserved T208 in the activation loop (24, 25). The conserved S212 was found to be phosphorylated in brain (11) but the responsible kinase was not known. As shown in this report, the site is phosphorylated by GSK3β, causing an inhibition of MARK2.
(B) Activation loops of selected Ser/Thr-kinases. The conserved threonines or serines corresponding to T208 of MARK2 are crucial for activation. Putative phosphorylation sites are underlined, known sites are boxed in grey. The residue equivalent to S212 is conserved, too. Numbers on the right are the genbank accession numbers.

**Fig. 2: GSK3β phosphorylates MARK and reduces its activity in vitro.**
(A) Effect of selected kinases on MARK activity. Recombinant MARK2 was incubated with different kinases and the activity towards the MARK-substrate peptide TR1 measured. Only the kinases MARKK and GSK3β had an influence on MARK activity. While MARKK activated MARK tenfold (1 unit = 55 nmol/min/mg), GSK3β significantly reduced MARK activity (Student’s t-test; p < 0.01 for MARKK and GSK3β compared to buffer). Note that the y-axis is not linear for better clarity.
(B) Recombinant MARK2 and GSK3β were pre-incubated for various times (upper x-axis), followed by incubation with the MARK-substrate peptide TR1 for 30 minutes (total incubation time on bottom x-axis). As a result the activity of MARK2 is reduced to 20% within two hours (grey curve). In contrast, MARK incubated alone does not change its basal activity of 55 nmol/min/mg (= 1 unit, black line).
(C and D) MARK2 wild type and mutants were incubated in the presence or absence of GSK3β. GSK3β reduces the activity of MARK2 (compare lanes 1, 2). The lower basal activity of the T208A mutant, which cannot be activated by MARKK or LKB1, is also reduced by GSK3β (lanes 3, 4). Mutation of S212 in the activation loop to Ala yields an inactive kinase which is not affected by GSK3β (lanes 5, 6). Asterisks indicate statistical significance (Student’s t-test; *p < 0.05; **p < 0.01, =p>>0.2).

The SDS-gel shows that equal amounts of MARK proteins were used in this assay. The autoradiograph (D, bottom panel) of the SDS gel shows that GSK3β can phosphorylate MARK2wt and MARK2T208A but not MARK2S212A. Note the increased signal in lanes 2 and 4 (MARK and GSK3β) compared to lanes 1 and 3 where MARK2 is only autophosphorylated (outside the catalytic domain).
(E) Western blot of GSK3β-treated MARK2wt and mutants with a phospho-S212-specific antibody (ABps212). Lane 1: partially purified MARK (MARKp110) from porcine brain, which was found to be phosphorylated on residues corresponding to both T208 and S212 in MARK2 (11). Note that this protein shows a higher molecular weight of approximately 110 kDa than MARK2, due to posttranslational modification. It is also prone to proteolytic degradation which is easily visible in this Western blot (2, 11). Samples in lanes 2-4 are aliquots of the samples shown in (C) lanes 2, 4 and 6. As expected, the S212A-mutant is not detected by this antibody whereas the wild type and the T208A-mutant give clear signals.
(F) GSK3β inhibits MARK2 (independently of its activation by MARKK) or constitutively active MARK2T208E. MARK is strongly activated by MARKK (lane 2) but this activation is counteracted by co-incubation with GSK3β (lane 3). As expected there is no effect on the S212A-mutant of MARK2 which has no basal activity and cannot be activated by MARKK (lanes 4-6). Mutating T208 in the activation loop to glutamic acid increases the activity of MARK significantly (lane 7) but not as strongly as by phosphorylation. However, GSK3β is able to reduce the activity of this mutant, too (lane 8). All experiments were performed in triplicate, graphs show mean values with standard errors.

