Regulation of the kinase MARK from *Rattus norvegicus (Brekenhout, 1769)* by GSK3

Thesis submitted to the Department of Biology, Faculty of Mathematics, Informatics and Natural Sciences of the University of Hamburg in partial fulfillment of the requirements for the degree of Ph.D.

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Hamburg, 2007

Genehmigt vom Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Professor Dr. E. MANDELKOW
Weiterer Gutachter der Dissertation:
Herr Priv.-Doz. Dr. H. QUADER
Tag der Disputation: 26. Januar 2007

Hamburg, den 30. Dezember 2006

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Synopsis

Summary

MARK (MAP/Microtubule Affinity Regulating Kinase), a kinase family related to PAR-1, was first discovered because of the ability to phosphorylate the neuronal microtubule-associated protein (tau/MAP2/MAP4) at the K*X*GS motifs located in the repeat domain. The consequence of this phosphorylation is that MAPs detach from microtubules and microtubules become highly dynamic (Drewes et al., 1997). This kind of phosphorylation of tau is enhanced in the brain of Alzheimer patients (Augustinack et al., 2002).

MARK2 isolated from mammalian brain is partly phosphorylated at both Thr_{208} and Ser_{212}, which reside in the activation loop. Activation of MARK2 is achieved by phosphorylation at Thr_{208} by the activating kinase MARKK or LKB1 (Timm et al., 2003; Lizcano et al., 2004). It requires Ser_{212} to be present, but not phosphorylated, because this phosphorylation is inhibitory as judged by mutational analysis (Timm et al., 2003). In the present study, GSK3ß is identified as the inhibitory kinase that phosphorylates $Ser_{212.}$ This is shown by *in vitro* kinase assays as well as in cells.

In N2a/F113 cells, overexpression of the constitutively active $MARK2^{T208E}$ together with the constitutively active $GSK3ß^{S9A}$, leads to the complete loss of phosphorylation at the K*X*GS motifs of tau indicating that MARK2 activity is inhibited.

To further characterize the influence of GSK3ß-MARK-Tau signaling cascade on the microtubule network, CHO cells are used as a cell model. Overexpression of MARK2 alone leads to phosphorylation and detachment of tau or other equivalent MAPs from the microtubules, leading to microtubule breakdown and eventually cell death. Overexpression of GSK3ß alone also leads to cell death probably due to apoptosis (Hetmann et al., 2000; Pap et al., 1998). In contrast, co-expression of GSK3ß with MARK2 inhibits the activity of MARK2 by phosphorylation of Ser_{212} and leads to a reduction of phosphorylation at the K*X*GS motifs of tau and rescue from microtubule break down and cell death. In PC12 cells, overexpression of MARK2 induces neurite outgrowth but when MARK2 is co-expressed together with GSK3ß no neurite outgrowth occurs indicating that GSK3ß is inhibiting MARK. These results reveal a novel relationship between signaling pathways regulating the neuronal cytoskeleton.

1.0 Introduction

Microtubules play a central role in a number of cellular processes like regulation of cell shape and polarity during differentiation, chromosome partitioning at mitosis, and intracellular transport (reviewed by Drubin et al., 1996; Hyman et al., 1996). During these processes the microtubules undergo rearrangements involving rapid transitions between the stable and dynamic states (Sammak et al., 1998), mediated by several factors including, microtubule associated proteins (MAP's) (Pryer et al., 1992).

The best-studied MAPs include MAP1b, MAP2, MAP4 and tau. MAP2 and tau are mostly neuronal where they stabilize microtubules in dendrites and axons, whereas MAP4 is ubiquitously expressed (Mattus, 1998). MAPs have an N-terminal projection domain, which serves as an anchor for enzymes and a C-terminal microtubule-binding domain, which contains imperfectly repeated motifs of approximately 31 amino acids each (Goedert et al., 1988). Phosphorylation of MAPs is an important factor in regulating the balance between the plasticity and the stability of the microtubule network (Lopez et al., 1995; Preuss et al., 1995; IIlenberger et al., 1998).

Among all MAPs, tau has received the most attention because it is the major component of the paired helical filaments in Alzheimer's disease and this pathological tau is abnormally phosphorylated (Grundke-Iqbal et al., 1986; Wood et al., 1986; Ishiguro et al., 1993). Phosphorylation sites in tau are broadly classified into 2 types:

1. Ser-Pro or Thr-Pro motifs – phosphorylated by proline-directed kinases such as GSK3ß, cdc2, Cdk5 or MAP kinase (Mandelkow & Mandelkow, 1998; Gail Johnson et al., 1999). The phosphorylation of these motifs outside tau's microtubule binding domain has only a moderate influence on microtubule binding (Biernat et al., 1993).

2. Other sites are located in the microtubule binding domain and can be phosphorylated by non proline-directed kinases like protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin dependent kinase II (Sironi et al., 1998), p70 S6K (Pei et al., 2006), SAD kinase (Kishi et al., 2005) and MARK (Drewes et al., 1995) (figure 1).

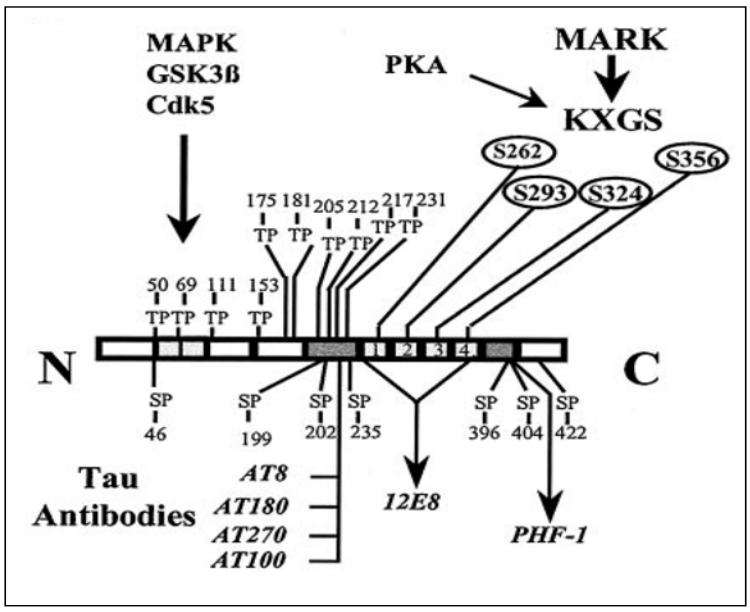

Figure 1: **Bar diagram of human tau, phosphorylation sites and antibody epitopes**. The main targets of cdk5 or cdc2 on htau40 are the double motifs Thr_{231}/Ser_{235} (epitope of antibody AT-180), Ser_{202}/Thr_{205} (antibody AT-8), and Ser_{404} (only weak reaction with PHF-1); the main targets of GSK3 are Ser_{396}/Ser_{404} (strong reaction with antibody PHF-1), and Ser_{202}/Thr_{205} (AT-8 epitope) (Illenberger *et al.*, 1998). The K*X*GS motifs (one per repeat) are the targets of MARK. (Adapted from Biernat et al., 2002)

1.1 MARK (MAP/Microtubule Affinity Regulating Kinase)

MARK (MAP/Microtubule Affinity Regulating Kinase) was originally discovered because of its ability to phosphorylate Ser residues (particularly Ser_{262}) within the K*X*GS motifs located in the repeat domain of tau and other MAPs (Drewes et al., 1995). Phosphorylation of these residues dramatically reduces the binding of MAPs to the microtubules and makes them dynamically unstable both under *in vitro* condition and in cells (Biernat et al., 1993; Drewes et al., 1997, 1998). This type of phosphorylation is one of the earliest events in the pathological process in Alzheimer's disease (AD) brain (Augustinack et al., 2002). In addition, Nishimura et al. proposed that phosphorylation at this site (Ser_{262}) in a *Drosophila* model primes tau for the hyperphosphorylation by other kinases like GSK3 and Cdk5, which in turn triggers the aggregation of tau into paired helical filaments, a hallmark in the AD neurofibrillary pathology (Nishimura et al., 2004).

MARK/Par-1 (partitioning-defective protein) kinases belong to the AMPK/Snf1 subfamily of the Ca^{2+}/calmodulin-dependent kinase II (CaMK) group (Hanks and Hunter, 1995). Homologous genes have been found in eukaryotes ranging from yeast to mammals, KIN1 and KIN2 in *S.cerevisiae* (Levin et al., 1987), kin1 in *S.Pombe* (Levin et al., 1990), dPAR-1 in *D.melanogater* (Tomancak et al., 2000), par-1 in *C.elegans* (Guo et al., 1995), Xpar-1A and Xpar-1B in *X.laevis* (Ossipova et al., 2002) and mPARs: p78, EMK and MARK in mammalian cells (Drewes et al., 1995; Bohm et al., 1997).

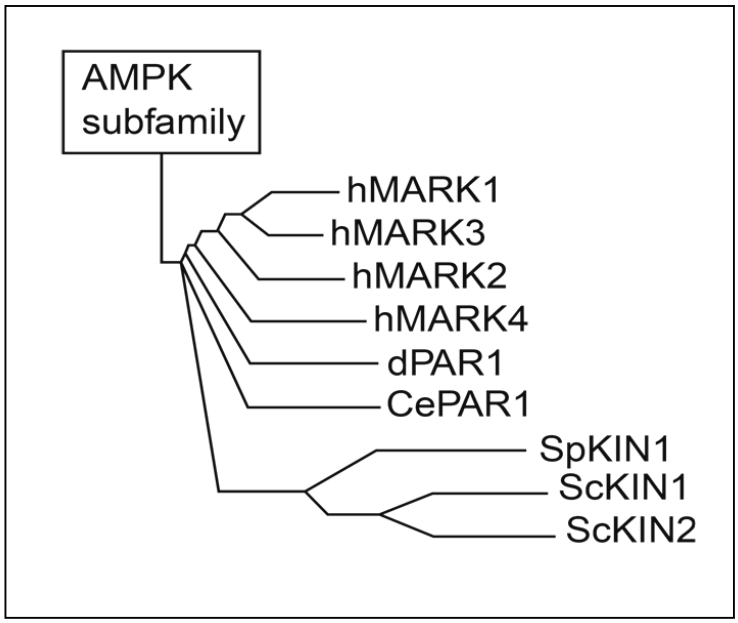

Figure 2: **MARK/PAR-1 kinases form a subfamily of the AMP-dependent protein kinase (AMPK) family**. In humans, four genes and 28 pseudogenes encode MARKs. The phylogenetic tree shows the relationship between the four human MARK gene products MARK1, MARK2, MARK3, MARK4, and their orthologs from *Drosophila melanogaster* (PAR-1), *Caenorhabditis elegans* (PAR-1, U22183), *Schizosaccharomyces pombe* (KIN1) and *Saccharomyces cerevisiae* (KIN1; KIN2, M69018). (Adapted from Drewes et al, 2004)

1.1.1 MARK isoforms

There are four different isoforms of MARK characterized from fetal human (MARK3, MARK4) and rat brain cDNA library (MARK1, MARK2) (Drewes et al., 1997). The sequences of MARKs are broadly subdivided into 5 domains: 1. an N-terminal header domain, 2. a 30 kDa kinase domain followed by a linker of about 20 amino acids that includes a four-residue motif (adjacent to the catalytic domain) that may serve as a common docking site (CD domain) for regulatory binding partners in analogy to MAP kinases (Tanoue et al., 2003), 3. UBA domain (ubiquitin associated), 4. an extended spacer domain and 5. a 110 residue C-terminal tail with KA domain (kinase associated domain 1) (Drewes et al. 1997). The functions of the UBA and KA domains are not well understood (Hoffmann and Bucher, 1996). The fact that most of the AMPK related protein kinases, including the yeast homologue Snf1, possess a UBA or UBA-like domain (Beullens et al., 2005; Jaleel et al., 2006) suggests a conserved function in structural stabilization or regulation of kinase activity.

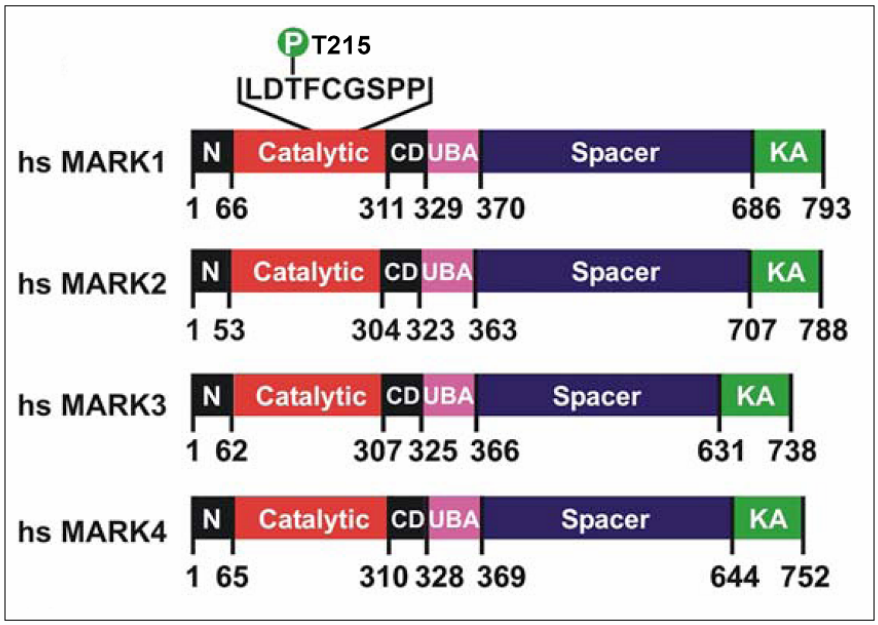

Figure 3: **Conserved domain structure of MARK**. MARK 1-4. Domains: N = header, C = catalytic, CD=common docking site, UBA=ubiquitin-associated, S=spacer, KA=kinase associated domain. All MARKs can be phosphorylated by MARKK at a conserved threonine in the catalytic domain (corresponding to T208 in MARK2). Site directed mutational analysis shows that phosphorylation at Ser_{212} is inhibitory. The kinase that phosphorylates Ser_{212} is unknown. (Adapted from Timm et al., 2003)

1.1.2 Function of MARK

One important function of MARK/Par1 is the regulation of MAP microtubule dynamics (Drewes et al., 1997; Timm et al., 2003). In CHO cells, expression of MARK leads to the phosphorylation of endogenous MAPs at the K*X*GS motifs. The result is that the MAPs detach from the microtubule network leading to microtubule breakdown. As a consequence the cells detaches from the substratum and ultimately undergoes cell death. The actin stress fibre network in these cells remains intact (Ebneth et al., 1999).

MARK/Par-1 plays a pivotal role in the establishment of cell polarity in different organisms, e.g., asymmetric distribution of P-granules in the *C. elegans* zygote (Guo et al., 1995), polar growth of *S. Pombe* (Levin et al., 1990), axis formation in the *D. melanogaster* embryo (Shulman et al., 2000; Tomancak et al., 2000), asymmetric organization of polarized epithelial cells (Bohm et al., 1997), polarized neurite outgrowth and neuronal polarity in neuroblastoma cells and hippocampal neurons (Biernat et al., 2002; Chen et al., 2006).

Par-1 in *D. melanogaster* functions as a positive regulator in the Wnt signaling pathway by phosphorylating Dsh and as a negative regulator in the JNK pathway (Sun et al., 2001). Deletion of Par-1 from *Drosophila* follicular cells results in defects in epithelial polarity (Cox et al., 2001; Doerflinger et al., 2003).

Kin1 and Kin2 are involved in the regulation of exocytosis in *S. cerevisiae*. Kin1 and Kin2 interact and induce the phosphorylation of t-SNARE Sec9, which in turn stimulates its release from the plasma membrane to the cytosol, promoting its recycling and availability for the incorporation into newly formed SNARE complexes (Elbert et al., 2005).

The function and substrates of mammalian MARK isoforms are represented in the table below:

Isoform GenBank ID and human gene locus	Synonyms	Expression (by northern blotting) and representation in EST databases	Substrates	Functional consequence of phosphorylation
MARK1 AB040910 1q42.11	EMK3; hPAR-1c	High in brain, spleen, skeletal. muscle, pancreas, kidney and heart; low in lung and liver; 89 ESTs available	Tau, MAP2, MAP4	Loss of microtubule binding and thinning of microtubule array
MARK2 X97630 11q12-q13	EMK1; hPAR-1b	Similar to MARK1: high in brain, spleen, skeletal muscle, pancreas, placenta, kidney and heart; low in lung and liver; 187 ESTs available	Tau, MAP2, MAP4	Loss of microtubule binding and thinning of microtubule array
			Dcx	Loss of Dcx-microtubule binding and increase microtubule dynamics in growth cone
MARK3 U64205 14q32.3	EMK2; hPAR-1a; KP78; C-TAK1	Highest in brain and pancreas; 417 ESTs available	PTPH1	14–3-3 binding , localization
			Cdc25C	14–3-3 binding, localization
			KSR1	14–3-3 binding, localization
			Plakophilin 2	14–3-3 binding, localization
			Dishevelled (?)	Increased β-catenin-dependent signalling
MARK4 AY057448 19q13.3	MARKL1; hPAR-1d	High in brain, glioma, testis; low in most other tissues; 162 ESTs available	tau, MAP2, MAP4	Microtubule bundling and thinning of microtubule array

Table.1. **MARK isoforms, distribution, substrates and functions**. (Adapted from Drewes et al., 2004)

1.1.3 Role of MARK in microtubule dependent transport

Microtubule dependent transport is achieved through motor proteins such as dynein or kinesin and their relatives (Waterman-Storer et al, 1997; Hirokawa, 1998; Lippincott-Schwartz, 1998). Kinesin is a microtubule plus-end directed motor, whereas dyneinis a minus-end directed motor. The transport of cargoes by kinesin towards the cell periphery is called anterograde transport, whereas the transport towards the cell interior is called retrograde transport (Brady, 1995; Vallee and Sheetz, 1996). The cargoes are attached to the motor proteins through adapter complexes.

Microtubules are covered with microtubule-associated proteins (MAPs), which contribute to the stabilization of the microtubule network. In addition, MAPs can compete with motors for microtubule binding (Lopez and Sheetz, 1993; Hagiwara et al., 1994). When CHO cells are transfected with tau, the plus-end directed kinesin dependent transport of vesicles and organelles along microtubules is preferentially inhibited, leading to the accumulation of mitochondria in the cell body and the retraction of the endoplasmic reticulum towards the cell interior (Ebneth et al., 1998; Trinczek et al., 1999). This suggests that the minus-end directed transport (by dynein)

dominates. The mechanism by which tau inhibits kinesin dependent transport is that it reduces the attachment frequency of the motors to microtubules (Seitz et al., 2002).

Transfection of primary retinal ganglion neurons with tau alone show strong axonal transport defects: whereas co-expression of tau with MARK2 rescues the axons from transport inhibition. This is due to phosphorylation of tau in the repeat region at the K*X*GS motifs by MARK2, which in turn decreases the affinity of tau for microtubules. This supports the attachment of kinesin to microtubules and facilitates anterograde transport (Mandelkow et al., 2004).

1.2 Regulation of MARK

There are three different modes of regulation of MARK known so far: phosphorylation, binding and autoinhibition.

1.2.1 Regulation by phosphorylation

The catalytic domain of the MARK family contains Ser/Thr residues in the activation loop (Thr_{208} and Ser_{212} in MARK2), spaced by three residues. Activation of MARK2 is achieved by phosphorylation of a single residue Thr_{208} by MARKK (Timm et al., 2003) or LKB1 (Lizanco et al., 2004). The MARK purified from the brain was phosphorylated at both Thr_{208} and Ser_{212} but judging from site directed mutational analysis the phosphorylation at Ser_{212} is inhibitory (Timm et al., 2003).

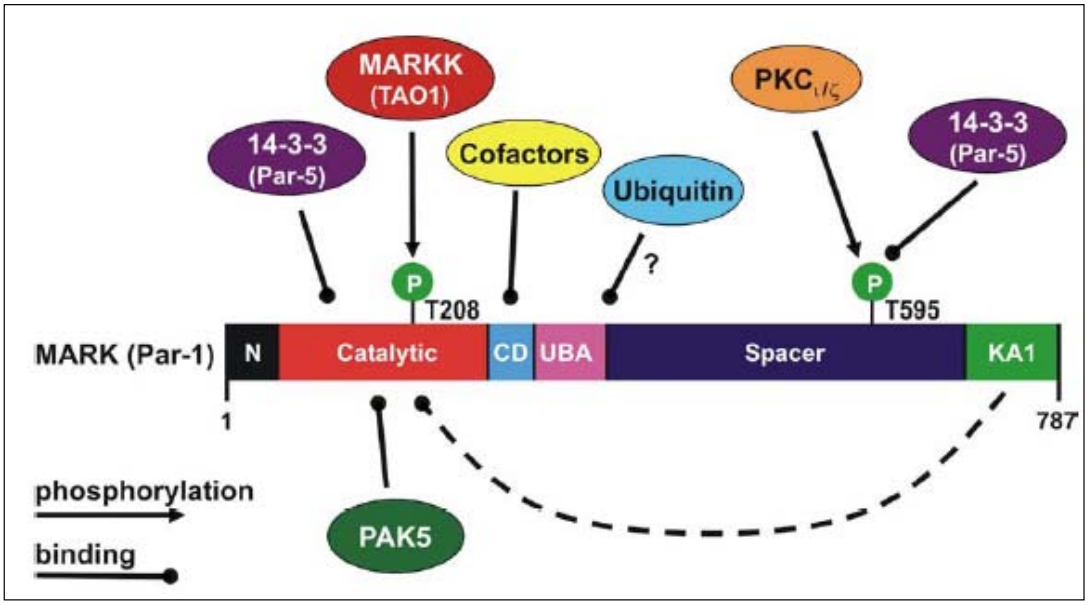

Figure 4: **Modes of regulation of MARK**. The diagram summarizes the known or plausible modes of regulation. All four human MARKs are activated by phosphorylation of a conserved threonine residue in the catalytic loop by MARKK or LKB1. The nearby Ser_{212} (numbering according to MARK2) has been found to be phosphorylated by an unknown kinase in MARKs purified from brain and this phosphorylation seems to confer inhibition. The threonine in the spacer domain is phosphorylated by protein kinase C-λ (PKC-λ) that induces the translocation of MARK from the membrane to cytosol. In flies and in humans, MARK/PAR-1 is in a complex with 14-3-3 family proteins, which bind to the catalytic domain with a region on 14-3-3 located outside the known phospho-ligand-binding pocket. A short diverse amino-terminal sequence is followed by the catalytic domain and an ubiquitin-associated (UBA) domain, which might be involved in interactions with other proteins in an ubiquitin-dependent fashion. The function of the carboxy-terminal kinase associated domain (KA1) is unknown. PAK5 inhibits the kinase activity of MARK by binding (Timm et al., 2006).

MARKK/TAO1 is a Ste20- related Ser/Thr kinase that together with the kinases PSK/TAO2 and JIK/KDS forms the kinase subfamily GCKVIII (Chen et al., 1999). MARKK activates MARK2 by phosphorylating Thr_{208} in the activation loop. In CHO cells, overexpression of MARKK triggers a cascade of activation of MARK, which in turn phosphorylates MAPs at K*X*GS motifs, leading to increased microtubule dynamics, subsequent breakdown of microtubules and cell death. In PC12 cells the activity of MARKK and MARK increases upon differentiation with NGF. This leads to the phosphorylation of tau at K*X*GS motifs, increased microtubule dynamics, and enables neurite outgrowth. This cascade NGF-MARKK-MARK-tau-microtubule dynamics is interrupted by siRNA against MARKK; as a result the cells cannot

differentiate. This clearly shows that the MARKK-MARK-MAPs pathway is functional in cells and plays a very important role in regulating microtubule dynamics (Timm et al., 2003). Apart from this function, MARKK/TAO1 also phosphorylates MKK3, which in turn activates the p38 MAPK pathway (Hutchison et al., 1998).

LKB1 is a Ser/Thr kinase belongs to the CaMK group of kinases. Loss-of-function mutations in this gene cause Peutz–Jeghers syndrome, which is associated with inherited susceptibility to colorectal and other cancers. LKB1 in complex with the pseudokinase STRAD (Bass et al., 2003) and the scaffolding protein MO25 (Boudeau et al., 2003) phosphorylates and activates 12 human kinases (NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4) that are related to AMP related protein kinases (AMPK) (Lizcano et al., 2004). AMPK is a sensor of the cellular energy pool that regulates the physiological processes that consume or regulate ATP to restore the energy pool in the cell (Hardie et al., 2003).

Phosphorylation of MARK in the regulatory loop by MARKK or LKB1 complex is not the only regulatory mechanism of MARK activity. Hurov et al. showed that human Par-1b (hPar-1b/MARK2) is phosphorylated by aPKC on Thr_{595}, a residue conserved in Par-1 orthologs in mammals, worms, and flies. aPKC negatively regulates the kinase activity and plasma membrane localization of hPar-1b *in vivo* (Hurov et al., 2004). In MDCK cells, the phosphorylation at this site enhances the binding of 14-3-3 to Par-1b, which in turn promotes the dissociation of PAR-1b from the lateral membrane (Suzuki et al., 2004). In hippocampal neurons, MARK2 functions downstream of the PAR-3/PAR-6/aPKC complex and aPKC in this complex phosphorylates MARK2 at Thr_{595} and negatively regulates the kinase activity of MARK2. This in turn causes dephosphorylation of microtubule associated proteins, such as tau, leading to the assembly of microtubules and elongation of axons (Chen at al., 2006).

1.2.2 Regulation by binding of PAK5

PAK5 is a member of Ste 20 family of kinases and belongs to the subfamily of p21-activated kinases group II (comprising PAK4-PAK6). It contains the following domains: header, P=PBD (p21-binding domain, including a variant of the CRIB motif), AID (auto-inhibitory domain), spacer and a catalytic domain. They are activated by small G-proteins such as Rac or Cdc42 in their GTP-bound state (Manser et al., 1994; reviewed by Etienne-Manneville et al., 2002). In N2a cells, PAK5 induces filopodia and neurite outgrowth (Dan et al., 2002; Bryan et al., 2004). PAK5 activates the JNK pathway and inhibits apoptosis by phosphorylating the pro-apoptotic protein BAD and preventing the localization of BAD to mitochondria (Pandey et al., 2002; Cotteret et al., 2003).

PAK5 inhibits MARK2 activity by binding and not by phosphorylation. The catalytic domain of PAK5 interacts with the catalytic domain of MARK2 and inhibits the kinase activity of MARK2, but the kinase activity of PAK5 remains unaffected. In CHO cells, MARK2 transfection alone destabilizes the microtubules and stabilizes the actin stress fibers, whereas co-transfection with PAK5 stabilizes the microtubule network and destabilizes the F-actin network (Matenia et al., 2005).

1.2.3 Regulation by autoinhibition

Kin1 and Kin2, orthologs of MARK in yeast functions in the secretory pathway. Kin1/2 architecture is similar to the MARKs. It has a kinase domain at the N-terminus of the protein, a regulatory domain at the C-terminus and a 42 amino acid stretch which is equivalent to the KA1 domain in MARK on the extreme carboxy terminus. The 42 amino acids are highly conserved throughout Par-1 orthologues from yeast to mammalian cells (Elbert et al., 2005).