**Fig. 3: Co-expression of active GSK3βS9A with MARK2wt or active MARK2T208E reduces the phosphorylation of tau at S262.**
(A) N2a/F113 cells stably expressing htau40 were transiently transfected with HA-tagged MARK2wt, MARK2T208A, MARK2S212A and MARK2T208AS212 with HA-tagged MARK2T208E, MARK2S212A and MARK2T208AS212A. 24 hours after transfection the cells were differentiated with retinoic acid for 6 hours. A Western blot of the lysates was probed with antibodies against tau phosphorylated at S262, total tau, MARK2
and actin as a marker for loading of similar protein amounts. The level of S262-phosphorylated tau is strongly increased in cells transfected with MARK2<sup>wt</sup> (lane 2) compared to non-transfected cells (lane 1). In cells with the non-activatable T208A-mutant, 12E8-staining is only slightly elevated (lane 3). Cells expressing the inactive S212A- or T208A/S212A-mutants show no 12E8-immunoreactivity.

(B) N2a/F113 cells stably expressing htau40 were transiently transfected with HA-MARK2<sup>wt</sup>, active ECFP-MARK2<sup>T208E</sup> alone and in presence of active ECFP-GSK3β<sup>S9A</sup>. 24 hours after transfection the cells were differentiated with retinoic acid for 6 hours. On a western blot the lysates were probed with antibodies against tau phosphorylated at S262, total tau, MARK2, GSK3β and actin as a marker for equal protein amounts. Expression of either MARK2<sup>wt</sup> or active MARK2<sup>T208E</sup> increases the phosphorylation of tau at S262 (lanes 2 and 3, compare non-transfected cells in lane 1). In contrast, co-expression of MARK2<sup>wt</sup> or active MARK2<sup>T208E</sup> with active GSK3β<sup>S9A</sup> results in the loss of 12E8-immunoreactivity (lanes 4, 5). Note that the level active MARK2<sup>T208E</sup> is threefold lower during co-transfection with GSK3β (lane 5) than without GSK3β (lane 3), but the reduction of Tau phosphorylation at S262 is more than tenfold as determined by densitometric analysis. Therefore this is a result of inactivation (as shown for the wildtype MARK in lanes 2 and 4), not of the reduced protein level.

**Fig. 4: Co-expression of GSK3β with MARK2 stabilizes the microtubule network.**

CHO cells were transfected with either wildtype MARK (EYFP-MARK2) or co-transfected with wild type MARK (EYFP-MARK2) and active GSK3β (mRFP-GSK3β<sup>S9A</sup>). In cells only transfected with MARK, the expression leads to the loss of the microtubule network. The cells round up and become smaller (upper row, arrows). In contrast, co-expression of EYFP-MARK2 and active mRFP-GSK3β<sup>S9A</sup> retains the microtubule network, indicating that GSK3β inhibits MARK2 in cells (lower row, arrows).

The graphs on the right show the classification of cells transfected with MARK alone (upper graph) or co-transfected with MARK and GSK3β (lower graph). Around 55% of the cells transfected with MARK alone lost their microtubule (MT) network, became round and small, whereas only 10% of the cells co-transfected with GSK3β exhibited that phenotype (white columns). On the other hand, only 15% of the MARK-transfected cells appeared normal, this was increased to 60% upon co-transfection with GSK3β (dark grey columns). The number of cells with reduced size but normal cytoskeleton did not change (30%; light grey columns). (Three independent experiments, 132 cells per series were counted).

**Fig. 5: GSK3β inhibits MARK2 in PC12 cells.**

PC12 cells were transiently transfected with either active mutants of MARK (ECFP-MARK2<sup>T208E</sup>) or GSK3β (mRFP-GSK3β<sup>S9A</sup>) or both. They were then differentiated with NGF for 48 hours. After fixation the cells were stained with phospho-S262 tau antibody (12E8). Cells transfected with active MARK alone show strong 12E8-staining and multiple processes (upper row, see arrows), whereas in cells transfected with active GSK3β the 12E8 staining is strongly reduced (middle row, arrows). In cells over-expressing both active MARK2<sup>T208E</sup> and active GSK3β<sup>S9A</sup> the phosphorylation of S262 in tau is comparable with untransfected cells and the transfected cells show no neurite outgrowth (lower row, arrows), indicating that MARK2-activity is inhibited. Upper left: Diagram of the effect of MARK2 on microtubule dynamics and neurite outgrowth, which is inhibited by GSK3β.