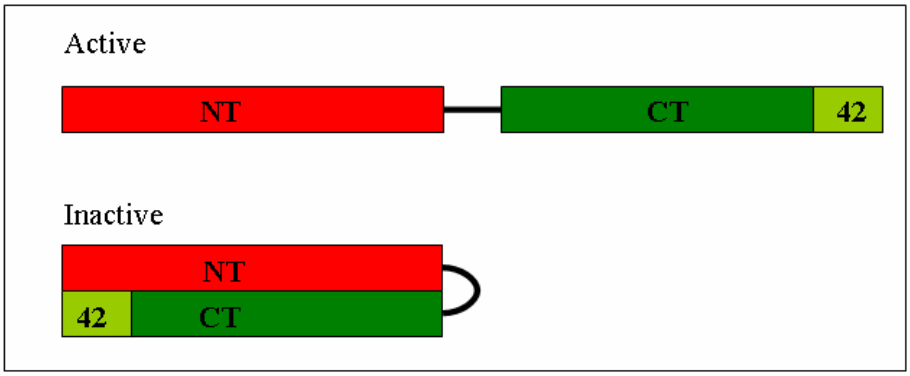

Figure 5: **Proposed autoinhibitory intramolecular interaction between the N-terminal and C-terminal domains of the Par1 family of kinases**. The C-terminal regulatory domain (KA1) interacts with the N-terminal kinase domain and mediates autoinhibition. NT: N-terminal Kin2 kinase domain, CT: C-terminal Kin2 regulatory loop, 42 amino acid tail (KA1). (Adapted from Elbert et al., 2005)

The C-terminal regulatory domain of Kin1/2 physically interacts with the N-terminal kinase domain and mediates autoinhibition. The 42 amino acid tail is critical for both the physical interaction and for the negative regulatory effect of the C-terminal domain (Elbert et al., 2005). This inhibition cannot be shown for MARKs (1/2) as the catalytic domain alone is two times more active than the full length protein (Marx et al., 2006).

1.3 Aim of this work

MARK (MAP/Microtubule Affinity Regulating Kinase) a kinase family related to PAR-1 is involved in establishing cell polarity. MARK phosphorylates microtubule-associated proteins (tau/MAP2/MAP4) at K*X*GS motifs in the repeat domain, causes detachment from microtubules, and renders them highly dynamic. This type of phosphorylation is enhanced in the tau aggregates from Alzheimer's brain tissue (Augustinack et al., 2002). It is therefore important to understand the regulation of MARK.

The amino acid sequence of MARK2 in the activation loop comprises of two phosphorylation sites Thr_{208} and Ser_{212}, which are partly phosphorylated when the kinase was purified from the porcine brain. Timm et al. showed that activation of MARK is achieved by phosphorylation of a single residue Thr_{208} by the activating kinase MARKK/TAO1. It requires Ser_{212} be present but not phosphorylated, because this phosphorylation is inhibitory as judged by mutational analysis (Timm et al., 2003).

The aim of this study is to identify the kinase that is responsible for the inhibitory phosphorylation of MARK2 at Ser_{212}.

2.0 Materials

2.1 Chemicals, Enzymes and Antibodies

2.1.1 Chemicals

The chemicals used for the experiments were purchased from: Amersham-Buchler, Biomol, Boehringer Mannheim, Difco, Gibco-BRL, Merck, Millipore, Pharmacia-LKB, Pharma Waldhof, NEN DuPont, Pierce, Riedel-de-Haen, Sigma and Roche. The radioactivity ($\gamma^{32}P$) was purchased from Amersham Life sciences.

2.1.2 Enzymes

Enzyme	Company
Restriction endonuclease	New England Biolabs
T4 DNA ligase	New England Biolabs
PfuUltra TM High-Fidelity DNA polymerase	Stratagene
Alkaline Phosphotase	Roche

2.1.3 Molecular weight marker

Protein marker

- Protein molecular weight marker (SM0431, MBI fermentas)

Marker protein	Molecular weight
β-galactosidase	116 kDa
BSA	66.2 kDa
Ovalbumin	45 kDa
lactate dehydrogenase	35 kDa
Bsp98I	25 kDa
β-lactoglobulin	18.4 kDa
lysozyme	14.4 kDa

- Protein marker for western blot analysis (Magic Marker™, Invitrogen)

DNA-Marker

Smart ladder (Fermentas)

Fragment size (bp) – 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600, 400, 200.

2.1.4 Antibodies

Antibody		**Dilution**	**Company**
Primary antibody	HA	1:1000	Cell Signalling
	HA-agarose conjugate	1:20	Santa Cruz
	GFP	1:2000	Clontech
	MARK	1:1000	Eurogentec
	MARK $pThr_{208}$	1:1000	Eurogentec
	MARK $pSer_{212}$	1:500	Eurogentec
	LKB1	1:1000	Cell signalling
	GST	1:2000	Zymed lab Inc
	GSK3β	1:1000	Biosource QBC
	GSK3β Ser_9	1:1000	Biosource QBC
	GSK3β Tyr_{216}	1:1000	Biosource QBC
	12E8	1:1000(WB) 1:200 (IF)	Elan Pharmaceuticals
	PHF1	1:500	Davies, AB
	K9JA	1:10000	DAKO
	Tubulin	1:250	Sigma
Secondary antibody	Goat anti-rabbit Immuoglobulins/HRP	1:2000	DAKO
	Goat anti-mouse Immunoglobulins/HRP	1:2000	DAKO
	Anti-mouse/rat-antibody/TRITC	1:200	Molecular Probes
	Anti-mouse/rat-antibody/Cy5	1:200	Molecular Probes

2.1.5 Kits

Kits	**Company**
BaculoGold™ Transfection kit	PharMingen
ECL Western Blotting Detection	Amersham
Plasmid Maxi kit	Macherey – Nagel
Plasmid Mini Kit	Invitek
Gel Extraction Kit (Ultra Clean™ Site Directed Mutagenesis)	Dianova
Quick Change TM Site Directed Mutagenesis	Stratagene
Gateway cloning kit	Invitrogen
Hyperfilm ECL	Amersham Biosciences
Effectene Transfection Reagent	Qiagen

2.2 Bacterial strains, viruses and cell lines

2.2.1 Bacterial strains

Bacterial strains	Genotype	Company
DH5α	F^-φ80lacY Δ*M15* Δ*(lacZYA/argF)U169 recAendA1 hsdR17($r_k m_k$+)phoA SupE44 thi/gzrA96relA1λ*	Gibco BRL
XL-2 blue	*Rec A1 endA1 GyrA96 thi1 hsdR17 SupE4 ReIA1 lac{F"proAB lac1^{q}ZΔM15 Tn10(Tet) Amz Cam^r}*	Stratagene
BL21	F – dcm ompT hsdS (r_B – m_B) gal	Stratagene

2.2.2 Yeast strain

Strain	Genotype	Company
AH 109	*MATa trp1-901 leu2-3, 112 ura3-52 his 3-200 gal4Δ gal8 LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2 URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	Clontech

2.2.3 Baculoviruses

MARKK	His-MARKKwt
MARK2	HA-MARK2
GSK3	His-GSK3β wt, His-GSK3β S9A
14-3-3	His-14-3-3ζ

2.2.4 Eukaryotic cell lines

Strains	Organism	Specification
Sf9	Spodoptera frugiperda	Insect cell line
PC12	Rattus norvegicus	Pheochromocytoma cells
CHO	Hamster	Chinese hamster ovary cell line
N2a	Mouse	Neuroblastoma cell line

2.3 Plasmids

Vectors	Features	Company
pEU	5,475 kb, Amp^R	MPA
pVL1392	9,6 kb, Amp^R, MCS, N-terminalHis-tag	MPA
pYFPC1	4,7 kb, Kan^R	Clontech

2.4 Media

2.4.1 Bacterial media

LB-medium	LB broth	25 g/l
LB-Agar	LB broth	25 g/l
	Bacto-Agar	25 g/l
SOC-medium	Bacto Trypton	20 g/l
	Yeast Extract	5 g/l
	NaCl	10 mM
	KCl	10 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	2%

2.4.2 Cell culture media

Sf9 medium	Grace's Insect medium	Gibco
	FCS	100 ml/l
	Penicillin/Streptomycin (100U/ml)	10 ml/l
CHO medium	HAM-medium	Gibco
	FCS	100 ml/l
	Penicillin/Streptomycin (100U/ml)	10 ml/l
	L-Glutamine	10 ml/l
N2a/F113 medium	MEM(Minimal Earle's medium)	Gibco
	FCS	100 ml/l
	L-Glutamine	10 ml/l
	Non essential amino acids	0.1 ml/0.1l
	Gentamycin 418	600 µg/ml
PC12 medium	DMEM (Dulbecco's Modified Eagle Medium)	Gibco
	Glucose	4500 mg/l
	L-Glutamine	10 ml/l
	FCS	50 ml/l
	HS	150 ml/l
	Penicillin/Streptomycin (100U/ml)	10 ml/l
Plating medium	DMEM (Dulbecco's Modified Eagle Medium)	Gibco
	FCS	100 ml/l
PC12 Differentiation medium	DMEM: F12	1:1
	FCS+HS	0.1 ml/0.1l
	NGF	100 ng/ml

2.4.3 Yeast medium

100x amino acid stock solution	Arginine	2 g/l
	Histidine	1 g/l
	Isoleucine	6 g/l
	Leucine	6 g/l
	Lysine	4 g/l
	Methionine	1 g/l
	Phenylalanine	6 g/l
	Threonine	5 g/l
	Tryptophan	4 g/l
Selection medium (SM)	Difco-yeast medium	8 g/l
	Tyrosine	55 mg/l
	Uracil	55 mg/l
	Adenine	55 mg/l
	100x amino acid stock soution	1:100
	Glucose	2%
Full medium (YEPD)	Yeast extract	11 g/l
	Bacto-Peptone	22 g/l
	Adenine	55 mg/l
	Glucose	2%

2.5 Equipment list

Apparatus	**Model**	**Company**
Blottingapparatus	Semi-Dry	VWR
DNA-Sequencing apparatus	ABI Prisma 310 Genetic Analyzer	Perkin Elmer
Scintillation counter	Tricarb 1900 CA	Packard Canberra Ltd
French Press	Pressure Cell 20 kpsi	SLM Aminco
PCR	Mastercycle personal	Eppendorf
Photometer	Ultrospec 300 Pro	Amersham Pharmacia Biotech
Incubator	Innova™ 4300	New Brunswick Scientific
Centrifuge	J2-21M/E	Beckman
Centrifuge	Minifuge A	Hereaus
Centrifuge	5402	Eppendorf
Rotor	JA-10, JA-20, TLA-45	Beckmann
Ultracentrifuge	TL-100	Beckmann
Gelelectrophoresis-System	SE200	Hoefer/Amersham Pharmacia Biotech
Ultracentrifuge	TL-100	Beckman
Ultracentrifuge	Optima™ LE-80K	Beckman Coulter
Homogenisator	DIAX 900	Heidolph
Phospho-Imager	BAS3000	Fuji
Fluorescence microscope	LSM 510 Meta	Zeiss
Scanner	DESKScanII V.2.4	Hewlett-Packard
Gel drier	Model 583	Bio-Rad
Incubator shaker	G25	New Brunswick Scientific
HPLC	Smart System	Pharmacia

2.6 Chromatographic columns

Mono S	Pharmacia
Ni-NTA beads	Qiagen
Phosphocellulose	Pharmacia
GST-beads	Amersham Pharmacia

2.7 Software's

TINA V.2.09f (1993)	Raytest Isotope GmbH
VECTOR NTI 9.0	Invitrogen
AIDA	Fuji

3.0 Methods

3.1 Cell biological methods

3.1.1 Sf9 cell culture

Sf9 (*Spodoptera frugiperda*) cells were grown in a 27°C incubator with 5% CO_2 in monolayer culture with Graces medium supplemented with 10% FCS and 100U/ml penicillin/streptomycin mixture. Confluent monolayers were sub-cultured by scraping the cells and diluting in the ratio of 1:4 in complete medium. Total cell counts were made with a haemocytometer.

A. Production of recombinant baculovirus

Sf9 cells were seeded at a density of $2x10^6$ cells per well in a 6 well plate and allowed the cells to attach firmly to the plate. 1µg of the recombinant baculovirus transfer vector (e.g. gene of interest cloned into pVL1392) was mixed 0.25µg of 'BV Baculo Gold DNA' (Pharmingen) and incubated at RT for 5 minutes. During the complex formation, the cells were washed with PBS and 1ml of Transfection Buffer A (Pharmingen) was added. 1ml of Transfection Buffer B (Pharmingen) was added to the transfection complex, mixed gently and added drop wise onto the cells. After every 2-3 drops the plate was swirled gently to ensure uniform mixing of the transfection complexes with Transfection buffer A and the plate was incubated at 27^oC for 4 hours. Then the transfection solution was replaced with 3ml of TNM-FH medium (Pharmingen) and incubated for 5 days at 27^oC. The viruses were collected and amplified by infecting the Sf9 cells.

B. Infection of Sf9 cells

For expression of proteins in Sf9 cells the actively growing cells (80% confluence) were infected with recombinant baculovirus. The MOI (Multiple of Infection) is 1-3. The cells were incubated with the virus at 27°C for 66-72 hours. The culture supernatant was filtered with 0.45µm filters and the filtrate was used for further infections. The protein was further purified from the cell pellet.

3.1.2 PC12 cell culture

PC12 cells ($3.5x10^6$ cells) were grown in a poly-D-lysine coated flask T-25 flask. The cells were incubated at 37°C incubator with 5% CO_2. The 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 (100U/ml) penicillin/streptomycin.

The differentiation of PC12 cells was carried out with a differentiation medium containing (DMEM: F12), 0.1% serum and 100ng/ml NGF for 24 hours.

For pull-down assays, the cells were washed once with 3ml of PBS. Then 1ml PBS was added, the cells were scraped and centrifuged at 14,000rpm for 2 minutes. The supernatant was removed and the cell pellets were used.

A. Trypsination

To split cells the old culture medium was removed and washed once with pre-warmed PBS. 0.5-1ml Trypsin EDTA (TE) solution was added and incubated at 37°C to facilitate trypsinization. Then fresh complete growth medium was added and the cells were resuspended by gentle pipetting. Aliquots of the cell suspension were added to new culture flasks.

3.1.3 CHO cell culture

CHO cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C incubator. The cells were grown in a medium containing HAM, 1% L-Glutamine, 10% FCS, and (100U/ml) penicillin/streptomycin. Confluent monolayers were sub-cultured by trypsinisation method as described in section 3.1.2a.

For immunoflurosence $1x10^4$ cells in 200µl medium were seeded on a cover slip and the cells were allowed to grow over night. The cells were then transfected with plasmids using Effectene method (Section 3.1.5)

3.1.4 N2a/F113 cell culture

N2a/F113 cells stably expressing htau40 was grown in a medium containing MEM (Minimal Earle's medium), 10% FCS, 1% L-glutamine, 1% nonessential aminoacid and 600µg/ml gentamycin 418. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37^oC.

The cells were sub-cultured by trypsinisation method as described in section (3.1.2a).

For Western blot analysis or immunoprecipitation, 1.5-3x10^6cells/well were grown in a 6 well plate for 24 hours and the cells were transiently transfected with appropriate plasmids using Effectene method (section 3.1.5). After 24 hours of post transfection, the cells were differentiated with 2ml of differentiation medium (MEM, 0.1% FCS, 0.1% nonessential amino acid, 2µM retinoic acid) for 6 hours. The cells were washed once with 3ml of PBS. Then 1ml of PBS was added, the cells were scraped and centrifuged at 14,000 rpm for 10 seconds. The cell pellet was used immediately or freezed in liquid nitrogen and stored at – 20^oC.

3.1.5 Effectene transfection

Effectene (Qiagen) was used to transfect CHO wt or N2a/F113 cells. Cells were seeded at a density of 0.2-2x10^6 cells in a 60mm dishes. DNA (1µg) was diluted in DNA condensation buffer EC to a final volume of 150µl and 8µl of Enhancer solution was added followed by vortexing and incubation at RT for 10 minutes. To the DNA enhancer mixture, 25µl of the Effectene reagent was added, and the solution was mixed thoroughly by pipetting and incubated at RT for 10 minutes for the transfection complex formation. During the complex formation, the cells were washed with PBS and 4ml of growth medium was added. 1ml of the growth medium was added to the transfection complex, mixed gently and added drop wise onto the cells. The dish was gently swirled to ensure uniform distribution of the transfection complexes.

3.1.6 Immunofluroscence

After 24 hours of post transfection, CHO 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 with PBS (3 times) and blocked with 10% goat serum at 37°C for 45 minutes. After blocking, the cells were treated with appropriate primary antibody for 1 hour at 37°C and then washed with PBS (3 times). Then secondary antibody was added and the cells were incubated at 37°C for 1 hour followed by washing with PBS (3 times). The cover slip was then mounted for microscopy.

3.1.7 Primary neuron culture

Hippocampus 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 100-200 neurons/mm^2 on a 6 well plate pre-coated over night with poly-D-lysine. After culturing for 4 hours, the media was changed to neuronal culture media (Neurobasal medium with 2% B-27) and the cells were grown for 7 days. For Western blot analysis and *in vitro* kinase assays, all inhibitors were added 3 hours before lysis.

3.1.8 Transfection of neurons

The cultures were grown for 7 days and the cells were infected with appropriate adenoviruses for 48 hours and the cells were fixed.

3.1.9 Yeast two-hybrid system

Protein-protein interaction study was performed in the yeast two-hybrid system. The system takes advantage of the composite nature of the GAL4 transcription factor. It needs both an activation domain (AD) and a DNA binding domain (DBD) for the transcription. The strategy employs the construction of a yeast strain containing a plasmid with the DBD fused to the (heterologous) protein for which the interactors are searched (the bait). A cDNA library containing an AD fused to heterologous cDNA is introduced into the bait strain. When the

cDNA interacts with the bait, transcription is initiated. The presence of the plasmid is selected by amino acid prototrophy. Two reporter genes (LEU2 and LacZ) are activated as a result of activation. To verify the specificity of the interaction the cDNA is typically introduced into another bait strain, where an interaction is not expected.

The yeast two-hybrid assay uses two plasmid constructs: the bait plasmid pGBK7, in which the protein of interest is fused to a GAL4 binding domain, and the hunter plasmid pGADT7, which is the potential binding partner fused to the GAL4 activation domain. The two plasmids were transformed into AH109 strain and plated in minimal medium to select for the bait plasmid and then on another minimal medium to select for the hunter. If the binding occurs between the proteins, transcriptional activity of the GAL4 promoter is restored and interactions are detected by blue versus white colonies. All of the yeast two-hybrid interactions studied in this work were based on MATCHMAKER Two-Hybrid System 3 (Clontech).

3.1.10 Transformation of *S.cerevisiae*

The yeast cells (AH109) were inoculated in a 50ml of YEPD medium and allowed to grow at 30°C in a shaker over night. Then 1.5ml of the over night culture was removed and centrifuged at 14,000 rpm for 10 seconds. The medium was removed carefully and the cells were resuspended in 100µl of the rest of the medium. To the resuspension 2µl of the carrier DNA (10mg/ml) and 1µg of plasmid DNA was added and vortexed. 500µl of the plating mixture (45% PEG 4000, 1M LiAc, 1M Tris-HCl pH 7.5, 0.5M EDTA) and 20µl of 1M DTT were added, vortexed briefly and incubated at RT for 6 hours to over night. Then heat shock was given at 42°C for 10 minutes. The cells were plated in the selective plate (-Leu, -Trp) and incubated at 30°C for 3 days.

The interaction between the bait and the hunter was further confirmed by streaking the cells on an X-gal selective plate (+/- Adenine). When there is interaction between two proteins blue colonies are seen.

3.2 Molecular biological methods

3.2.1 Site-directed mutagenesis

All of the site-directed mutagenesis was performed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The components of the reaction mixture were as follows:

10x Pfu Ultra High Fidelity buffer	2µl
dsDNA template (25ng/µl)	5µl
dNTPs (2.5mM)	2 µl
Primer sense (0.5pmole/µl)	1µl
Primer anti-sense (0.5pmole/µl)	1µl
Pfu polymerase (2.5U/µl)	0.5µl
H_2O to a final volume of	20µl

PCR program

Step	Time	Tempurature	Cycles
Initial denaturation	30 seconds	95°C	1
Denaturation	30 seconds	95°C	16
Annealing	1 minute	55-5°C	
Extension	1 minute/kb	68°C	
Final extension	10 minutes	68°C	1

The primer annealing temperature was calculated according to the melting temperature (Tm) of the primers and the extension time was calculated according to the length of the plasmid.

The PCR products were then treated with DpnI in order to digest the template plasmid DNA.

DpnI digestion:

PCR product	20µl
10x DpnI buffer	(1x) final concentration
DpnI	10 units

The mixture was incubated at 37°C for 1 hour (for digestion) and then at 60°C (for DpnI heat inactivation). The PCR products were then analyzed by agarose gel electrophoresis.

3.2.2 Preparation of agarose gels

To 0.7g-1g of electrophoresis grade agarose, 100ml of 1xTAE buffer (40mM Tris-Acetate pH 8.0, 2mM EDTA) was added and heated until a clear, transparent solution was obtained. The melted solution was then poured into a mold and allowed to harden (30-45 minutes). The comb and the tape were carefully removed and mounted in the electrophoresis tank, filled with 1xTAE.

3.2.3 Preparation of ethidium bromide

The agarose gel was stained with EtBr (ethidium bromide) solution for 30 minutes at RT (EtBr solution was prepared by dissolving 80µl of 1% ethidium bromide in 200ml of H_2O). The stained gel was soaked in water for 10 minutes at RT to reduce the background fluorescence caused by unbound EtBr.

3.2.4 Restriction digestion

DNA samples were analyzed by restriction digestion. The DNA sample (1µg) was mixed with 1U of enzyme with appropriate 10xNEB buffer (a final concentration of 1x) in a total reaction volume of 50µl and incubated at 37°C for 1 hour. After digestion, 10µl from the total reaction mix was removed and mixed with 2µl of 6xDNA loading buffer (15% Ficoll in TAE, 0.25% Xylenexyanol FF) and loaded onto a 1% agarose gel and the fragments were separated at 80V.

3.2.5 Elution of DNA from the agarose gel

The gel was exposed to a long wavelength UV illumination and the desired fragment was excised with smallest possible volume of agarose using a sterile scalpel to a clean 1.5ml micro centrifuge tube. The elution of DNA was performed using the 'Qiagen Gel elution kit'.

3.2.6 Ligation

The components of the ligation reaction were mixed in the following order and incubated at 16°C over night. The molar ratio of the purified vector backbone and the insert was 1:3.

10xligase buffer	1x
Vector	200ng
Insert	600ng
Ligase	5U
H_2O	- to a final volume of 10µl

3.2.7 Transformation

a. Chemical Transformation

Ultra competent XL-2 Blue cells (Stratagene) were thawed on ice and 2µl of Stratagene's ß-mercaptoethanol was added (final concentration of 25mM). ß-mercaptoethanol was added to increase the efficiency of transformation. The cells were aliquoted into a sterile pre-cooled 1.5ml microcentrifuge tubes and 3µl of the ligation mix was added and tapped gently. The cells were incubated on ice for 30 minutes and heat pulsed at 42°C for 30 seconds. 200µl of pre-warmed (37°C) SOC medium was added and incubated in a shaker at 37°C for 1 hour. Then 50µl of cells were plated on agar plates with appropriate antibiotics and incubated at 37°C over night.

b. Electroporation

DH5α and BL21DE3 cells were transformed using the electroporation method: 50ng of DNA was added to the pre-thawed electro-competent cells. The cells were carefully transferred to the cuvette and electric shock was given. Then 200µl of pre-warmed (37°C) SOC medium was added and incubated in a 37°C shaker for 45 minutes. Two different dilutions of cells were plated on agar plates with appropriate antibiotics and incubated at 37°C over night.

3.2.8 Plasmid preparation

Single colonies were picked from the agar plates, inoculated in 5ml of LB medium containing the appropriate antibiotic and incubated at 37°C over night. Plasmid preparation (Invitrogen kit) was carried out according to the instruction manual. For sequencing the amount of DNA obtained from a mini preparation was not always sufficient, so midi preparation (Nucleobond) was done from 100ml cultures according to the instruction manual.

3.2.9 Estimation of DNA concentration

a. Spectrophotometric method

The concentration and the degree of purity of double-stranded plasmid DNA were determined based on the Beer/Lambert law by measuring the absorbance at 260nm and 280nm.

$\mathbf{A_{260}} = \boldsymbol{E_{260}\, c\, l}$. A_{260} is the absorbance at 260nm, E_{260} is the molar coefficient, c is the molar concentration and l is the optical length.

1µl of DNA sample was diluted in 119µl of water, mixed well and the readings were taken at 260nm and 280nm. Pure preparations of DNA have an OD_{260}/OD_{280} ratio of 1.8. An OD_{260}/OD_{280} of 1 corresponds to approximately 50µg/ml for double-stranded DNA and 40µg/ml of single-stranded DNA.

b. Ethidium bromide method

When the concentration of DNA is less than 250ng/ml or heavily contaminated with other substances it is difficult to estimate the concentration spectrophotometrically. The amount of DNA in such samples is estimated by the fluorescence emitted by ethidium bromide intercalated into the DNA. 5µl of DNA was mixed with 1µl of 6xDNA gel loading buffer and loaded onto a 0.7% agarose gel (Sambrook et al., 1989).

3.2.10 Sequencing

DNA sequencing reactions were performed using the fluorescent dye labeling method (Sanger et al., 1977) in a Robocycler Gradient 96 PCR machine. The components of the sequencing reaction were mixed as follows:

Terminator ready reaction mix	8µl
dsDNA	500ng
Primer	10pmol
H_20 to a final volume of	20µl

The PCR program for the sequencing is as follows:

1. Denaturation	96°C	10 seconds
2. Annealing	45°C	5 seconds
3. Elongation	60°C	4 minutes

(x30 cycles). Pellet Paint NF Co-Precipitant was added to precipitate the DNA.

To the 20µl reaction mixture, 1µl of Pellet Paint NF Co-Precipitant and 80µl of 70% ethanol was added. The sample was then mixed gently and centrifuged at 13,000 rpm for 10 minutes at RT. The supernatant was carefully removed. To the pellet, 250µl of 70% ethanol was added to remove traces of salt and centrifuged at 13,000 rpm for 10 minutes at RT. The pellet was then air dried and resuspended in 30µl of HPLC-grade ddH_2O.

The ABI PRISM 310 Genetic analyzer was used to sequence the DNA. The analysis of the sequences was performed with the Vector NTI software package.

3.3 Biochemical methods

3.3.1 SDS-PAGE

SDS-PAGE was performed following a modified protocol in our lab (Laemmli, 1970; Matsudaira et al, 1978). The stacking gel was 4% and the resolving gel was 10% or 17%.

The composition of the resolving and stacking gel were as follows:

Components	**Resolving gel (ml)**		**Stacking gel (ml)**
	10%	**17%**	**4%**
40% Acrylamide/ Bis-Acrylamide (37.5:1)	15.0	25.6	5.4
Tris HCl (1M pH 8.8)	22.0	22.0	-
Tris HCl (0.25M pH 6.8)	-	-	27.0
10% SDS	0.6	0.6	0.54
TEMED	0.12	0.12	0.108
10% APS	0.065	0.065	0.15
H_2O	22.0	11.4	20.9

Protein samples were mixed with 6xSDS sample buffer (500mM Tris pH 6.8, 20% (w/v) SDS, 50% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.03% (w/v) bromophenol blue) to a final concentration of 1x and heated at 90°C for 3 minutes. The electrophoresis was carried out at 150V and 35mA in 1xSDS PAGE running buffer (0.025M Tris, 0.192M Glycine, 0.1% SDS pH 8.3).