**Fig. 6: Colocalization of endogenous inactive GSK3β and active MARK in the neurite tips of differentiated PC12 cells.**

PC12 cells were differentiated with NGF for 72 hours, fixed and stained with antibodies against inactive GSK3β (phosphorylated at S9), active MARK (phosphorylated at T208), Tau phosphorylated at S262 and actin. Colocalization of active MARK (A) and inactive GSK3β (B) is seen predominantly at the tips of neurites (arrows) and the cell periphery. Magnified images of the tips labeled by arrows are shown below. MARK is highly active at the tips of the neurites, seen by co-staining of active MARK (D) and phospho-S262 Tau (E) (merge in F). Magnified images of the tips labeled by arrows are shown below. Note the strong overlap of active MARK and phospho-S262 Tau at the very tips (F1 – F3). The staining for active MARK (G) overlaps with the actin signal (H) at the tips of the neurites (I, magnified images below).
Fig. 7: Colocalization of endogenous inactive GSK3β and active MARK in the growth cones of cortical neurons.
Active MARK (A) as well as inactive GSK3β (B) are concentrated in the growth cones of stage three neurons (arrow and arrowhead, magnification of arrow-labeled growth cone below). Activity of MARK is highest at the growth cone as indicated by phosphorylation of Tau at S262 (D, E and F; see arrow, magnification below). The highest activity of MARK also coincides with strong actin staining in the growth cone (G, H and I; see arrow, magnification below), the site of the highest microtubule and actin dynamics.

Fig. 8: Model of conformational changes in the catalytic domain of MARK2.
The model is based on our X-ray structure of MARK2 (ref. 7, PDB-ID 1Y8G). The catalytic loop (grey) is located deep in the cleft between the small and the large lobes of the kinase domain (blue). The UBA domain (red) in the back is linked to the large lobe by a stretch that contains the CD domain (green).
(A) In the inactive state the catalytic loop (yellow; partly disordered and modelled here as dotted line) is folded back into the cleft and resides underneath the ATP-binding loop (P-loop). Both T208 and S212 point to the right and are accessible for kinases, e.g. T208 to MARKK and S212 to GSK3β.
(B) In the activated state, phosphorylation of the T208 (indicated by red sphere) results in a reorientation of the activation loop. It becomes folded to the right and stabilized by interactions of the pT208 to residues in helix C. S212 is now fixed by hydrogen bonds towards K177 and D175 in the catalytic loop (grey). The catalytic pocket opens up and allows entry of ATP (violet) and substrate (Tau peptide, cyan) which aligns with the catalytic (grey) and the activation loop (yellow).
(C) Phosphorylation of S212 or mutation to Glu or Ala disrupts the stabilizing interaction between the activation loop (yellow) and the catalytic loop (grey) resulting in an inactive kinase. Furthermore it is likely that the phosphate of pS212 (indicated by red sphere) will interfere with the correct alignment of the substrate within the catalytic cleft.
**Figure 1**

A.

B.

T208  S212

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<td>M KK3</td>
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Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Glycogen synthase kinase (GSK) 3-beta directly phosphorylates serine 212 in the regulatory loop and inhibits microtubule affinity regulating kinase (MARK) 2
Thomas Timm, Kiruthiga Balusamy, Xiaoyu Li, Jacek Biernat, Eckhard Mandelkow and Eva-Maria Mandelkow

J. Biol. Chem. published online April 18, 2008

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