3.3.2 Staining of SDS gels

a. Coomassie staining

The gels were stained in a staining solution (0.1% (w/v) Coomassie Blue R-250, 45% methanol and 9% acetic acid) for 20 minutes on a shaking platform. After staining the gels were destained in an intensive destaining solution (50% (v/v) methanol and 10% (v/v) acetic acid) for 30 minutes and then transferred to a normal destaining solution (5% (v/v) methanol and 7.5% (v/v) acetic acid) for 2 hours.

b. Silver staining

The protocol for silver staining was as follows:
1. The gel was first fixed with the fixation solution (30% ethanol and 10% acetic acid) for 20 minutes. 2. After fixation, the proteins were cross-linked for 30 minutes to over night with the cross-linking solution (30% ethanol, 0.5M sodium acetate, 0.5% glutaraldehyde and 0.2% sodium thiosulphate). 3. The gel was washed with millipore water for 10 minutes (3 times). 4. The gel was stained in the silver staining solution (0.1% silver nitrate and 0.02% formaldehyde) for 30 minutes. 5. The gel was washed with millipore water for 20 seconds. 6. Then the gel was developed with the developing solution (2.5% sodium carbonate and 0.01% formaldehyde). 7. The reaction was stopped with 0.05M EDTA.

c. Roti-Blue staining

The radioactive gels were stained with Roti-Blue staining solution (20ml Roti-Blue, 20ml methanol and 60ml of H_2O) for over night. After staining the gels were destained in an intensive destaining solution (50% (v/v) methanol and 10% (v/v) acetic acid) for 10 minutes and then transferred to a normal destaining solution (5% (v/v) methanol and 7.5% (v/v) acetic acid) for 2 hours.

3.3.3 Estimation of protein concentration

a. Bradford Method

Protein concentration was estimated using the Bradford method (Bradford, 1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when bound to protein. 1µg to 5µg of BSA solution in 10µl were used as standards. The samples were taken in two different dilutions. Then 200µl of Bradford reagent was added to each probe and mixed. The absorbance at 595nm was measured in a microtitre plate and the concentration of the protein was calculated from the standard curve.

b. BCA method

Protein concentration in total cell lysates (cortex/hippocampus) was estimated by this method. BCA (Bicinchoninic acid solution) mix was prepared (10ml of BCA + 200µl $CuSO_4$). Total cell lysate (2µl) was mixed with 48µl H_2O and 1ml of BCA mix. The sample was mixed gently and incubated at 60°C for 30 minutes then centrifuged at 14,000 rpm for 30 seconds and measured at OD_{595}.

3.3.4 Western blot analysis

Western blotting was performed following a modified method of Towbin et al., 1979. The proteins were electrophoresed on SDS gels and then electro-transferred to PVDF membranes (1mA/cm², 1 hour). The membranes were blocked with 5% milk (5g milk powder in 100ml of 1xTBST) for 1 hour at RT and then treated with appropriate primary antibody (diluted in 1xTBST) at 37°C for 1 hour. The membranes were washed with 1xTBST (3 times). The secondary antibody (diluted 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. The ECL Western blotting system is a chemiluminescent, non-radioactive method to detect antigens that have been immobilized onto membranes. This system makes use of a horseradish peroxidase (HRP) conjugated secondary antibody that, in conjugation with a chemiluminescent substrate, luminol generates a signal that can be captured on the film and the bands can be visualized using LAS 3000.

3.3.5 Purification of His-tagged proteins from Sf9 cells

Sf9 cells expressing transfected protein were harvested and resuspended in phosphate buffer A (50mM sodium phosphate (pH 8.5), 100mM NaCl, 25mM imidazole, 1mM ß-mercaptoethanol, 1mM benzamidine, 5mM CHAPS) (1ml/T-75 flask). The cells were disrupted with a small French press unit and the total cell lysate was centrifuged for 10 minutes (14,000 rpm, 4°C) and the cleared lysate was incubated with 75µl of Ni-NTA beads for 30 minutes at 4°C. Centrifuged at 4000 rpm for 5 minutes. The supernatant was carefully removed; 500µl of buffer A was added to the beads, mixed gently and centrifuged at 4000 rpm for 5 minutes (3 times). To elute the protein from the beads 500µl of buffer B (buffer A with 500mM imidazole) was added, mixed gently and incubated on ice for 10 minutes. Then centrifuged at 4000 rpm for 5 minutes (3 times). The eluates were dialyzed against buffer C (50mM Tris-HCl pH 8.2, 200mM NaCl, 1mM DTT, 50% glycerol) for 4 hours to over night and stored at –20°C. The purity of the protein was determined by SDS-PAGE with Coomassie blue staining.

3.3.6 Expression of protein in *E.coli*

a. Expression of protein

The pre-inoculum was prepared by growing the strain expressing the protein of interest in 5ml LB medium with appropriate antibiotics at 37°C over night. 2-5% of the pre-inoculum was inoculated into 100ml LB medium with antibiotic and the culture was grown at 37°C, until the OD reach 0.6. The culture was cooled down at 4°C for 2 hours before IPTG induction. 500µl of the culture was saved as an uninduced control. Then IPTG was added to a final concentration of 0.2mM and the culture was grown at 25°C over night. Cells were then harvested by centrifugation at 8,000 rpm for 10 minutes.

b. Cell lysis

The cell pellet was resuspended in lysis buffer (50mM Tris pH 7.4, 100mM NaCl, 1mM ß-mercaptoethanol, 1mM benzamidine, 5mM CHAPS (2ml/100ml) for the GST fusion protein. For tau protein the lysis buffer components were 50mM PIPES, 500mM NaCl, 1mM $MgCl_2$, 1mM EGTA, 5mM DTT pH 6.9. Then the cell suspension was carefully transferred to the

French press and a pressure of 10,000 PSI was applied (2 times). The lysates were then centrifuged at 14,000 rpm for 20 minutes at 4°C (2 times). The supernatant was carefully removed and processed further.

3.3.7 Purification of GST-tagged protein from *E.coli*

The supernatant (section 3.3.6) was incubated with 75µl of glutathione 4B sepharose beads for 30 minutes at 4°C. The beads were washed with buffer A (3 times), and the proteins were eluted from the beads with 20mM L-reduced glutathione in buffer A. The eluates were dialyzed against buffer C (50mM Tris-HCl pH 8.2, 200mM NaCl, 1mM DTT, 50% glycerol) for 4 hours to over night and stored at –20°C.

3.3.8 Purification of tau protein

The cell lysate (from step 3.3.6) was boiled for 20 minutes and centrifuged at 127,000 xg for 40 minutes at 4°C. An aliquot of 50µl was saved in each and every step for the gel. The supernatant was dialysed against a cation exchange buffer A (20mM MES, 50mM NaCl, 1mM $MgCl_2$, 1mM EGTA, 2mM DTT, 0.1M PMSF, pH 6.8) at 4°C under constant stirring. The dialyzed protein was again centrifuged at 127,000 xg for 40 minutes at 4°C. Then the clear supernatant was loaded onto the cation exchange chromatographic column. The column was washed with 3-4 column volumes of buffer A. The protein was eluted with a linear gradient of buffer B (20mM MES, 1M NaCl, 1mM $MgCl_2$, 1mM EGTA, 2mM DTT, 0.1M PMSF, pH 6.8). The sample from each step and the fractions were loaded onto the gel and the fraction with proteins were pooled and concentrated in a 10kDa cut off concentrator.

The concentrated tau protein was applied to the gel filtration column (Superdex^{TM} 75) with a flow rate of 0.5ml/minute. The buffer used for the gel filtration column was PBS. The fractions were checked by SDS-PAGE.

3.3.9 Co-immunoprecipitation

In this method, an antibody specific for one protein is incubated with a cell lysate to form a complex with the target protein.

a. Preparation of cell lysate

The cell pellet from N2a/F113 or Sf9 cells were lysed in 300µl of lysis buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 3mM Na-EGTA, 3mM $MgCl_2$, 0.1% NP-40, 5mM CHAPSO, 1mM DTT, 2mM Benzamidine, 1mM PMSF, 1mM Na_3VO_4, 2µM MC_{LR}, 1x protease cocktail inhibitor (Sigma). The cell lysate was placed on ice for 30 minutes and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant was carefully transferred to a 0.5ml tube and the pellet was resuspended in 300µl of 1xSDS sample buffer. Each step 10µl of the sample was saved for the Western blot analysis. The amount of protein in the supernatant was estimated by BCA method (3.3.3b).

b. Immunoprecipitation

To the supernatant, HA antibody agarose conjugate (Santa Cruz) was added to a final concentration of 50µg/ml and incubated end over end in a rotator for 4 hours at 4°C. Then centrifuged at 14,000 rpm for 20 seconds. The supernatant was carefully removed and to the beads 100µl of wash buffer (lysis buffer without CHAPSO) was added, mixed gently and centrifuged at 14,000 rpm for 20 seconds (3 times). Finally the beads were resuspended in 50µl of 1xkinase assay buffer (50mM Tris-HCl pH 7.4 (RT), 5mM MgCl2, 2mM benzamidine, 2mM EGTA, 0.5mM DTT, 0.5mM PMSF). 7µl of the immunoprecipitated protein was used for the *in vitro* kinase assay. For Western blot analysis, the sample was mixed with 2xSDS sample buffer and boiled at 95^{o}C for 3 minutes.

3.3.10 GST pull-down assay

The cell pellet was processed as in section 3.3.10a. The supernatant (300µl) was incubated with a 300µl of 30µM GST constructs over night at 4^{o}C. Then 100µl of glutathione 4B Sepharose beads (50%) were added and incubated for further 2 hours at 4^{o}C. As a negative control, lysates were incubated with the beads alone. Centrifuged at 4,000 rpm for 20 seconds.

The supernatant was carefully removed and to the beads 100µl of PBS was added, mixed gently and centrifuged at 4,000 rpm for 20 seconds (3 times). The beads were then resuspended in 50µl of 1xSDS sample buffer and boiled at 95^{o}C for 3 minutes. Then the sample was resolved in SDS-PAGE.

3.3.11 Sub-cellular fractionation by iodixanol gradient

This technique has been used to study the localization of specific proteins in sub-cellular compartments.

Sub-cellular fractionation was performed using iodixanol as medium according to previously described methods with some modifications (Xia et al., 1998; Iwata et al., 2001). Rat brain (0.2g) was homogenized in 2ml of the homogenization buffer (10mM HEPES (pH 7.4), 1mM EDTA, 0.25M sucrose, complete protease inhibitor mixture). All of the following steps were carried out at 4°C. Tissues were disrupted using a polytron homogenizer. Nuclei and unbroken cells were pelleted down by centrifugation at 1,500 xg for 10 minutes. The post nuclear supernatant was again centrifuged for 1 hour at 65,000 xg. The resultant vesicle pellets were resuspended in 0.8ml of homogenization buffer with 1% tritonX-100. The resuspended vesicle fractions were loaded on the top of the gradients and centrifuged in a SW41 rotor at 40,000 rpm for 2.5 hours. A clear gradient was noticed (alternative thin and thick bands). The thin and thick bands were collected separately. The volume each fraction collected was approximately 500µl. 10µl was mixed with 2µl of the 2xSDS buffer and loaded onto a 10% for Western blot analysis.

Preparation of iodixanol gradient:

Gradients were set up in 13ml Beckman SW41 centrifuge tubes by diluting the iodixanol (Optiprep, 60% w/v). Iodixanol was diluted to the following concentrations with homogenization buffer.

Iodixanol concentration (%)	2.5	5	7.5	10	12.5	15	17.5	20	30
Volume in gradient (ml)	0.8	1.6	1.6	0.4	1.6	0.4	0.4	0.4	0.24

3.3.12 Kinase assay

5xkinase assay buffer-250mM Tris-HCl pH 7.4 (RT), 25mM $MgCl_2$, 10mM benzamidine, 10mM EGTA, 2.5mM DTT, 2.5mM PMSF. The kinase assays were carried out as follows:

Reaction mix:

5x kinase assay buffer	1x
25x protease cocktail inhibitor	1x
100x BSA	2x
10mM ATP	100µM
γ^{32}P ATP	5µCi

the final volume was made up to 10µl with H_2O.

a. The activity of GSK3ß was assayed by incubating the reaction mix I with 0.2-0.3µg of GSK3ß, and different substrates (100µM pCREB peptide or 1µg htau40 or 150µM TR1 peptide) at 30^oC for 2 hours. MARK used in the following kinase assays was prepared and purified from *E.coli* by Dr.Thomas Timm from our laboratory.

b. For the inhibitor assays, 2µl of the inhibitor (required concentration) was mixed with 2µl of 300nM MARK2 or 300nM MARKK for 30 minutes on ice. Then the reaction mix and substrate was added and incubated at 30^oC for 2 hours.

c. For kinase assays with MARK2 mutants and GSK3ß +/- MARKK, the kinases were incubated with reaction mix at 30^oC for 2 hours. Then the substrate (TR1 peptide) was added incubated for further for 30 minutes at 30^oC. In case of time course experiment the kinases were not pre incubated.

d. HA MARK2 immunoprecipitates were incubated with reaction mix and substrate together at 30^oC for 2 hours.

The reaction was stopped by adding half the volume of 30% TCA (w/v). The sample was incubated on ice for 15 minutes and centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully applied to phosphocellulose discs. Then washed with 0.1M H_3PO_4 5 times at an interval of 5 minutes. The disc was air-dried and the radioactivity was measured

using the scintillation counter. The pellet was resuspended in 10µl of 1xSDS sample buffer, heated for 3 minutes at 95^{o}C and loaded onto a 10% gel. The gel was stained with Roti-blue (section 3.3.2c), destained, dried and autoradiogram was performed.

4.0 Results

Alzheimer's disease is a severe, progressive neurodegenerative disease characterized by two pathological features – neurofibrillary tangles (NFTs) and amyloid plaques. (Yamaguchi et al., 1996). The microtubule-associated protein tau is a major component of neurofibrillary tangles, which are the intra neuronal aggregates of paired helical filaments (PHFs). The tau protein in the tangles is hyperphosphorylated at more than 20 Ser and Thr residues. Among the many phosphorylated sites in tau, Ser_{262} at the K*X*GS motif in the repeat region is enhanced in Alzheimer's disease. MARK (MAP/Microtubule Affinity Regulating Kinase) is known to phosphorylate tau at this site Ser_{262} in the K*X*GS motif and other K*X*GS motifs in the repeat domain and as a result tau detaches from the microtubules and the microtubules break down (Drewes et al., 1997).

MARK isolated from adult porcine brain is partly phosphorylated at both Thr_{208}/Ser_{212} (numbering according to MARK2) in the activation loop of the catalytic domain. Activation of MARK2 is achieved by the phosphorylation of a single residue, Thr_{208}, by either MARKK or LKB1 (Timm et al., 2003; Lizcano et al., 2004). It requires Ser_{212} to be present, but the phosphorylation at this site seems to be inhibitory as judged by mutating this Ser_{212} to Glu or Ala (Timm et al., 2003). Since the Ser_{212} in the activation loop is followed by a proline, a proline directed kinase was thought to phosphorylate MARK2 at Ser_{212}.

Among many proline directed kinase GSK3ß was chosen because of the following reasons:

1. GSK3ß co-purifies with MARK2 through several steps of purification from rabbit skeletal muscles (Drewes et al., 1995).
2. GSK3ß is particularly interesting in the context of AD because it phosphorylate tau efficiently at Ser/Thr-Pro motifs that are elevated in AD (Hanger et al., 1992; Mandelkow et al., 1992; Ishiguro et al., 1993; Lovestone et al., 1994; Song et al; 1995; Moreno et al., 1996; Hong et al., 1997; Sing et al., 1999).

3. Especially in the light of recent reports in the literature, it was suggested that GSK3ß is a kinase that phosphorylates MARK2 at Ser_{212} and activates MARK2 (Kosuga et al., 2006).

So in this study the role of GSK3ß as a possible upstream or downstream kinase of MARK was investigated.

Different constructs of recombinant GSK3ß were cloned, expressed and purified from *E.coli* and Sf9 cells and the activity was checked with htau40 or pCREB peptide as a substrate. The effect of different constructs of GSK3ß on different MARK2 constructs was checked both *in vitro* with the TR1 peptide as well as in cells with htau40 as a substrate. To further address the functional relationship between GSK3ß and MARK2, the proteins were co-expressed in CHO wt (Chinese Hamster Ovary) cells and the effects on the stability of the microtubule network were analyzed. After that the expression patterns of endogenous MARK and GSK3ß were studied in PC12 cells.

4.1 Expression and purification of proteins

4.1.1 Expression and purification of GSK3ß wild type from *E.coli*

In order to express GSK3ß in *E.coli*, the gene was cloned into pET28a vector, a construct allowing the addition of a poly-histidine extension to the recombinant protein. The vector was transformed into BL21DE3 cells following the electroporation protocol (section 3.2.7b). Transformed cells were initially grown at 37°C over night and then induced at 37°C for 3 hours by the addition of 0.1mM IPTG. The protein was purified in two steps: with a cation exchange phosphocellulose column and with a Ni-NTA affinity column (section 3.3.6). The yield of purified protein was estimated to be 1-2mg per liter.

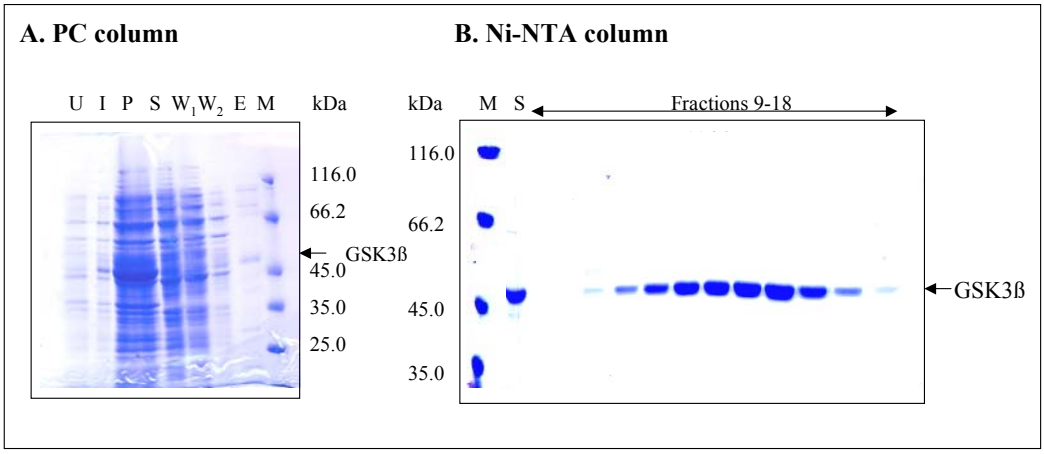

Figure 6: **Purification of GSK3ß^{wt} from *E.coli***. A. Phosphocellulose column (PC column). U=uninduced cells, I=induced with 0.1mM IPTG, P=pellet, S-supernatant, W^1=1st wash, W^2=2nd wash, E=eluate, M=protein marker. B. Ni-NTA column fractions. The eluate from the PC column was centrifuged and the supernatant was loaded. Fractions 12-19 represent the protein peak. The volume of each fraction collected was 100µl. 10µl was loaded onto a 10% gel. These fractions were pooled and concentrated with 30-kDa cut off concentrator. The yield of purified protein was estimated to be approximately 1-2mg per liter.

4.1.2 Expression and purification of GSK3ß from Sf9 cells

GSK3ß^{wt} and GSK3ß^{S9A} were cloned in pVL vector to express the protein in Sf9 cells. The vectors containing GSK3ß^{wt} or GSK3ß^{S9A} gene were transfected with Baculo platinum viral DNA in Sf9 cells and the viruses were collected after 5 days of

transfection. The viruses obtained were used for further infection. In order to express the protein, Sf9 cells were infected with the recombinant virus for 3 days, the cells were collected, lysed and the expressed protein was purified (section 3.3.5).

GSK3ß^{wt} with the N-terminal His_6 tag was purified by affinity chromatography using a Ni-NTA column, as described in materials and methods. The purification conditions were standardized by optimization of pH, salt, and imidazole concentration. The Ni-NTA column was pre-equilibrated with buffer-A containing 20mM imidazole to remove most of the non-specific binding proteins. In addition, a gradient elution of imidazole removed more tightly bound non-specific contaminant proteins in the early fractions and released the pure protein in the following fractions. The fractions were analyzed by SDS-PAGE (figure 7), and the band in lanes 5-10 corresponds to the expected size of the GSK3ß. Fractions 10-15 were pooled, concentrated and used for further analysis. The same method was employed for the purification of the constitutively active mutant of GSK3ß (GSK3ß^{S9A}). When GSK3ß is phosphorylated at Ser_9, the enzyme becomes inactive. So mutating Ser_9 to Ala generates a constitutively active enzyme.

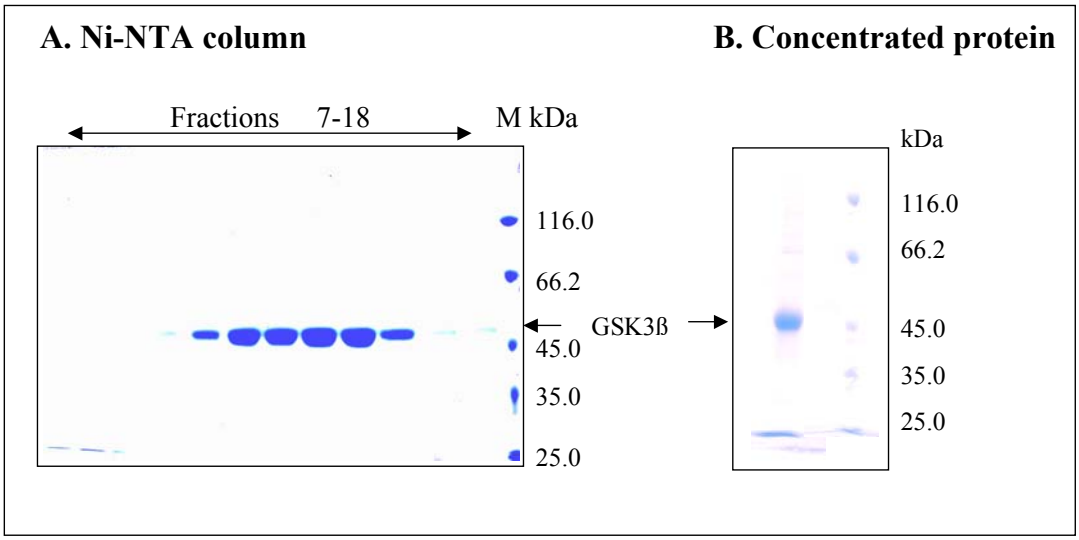

Figure 7: **Purification of GSK3ß from Sf9 cells**. The eluted fractions of the Ni-NTA column were analyzed with SDS-PAGE. P=pellet, S=supernatant, FT=flow through, M=protein marker. The volume of each fraction collected was 100µl. 10µl was loaded onto a 10% gel. Fractions 10-15 were pooled and concentrated with 5-kDa cut off concentrator to 100µl and dialysed with dialysis buffer over night and used for further analysis. B. 2µl of the concentrated protein was loaded onto a 10% gel. The concentration of the protein was estimated to be approximately 0.5µg/µl.

4.1.3 Expression and purification of htau40 from *E.coli*

BL21DE3 cells were transformed with the htau40 expression plasmid by using electroporation method. Transformed cells were initially grown at 37°C over night and then induced at 37°C for 3 hours by the addition of 0.1mM IPTG. The cells were harvested by centrifugation and lysed. The protein was purified in two steps: with a cation exchange SP sepharose column and a gel-filtration column (section 3.3.8). The numerous bands below the expected size of htau40 are degradation products of the protein (figure 8).

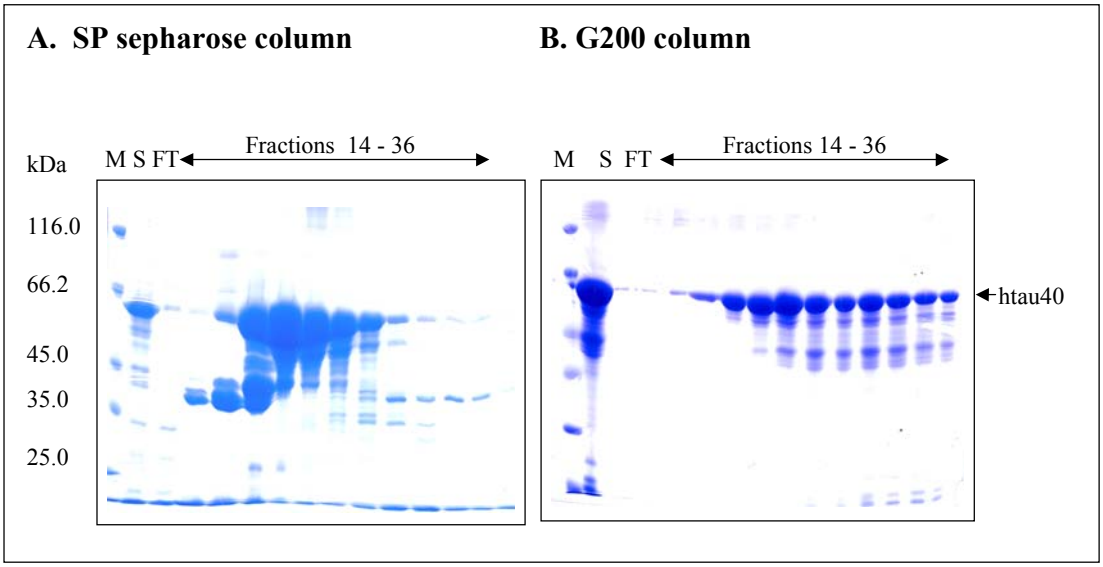

Figure 8: **Purification of htau40 from *E.coli***. A. SP sepharose column. Fractions 14-36 represent the protein peak (only even number fractions were loaded). B. G200 gel-filtration column. Fractions 14-36 represent the protein peak. The volume of each fraction collected was 2ml. 5µl was loaded onto a 10% gel. The fractions (14-36) from the gel-filtration column were pooled and concentrated for further experiments. S=supernatant, FT=flow through, M=protein marker. The other bands in the gel are degradation products of htau40.

4.2 Activity assay for recombinant GSK3ß

The activity of GSK3ß purified from *E.coli* or Sf9 cells were tested with different substrates like htau40, pCREB peptide and TR1 peptide. TR1-$_{255}$NVKSKIGSTENLK$_{268}$, a specific substrate of MARK is derived from the first repeat of the microtubule-binding domain of tau. pCREB-$_{123}$KRREILSRRP(pS)YRK$_{136}$, a specific substrate of GSK3, is derived from the CREB protein.

The activity of GSK3ß was assayed by incubating GSK3ß (0.5µg) with substrate (htau40 or pCREB peptide or TR1 peptide) and radioactive ATP. The substrate and radioactive ATP without GSK3ß serves as a negative control. The reaction mixture was incubated at 30^{o}C for 2 hours. Then TCA was added to a final concentration of 15% to precipitate the proteins (GSK3ß and htau40). The precipitated proteins were separated from the peptide by centrifugation. The supernatant was loaded onto the phosphocellulose discs, air-dried, washed with H3PO4 (0.1M) and the amount of phosphate incorporated into the peptide was counted using liquid scintillation counter (section 3.3.12). The precipitated protein was mixed with 2xSDS buffer and loaded onto a 10% gel. The proteins were excised from the gel and the amount of radioactivity incorporated was counted by the liquid scintillation counter.

The activity of GSK3ß was calculated by subtracting the values obtained from the two combinations (GSK3ß+substrate – substrate).

4.2.1 Phosphorylation of recombinant tau by GSK3ß

GSK3 is primarily regulated by phosphorylation. When GSK3ß is phosphorylated at Ser_9 the enzyme becomes inactive, because it acts as a competitive pseudosubstrate and occupies the substrate-binding site of GSK3ß (Cross et al., 1995). So mutating Ser_9 to Ala the enzyme becomes constitutively active.

GSK3ß phosphorylates htau40, the longest human tau isoform (441 residues) at Ser_{396}, Ser_{404}, Ser_{46}, Ser_{50}, Ser_{202}, and Thr_{205} (Godemann et al., 1999). The amount of phosphate incorporated by GSK3ß wild type and S9A mutant is approximately 2-mol phosphate per mol htau40. The result shows that the activities of the recombinant GSK3ß wild type and the constitutively active mutant ($GSK3ß^{S9A}$) purified from Sf9 cells were similar.

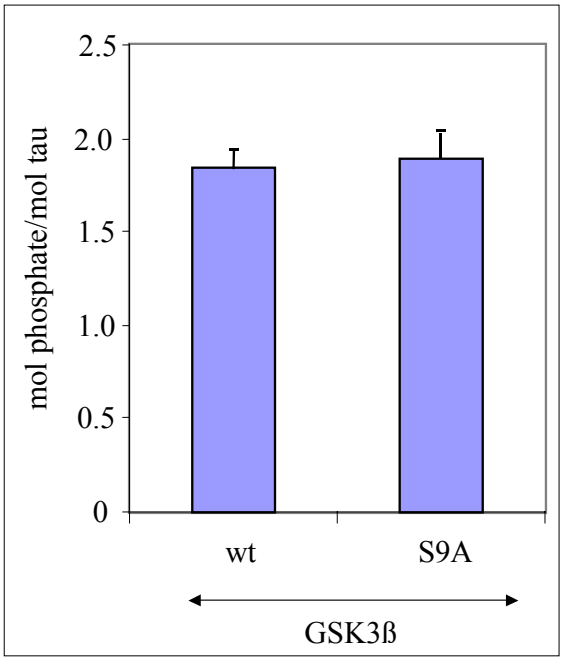

Figure 9: **Activity assay for $GSK3ß^{wt}$ and $GSK3ß^{S9A}$ purified from Sf9 cells**. The activities of recombinant His-$GSK3ß^{wt}$ and His-$GSK3ß^{S9A}$ purified (Ni-NTA column) from Sf9 cells were assayed for its ability to phosphorylate htau40. The reaction products were loaded onto a 10% SDS gel and the bands were excised and counted in the liquid scintillation counter. The amount of Pi incorporated in htau40 was calculated.

4.2.2 Phosphorylation of pCREB peptide by GSK3ß

The *in vitro* activities of wild type and constitutively active mutant of GSK3ß (purified from Sf9 cells and *E.coli*) were examined with pCREB peptide as a substrate The amount of phosphate incorporated by GSK3ß^{wt}, GSK3ß^{S9A} (purified from Sf9 cells) and GSK3ß^{wt} (*E.coli*) was approximately 0.58, 0.56 and 0.57-mol phosphate per mol pCREB peptide. Figure 10 shows that the activities of GSK3ß^{wt} (purified from Sf9, *E.coli*) and GSK3ß^{S9A} (purified from Sf9 cells) are similar.

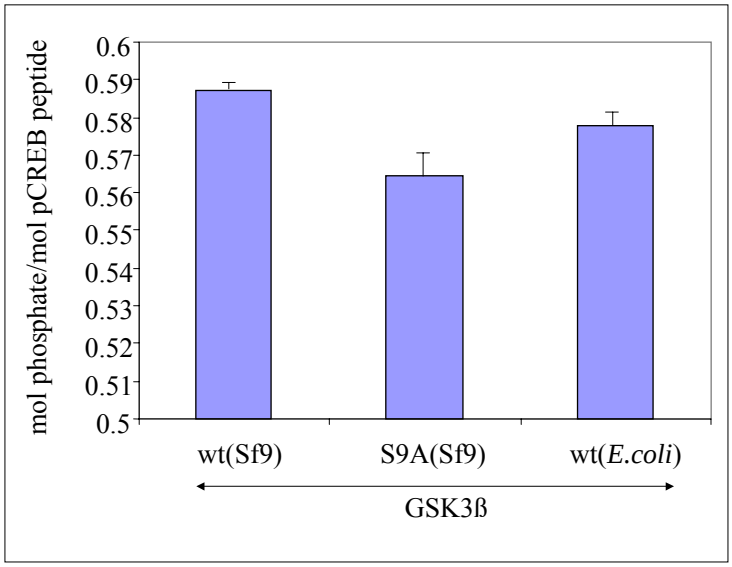

Figure 10: **Phosphorylation of pCREB peptide by GSK3ß purified from *E.coli* and Sf9 cells**. The activities of recombinant GSK3ß^{wt} and GSK3ß^{S9A} (expressed in *E.coli* or Sf9cells) were assayed for its ability to phosphorylate pCREB peptide. The amount of phosphate incorporated by GSK3ß^{wt}, GSK3ß^{S9A} (purified from Sf9 cells) and GSK3ß^{wt} was 0.58, 0.56 and 0.57-mol phosphate per mol pCREB peptide. The activity of GSK3ß purified from *E.coli* or Sf9 cells were similar.

4.2.3 Phosphorylation of TR1 peptide by GSK3ß

The activity of GSK3ß (prepared from *E.coli* and Sf9 cells) towards TR1 peptide (which is a specific substrate of MARK2) was examined. So, MARK2 with TR1 peptide was used as a positive control. The amount of phosphate incorporated by MARK2 is 0.016-mol phosphate, whereas the amount of phosphate incorporated by GSK3ß is negligible. This result indicates that only MARK2 can phosphorylate TR1 peptide and not GSK3ß, confirming that TR1 peptide is not a substrate for GSK3ß.

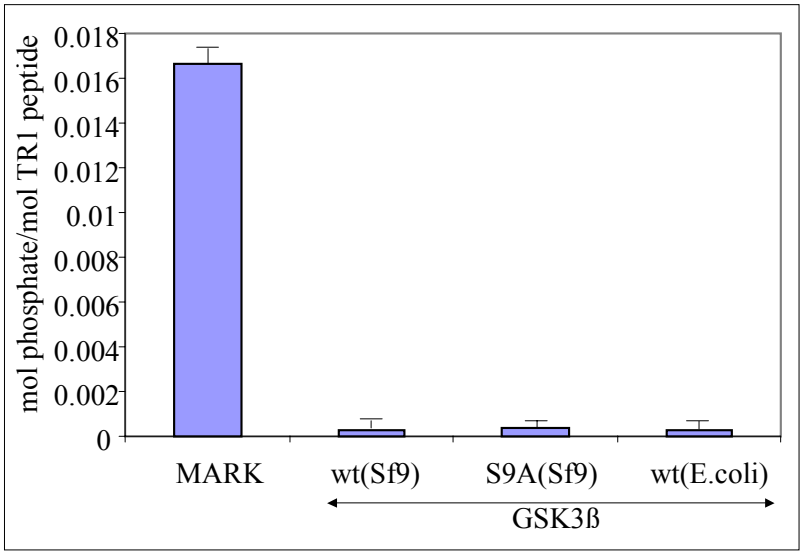

Figure 11: ***In vitro* kinase assay with TR1 peptide as a substrate**. The activities of recombinant HA-MARK2^{wt} (expressed in *E.coli*) or His-GSK3ß^{wt} (purified from Sf9 cells or *E.coli*) or His-GSK3ß^{S9A} (from Sf9 cells) were assayed for its ability to phosphorylate TR1 peptide. The amount of phosphate incorporated into TR1 peptide by MARK2 is 0.016-mol phosphate, whereas the phosphate incorporated by GSK3ß into TR1 peptide is negligible. This result shows that only MARK2 can phosphorylate TR1 peptide and not GSK3ß, indicating that TR1 peptide is not a substrate for GSK3ß.

4.2.4 Effect of GSK3ß inhibitors on MARK and MARKK

The effect of GSK3ß inhibitors lithium chloride, staurosporine and SB-216763 on MARK2 and MARKK activity was tested *in vitro*. The kinase assays were carried out in the presence of either synthetic TR1 peptide (substrate for MARK), or a synthetic G23KY peptide (a substrate for MARKK). The synthetic G23KY peptide ($_{203}$GNKLDTFCGSPPYAAPELFQGKKY$_{226}$), a specific substrate for MARKK, was derived from the activation loop of MARK2.

The kinase (MARK or MARKK) was pre-incubated with the inhibitor staurosporine (3nM) or lithium chloride (2mM or 20mM) SB-216763 (concentration 1µM) for 30 minutes on ice. Then radioactive ATP and appropriate substrate (TR1 or G23KY) was added and incubated for further 2 hours at 30^{o}C. The amount of phosphate incorporated into peptide was analyzed by scintillation counter (section 3.3.12)

Staurosporine (a relatively non-selective protein kinase inhibitor, inhibits many kinases to different degrees) (Kabir et al., 2002) and lithium chloride (a specific inhibitor of GSK3) (Klein et al., 1996) were tested for the inhibition of MARK and MARKK. The activities of MARK and MARKK in the presence of staurosporine or lithium chloride were assayed using TR1 peptide or G23KY peptide as a substrate.

Figure 12A shows the activity of MARK2 or MARKK was completely lost in the presence of 3nM staurosporine. This result indicates that staurosporine strongly inhibits MARK and MARKK activity.

Figure 12B shows the activity of MARK2 or MARKK was not affected in the presence of 2mM or 20mM lithium chloride. The activity of GSK3ß was reduced to 50% and 10% in the presence of 2mM and 20mM lithium chloride concentration. This result indicates that lithium chloride inhibits GSK3ß but not MARK or MARKK.

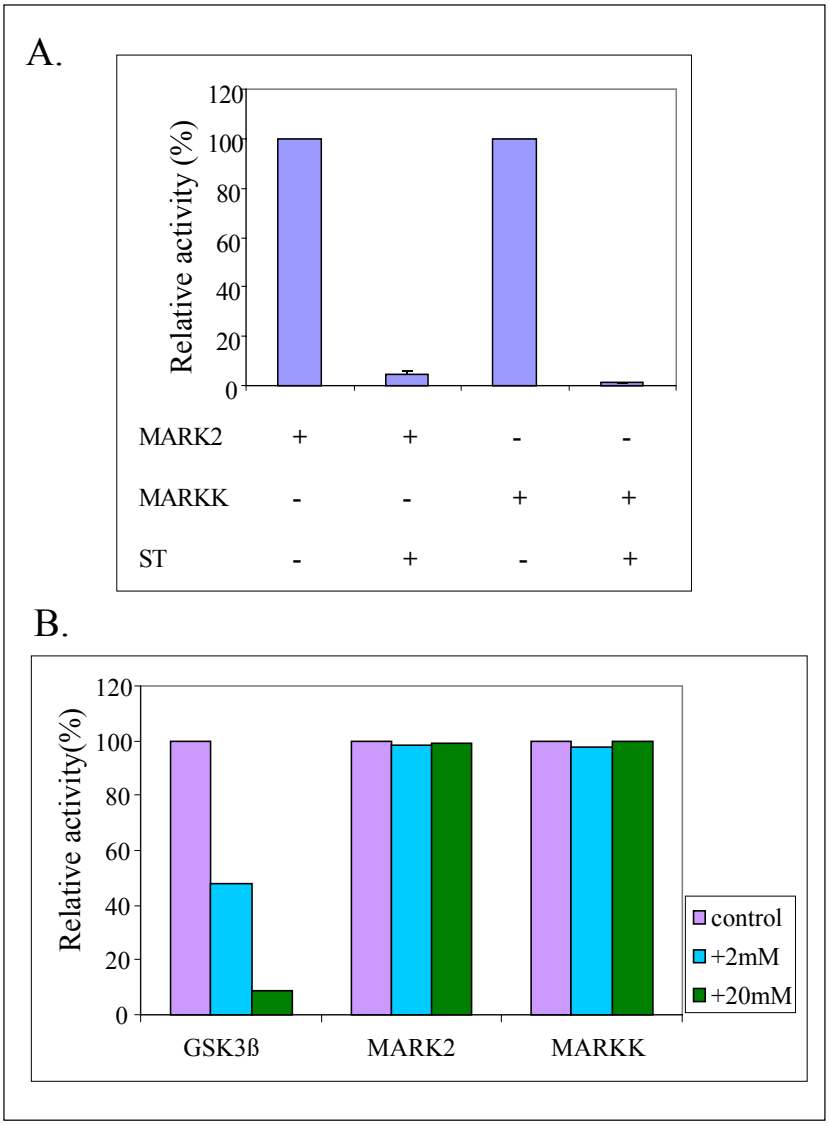

Figure 12: **Effect of staurosporine and lithium chloride on MARK and MARKK**. Recombinant MARKK (300nM) or MARK2 (300nM) was assayed for its ability to phosphorylate G23KY peptide (for MARKK) or TR1 peptide (for MARK2) in the presence of staurosporine (3nM) or lithium chloride (2mM or 20mM). A. The activities of MARK2 and MARKK were drastically reduced in the presence of staurosporine. ST=staurosporine. B. The activities of MARK2 or MARKK were not affected in the presence of 2mM or 20mM lithium chloride.

SB-216763 compound was originally designed as an inhibitor of GSK3ß (Cross et al., 2001). Under our experimental condition, the inhibition of GSK3ß by SB-216763was measured to be around 750nM (IC50=750nM). So, the activities of MARK2 and MARKK were checked in the presence of 1µM inhibitor. As seen in the figure 13, the activity of MARK2 and MARKK was reduced to approximately 50% and 30% respectively.

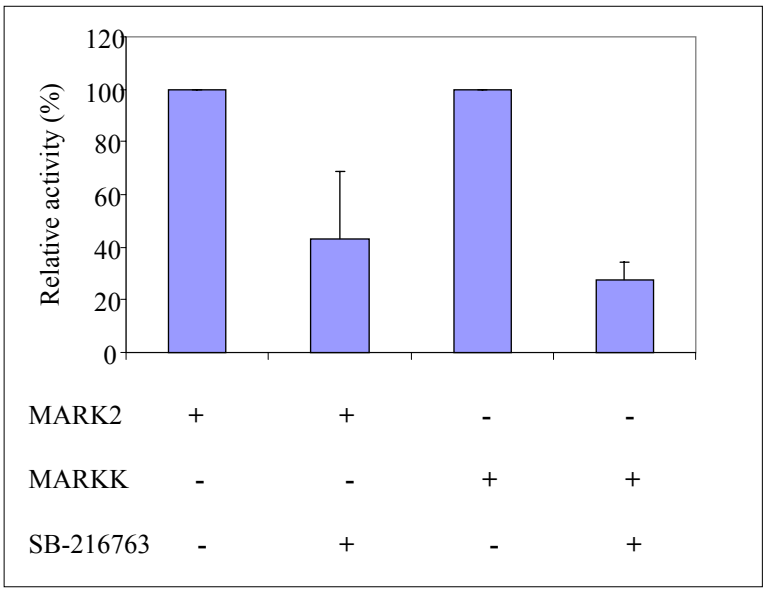

Figure 13: **Effect of SB-216763 on MARK2 and MARKK**. The activities of recombinant MARK2 (*E.coli*) and MARKK (Sf9) were assayed for its ability to phosphorylate htau40 or G23KY peptide in the presence of 1µM inhibitor concentration. The activity of MARK2 and MARKK was reduced to approximately 50% and 30%.

The above results indicate that the inhibitor staurosporine significantly reduced the activity of MARK and MARKK *in vitro*. SB-216763, a specific GSK3ß inhibitor reduced the activity of MARK2 and MARKK *in vitro*. Lithium chloride, which is known to be a specific inhibitor of GSK3, did not affect the activity of either MARK or MARKK up to a concentration of 20mM. So, lithium chloride was selected to study the relationship between MARK and GSK3ß in cells.

4.3 Interaction between GSK3ß and MARK2

The interaction between GSK3ß and MARK2 was tested by two methods: yeast two-hybrid system and the GST pull-down assay.

4.3.1 Yeast two-hybrid system

Clontech Matchmaker Gal4 two-hybrid system was used to check the interaction between MARK2 and GSK3ß. The constructs used were: $MARK2^{wt}$, $MARK2^{T208A/S212A}$, N-terminal (amino acids 1-370) and C-terminal (amino acids 324-722) and full length GSK3ß. The result indicates that there is no interaction between different MARK2 constructs and full length GSK3ß.

No.	Plasmid (A)	Plasmid(B)	Interaction
1	$MARK2^{wt}$	GSK3ß	-
2	$MARK2^{T208A/S212A}$	GSK3ß	-
3	MARK2 N-terminus (1-370 amino acids)	GSK3ß	-
4	MARK2 C-terminus (324-722 amino acids)	GSK3ß	-
5	pGADT7	GSK3ß	-

Figure 14: **Interaction between GSK3ß and MARK2 in yeast two-hybrid system**. $MARK2^{wt}$, $MARK2^{T208A/S212A}$, N-terminal (amino acids 1-370) and C-terminal (amino acids 324-722) constructs were tested with full length $GSK3ß^{wt}$ in yeast two-hybrid screens. Data was collected under the most stringent growing conditions for yeast. Results show that there is no interaction between full length GSK3ß and different constructs of MARK2.

4.3.2 GST pull-down assay

The aim of this experiment was to check whether there is an interaction between the GST-tagged MARK2 constructs and the endogenous GSK3ß expressed in differentiated and undifferentiated PC12 cells.

The recombinant GST-tagged MARK2 constructs (GST-MARK2 NCU^{wt} and GST-MARK2 $NCU^{T208A/S212A}$) were expressed and purified from *E.coli*. The length of MARK2 NCU construct used is 1-362 amino acids. GST-MARK2 $NCU^{T208A/S212A}$ mutant of MARK2 was chosen because it eliminates the phosphorylation sites in the activation loop of MARK2 ($MARK2^{T208A/S212A}$) and thus stabilizes the interactions with the potential kinase phosphorylating these sites, as has been shown for the example of the activating kinase MARKK (Timm *et al.*, 2003).

For the pull-down assay equal amounts (30µM) of different recombinant GST-MARK2 constructs were mixed with PC12 cell lysate expressing endogenous GSK3ß and incubated over night. The proteins were precipitated for 2 hours using glutathione-sepharose 4B beads (section 3.3.11). GST protein or the beads alone with the lysate serve as a negative control. After centrifugation the beads were collected, washed twice with PBS and then resuspended in 50µl of 2xSDS sample buffer. The samples were resolved in SDS-PAGE followed by Western blotting with GSK3ß and GST antibodies.

The expression of GSK3ß is similar in the undifferentiated and differentiated cells (figure 15A, lanes 1, 2). The GST proteins pulled down different amount of GSK3ß (lanes 5, 6, 8, 9). Though the amount of GST proteins used was similar but after pull-down the amount of the GST proteins retained were not the same (lanes 5, 6, 8, 9). So the amount of GSK3ß pulled down with GST constructs were quantified using AIDA software.

Figure 15B shows that there is a strong interaction between MARK2 $NCU^{T208A/S212A}$ and GSK3ß in undifferentiated cells compared to the differentiated PC12 cells. In contrast, there is only a very weak interaction between GST-tagged MARK2 NCU^{wt} and GSK3ß in both undifferentiated cells and the differentiated PC12 cells.

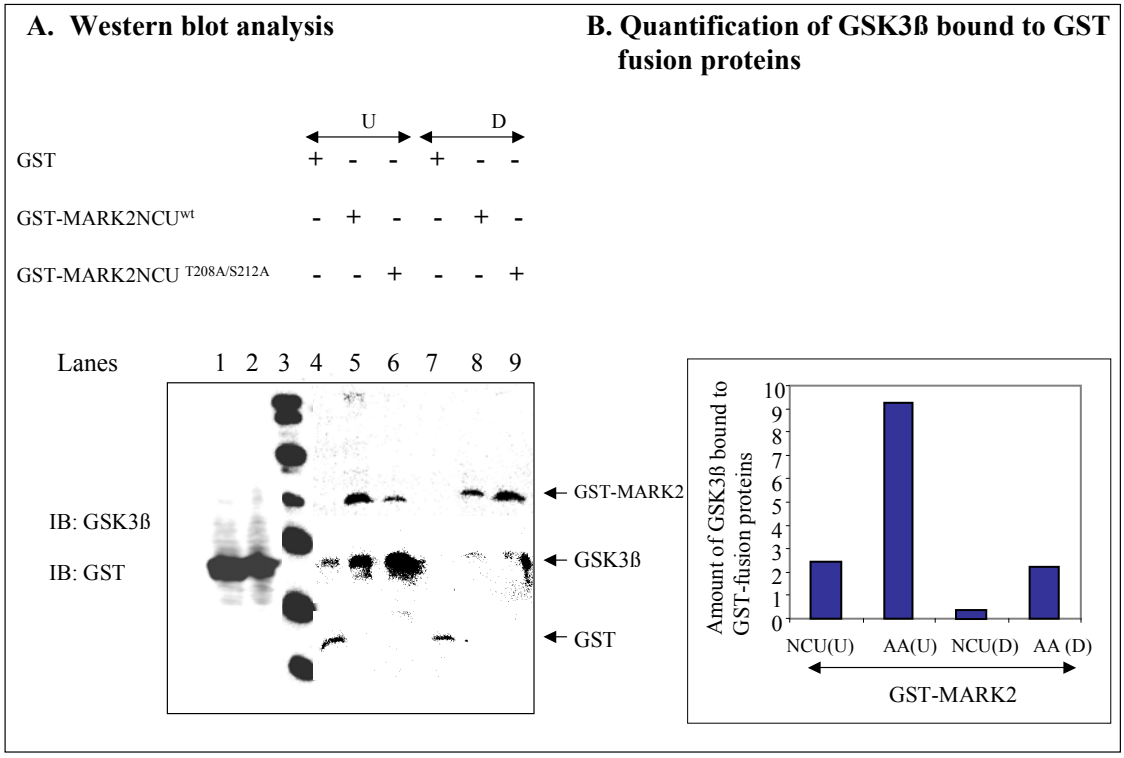

Figure 15: **GST pull-down assay**. A. PC12 cells ($3.5x10^6$ cells) were plated in a T-25 flask coated with poly-D-Lysine and cultured for 24 hours and then differentiated with 100ng/ml NGF for 24 hours. Undifferentiated cells were used as control. The cells were collected, lysed in 300µl lysis buffer and the lysates were cleared by centrifugation. The bacterially expressed and purified GST-$MARK2^{T208A/S212A}$ (30µM) and GST-$MARK2^{wt}$ (30µM) were added to the supernatant (to pull down the endogenous GSK3ß) and the proteins were precipitated with gluthathione-4B sepharose beads. The beads were washed with PBS to remove non-specific binding and the beads were finally resuspended in 50µl of 2xSDS buffer (section 3.3.10). The pull-down fractions (10µl) were resolved in SDS-PAGE followed by Western blot analysis with GSK3ß and GST antibodies. The amounts of GSK3ß expressed in the undifferentiated and differentiated cells are similar as shown in lanes 1, 2. The blot shows the amount of GST-fusion proteins that are retained. The inputs were similar but after pull down the amount of GST proteins that retained was not the same. B. The intensity of the bands (GST-$MARK2^{T208A/S212A}$ and GSK3ß) was measured using AIDA software. The figure 15B shows that $MARK2^{T208A/S212A}$ interacts strongly with GSK3ß in undifferentiated cells compared to the differentiated cells. U=undifferentiated, D=differentiated, AA=$MARK2^{T208A/S212A}$.

4.3.3 Sub-cellular fractionation of adult and embryo (E18) rat brain

To examine the sub-cellular localization of MARK and GSK3ß from *Rattus norvegicus*, embryonic and adult brains were used.

Adult and embryonic rat brain (0.2g) was homogenized in 2ml of homogenization buffer and the tissues were disrupted using a polytron homogenizer. Nuclei and unbroken cells were pelleted down by centrifugation. The post nuclear supernatant was again centrifuged and the resultant vesicle pellet was resuspended in 0.8ml of homogenization buffer with 1% triton X-100. The resuspended vesicle fractions was loaded on the top of an iodixanol gradient and centrifuged. The gradient was clearly noticed (alternative thick and thin bands). The thick and thin bands were marked and collected separately (section 3.3.11). A total of 12 fractions (approximately 500µl each) were collected and 10µl of the fraction was loaded onto a 10% gel for Western blot analysis.

As shown in the figure 16B&C, both in embryo and in the adult rat brain, GSK3ß was found uniformly distributed in all the fractions. On the other hand MARK2 was differentially distributed. In the embryonic brain MARK2 was found in fractions 1-7 (figure 16B). In the adult brain, MARK2 was mostly found in ER fractions 8-12 (figure 16C) detected with the MARK2 specific antibody. The antibody was raised against the N-terminal peptide (19-33 amino acids). The two bands were found in the MARK2 blot. The lower band might be the degradation product of MARK2.

Next, localisation of the activators of MARK2 (MARKK and LKB1) was checked. In the early developmental stage active MARK ($pThr_{208}$) was found to co-localize with MARKK (figure 16B, fractions 1-7), whereas LKB1 was found in completely different fraction (fractions 9-12). In the case of adult brain the amount of active MARK2 in lane 12 is less compared to lane 11, which in turn correlates with MARKK and not with LKB1. The active MARK is detected with a specific antibody against phosphorylated Thr_{208} (numbering with respect to MARK2) in the activation loop. This data clearly suggest that MARKK is responsible for the activation of MARK2 in both embryonic and in adult brain.

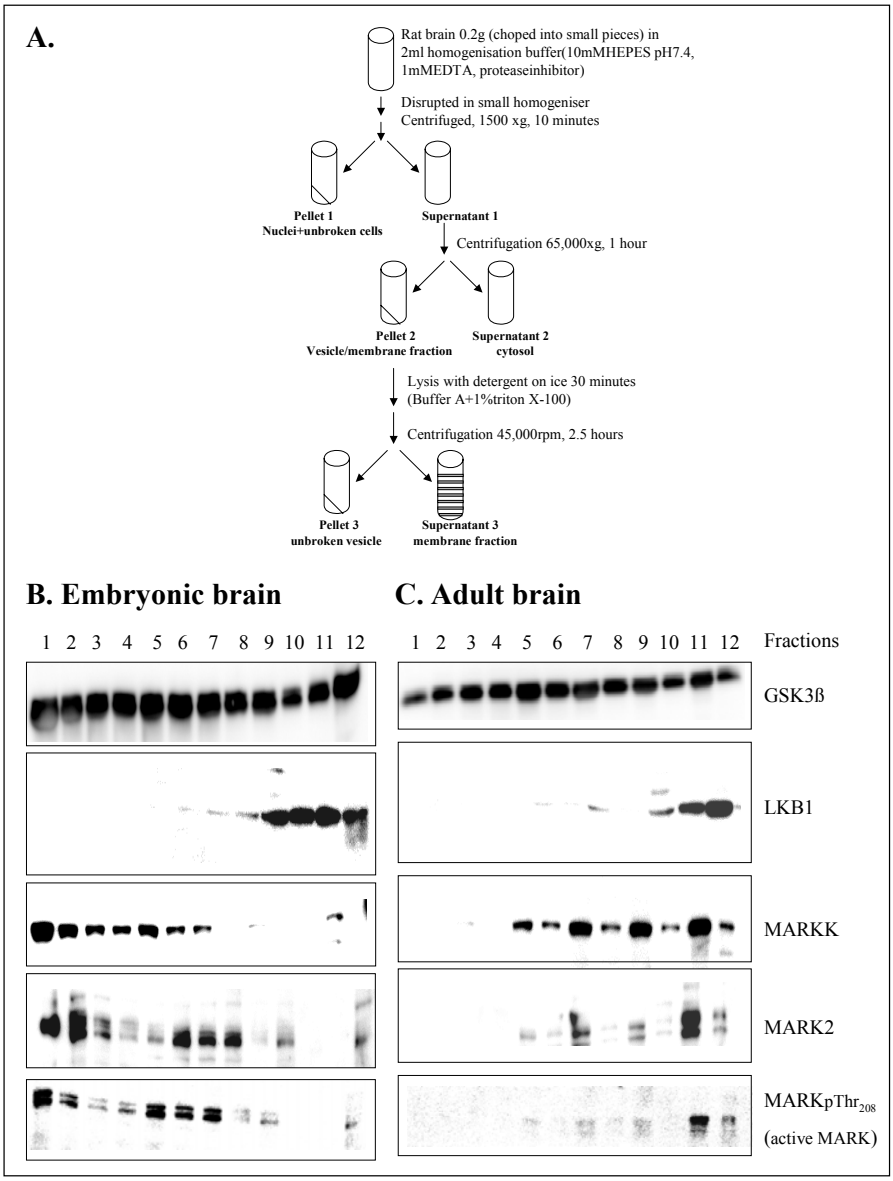

Figure 16: **Sub-cellular fractionation of rat brain by iodixanol gradient method**. A. Schematic representation of the fractionation of rat brain by Iodixanol gradient. Adult and embryonic rat brain (0.2g) was homogenized separately in 2ml of the homogenization buffer and the tissues were disrupted using a polytron homogenizer. Nuclei and unbroken cells were pelleted down by centrifugation at 1,500 xg for 10 minutes. The post nuclear supernatant was again centrifuged for 1 hour at 65,000 xg. The resultant vesicle pellets were resuspended in 0.8ml of homogenization buffer with 1% triton X-100. The resuspended vesicle fractions was loaded on the top of an iodixanol gradient and centrifuged at 40,000 rpm for 2.5 hours. The gradient was clearly noticed (alternative thick and thin bands). The bands were marked and collected separately. A total of 12 fractions were collected (approximately 500µl each). B and C. 10µl of the fractions were loaded onto a 10% gel followed by Western blot analysis using battery of antibodies like GSK3ß, MARKK, LKB1, MARK2 and MARK2 $pThr_{208}$. GSK3ß is uniformly distributed in all the fractions. MARKK co-localizes with active MARK2 in the embryonic brain (figure 16B, lanes 1-7), whereas the LKB1 is localized in different fractions (figure 16B, lanes 9-12). In the case of adult brain MARKK, LKB1 and MARK2 were found in the same fraction (figure 16C, lanes 11, 12), but the amount of active MARK correlates with MARKK and not with LKB1. This result indicates that MARKK is responsible for regulating MARK activity and not LKB1 in the brain.

4.4 Inhibition of MARK activity by GSK3ß *in vitro*

The kinase MARK was initially discovered because of its ability to phosphorylate tau and related MAPs (MAP2, MAP4) at serines within the K*X*GS motifs in the microtubule-binding domain, leading to the detachment of tau from the microtubules and as a result disrupting the microtubule network.

The activation loop of MARK has two phosphorylation sites, Thr_{208} and Ser_{212} (numbering according to MARK2). Activation of MARK2 is achieved by phosphorylation of a single residue, Thr_{208} by MARKK (Timm et al., 2003) or LKB1 (Licanzo et al., 2004). It was shown under *in vitro* condition that when Thr_{208} was mutated into Ala (T208A), the activation by MARKK was lost and only basal activity remained. This means that Thr_{208} is important for activation, and that the basal activity does not depend on it. By contrast, when Ser_{212} or both Thr_{208} and Ser_{212} were mutated into Ala (S212A, T208A/S212A), the intrinsic and the activatable activities of MARK2 were completely abolished. When Thr_{208} was mutated into Glu (T208E), the basal activity was increased 4 fold but no further activation by MARKK took place. By changing Ser_{212} of MARK2 into Glu the basal activity and the activation by MARKK was lost, independently of whether Thr_{208} was mutated to Glu. Since E often mimics phospho-ser or -thr, this also suggests that phosphorylation of Ser_{212} is not essential for activity, but on the contrary is inhibitory (Timm et al., 2003).

Since proline residues follow Ser_{212} in the catalytic loop, the proline-directed kinase GSK3ß was checked for the phosphorylation of Ser_{212} and effect on MARK2 activity. The activity of MARK2 can be measured by the phosphorylation of TR1 peptide derived from the first repeat sequence of tau protein.

4.4.1 Effect of recombinant GSK3ß on MARK2 activity

The activity of recombinant MARK2 in the presence of recombinant GSK3ß was checked at different time points with TR1 peptide as a substrate.

The kinase MARK2 (1.5µg) alone or MARK2 with GSK3ß (1.5µg + 1.0µg) was mixed with radioactive ATP. After 30, 60, 90, 120 and 150 minutes, the reaction solution was mixed with TR1 peptide and incubated at 30^{o}C for 30 minutes (section 3.3.12). The amount of radioactivity incorporated into TR1 peptide was calculated.

To check whether MARK2 has any effect on GSK3ß, MARK2 (0.5µg) was incubated with GSK3ß (0.5µg) and radioactive ATP at 30^{o}C for 2 hours. pCREB peptide, a specific GSK3ß substrate was added and incubated for further 30 minutes at 30^{o}C. The amount of radioactivity incorporated into pCREB peptide was calculated.

As shown in figure 17A, in the presence of GSK3ß, the activity of MARK2 was reduced as the time increases, whereas the activity of MARK2 alone without GSK3ß remains nearly constant (figure 17A, red circles). The activity of MARK was calculated to be 55 nmol/min/mg. Treatment of MARK2 with GSK3ß leads to 3-fold decrease in activity (~15 nmol/min/mg; figure 17A, black circles). On the other hand, the activity of GSK3ß was not reduced in the presence of MARK2 (figure 17B). These data indicate that GSK3ß inhibits the activity of MARK2 *in vitro,* but the reverse does not hold.

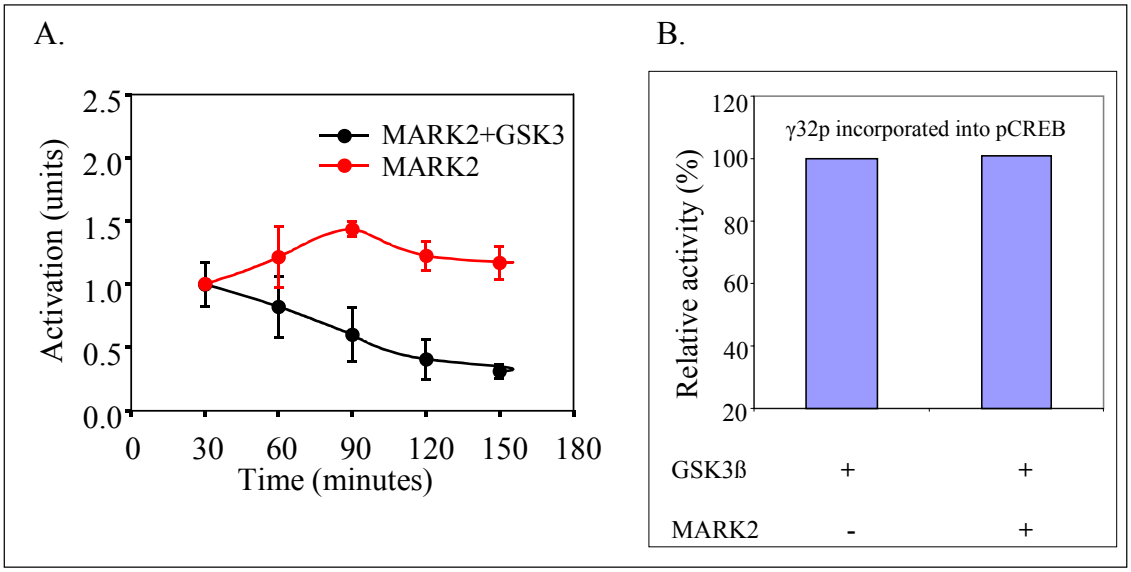

Figure 17: **Activity of recombinant MARK2 in the presence of GSK3ß**. A. Kinase assay was performed with MARK2 (1.5µg) alone or MARK2 with GSK3ß (1.5µg+1.0µg) and radioactive ATP in a total volume of 30 µl each. After 30, 60, 90, 120 and 150 minutes, 5µl of the reaction solution was mixed with TR1 peptide and incubated at 30°C for 30 minutes (section 3.3.12). B. MARK2 (0.5µg) was incubated with GSK3ß (0.5µg) and radioactive ATP and incubated at 30°C for 2 hours. Then pCREB peptide was added and incubated for further 30 minutes at 30°C. Reactions were stopped by addition of half the volume of 30% (w/v) TCA. After centrifugation the supernatant was applied to phosphocellulose paper discs, washed with phosphoric acid (0.1M), dried by air and radioactivity was measured in a scintillation counter. Figure 17A shows that the activity of MARK2 was reduced in the presence of GSK3ß as the time increases (black circles), whereas the activity of MARK alone remains nearly constant (red circles). Figure 17B shows that the activity of GSK3ß was not affected in the presence of MARK2. This result confirms that only GSK3ß inhibits MARK2 but the reverse does not hold.

4.4.2 GSK3ß inhibits MARK2 by phosphorylating at Ser_{212}

Next, we checked whether the reduction in MARK2 activity in the presence of GSK3ß is through phosphorylation at Ser_{212}. It was reported that Thr_{208} and Ser_{212} residues in the activation loop of MARK2 are important for the activity of the kinase (Timm et al., 2003). So the two residues were mutated to non-phosphorylatable Ala or Glu residues ($MARK2^{T208A}$, $MARK2^{S212A}$). The activity and the phosphorylation of these mutants were checked in the presence of GSK3ß.

For the activity assay, 0.5µg of wild type and the mutants of MARK2 ($MARK2^{T208A}$, $MARK2^{S212A}$, $MARK2^{T208E}$) were pre-incubated with 0.3µg of GSK3ß and radioactive ATP at 30°C for 2 hours. Then the substrate was added and incubated for further 30 minutes. The reaction was stopped by the addition of half the volume of 30% (w/v) TCA. After centrifugation, the supernatant was applied to phosphocellulose paper discs, washed with phosphoric acid (0.1M), dried by air and radioactivity was measured in a scintillation counter (section 3.3.12). The pellet was resuspended in 10µl of 1xSDS buffer. 5µl of the sample was resolved in SDS-PAGE, stained with Roti-blue (section 3.3.2) and an autoradiogram was performed. Western blot analysis of the other half of the GSK3ß treated MARK samples was performed with $pSer_{212}$ antibody.

As shown in figure 18A, in the absence of GSK3ß (lanes 1, 3, 5, 7) MARK2 wild type phosphorylates the TR1 peptide at a rate of 55 nmol/min/mg (1 unit = 55 nmol/min/mg) and $MARK2^{T208A}$ at a rate of approximately 22 nmol/min/mg (figure 18A, lane 3). The activity of $MARK2^{T208E}$ mutant was increased to 6 fold (figure 18A, lane 7) compared to $MARK2^{wt}$. The mutant $MARK2^{S212A}$ was completely inactive. In the presence of recombinant GSK3ß, the activity of $MARK2^{wt}$, $MARK2^{T208A}$ and $MARK2^{T208E}$ was reduced to approximately 3-4 fold (figure 18A, lanes 2, 4, 8).

SDS-PAGE (figure 18B) shows the amount of proteins used for the kinase assay was similar. The other bands in between MARK2 and GSK3ß might be the degradation product of MARK2 and in the case of wild type, T208A and S212A we identified bacterial heat shock protein.

The autoradiogram (figure 18C) shows that in the absence of GSK3ß, $MARK2^{T208A}$ (lane 3) there is weak autophosphorylation compared to $MARK2^{T208E}$. In the presence of GSK3ß, there is strong incorporation of phosphate in $MARK2^{wt}$ and $MARK2^{T208A}$ whereas no increase in phosphorylation was noticed in the case of $MARK2^{T208E}$ (figure 18C, lanes 7, 8). This might be because the reduction in autophosphorylation is equal to the amount of phosphate incorporated by GSK3ß. No autophosphorylation or phosphate was incorporated into $MARK2^{S212A}$ mutant in the presence of GSK3ß (figure 18C, lanes 5, 6). This in turn indicates that increase in phosphorylation of $MARK2^{wt}$, $MARK2^{T208A}$ is due to the phosphorylation of GSK3ß at Ser_{212} and not at other amino acid residues. This clearly shows that the phosphorylation of GSK3ß remains unaffected even when Thr_{208} was mutated to Ala.

The data was further confirmed by Western blot analysis using a specific phosphorylation dependent antibody (MARK2 $pSer_{212}$ antibody). As seen in the figure 18D, in the presence of GSK3ß this antibody recognizes $MARK2^{wt}$ weakly and the $MARK2^{T208A}$ mutant strongly whereas no signal was detected with $MARK2^{S212A}$ mutant. GSK3ß phosphorylates MARK2 at Ser_{212} and this phosphorylation is even stronger when Thr_{208} was mutated to Ala. The positive control is a partially purified porcine brain extract where MARK was doubly phosphorylated at Thr_{208} and Ser_{212} (numbering according to MARK2). The other bands in this lane might be other proteins.

These data collectively indicates that GSK3ß phosphorylates MARK2 at Ser_{212} and inhibits the activity of MARK2.

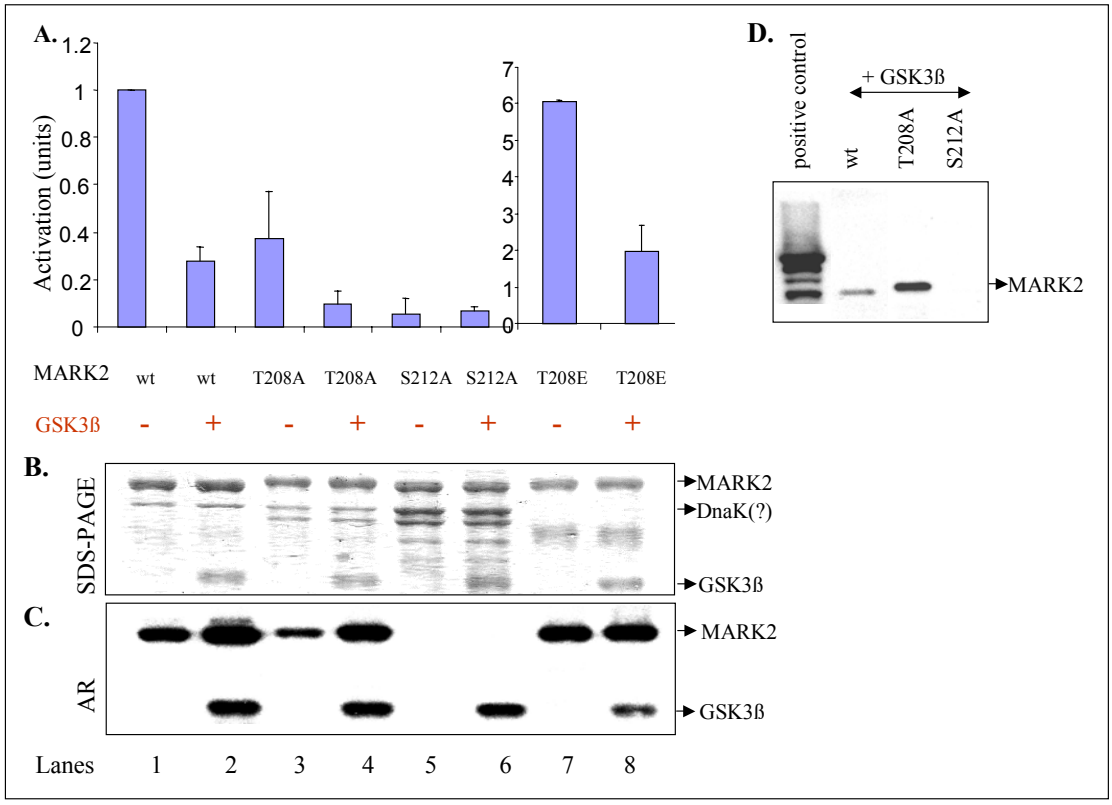

Figure 18: **Effect of GSK3ß on different MARK mutants *in vitro***. A. *In vitro* kinase assay. Wild type and the mutants of MARK2 ($MARK2^{T208A}$, $MARK2^{S212A}$, $MARK2^{T208E}$) (0.5µg) were pre-incubated in the presence and absence of recombinant GSK3ß (0.3µg) purified from *E.coli* and radioactive ATP at 30°C for 2 hours. Then TR1 peptide was added and incubated for further 30 minutes at 30°C. The amount of phosphate incorporated was measured in a scintillation counter (section 3.3.12). In figure 18A, lanes 1, 3, 5, 7 show the activity of MARK2 alone. Lanes 2, 4, 6, and 8 show the activity of MARK2 in the presence of GSK3ß. The activity of $MARK2^{wt}$ was set to 1 unit (1 unit = 55 nmol/min/mg). The activity of $MARK2^{T208A}$ is 3 fold less and $MARK2^{T208E}$ shows 6 fold higher activity compared to $MARK2^{wt}$, whereas $MARK2^{S212A}$ is inactive. The activity of the $MARK2^{wt}$, $MARK2^{T208A}$, and $MARK2^{T208E}$ was reduced 3-4 fold in the presence of GSK3ß. B. SDS-PAGE. The precipitated proteins were mixed with 10µl of 1xSDS buffer. 5µl of the sample was loaded onto a 10% gel and the proteins were separated. The amount of MARK used for the kinase assay was similar in each sample (figure 18B). C. Autoradiogram. The amount of radioactive ATP incorporated into the proteins was read using the BAS reader. $MARK2^{T208A}$ alone show weak autophosphorylation (figure 18C, lane 3) compared to $MARK2^{wt}$ and $MARK2^{T208E}$ (figure 18C, lanes 1, 7) this in turn correlates with the kinase activity. But in the presence of GSK3ß, $MARK2^{wt}$ and $MARK2^{T208A}$ show strong increase in phosphorylation (figure 18C, lanes 2, 4). For $MARK2^{T208E}$, there is no increase in the phosphorylation of the protein in the presence of GSK3ß, though there is a strong reduction in the activity of kinase (compare figure 18A&C, lanes 7, 8). May be here the reduction in autophosphorylation is equal to the amount of phosphorylation by GSK3ß. On the other hand in the case of $MARK2^{S212A}$, there is no autophosphorylation and phosphorylation by GSK3ß. This clearly indicates that GSK3ß phosphorylates the site Ser_{212}. D. Western blot analysis was performed for MARK2 samples that have GSK3ß in the reaction mixture (lanes 2, 4, 5) with $pSer_{212}$ antibody.

4.4.3 Activity assay for MARK2 in the presence of GSK3ß and MARKK

The activity of MARK2 was checked in the presence of MARKK and GSK3ß. MARKK activates MARK2 by phosphorylation at Thr_{208} (Timm et al., 2003).

MARK2 wild type (0.5µg) and the mutant S212A (0.5µg) were pre-incubated with MARKK (0.3µg), GSK3ß (0.3µg) and radioactive ATP for 2 hours at 30°C. Then the substrate TR1 peptide was added and incubated for further 30 minutes. TCA (to a final concentration of 15%) was added to precipitate the proteins. The sample was centrifuged and the supernatant was loaded onto the phosphocellulose disc and the amount of phosphate incorporated into TR1 peptide was counted using the liquid scintillation counter (section 3.3.12). The pellet was mixed with 1xSDS buffer and loaded onto a 10% gel and an autoradiogram was performed.

$MARK2^{wt}$ alone showed a low basal activity (figure 19A, lane 1), but S212A mutant showed no basal activity (figure 19A, lane 2). In the presence of MARKK the activity of $MARK2^{wt}$ was increased to approximately 7 fold (figure 19A, lane 3). On the other hand in the case of S212A mutant there is no intrinsic activity and activation by MARKK (figure 19A, lane 4). The activity of $MARK2^{wt}$ was lost completely in the presence of GSK3ß (figure 19A, lane 5). Upon simultaneous incubation of $MARK2^{wt}$ with MARKK and GSK3ß, the activity of $MARK2^{wt}$ was reduced 3 fold (figure 19A, lane 7) compared to activation of MARK2 by MARKK (figure 19A, lane 3).

In the autoradiogram (figure 19B), $MARK2^{wt}$ showed autophosphorylation compared to the S212A mutant (figure 19B, lanes 1, 2). By incubating $MARK2^{wt}$ or $MARK2^{S212A}$ with MARKK there is a strong incorporation of the phosphate in both wild type and the S212A mutant indicating that the phosphorylation of MARK2 at Thr_{208} by MARKK is not affected by the mutation of Ser_{212} to Ala (figure 19B, lanes 7, 8). In contrast, incubation of $MARK2^{wt}$ or $MARK2^{S212A}$ with $GSK3ß^{wt}$, there is strong incorporation of phosphate in $MARK2^{wt}$ (figure 19B, lane 5) whereas no phosphate was incorporated into S212A mutant (figure 19B, lane 6), indicating that when Ser_{212} is mutated to Ala, GSK3ß cannot phosphorylate MARK2. This result

indicates that when Ser_{212} is mutated to Ala, there is no basal activity and activation by MARKK, independently of the phosphate incorporated at Thr_{208} in S212A mutant by MARKK. The activation of MARK2 in the presence of MARKK was reduced in the presence of GSK3ß, further confirming the inactivation of MARK2 by GSK3ß even in the presence of the activator MARKK.

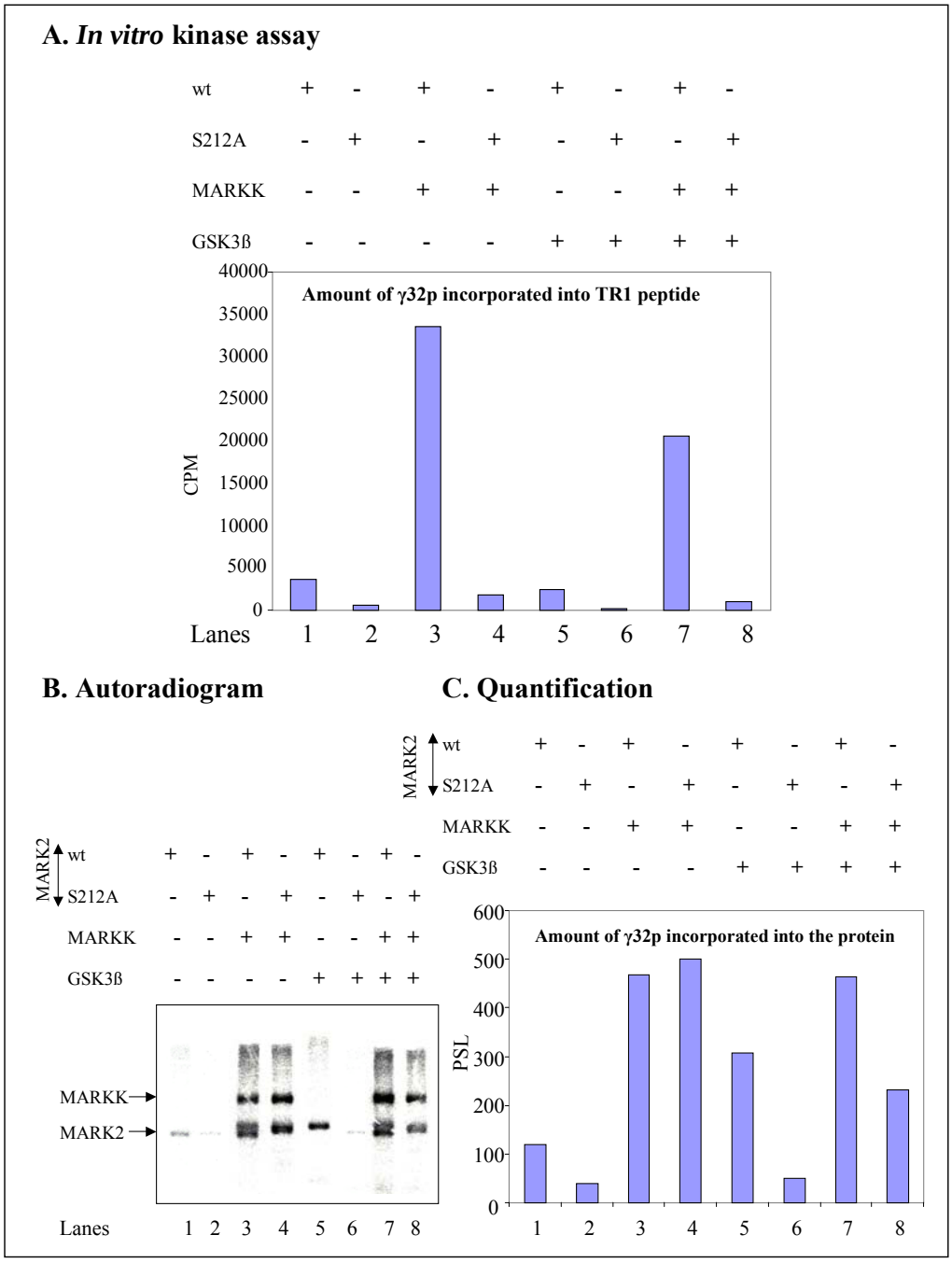

Figure 19: **Activity of MARK2 in the presence of MARKK and GSK3ß**. A. *In vitro* kinase assay. $MARK2^{wt/S212A}$ were pre-incubated with MARKK, GSK3ß and radioactive ATP at 30°C for 2 hours. Then the substrate TR1 peptide was added and incubated for further 30 minutes at 30°C. The amount of phosphate incorporated into the peptide was counted using scintillation counter (section 3.3.12). B. Autoradiogram. The pellet was mixed with 10µl of 1xSDS buffer and loaded onto a 10% gel. The proteins were separated and the amount of radioactive ATP incorporated was detected using the BAS reader. The activity of MARK2 was reduced in the presence of MARKK and GSK3ß. MARK2 was phosphorylated at Thr_{208} independently of the mutation at Ser_{212} to Ala (lanes 4, 8). C. The intensity of the band from the autoradiogram was calculated using AIDA software PSL-photo stimulated luminescence.

4.4.4 Activity of immunoprecipitated MARK2 in the presence of recombinant GSK3ß added *in vitro*

The *in vitro* activity towards the TR1 peptide by immunoprecipitated MARK2 from transfected Sf9 cells was examined in the presence and absence of recombinant GSK3ß.

Sf9 cells were infected with the baculovirus encoding HA-MARK2 for 72 hours. Lysates were prepared and HA antibody agarose conjugate (monoclonal) was added to immunoprecipitate the HA-tagged proteins. The immunoprecipitates were resuspended in 50µl of 1xkinase assay buffer (section 3.3.9). The immunoprecipitated MARK2 (7µl) was incubated with radioactive ATP and TR1 peptide in the presence and absence of GSK3ß at 30^{o}C for 2 hours.

As shown in figure 20, the immunoprecipitate HA-MARK2 phosphorylated the TR1 peptide strongly; however in the presence of recombinant GSK3ß the phosphorylation of TR1 peptide was reduced to less than 50%. This data indicate that addition of recombinant GSK3ß to MARK2 immunoprecipitate inhibits the activity of MARK2.

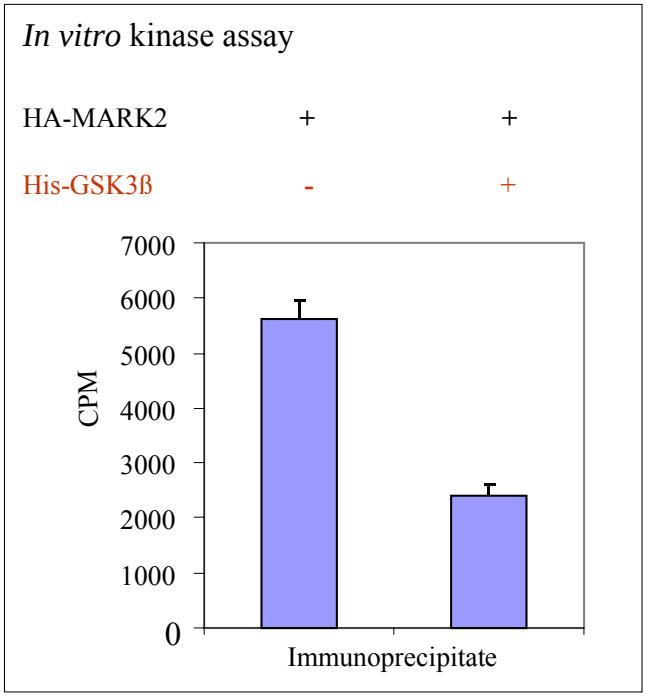

Figure 20: **Recombinant GSK3ß reduces the activity of MARK2 *in vitro*.** Sf9 cells (80% confluent) were infected with HA-MARK2 baculovirus for 72 hours. The cells were lysed in 300µl lysis buffer, and cleared by centrifugation. HA agarose conjugate antibody (monoclonal) was added to the supernatant in the ratio of 1:20 and incubated at 4°C for 4 hours to immunoprecipitate HA-MARK2. Centrifuged, pellet was collected and washed with wash buffer (3 times) (section 3.3.19). Finally, the pellet was resuspended in 50µl of 1xkinase assay buffer. 7µl of the immunoprecipitate MARK2 was incubated with radioactive ATP and TR1 peptide in the presence and absence of 0.5µg of GSK3ß. The amount of phosphate incorporated into TR1 peptide by MARK2 immunoprecipitate was reduced to less than 50% in the presence of recombinant GSK3ß.

4.5 Effect of GSK3ß inhibitors on 12E8 phosphorylation of tau in cortical neurons

It was shown that GSK3ß phosphorylates htau40 (longest human tau isoform) at multiple sites including Ser_{396}, Ser_{404}, Ser_{46}, Ser_{50}, Ser_{202}, and Thr_{205} (Godemann et al., 1999). Both *in vitro* and *in vivo* experiments have shown that lithium chloride inhibits GSK3ß activity (Klein et al., 1996). Since the other inhibitors of GSK3ß like SB-216763 and staurosporine also affect MARK2 and MARKK activity, lithium chloride was used to examine the relationship between MARK2 and GSK3ß. Cortical cells were treated with the inhibitors (lithium chloride, staurosporine, 14-2A) along with

phosphatase inhibitor okadaic acid (OA) to prevent the rapid dephosphorylation of tau protein by phosphatases for 3 hours. The effects of the inhibitors on tau phosphorylation were checked with tau antibodies (K9JA, 12E8 and PHF1).

The K9JA antibody raised against the four repeats and C-terminal tail of tau detects tau regardless of the phosphorylation and serves as a standard for the protein concentration. The 12E8 antibody clearly discriminates between the phosphorylated and non-phosphorylated proteins, despite the fact that 12E8 was raised against a synthetic phosphopeptide corresponding to amino acids 257-270 of tau protein (according to the numbering of human tau40). PHF1 antibody recognizes tau when it is phosphorylated at Ser_{396} and Ser_{404}.

Inhibition of GSK3 activity by lithium chloride treatment reduced the phosphorylation at PHF1 site (IB: PHF1, lane 3) and partially reduced the phosphorylation of Ser_{262} in the K*X*GS motif compared to OA treatment (IB: 12E8, lanes 2, 3). Since GSK3 cannot phosphorylate Ser_{262} of tau directly, the finding raised the possibility that GSK3 phosphorylates and activates a K*X*GS motif kinase, leading to the phosphorylation of tau at Ser_{262}. MARK mainly mediates the phosphorylation of tau at Ser_{262} in the K*X*GS motif, but there are some reports, which implicates that PKA (Schneider et al., 1999), SAD (Kishi et al., 2005) or p70S6K (Pei et al., 2006) can also phosphorylate at this site. To rule out the possibility that PKA phosphorylates Ser_{262}, cells were treated with PKA inhibitor (14-2A). Inhibition of PKA clearly showed that there was no change in the 12E8 staining, indicating that MARK2 or other kinases like SAD (Kishi et al., 2005) or p70S6K (Pei et al., 2006) phosphorylate Ser_{262} in the K*X*GS motif and not PKA. K9JA antibody shows that the amount of tau in each sample is similar.

To confirm the above data, the activity of MARK in the total cell lysate from cells treated with different inhibitors was checked *in vitro* with TR1 peptide as a substrate (figure 21B). *In vitro* kinase assay showed that inhibition of GSK3ß in cells with lithium chloride reduced the phosphorylation of TR1 peptide, which can be phosphorylated by MARK (Drewes et al., 1995) but not by GSK3ß. Similar to lithium chloride, staurosporine also strongly inhibited the MARK activity, whereas in the presence of okadaic acid the phosphorylation of TR1 peptide was increased. This result indicates that MARK might be regulated through GSK3ß.

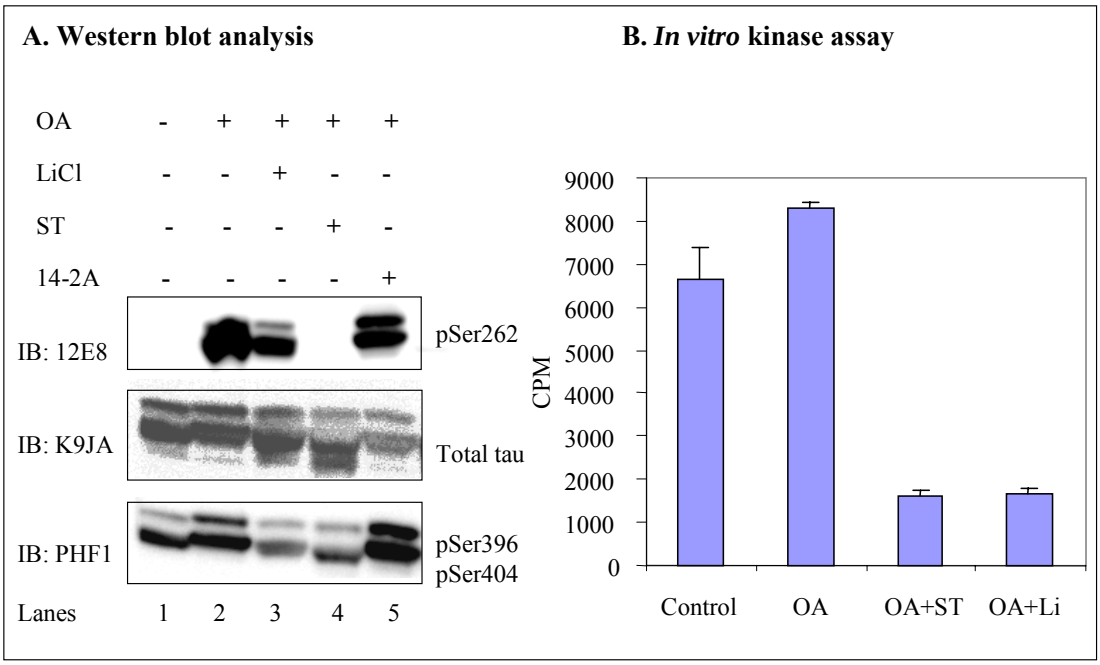

Figure 21: **Inhibition of GSK3ß with lithium chloride partially decreases 12E8 staining**. Cortical neurons ($2.4x10^6$ cells/well) were plated in a 6 well plate coated with poly-D-Lysine, cultured for 7 days and then treated with the inhibitors for 3 hours. Lysates were collected and the concentration of protein was estimated by BCA method (section 3.3.3b). A. Western blot analysis. 20µg of the total cell lysate was loaded onto a 10% gel and the Western blot analysis was performed with the following antibodies: K9JA, 12E8 and PHF1. The phospho-independent tau antibody K9JA shows that the total tau levels in all the lanes were approximately the same. The 12E8 antibody shows that the amount of phosphorylated tau is higher in lane 2 compared to lanes 4, 5 and there is a partial reduction in lane 3 where the cells are treated with lithium chloride. The amount of inhibitors used is: okadaic acid (OA-100nM), lithium chloride (Li-20mM), staurosporine (ST-50µM), 14-2A (10µM). 14-2A is a PKA inhibitor. B. Total cell lysate (10µg) was incubated with radioactive ATP and TR1 peptide at 30°C for 2 hours. The amount of radioactivity incorporated into the peptide was analyzed with the liquid scintillation counter. The amount of phosphate incorporated into TR1 peptide was higher in cells treated with okadaic acid compared to either lithium chloride or staurosporine treatment.

4.6 Effect of GSK3ß on MARK2 in cells

The inhibition of MARK2 by GSK3ß was checked under *in vivo* condition in the following cell lines N2a/F113, CHO and PC12 cells using techniques like immunoprecipitation and immunofluroscence.

MARK phosphorylates the microtubule-associated proteins in their conserved K*X*GS motifs in the repeat region (particularly Ser_{262}) (Ebneth et al., 1999). Phosphorylation at these sites regulates the binding of tau to the microtubules. The 12E8 antibody recognizes tau only when it is phosphorylated at Ser_{262}. It has been shown that GSK3ß can't phosphorylate tau at Ser_{262} in the K*X*GS motif (Godemann et al., 1999).

In N2a/F113 cells, different mutants of MARK2 were expressed alone or in the presence of GSK3ß and the activity of MARK2 was checked with endogenous tau protein as a substrate or the expressed proteins were immunoprecipitated and *in vitro* kinase assay was performed with TR1peptide as a substrate.

In CHO cells, MARK2 and GSK3ß were expressed alone or in combination and the effect on microtubule network was observed.

In PC12 cells, upon differentiation MARK2 leads to the phosphorylation of tau at K*X*GS motifs, increases microtubule dynamics, and enables the initiation of neurite outgrowth. (Biernat et al., 2003). GSK3ß was expressed in combination with MARK2 and the phosphorylation of endogenous tau at Ser_{262} and the neurite outgrowth was observed.

4.6.1 Wild type and mutants of MARK2 phosphorylate tau differentially

The aim of this experiment was to check the activity of different mutants of MARK2 under *in vivo* condition with endogenous tau protein as a substrate.

N2a/F113 cells stably expressing htau40 were transiently transfected with plasmids encoding for HA-tagged $MARK2^{wt}$ and the MARK mutants ($MARK2^{T208A}$ or $MARK2^{S212A}$ or double mutant $MARK2^{T208A/S212A}$) for 24 hours and differentiated with retinoic acid for 6 hours. Cells were collected and lysates were prepared (section 3.3.9a). 8µg of the lysate were resolved in the SDS-PAGE and Western blot analysis was performed.

The expression of MARK2 wild type and the mutants were confirmed with the HA antibody. The expression of $MARK2^{wt}$ increased the phosphorylation of tau at Ser_{262} (figure 22, lane 2). On the other hand, the expression of $MARK2^{T208A}$, $MARK2^{S212A}$ or $MARK2^{T208A/S212A}$ mutant drastically reduced the phosphorylation at Ser_{262} compared to $MARK2^{wt}$ (figure 22, lanes 3, 4, 5). K9JA antibody recognizes tau regardless of phosphorylation and serves as a standard for protein concentration (figure 22A, IB: K9JA). The ratio of phosphorylated tau to total tau was quantified using AIDA software. The ratio of total tau to phosphorylated tau was quantified using AIDA software. As seen in figure 22B, $MARK2^{wt}$ phosphorylated tau strongly at 12E8 epitope compared to the mutants. This result confirms that mutation of either Thr or Ser or Thr/Ser to Ala in the activation loop reduces the activity of the kinase in cells.

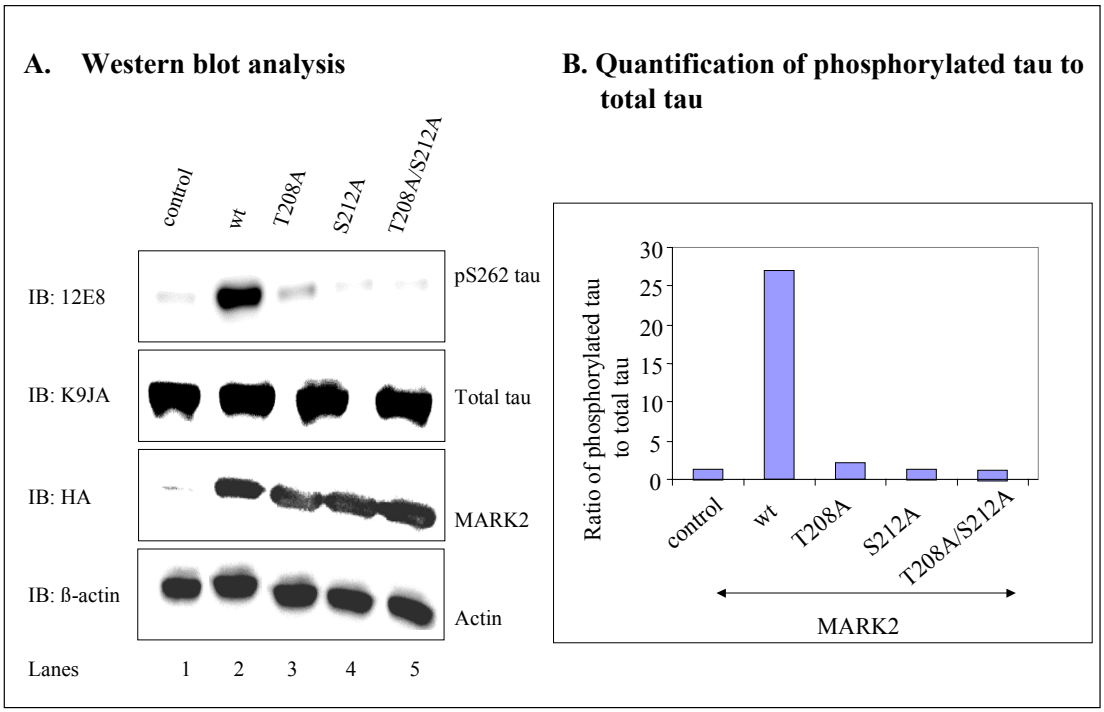

Figure 22: **Phosphorylation of tau by different MARK2 mutants**. A. N2a/F113 stably expressing htau40 ($2x10^5$ cells/well) were grown in a 6 well plate for 24 hours and the cells were transiently transfected with plasmids encoding for HA-MARK2^{wt}, HA-MARK2^{T208A}, HA-MARK2^{S212A} or HA-MARK2$^{T208A/S212A}$ and control without any transfection (lane 1). The transfections were carried out with Effectene reagent (section 3.1.5). After 24 hours of transfection, the cells were differentiated with 2µM retinoic acid for 6 hours. Lysates were prepared and 8µg of the total cell lysate was loaded onto a 10% SDS gel followed by Western blot analysis. HA-tagged MARK2 wt, T208A, S212A, T208A/S212A protein were expressed in similar levels as detected with the HA antibody. The K9JA antibody shows that the amount of total tau in control and in the transfected cells is almost similar. The phospho-dependent tau antibody 12E8 recognizes tau when it is phosphorylated at Ser_{262}. In cells overexpressing HA-MARK2 the 12E8 immunoreactivity was stronger; in contrast, expression of either HA-MARK2^{T208A} or HA-MARK2^{S212A} or HA-MARK2$^{T208A/S212A}$ resulted in significant loss of 12E8 immunoreactivity. Actin blots are included to show the amount of protein loaded for all the samples. B. The amount of total tau (detected with K9JA antibody) and phosphorylated tau (detected with 12E8 antibody) was quantified using AIDA software. The quantitative data demonstrate that there is an increase in phosphorylated tau in cells transfected with HA-MARK2^{wt} compared to cells transfected with HA-MARK2 mutants.

4.6.2 Effect of immunoprecipitated MARK2 mutants on recombinant TR1 peptide

To confirm the above data, the *in vitro* activity of the different immunoprecipitated MARK2 mutants towards the TR1 peptide was examined. Cells were transfected with HA-MARK2^{wt}, HA-MARK2^{T208A}, HA-MARK2^{S212A}, or without plasmid for 24 hours. Lysates were prepared and immunoprecipitation was carried out with HA antibody agarose conjugate (section 3.3.9). The immunoprecipitates were resolved in SDS-PAGE followed by Western blot analysis with HA antibody to confirm the expression of the proteins (HA-MARK2^{wt}, HA-MARK2^{T208A} and HA-MARK2^{S212}). The phosphorylation dependent MARK antibody (pThr$_{208}$) was used to check whether MARK is active. As seen in figure 23A, the antibody recognizes HA-MARK2^{wt} and HA-MARK2^{S212A} but not HA-MARK2^{T208A} indicating that the antibody is specific and the upstream kinases can phosphorylate MARK even when Ser$_{212}$ is mutated to Ala.

The immunoprecipitates were mixed together with TR1 peptide, radioactive ATP and the phosphorylation of TR1 peptide was measured by the incorporation of radioactive phosphate. The *in vitro* kinase assay shows that HA-MARK2^{wt} phosphorylated the TR1 peptide strongly compared to HA-MARK^{T208A} or HA-MARK2^{S212A} mutant (figure 23B).

This result suggests that even when HA-MARK2^{S212A} is phosphorylated at Thr$_{208}$ (figure 23, lane 4), it cannot phosphorylate TR1 peptide indicating that the enzyme is inactive. In the case of HA-MARK^{T208A}, the amount of T208A immunoprecipitated with the HA antibody is roughly 3 times higher than HA-MARK2^{wt} and HA-MARK2^{S212A} but the activity of HA-MARK2^{T208A} is as low as HA-MARK2^{S212A}. This data clearly indicate that mutation in the catalytic loop of MARK2 destroys the activity of the kinase.

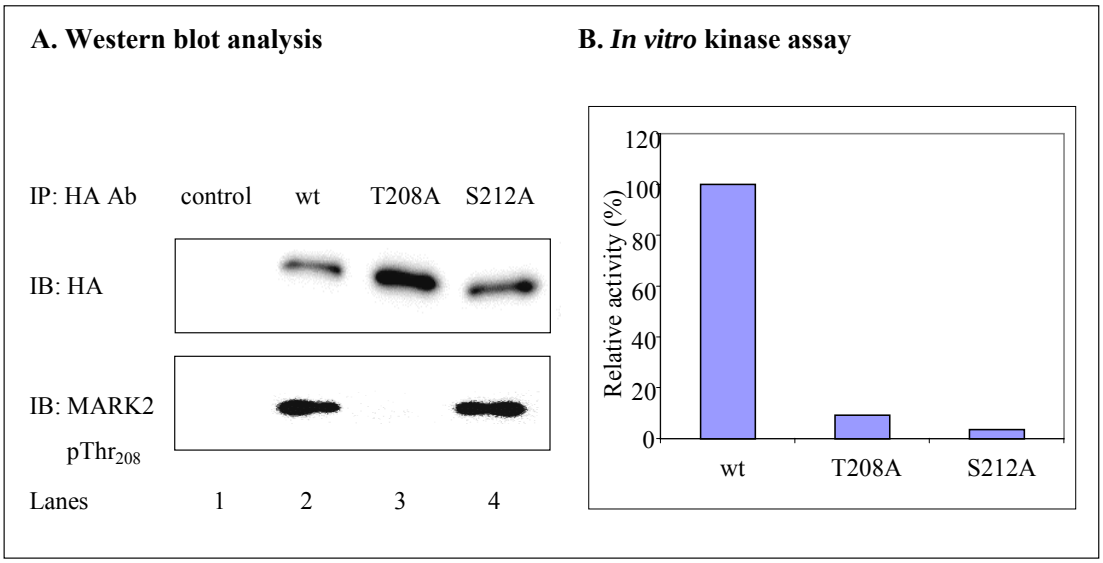

Figure 23: **MARK2^{wt} phosphorylates TR1 peptide efficiently compared to the mutants (HA-MARK2^{T208A} or HA-MARK2^{S212A})**. N2a cells (2x10^5 cells/well) were grown in 6 well plates for 24 hours and were transiently transfected with 1µg of plasmid encoding HA-MARK2^{wt}, HA-MARK2^{T208A}, HA-MARK2^{S212A} or HA-MARK2$^{T208A/S212A}$ and control without any transfection. The transfections were carried out with Effectene reagent (section 3.1.5). After 24 hours of transfection, the cells were differentiated with 2µM retinoic acid for 6 hours. Lysates were prepared and HA antibody agarose conjugate (monoclonal) was added to immunoprecipitate the HA-tagged proteins. The immunoprecipitates were resuspended in 50µl of 1xkinase assay buffer (section 3.3.9). The immunoprecipitates (8µl) were resolved in 10% SDS-gel followed by Western blot analysis. A. The expression of HA-MARK2^{wt}, HA-MARK2^{T208A}, or HA-MARK2^{S212A} proteins were confirmed with HA antibody. MARK2 pThr$_{208}$ antibody was used to check the presence of active MARK. The blot shows that the MARK2^{wt} and MARK2^{S212A} mutant were phosphorylated at Thr$_{208}$ but not HA-MARK2^{T208A} mutant. B. *In vitro* kinase assay. The immunoprecipitates (7µl) were mixed together with TR1 peptide, radioactive ATP and the phosphorylation of TR1 peptide was measured by the incorporation of radioactive phosphate. Immunoprecipitates from cells expressing HA-MARK2^{wt} showed increased phosphorylation of the TR1 peptide compared to immunoprecipitates from cells expressing HA-MARK2^{T208A}, HA-MARK2^{S212A} mutant. Though the amount of T208A immunoprecipitated with the HA antibody is roughly 3 times higher than the others the activity of HA-MARK2^{T208A} is as low as HA-MARK2^{S212A}.

4.6.3 Phosphorylation of tau in cells by MARK2^{wt} and MARK2^{T208E} in the presence and absence of GSK3ß^{S9A}

To aim of this experiment was to check the inhibition of MARK2 activity by GSK3ß in cells. The activity of MARK2 was assayed by the phosphorylation of endogenous tau at Ser_{262}, which in turn can be recognized by 12E8 antibody.

GSK3ß, a Ser/Thr kinase is normally active in cells and is primarily regulated through inhibition of its kinase activity. The activity of GSK3ß is regulated by phosphorylation. Very little is known about the activation of the kinase, whereas when GSK3ß is phosphorylated at Ser_9 the enzyme becomes inactive (reviewed in Jope et al., 2004). The kinases that are known to phosphorylate Ser_9 are Akt (Cross et al., 1995), PKA (Tanji et al., 2002), PKC (Fang et al., 2002), LKB1 and p90RSK (Saito et al., 1994; Brady et al., 1998). So when Ser_9 is mutated to Ala the enzyme becomes constitutively active. The constitutively active mutant of GSK3ß (GSK3ß^{S9A}) was used in this experiment to prevent the inactivation of GSK3ß during expression.

MARK2 can be activated by phosphorylation at Thr_{208} by MARKK. MARK2^{T208E} is a constitutively active mutant of MARK2, because E often mimics the phospho-serine or phospho-threonine.

N2a/F113 cells stably expressing tau were transiently transfected with HA-MARK2^{wt}, ECFP-MARK2^{T208E} in the presence and absence of ECFP-GSK3ß^{S9A}. After 24 hours of transfection, the cells were differentiated with retinoic acid for 6 hours before harvesting. Lysates were collected and the concentration of the protein was estimated by BCA method (section 3.3.9a, 3.3.3b). Equal amounts of proteins were loaded and immunoblotted with different tau antibodies, GFP antibody, HA antibody or ß-actin antibody. The K9JA antibody shows that the total amount of tau in each sample is almost similar (figure 24). The expression of MARK2^{wt} was confirmed by Western blot analysis with the HA antibody. Since the GFP antibody can recognize GFP, CFP, and YFP tags, this antibody was used to detect the exogenously expressed GSK3ß^{S9A}

and MARK2^{T208E}, which are both CFP tagged. ß-actin antibody confirms that equal amount of protein was loaded.

The phosphorylation of tau at Ser_{262} by MARK2^{wt} and MARK2^{T208E} was checked in the presence and absence of GSK3ß^{S9A} with the phosphorylation sensitive antibody 12E8. It has been shown that GSK3ß cannot phosphorylate tau at Ser_{262} in the K*X*GS motif (Godemann et al., 1999).

As seen in figure 24, expression of MARK2^{wt} and MARK2^{T208E} increased the phosphorylation of tau at Ser_{262} compared to the control. On the other hand, co-expression of GSK3ß^{S9A} along with the MARK2^{wt} or MARK2^{T208E} strongly reduced the phosphorylation of tau at Ser_{262}. These results strongly imply that GSK3ß inhibits MARK2 activity in cells.

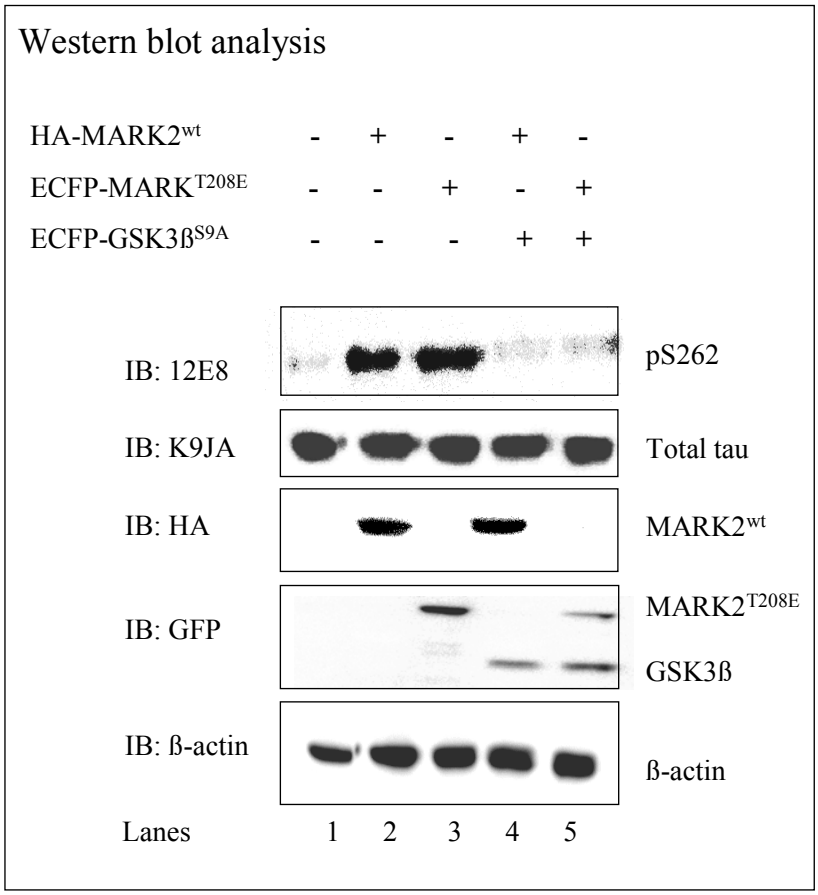

Figure 24: **Co-expression of GSK3ß^{S9A} with MARK2^{wt} and MARK2^{T208E} reduces the phosphorylation at Ser$_{262}$.** N2a/F113 cells stably expressing htau40 (2x10^5 cells/well) were grown in a 6 well plate for 24 hours and were transiently transfected with 1µg of plasmid encoding for HA-MARK2^{wt}, ECFP-MARK2^{T208E} alone or in the presence of ECFP-GSK3ß^{S9A}. After 24 hours of transfection, the cells were differentiated with 2µM retinoic acid for 6 hours. The cells were collected, lysed in 100µl of lysis buffer and the concentration of the protein in the total cell lysate was estimated by BCA method (section 3.3.9a, 3.3.3b). 20µg of protein was loaded onto a 10% gel for Western blot analysis. Western blot analysis shows that HA-MARK2^{wt}, ECFP-MARK2^{T208E} and ECFP-GSK3ß^{S9A} were expressed at similar levels as detected with HA antibody or GFP antibody. The phospho-independent tau antibody K9JA shows that the total tau levels in the transfected and control cells were approximately the same. The 12E8 immunoblotting shows that the expression of either the HA-MARK2^{wt} or ECFP-MARK2^{T208E} increased the phosphorylation of tau at Ser$_{262}$. In contrast, co-expression of HA-MARK2^{wt} and ECFP-MARK2^{T208E} with ECFP-GSK3ß^{S9A} resulted in a significant loss of 12E8 immunoreactivity. Actin blot is included to show equal amount of protein was loaded for all the samples.

4.6.4 Activity of immunoprecipitated MARK decreases in the presence of GSK3ß^{S9A}

To validate the above data, the *in vitro* activity of immunoprecipitate HA-MARK2 from cells expressing HA-MARK2 alone or together with GSK3ß^{S9A} towards TR1 peptide was examined. N2a/F113 cells stably expressing tau were transiently transfected with HA-MARK2^{wt} alone or in the presence of ECFP-GSK3ß^{S9A}. After 24 hours of transfection, the cells were differentiated with retinoic acid for 6 hours before harvesting. Cells were collected and lysates were immunoprecipitated with the HA antibody agarose conjugate (section 3.3.9). The immunoprecipitates were resolved in SDS-PAGE followed by Western blot analysis with HA and GSK3ß antibody.

The expression of HA-MARK2^{wt} was confirmed with the HA antibody (figure 25A, IB: HA). GSK3ß antibody recognizes both the endogenous and the transfected GSK3ß (GSK3ß^{S9A}). The thick band in the IP sample (lanes 9, 10) is the heavy chain of the HA antibody, recognized by the anti-mouse secondary antibody (figure 25A, lanes 9, 10).

As seen in the figure 25B, the immunoprecipitates from cells expressing HA-MARK2 alone phosphorylated the TR1 peptide strongly. In contrast, the activity of HA-MARK2 immunoprecipitated from cells co-expressing CFP-GSK3ß^{S9A} was reduced to approximately 50%. This result further confirms that GSK3ß inhibits MARK activity in cells.

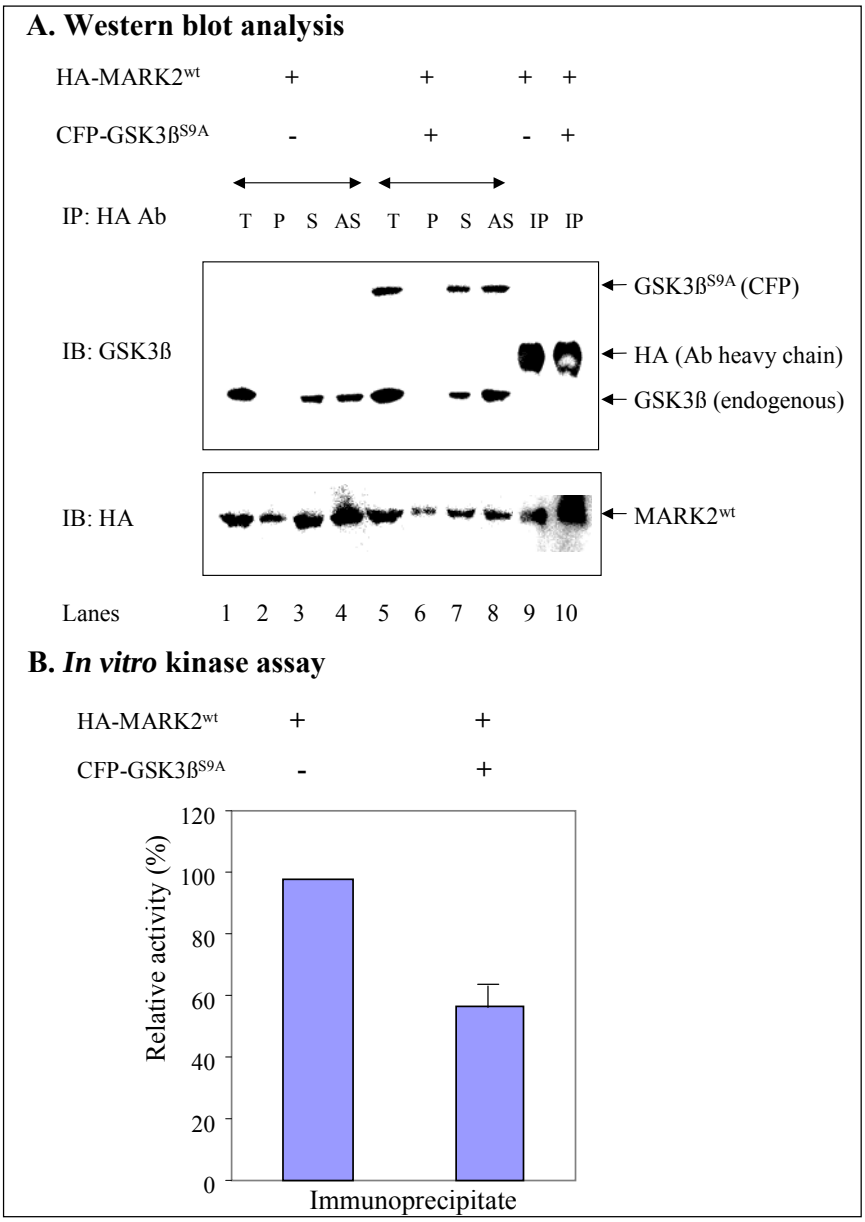

Figure 25: **Activity of immunoprecipitated MARK2 from cells co-expressing ECFP-GSK3ß**. HA-MARK2^{wt} alone or along with ECFP-GSK3ß was expressed in N2a/F113 cells for 24 hours. Lysates were prepared and HA antibody agarose conjugate (monoclonal) was added to immunoprecipitate the HA-tagged protein. The immunoprecipitates were resuspended in 50µl of 1xkinase assay buffer (section 3.3.9). A. The total cell lysate (5µl), pellet (5µl), supernatant (5µl), immunoprecipitates (8µl) were resolved in 10% SDS-gel followed by Western blot analysis with HA and GSK3ß antibody. T=total cell lysate, P=pellet, S=supernatant and IP=immunoprecipitate. The HA antibody shows the expression of the exogenous protein HA-MARK2 in both the single (HA-MARK2 - lanes 1-4, 9) and double transfected cells (HA-MARK2+ECFP-GSK3ß^{S9A} - lanes 5-8, 10). GSK3ß antibody, a mouse monoclonal antibody, recognizes both the exogenous and endogenous protein. The thick band in the immunoprecipitates (lanes 9, 10) of the GSK3ß blot is the heavy chain of the HA antibody which was recognized by the anti-mouse secondary antibody. B. HA-MARK2 phosphorylated the TR1 peptide to a significantly greater extent than the HA-MARK2 immunoprecipitate from cells where ECFP-GSK3ß was co-expressed.

4.7 GSK3ß-MARK-Tau cascade in cells

4.7.1 Co-transfection of MARK2 and GSK3ß^{S9A} in CHO cells stabilizes the microtubule network

To examine the relationship between GSK3ß, MARK2, MAPs and the microtubule network, CHO wild type cells were used. CHO wild type cells contain endogenous MAP4 but not tau, phosphorylatable at K*X*GS motifs in the repeat domain. CHO cells were transfected with constitutively active mutant of GSK3ß^{S9A} (mRFP) and MARK2^{wt} (YFP). The untransfected cells have an extended shape with a clear microtubule network. Transfection of CFP-MARK2 alone showed membrane localization, led to the loss of microtubules, shrinkage of the cells and eventually cell death (figure 26). Transfection of GSK3ß^{S9A} showed cytoplasmic localization and also leads to cell death. In contrast, co-transfection of CFP-MARK2 and mRFP-GSK3ß^{S9A} mutant showed co-localization and rescued the cells from the loss of the microtubule network. This result indicates that GSK3ß inhibits the microtubule destabilizing effect of MARK.

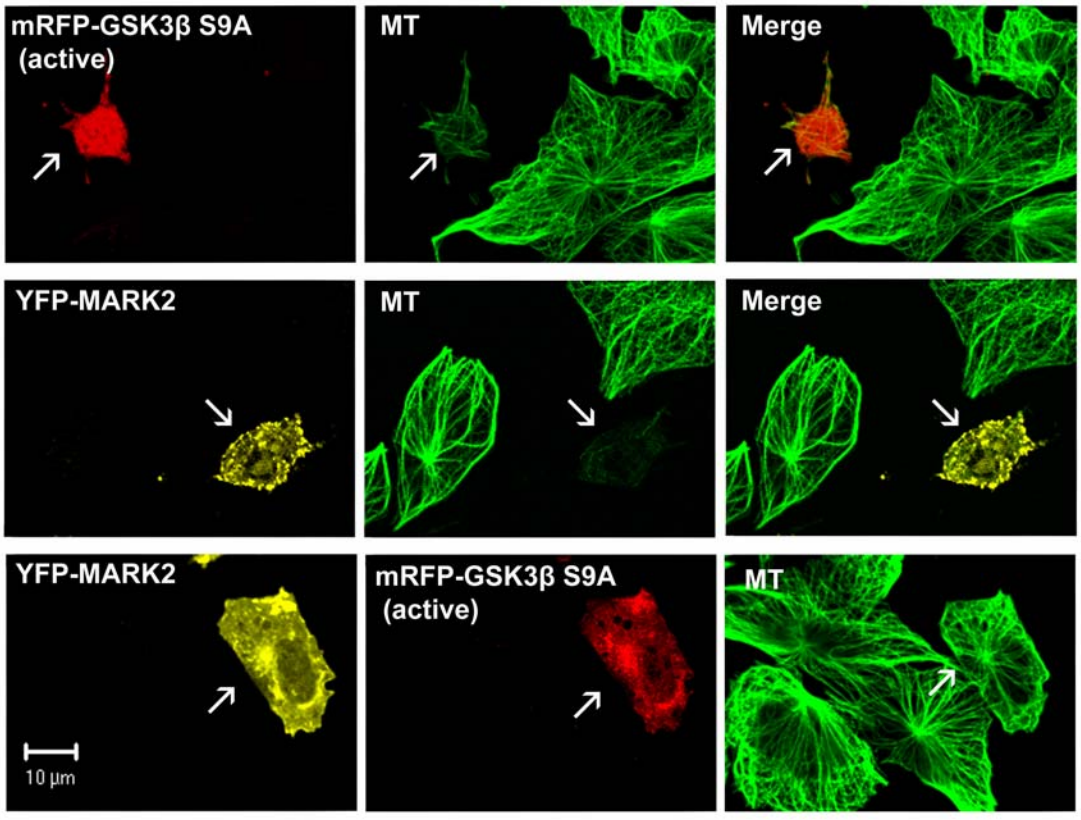

Figure 26: **Co-expression of GSK3ß with MARK2 stabilizes the microtubule network**. CHO cells were transfected with YFP-MARK2^{wt}, mRFP-GSK3ß^{S9A}, or co-transfected with YFP-MARK2^{wt}and mRFP-GSK3ß^{S9A}. After 16 hours, cells were fixed and stained for microtubules with antibody YL1/2 and Cy5-secondary antibody. Note, the cell transfected with YFP-MARK2^{wt} or mRFP-GSK3ß^{S9A} led to the loss of the microtubule network. The cells round up and appear smaller. In contrast, co-expression of YFP-MARK2^{wt} and mRFP-GSK3ß^{S9A} retains the microtubule network, indicating that GSK3ß inhibits MARK2 in cells. The arrows indicate the transfected cells.

4.7.2 Differential regulation of MARK2 and GSK3β in PC12 cells

To further understand the regulation of MARK2 by GSK3ß in cells the expression pattern of endogenous MARK2 and GSK3ß in undifferentiated and differentiated PC12 cells were checked.

It has been already shown that differentiation of neuronal cells (PC12, N2a) leads to increased MARK activity. This in turn leads to the phosphorylation of tau at K*X*GS motifs, increased microtubule dynamics, and promotes the initiation of neurite outgrowth (Biernat et al., 2002).

PC12 cells were differentiated with NGF for different time points (0, 1, 4, 24 hours) and the expression pattern of MARK and GSK3β were examined. As shown in the figure 27, the amount of active GSK3β (detected with GSK3β pTyr216 antibody) is significantly higher in undifferentiated compared to cells that were differentiated for 1 hour. On the other hand, the amount of active GSK3ß is significantly reduced when differentiated for 4 or 24 hours, but the amount of total GSK3β remains unchanged. In contrast, the expression level of MARK2 is highest after 24 hours.

This result indicates that the amount of active GSK3ß (detected with the $pTyr_{216}$GSK3ß antibody) decreases with the time of NGF treatment and the expression of MARK increases after 24 hours. This in turn supports the notion that the cells posses an innate mechanism to control the activity of GSK3ß, which negatively regulates MARK2 activity needed for neurite outgrowth.

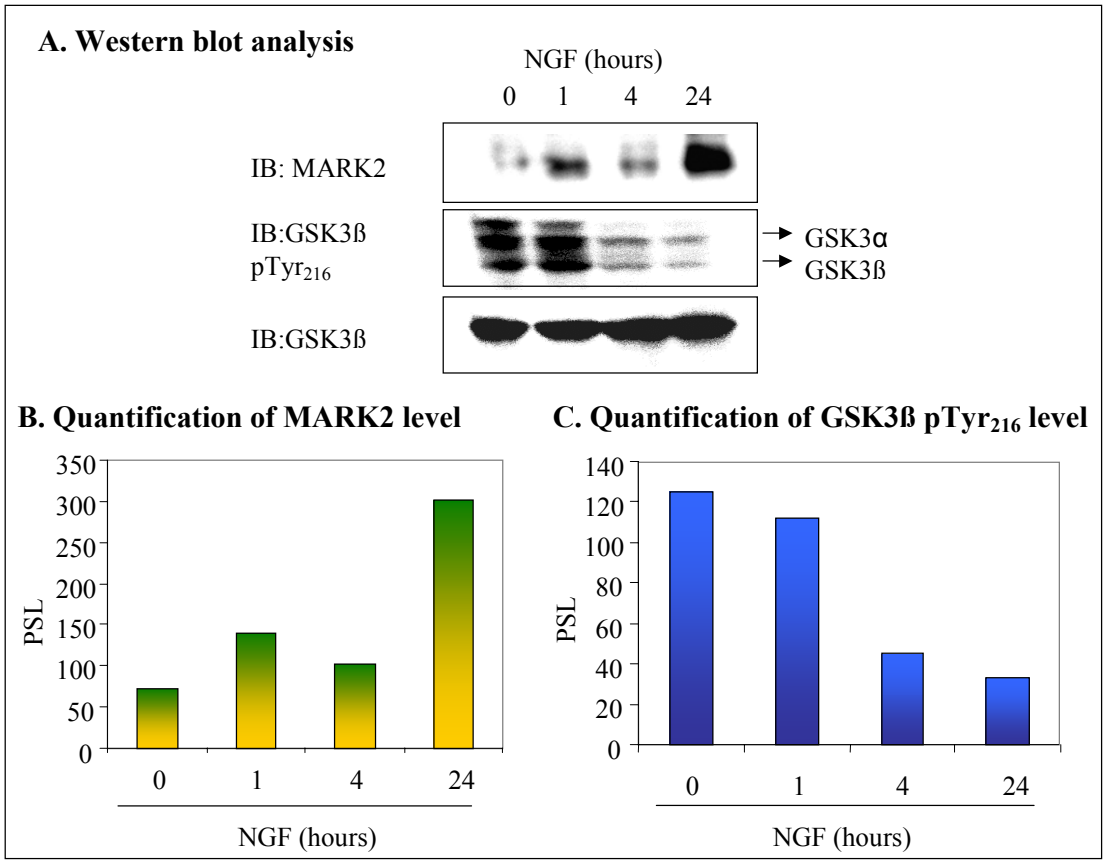

Figure 27: **Differential regulation of MARK2 and GSK3ß in PC12 cells**. PC12 cells were treated with 100ng/ml NGF, harvested after different time points (0, 1, 4, 24 hours) and lysed in 100µl of 1xSDS buffer. A. Western blot analysis. Cell lysates were loaded in 10% SDS gel and immunoblotted for MARK2, total GSK3ß and GSK3ß pY216. B and C. Quantification of MARK2 and $pTyr_{216}$ GSK3ß. The intensity of the signal in Western blot was quantified using AIDA software. MARK2 is up regulated and the active GSK3ß is down regulated with the time of NGF treatment. PSL-photo stimulated luminescence.

4.7.3 Effect of overexpression of GSK3ß and MARK2 on 12E8 staining and neurite outgrowth in PC12 cells

To study the effect of GSK3ß on MARK2, the proteins were overexpressed in PC12 cells. Phosphorylation of endogenous tau at Ser_{262} and the neurite outgrowth was observed. ECFP-$MARK2^{T208E}$ and mRFP-$GSK3ß^{S9A}$ plasmids were transfected in PC12 cells and the cells were differentiated with NGF for 48 hours. Transfection of $MARK2^{T208E}$ alone showed strong 12E8 staining and the formation of neurite outgrowth, whereas transfection of $GSK3ß^{S9A}$ showed no 12E8 staining and no neurite outgrowth indicating that GSK3ß inhibits the endogenous MARK2 activity. Moreover co-expression of $MARK2^{T208E}$ and GSK3ß showed no neurite outgrowth. This result indicates that GSK3ß cannot phosphorylate 12E8 epitope and overexpression of GSK3ß inhibits MARK2 activity and hence there is no neurite outgrowth in PC12 cells.

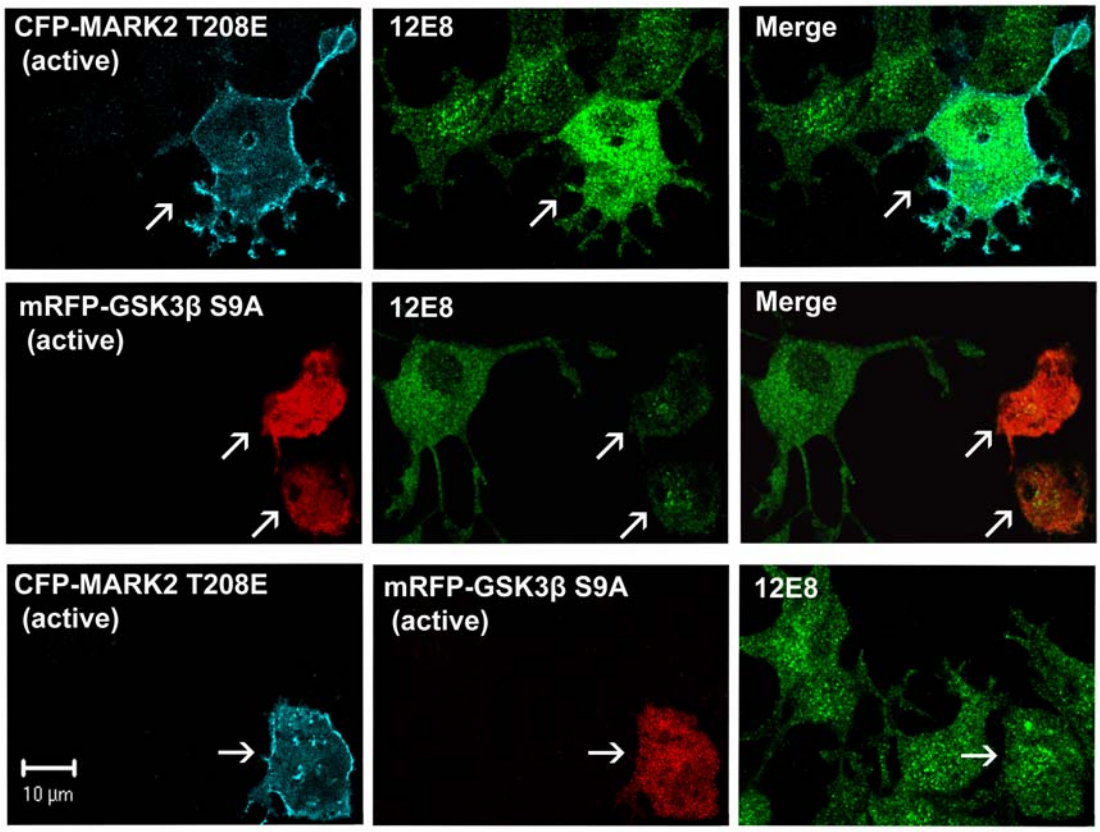

Figure 28: **Effect of GSK3ß on MARK2 in PC12 cells**. PC12 cells were transiently transfected with ECFP-MARK2^{T208E} or mRFP-GSK3ß^{S9A} or in combination and the cells were differentiated with NGF for 48 hours. The cells were fixed and stained for phospho-tau antibody 12E8 (Cy5). Transfection of MARK2^{T208E} alone showed strong 12E8 staining whereas transfection of GSK3ß alone showed no 12E8 staining. In contrast, co-expression of MARK^{T208E} and GSK3ß^{S9A} showed no neurite outgrowth.

5.0 Discussion

The microtubule associated protein tau is a phosphoprotein that functions to stabilize microtubules, the tracks for axonal transport in healthy neurons. In degenerating neurons, tau is hyperphosphorylated, detaches from microtubules, which in turn leads to the breakdown of microtubules and transport inhibition in neurons. The hyperphosphorylated tau that cannot bind to the microtubules anymore aggregates into paired helical filaments (PHFs), which deposit as neurofibrillary tangles (NFTs) in the neurons. The detachment of tau from microtubules is preferentially achieved by phosphorylation at K*X*GS motifs located in the microtubule-binding repeat region of tau. MAP/Microtubule affinity regulating kinase (MARK) has been identified as a Ser/Thr kinase that phosphorylates tau and other MAPs at the K*X*GS motifs within the repeats, abolishes the binding of tau to microtubules thus disrupting the microtubule stability (Drewes et al., 1997). An important aspect is that this type of phosphorylation is enhanced in the early stage of Alzheimer's disease (Augustinack et al., 2002). Further, Nishimura et al. proposed that activation of MARK in a *Drosophila* model primes tau for the hyperphosphorylation by other kinases like GSK3 and Cdk5, which in turn triggers the aggregation of tau into paired helical filaments, a hallmark of AD neurofibrillary pathology (Nishimura et al., 2004).

MARK and its homologue Par-1 in *C.elegans* and *Drosophila* are involved in generating and maintaining cell polarity during development (Guo et al., 1995). MARK is involved in neurite outgrowth, establishment of neuronal polarity in neuroblastoma cells and hippocampal neurons (Biernat et al., 2002; Chen et al., 2006), in cell cycle regulation (Müller et al., 2003), in Wnt signaling pathway (Sun et al., 2001) and in the regulation of exocytosis in yeast (Elbert et al., 2005). So, it is therefore important to understand the regulation of MARK.

5.1 Inhibition of MARK activity by GSK3ß

MARK isolated from rat brain showed some phosphorylation at Thr_{208} and Ser_{212} (numbering according to MARK2) in the activation loop (Drewes et al., 1997). Activation of MARK2 is achieved by phosphorylation of a single residue, Thr_{208} by

MARKK or LKB1 (Timm et al., 2003; Spicer et al., 2001). It requires Ser_{212} to be present but not phosphorylated. Site-directed mutational analysis shows that when Thr_{208} is mutated to Ala, the activation by MARKK was lost and only the basal activity remained, implying that Thr_{208} is important for the activation by upstream kinases MARKK or LKB1. In contrast, when Ser_{212} or both Thr_{208} and Ser_{212} were mutated to Ala or Glu, the intrinsic and activatable activities of MARK2 were completely abolished (Timm et al., 2003).

The above findings were confirmed under *in vivo* condition using N2a/F113 cells, stably transfected with a gene encoding htau40. Different MARK2 constructs (wt, T208A, S212A) were expressed in N2a/F113 cells and the phosphorylation of tau at Ser_{262}/Ser_{356} was detected with the 12E8 antibody (figure 22). The 12E8 antibody clearly discriminates between the phosphorylated and unphosphorylated protein, despite the fact that 12E8 was raised against a synthetic phosphopeptide corresponding to amino acids 257-270 of tau protein (according to the numbering of human tau40). This result shows that only wild type MARK2 phosphorylated the 12E8 epitope strongly and efficiently but not MARK T208A or S212A mutant. In contrast, the *in vitro* data showed $MARK2^{wt}$ and $MARK2^{T208A}$ have a similar amount of basal activity. The difference in the activity of $MARK2^{T208A}$ *in vitro* and in cells compared to $MARK2^{wt}$ could be that in cells the activity of $MARK^{wt}$ is increased several fold by phosphorylation at Thr_{208} by MARKK or LKB1. In the case of T208A mutant there is no activation by MARKK, which is in line with the *in vitro* site-directed mutational analysis. These data confirm that the mutation of either Thr_{208} or Ser_{212} to Ala abolishes the activity of MARK2. Since the activation loop of the four MARK isoforms is similar, this result holds for all isoforms (figure 29).

The aim of this study was to identify the kinase responsible for the phosphorylation of Ser_{212} in MARK2. The following figure shows the amino acid comparison of the catalytic loops of the four isoforms of MARK:

rMARK2	202 - FGNKLDTFCGSPPYAAPE - 219
hMARK1	210 - VNNKLDTFCGSPPYAAPE - 227
hMARK3	205 - VGNKLDTFCGSPPYAAPE - 222
hMARK4	208 - LSNKLDTFCGSPPYAAPE - 225

Figure 29: **Sequence alignment of the activation loop of the four MARK isoforms**. Invariant residues are highlighted in blue, Thr in red is phosphorylated by MARKK or LKB1.

Since Ser_{212} in the activation loop of MARK2 is followed by a proline, proline-directed kinases are considered to be the potential targets. Among many proline-directed kinases, GSK3ß was chosen because of the following reasons. GSK3ß co-purifies with MARK2 through several steps of purification from rabbit skeletal muscles (Drewes et al., 1995). GSK3ß has been discussed as a kinase that activates MARK2 by phosphorylation at Ser_{212} (Kosuga et al., 2006). GSK3ß is particularly interesting in the context of AD because it phosphorylates tau efficiently at Ser/Thr-Pro motifs that are elevated in AD (Hanger et al., 1992; Mandelkow et al., 1992; Ishiguro et al., 1993; Lovestone et al., 1994; Song et al; 1995; Moreno et al., 1996; Hong et al., 1997; Sing et al., 1999). GSK3 is involved in the Wnt signaling pathway, which may be perturbed in AD due to mutations in the PS1 gene, which are related to familial AD (Takashima et al., 1998; Zhang, et al., 1998; Zhou et al., 1997). Thus, GSK3ß may link different pathways that are important for neurodegeneration in AD. Because of this it's important to find out whether GSK3ß would qualify as a kinase that phosphorylates Ser_{212} in the activation loop.

As a first step to understand the relationship between GSK3ß and MARK2, the activity of MARK2 and $GSK3ß^{wt}$ or $GSK3ß^{S9A}$ (constitutively active mutant) was studied *in vitro* in the presence of TR1 peptide or pCREB peptide as a substrate (figure 11, figure 10). The TR1 peptide, derived from the first repeat of the microtubule-binding domain of tau, can be phosphorylated only by MARK but not by GSK3ß (figure 11). The result of figure 17A shows that the activity of MARK2 was reduced in the presence of GSK3ß, whereas the activity of GSK3ß was unaffected in the presence of MARK2 (figure 17B), indicating that GSK3ß inhibits MARK2, but the reverse does not hold.

A more detailed analysis shows that the basal activities of MARK2^{wt} and MARK2^{T208A} were reduced in the presence of recombinant GSK3ß (figure 18A). Western blot analysis shows that the reduction in the activities of MARK2^{wt} and MARK2^{T208A} mutant is due to the phosphorylation of MARK2 at Ser_{212} by GSK3ß as detected with the specific Ser_{212} phosphorylation dependent antibody (figure 18D). No signal was detected in the case of MARK2^{S212A} mutant. This result shows that GSK3ß directly phosphorylates MARK2 at Ser_{212} and inhibits the activity of MARK2.

To confirm the inhibition of MARK2 by GSK3ß in cells, MARK2^{wt} and MARK2^{T208E} were expressed alone or together with GSK3ß^{S9A} and the effects were observed as phosphorylation of tau at Ser_{262} (figure 24). It has been shown that GSK3ß cannot phosphorylate tau at Ser_{262} in the K*X*GS motif (Godemann et al., 1999), so phosphorylation at this site is due to MARK or SAD kinase (Kishi et al., 2005) or p70 S6K (Pei et al., 2006) and not by GSK3ß. The reason to use a constitutively active mutant of GSK3ß (GSK3ß^{S9A}) is to prevent the inactivation of GSK3ß in the cells. As a result, expression of MARK2^{wt} or MARK2^{T208E} increased the phosphorylation of tau at Ser_{262} compared to the control. On the other hand, co-expression of GSK3ß^{S9A} along with MARK2^{wt} or MARK2^{T208E} significantly reduced the phosphorylation of tau at Ser_{262}. This result strongly suggests that GSK3ß inhibits MARK2 activity in cells (figure 24).

Since the activity of MARK2 was reduced in the presence of GSK3ß, it was interesting to know whether these two proteins interact with each other. The *in vitro* pull-down assay shows a strong interaction between constitutively inactive mutant MARK2$^{T208A/S212A}$ and the endogenous GSK3ß expressed in undifferentiated but not in the differentiated PC12 cells (figure 15). This MARK2$^{T208A/S212A}$ mutant was chosen because it eliminates the phosphorylation sites in the activation loop of MARK2 and thus may stabilize the interaction with an upstream kinase, as shown for the example of the activating kinase MARKK (Timm et al., 2003). Immunoprecipitation of MARK2^{wt} from Sf9 cells co-expressing GSK3ß^{wt} showed no interaction, implying that the interaction between the two kinases is very transient and the interaction is strengthened with the non-phosphorylatable form of MARK2$^{T208A/S212A}$. Further, the localization of GSK3ß and MARK2 in adult and

embryo (stage E18) brain was studied fractionating the brain extract by an iodixanol gradient. The result shows that GSK3ß is uniformly distributed in all the fractions. In the early developmental stage (embryo stage E18) active MARK $pThr_{208}$ co-localizes with the upstream activator MARKK, whereas in the adult brain both the known activators of MARK, MARKK and LKB1, co-localize with active MARK although MARKK predominates (figure 16, fraction 11). This result indicates that MARKK regulates MARK activity in the early developmental stage and in adult brain predominantly (figure 16).

To further address the functional relationship between GSK3ß and MARK2, the proteins were co-expressed in CHO wt cells and the cellular effects were described in terms of their effects on the microtubule cytoskeleton (figure 26). Overexpression of MARK2 leads to phosphorylation of tau or related MAPs at the K*X*GS motifs, their detachment from microtubules, breakdown of microtubules and finally cell death as well (Drewes et al., 1997). Overexpression of GSK3ß alone leads to cell death. The reason might be that GSK3ß is involved in a broad number of other pathways and overexpression might lead to the imbalance in the cellular milieu. One mechanism of action that was reported due to GSK3ß overexpression in neuronal cells was apoptosis (Hetmann et al., 2000). Brownlees et al. showed that overexpression of GSK3ß in mice during embryonic and postnatal development lead to the death of mice (Brownlees et al., 1997). The proposed mechanism of action is that the survival pathway involving PI3-kinase negatively regulates GSK3ß; therefore overexpression of GSK3ß overrides the survival pathway and leads to cell death. It has also been shown that challenging cultured neurons with trophic factor withdrawal or with PI3-kinase inhibitor leads to the stimulation of GSK3ß activity that results in apoptosis (Pap et al., 1998). Moreover, the cellular effects of GSK3ß on the cytoskeleton are only partly understood. GSK3ß phosphorylates both neurofilament subunits (Guidato et al., 1996) and acts on microtubules via their MAPs. GSK3ß can phosphorylate tau both at primed and unprimed sites (Foil et al., 1987). Unprimed sites are proline rich regions of a protein, in the case of primed sites phosphorylation of a Ser or Thr, located four residues to the C-terminal side of the GSK3 phosphorylation site should be first pre-phosphorylated by other kinases (Fiol et al., 1987). The phosphorylation sites of GSK3ß in tau are the Ser-Pro/Thr-Pro motifs that lie outside the microtubule-binding region. Extensive *in vitro* analysis by Biernat et al. showed that

phosphorylation of tau at the Ser-Pro/Thr-Pro motifs by proline-directed kinases has only moderate effect on tau-microtubule interaction, whereas phosphorylation of Ser_{262}, which resides in the microtubule-binding region strongly reduces the binding of tau to microtubules. So it is clear that phosphorylation of a single residue Ser_{262}, mediated by the non-proline directed kinase MARK, affects the binding of tau to microtubules (Biernat et al., 1993; Illenberger et al., 1998).

Transfection of either GSK3ß or MARK2 in CHO cells leads to cell death whereas co-expression of MARK2 with GSK3ß rescues the cell from microtubule loss and cell death (figure 26). This data can be interpreted in two different ways:
1. GSK3ß inhibits MARK2 activity, 2. MARK2 inhibits GSK3ß activity. But, from the *in vitro* studies it's clear that only GSK3ß inhibits MARK2 activity by phosphorylation at Ser_{212} whereas MARK2 has no effect on GSK3ß activity. The difference in the effect of combination of these two kinases in cells can be explained in the following ways:

1. A change in the intracellular localization might affect the properties of a kinase. Co-expression of the two kinases changes the intracellular localization and thereby preventing the access to the substrates. Transfection of CHO cells with MARK2 alone showed membrane localization and GSK3ß overexpression mainly showed the cytoplasmic localization, whereas in the case of double transfection MARK2 co-localized with GSK3ß in the cytoplasm.

2. In the case of double transfection, MARK2 and GSK3ß may form a complex with the adapter protein 14-3-3 or other proteins thereby partly preventing the access of GSK3ß to its other substrates. It's known from the literature that both GSK3ß and MARK2 can bind to 14-3-3 independently (Agarwal-Mawal et al., 2003; Morton et al., 2002). It remains to be examined whether GSK3ß, MARK2 and 14-3-3 forms a complex in cells. In MDCK cells, when Par-1b is phosphorylated at Thr_{595} by aPKC, it binds to 14-3-3 and is translocated from the lateral membrane to the cytosol (Suzuki et al., 2004).

Kinases are involved in more than one signaling cascade. For example, MARK isoforms are not only involved in regulating the microtubule dynamics, cell polarity, Wnt signaling but also in neurite outgrowth (Biernat et al., 2002). So it's important to know whether there is a differential regulation of MARK and GSK3ß in cells. For this experiment PC12 cells were chosen because both the proteins were expressed endogenously at a detectable level. As shown in figure 27 the amount of active GSK3ß (detected with the $pTyr_{216}$GSK3ß antibody) decreases with the time of NGF treatment. Interestingly after 24 hours of NGF treatment, the activity of GSK3ß goes down and the activity of MARK2 gets up regulated. This result supports the notion that the cells possess an innate mechanism to control the activity of GSK3ß, which negatively regulates MARK2 activity needed for neurite outgrowth.

In neurons the dynamics of microtubules are spatially regulated (Ahmad et al., 1993). The ability of GSK3ß to control axonal growth presumably relies in its ability to regulate a number of different microtubule binding proteins like CRMP-2 (collapsing response mediator protein-2), APC (Adenomatous polyposis coli) and MAP1B. The ability of CRMP-2 or APC to bind to tubulin dimers or microtubules is abolished when phosphorylated by GSK3ß (Fukata et al., 2002; Yoshimura et al., 2006; Akhmanova et al., 2005). Thus, local inactivation of GSK3ß (like in the tip of the axon) results in the dephosphorylation of CRMP-2 and APC, which leads to enhanced polymerization and stabilization of microtubules and leads to the growth of the axon. GSK3ß is inactive in the tip of the axon, and thus MARK is more active, indicating that dynamic microtubules are needed in the growth cone (Zhou et al., 2004). This also supports the phenomenon that both the kinases are differentially regulated under *in vivo* condition.

Inhibition of GSK3ß by lithium chloride in cortical neurons lead to the partial reduction in 12E8 staining (figure 21). One might conclude from this experiment that GSK3ß activates MARK2. But the major drawback with lithium chloride is that it has multiple cellular targets. It inhibits polyphosphate 1-phosphatase, inositol monophosphatase, casein kinase-II (CKII), MAP kinase-activated protein kinase-2 (MAPKAPK2), p38-regulated/activated kinase (PRAK) (Berridge et al. 1989; Davies et al. 2000), protein kinase C substrates like MARCKs (Manji et al., 1999) and activates PI3-kinase/PKB and c-jun N-terminal kinase (JNK) in cellular assays

(Chalecka-Franaszek et al., 1999; Yuan et al. 1999a). The best alternative would be to silence the endogenous GSK3ß activity in cells and check the activity of endogenous MARK2 with tau as a substrate. This experiment is quite challenging because it's important to find a cell line that expresses GSK3ß, MARK2 and tau at a detectable level, because overexpression of either of these two kinases would lead to cell death. From the previous experiment (figure 27) it is known that the PC12 cell itself fine-tunes the regulation of MARK and GSK3ß: Upon differentiation with NGF the activity of GSK3ß is down regulated, whereas MARK expression is up regulated. So in this cell line when GSK3ß is silenced without differentiation, the effect on MARK cannot be checked because there is no expression of MARK. On the other hand, silencing GSK3ß and differentiation would not make sense because upon differentiation with NGF or BDNF, GSK3ß will be down regulated in any case.

When this work was carried out in our laboratory, Kosuga et al. reported that GSK3ß activates MARK2 by phosphorylation at Ser_{212}. Their results contradict our data. (Kosuga et al., 2006). The major points that differ from our data and the drawbacks of the reported paper are discussed below:

1. The basis for the activation of MARK2 by GSK3ß originated from the Western blot analysis where the authors showed that the overexpression of MARK2 wild type and the T208A mutant in N2a/F113 cells increased the phosphorylation of Ser_{262}, but overexpression of the S212A mutant of MARK2 did not show any effect. So they concluded that phosphorylation of Ser_{212} is needed for the activation of MARK2. Careful examination of this Western blot shows that the amount of wild type MARK2 expressed is at least three times less compared to $MARK2^{T208A}$. From our analysis (figure 23), even three-fold increase in the expression of $MARK2^{T208A}$ showed no increase in the activity compared to the wild type MARK2. Moreover S212A is completely inactive and cannot be activated (Timm et al., 2003), indicating their conclusion is not right.

2. Important data are missing i:e the activity assay for recombinant MARK2 in the presence of recombinant GSK3ß under *in vitro* condition. The authors performed the *in vitro* kinase assays with immunoprecipitated MARK from

cells and not with the recombinant proteins from *E.coli*, so these proteins may already be modified. The authors showed only the phosphorylation of MARK (by autoradiogram) but this is not directly related to the activation (could also mean inhibition).

3. Another major drawback is that the phosphorylation of MARK2 at Ser_{212} by GSK3ß was never shown with a phosphorylation dependent antibody against $pSer_{212}$.

4. With respect to the GSK3ß siRNA experiment in HEK293 cells, it was shown that as the concentration of GSK3ß siRNA increases there is significant reduction in the phosphorylation signal of Ser_{262}. But the authors failed to show the expression of MARK in this cell line. So, in this cell line probably other kinases like SAD and p70RSK might be involved in phosphorylating tau at Ser_{262}.

5.2 Structural basis for the inhibition of MARK by GSK3ß

Cells use several control mechanisms to regulate the kinases and phosphatases that are involved in regulating metabolism, gene expression, cell growth, cell motility, cell differentiation and cell division. The regulation of kinases can be brought about by the following mechanisms: either by binding of additional subunits e.g. cAMP-binding to the regulatory subunit of cAMP-dependent protein kinases or inhibition by an auto-regulatory process, e.g. myosin light chain kinase or different sub cellular localization or by phosphorylation and dephosphorylation. The conversion of an inactive kinase to an active kinase involves conformational changes in the protein that lead to the correct positioning of substrate binding and catalytic groups and relief of steric blocking to allow access of the substrate to the catalytic site. Activation by phosphorylation in the activation segment (the region spanning the conserved sequences DFG and APE) was identified as one of the key mechanisms of regulation in recent years. The first observation of Thr_{197} phosphorylation in the activation loop of PKA was reported in 1979 (Shoji et al., 1979).

Figure 30 shows the ribbon diagram of MARK2 catalytic domain in combination with the UBA domain that was solved in our laboratory (Panneerselvam et al. 2006; Marx et al., 2006). Most of the regulatory loop is disordered and invisible in the inactive state; only the C-terminal part including residues Thr_{208} and Ser_{212} (yellow) is visible in the known MARK structures. In the active state, the regulatory loop is thought to point to the right side (orange loop) according to the known structures of active kinases. In both conformations, the secondary phosphorylation site Ser_{212} (highlighted in red) is located at the same place, close to the catalytic center (Panneerselvam et al. 2006; Marx et al., 2006). Phosphorylation at this site might prevent the activation loop to adopt the active conformation.

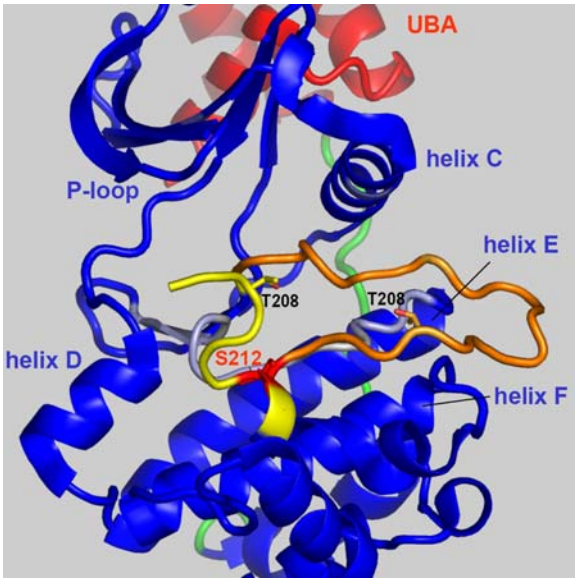

Figure 30: **Structure of MARK2**. It is a ribbon diagram of MARK2 catalytic and UBA domain (PDB-ID: 1Y8G, inactive state; Panneerselvam et al. 2006). Blue=catalytic domain, red=UBA domain, green=linker between catalytic domain and UBA domain and grey=catalytic loop. Most of the regulatory loop is disordered and invisible in the inactive state; only the C-terminal part including residues Thr_{208} and Ser_{212} (yellow) is visible in the known MARK structures. In the active state, the regulatory loop is thought to point to the right side (orange loop) according to the known structures of active kinases. As shown in figure 30, the position of Ser_{212} is similar in both the states (active and inactive). Phosphorylation at this site might prevent the activation loop to adopt the active conformation.

A mutation of the residue equivalent to Ser_{212} in JIK results in a dramatic decrease in the activity (Tassi et al., 1999).

5.3 Properties of GSK3

GSK3 is a multi functional Ser/Thr kinase found in all eukaryotes. It was first isolated and purified as an enzyme capable of phosphorylating and inactivating the enzyme glycogen synthase involved in glycogen metabolism (Embi et al., 1980; Woodgett et al., 1984). Apart from its role in glycogen metabolism, GSK3 acts as a regulatory switch that determines the output of numerous signaling pathways initiated by diverse stimuli (Frame et al., 2001; Woodgett, 2001).

There are two mammalian isoforms of GSK3 encoded by distinct genes-GSK3α and GSK3ß (Woodgett, 1990). GSK3α has a molecular mass of 51 kDa, where as GSK3ß is a 47 kDa protein. The difference in size is due to a glycine rich extension at the N-terminus of GSK3α. A minor (15% of total) splice variant of GSK3ß, GSK3ß2 has a 13 amino acid residue insert in the kinase domain. Of these three GSK3 isoforms GSK3ß2 has a reduced activity towards tau compared to unspliced GSK3ß (Mukai et al., 2002).

GSK3 can phosphorylate both primed and unprimed substrates. Primed substrates are the ones that have to be first pre-phosphorylated by other kinases at Ser or Thr residues, which are located four residues C-terminal to the GSK3 phosphorylation site. In the case of unprimed substrates the prior phosphorylation is not needed (Fiol et al., 1987).

GSK3 can be regulated either by phosphorylation, intracellular localization or by interaction with other proteins. GSK3 is normally active in cells and is primarily regulated through inhibition of its kinase activity. GSK3 activity is significantly reduced by phosphorylation of an N-terminal Ser, Ser_9 in GSK3ß and Ser_{21} in GSK3α (Cross et al., 1995). Phosphorylation at this site is mediated by a variety of extracellular stimuli including insulin, EGF (Epidermal Growth Factor), FGF (Fibroblast Growth Factor), wnt, phorbolesters, forskolin and growth factor depletion. The kinases that are known to phosphorylate these serines are Akt (Cross et al., 1995), PKA (Tanji et al., 2002), PKC (Fang et al., 2002), LKB1 and p90RSK (Brady et al.,

1998; Saito et al., 1994). GSK3 is predominantly a cytosolic protein, but it is also present in the nuclei and mitochondria (Bijur et al., 2003).

The best example for regulation of GSK3 by interaction with other proteins is the canonical wnt-signaling pathway. In the absence of the wnt signal, GSK3ß is bound to axin in a complex with ß-catenin, casein kinase (CK1), and adenomatous polyposis coli protein (APC). As a result, CK1 phosphorylates ß-catenin to prime it for phosphorylation by GSK3ß, resulting in the proteosomal degradation of ß-catenin. Upon wnt stimulation, wnt binds to the receptor Fzl-R and LRP5/6, results in the recruitment of FRAT and Disheveled which is phosphorylated by PAR-1, into the GSK3ß complex and displaces ß-catenin. This prevents the phosphorylation of ß-catenin by GSK3ß, enabling ß-catenin to accumulate and translocate into the nucleus where it is a co-transcriptional activator of TCF/LEF facilitating gene expression (reviewed in Jope et al., 2004).

GSK3 plays an important role in many cellular processes like glycogen metabolism, mitotic spindle dynamics (Wakefield et al., 2003), cell fate specification, microtubule dynamics (Wittmann et al., 2005), organelle transport (Mudher et al., 2004; Morfini et al., 2002; Morfini et al., 2004), cell polarization (Etienne-Manneville et al., 2003), neuronal polarity (Yoshimura et al., 2005; Jiang et al., 2005), oncogenesis, cell survival and death (reviewed in Jope et al., 2004).

GSK3 has been implicated in wide variety of human disease like diabetes, muscle hypertrophy, cancer, bipolar mood disorder, schizophrenia and Alzheimer's disease (reviewed in Jope et al., 2004).

GSK3ß was found elevated in the AD human brain (Yamaguchi et al., 1996). GSK3ß phosphorylates tau at several sites (Ser/Thr-Pro motifs) outside the microtubule-binding region that are elevated in the AD brains. Immuno histochemical studies show that GSK3ß is located in neurofibrillary tangles (Pei et al., 1999). The role of GSK3ß in AD remains highly elusive. Mudher et al. proposed that inhibition of GSK3ß in tau overexpressing *Drosophila* model leads to the inhibition of anterograde transport (Mudher et al., 2004). In contrast, Spittaels et al. proposed that in double transgenic mice overexpressing GSK3ß and tau, there is less motor impairment

compared to the transgenics overexpressing human tau alone (Spittaels et al., 2000). On the other hand, Lucas et al. showed that tau was hyperphosphorylated and exhibited neurodegeneration in conditional transgenic mice overexpressing GSK3ß in the brain (Lucas et al., 2001).

In summary, our data presented here support the idea that GSK3ß phosphorylates MARK2 at Ser_{212} in the activation loop, and thereby inhibits the kinase activity of MARK2 both under *in vitro* and *in vivo* condition. Inhibition of MARK activity by GSK3ß was investigated under *in vivo* condition in cell lines confirming that this pathway also operates in cells.

6.0 References

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7.0 Appendix

7.1 Abbreviations

° C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	micrometer
A260	Absorbance at 260nm
A280	Absorbance at 280nm
AD	Alzheimer's disease
Ala, A	Alanine
APS	Ammoniumpersulfate
ATP	Adenosine triphosphate
Aβ	Amyloid β-peptide
Bq	Becquerel
BSA	Bovine Serum Albumin
CFP	Cyan Fluorescent Protein
CHAPS	Cholamidopropyldimethylaminopropansulfonate
CHAPSO	3-[(3-Cholamidopropyl) Dimethylammonio]-2-Hydroxy-1-Propanesulfonate
CPM	counts per minute
CREB	cAMP response element-binding protein
C-TAK	Cdc25C-associated kinase
Da	Dalton
DMSO	Dimethyl-Sulfoxide
DNA	Deoxy ribonucleic acid
dNTP	Deoxynucleotidetriphosphate
DTT	1,4 Dithiothreitol
E.coli	*Escherichia coli*
e.g.	for example
ECL	Enhanced Chemoluminiscence
EDTA	Ethylendiamine-tetraacidicacid
EGTA	Ethylenglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacidicacid
FCS	Fetal calf serum
FTDP-17	Frontotemporal Dementia with Parkinsonism linked with chromosome 17
Glu, E	Glutamic acid
GSK	Glycogen synthase kinase
GST	Glutathion S-Transferase
GTP	Guanosine-5'-triphosphate
HA	Hemagglutinin
HCl	Hydrochloric acid
HEPES	N-2-Hydroxyethyl-piperazine-N´-2- ethanesulfonicacid
His	Histidine
HPLC	High performance liquid chromatography
HRP	horse radish peroxidase
IF	Immunofluroscence
IP	Immunoprecipitate
IPTG	Isopropyl-1-thio-ß-D-galaktopyranoside
kDa	Kilo Dalton

Kin 1/2	Kinase gene product ½
LB	Luria-Bertani
Leu	Leucine
M	Molar
MAPK	Mitogen activated protein kinase
MAPs	Microtubule associated proteins
McLR	Microcystin-LR
MEK	MAP/ERK (Extracellular Signal-regulating Kinase) Kinase
mg	Milligram
ml	millilitre
MOPS	4-morpholinopropanesulfonic acid
NaCl	Sodium chloride
NaF	Sodium fluoride
NGF	Nerve Growth Factor
nM	nano molar
OD600	Optical density at 600
P/S	Penicillin/Streptomycin
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activating Kinase
PAR-1	Partitioning defective-1
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PHF	Paired Helical Filament
PIPES	Piperazine- N, N'- bis- (2-ethane sulfonic acid)
PKA	Proteinkinase A
PKC	Proteinkinase C
PMSF	Phenyl methyl sulfonyl fluoride
PP2A	protein phosphotase 2a
rpm	Rotation per minute
RT	Room Tempurature
SDS	Sodium dodecyl sulphate
Ser, S	Serine
Sf9	*Spodoptera frugiperda*
TAE	Tris-Acetate-EDTA buffer
TAO	'Thousand And One amino acids' kinase
TBST	Tris- buffered saline Tween-20
TCA	Trichloro acetic acid
TEMED	N,N,N´,N´-Tetramethylethylen-diamine
Thr, T	Threonine
TR1	Tau peptide from repeat1
Tris	Tris (hydroxy methyl) amino methane
TRITC	Tetramethylrhodaminisothiocyanate
Triton X-100	Polyoxyethylen- (9-10)-p-t-octylphenol
Tween 20	Polyoxyethylen-sobitanmonolaurate
Tyr, Y	Tyrosine
U	units
UBA	Ubiqutin-associated domain
UV	Ultraviolet
V	voltage
v/v	volume / volume
w/v	weight / volume
wt	wildtype
YFP	Yellow Fluorescent Protein

7.2 Amino acid and oligonuleotide sequences

A. MARK2 protein sequence

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1   MSSARTPLPTLNERDTEQPTLGHLDSKPSSKSNMLRGRNSATSADEQPHIGNYRLLKTIG
                                                    CAT
61  KGNFAKVKLARHILTGKEVAVKIIDKTQLNSSSLQKLFREVRIMKVLNHPNIVKLFEVIE
                                                    208 212
121 TEKTLYLVMEYASGGEVFDYLVAHGRMKEKEARAKFRQIVSAVQYCHQKFIVHRDLKAEN
                                                    UBA
181 LLLDADMNIKIADFGFSNEFTFGNKLDTFCGSPPYAAPELFQGKKYDGPEVDVWSLGVIL
                                                    C-Terminus
241 YTLVSGSLPFDGQNLKELRERVLRGKYRIPFYMSTDCENLLKKFLILNPSKRGTLEQIMK
                                                    SPACER
301 DRWMNVGHEDDELKPYVEPLPDYKDPRRTELMVSMGYTREEIQDSLVGQRYNEVMATYLL
361 LGYKSSELEGDTITLKPRPSADLTNSSAPSPSHKVQRSVSANPKQRRSSDQAVPAIPTSN
421 SYSKKTQSNNAENKRPEEETGRKASSTAKVPASPLPGLDRKKTTPTPSTNSVLSTSTNRS
481 RNSPLLDRASLGQASIQNGKDSTAPQRVPVASPSAHNISSSSGAPDRTNFPRGVSSRSTF
541 HAGQLRQVRDQQNLPFGVTPASPSGHSQGRRGASGSIFSKFTSKFVRRNLNEPESKDRVE
601 TLRPHVVGGGGTDKEKEEFREAKPRSLRFTWSMKTTSSMEPNEMMREIRKVLDANSCQSE
661 LHERYMLLCVHGTPGHENFVQWEMEVCKLPRLSLNGVRFKRISGTSMAFKNIASKIANEL
721 KL
```

B. GSK3ß protein sequence

```
1   MSGRPRTTSFAESCKPVQQPSAFGSMKVSRDKDGSKVTTVVATPGQGPDRPQEVSYTDTK
61  VIGNGSFGVVYQAKLCDSGELVAIKKVLQDKRFKNRELQIMRKLDHCNIVRLRYFFYSSG
121 EKKDEVYLNLVLDYVPETVYRVARHYSRAKQTLPVIYVKLYMYQLFRSLAYIHSFGICHR
181 DIKPQNLLLDPDTAVLKLCDFGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSIDV
241 WSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFPQIKAHP
301 WTKDSSGTGHFTSGVRVFRPRTPPEAIALCSRLLEYTPTARLTPLEACAHSFFDELRDPN
361 VKLPNGRDTPALFNFTTQELSSNPPLATILIPPHARIQAAASTPTNATAASDANTGDRGQ
421 TNNAASASASNST
```

C. Primers for site-directed mutagenesis

GSK3_S9A_s : 5’-ATG TCA GGA CGT CCC AGA ACC ACC GCC TTT GCG GAG-3’

GSK3_S9A_as: 5’-CAG CTC TCC GCA AAG GCG GTG GTT CTG GGA CGT CCT-3’

7.3 List of figures

7.4 Curriculum Vitae

Name	**:**	Kiruthiga Balusamy
Date of Birth	**:**	18.05.1979
Nationality	**:**	Indian

Educational Qualification

1996-1999	:	Bachelor of Science (Biotechnology/Biochemistry), Avinashilingam Deemed University, Coimbatore, India.
1999-2001	:	Master of Science (Microbial Gene Technology), Madurai Kamaraj University, Madurai, India. Supervisor: Dr. Shanmuga Sundaram Project Title: PCR based detection of Hepatitis B virus in human serum.
2001-2002	:	Junior Research Assistant, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India. Supervisor: Dr. Namita Surolia Project Title: Cloning and Overexpression of Beta keto Acyl ACP synthase III of *Plasmodium falicparum*.
01.2003-12.2006	:	PhD Student Max-Planck Unit for Structural Molecular Biology, DESY, Hamburg, Germany Supervisor: Prof. Dr. Eckhard Mandelkow Thesis title: Regulation of the kinase MARK from *Rattus norvegicus* by GSK3.

7.5 Acknowledgement

I am grateful to Prof. Dr. Eckhard Mandelkow and Dr. Eva-Maria Mandelkow for giving me the opportunity to work on this interesting project and for their critical comments and constant support throughout the whole study.

I express my thanks to Dr. Jacek Biernat for active discussions and helping me to view things in an optimistic way.

I owe my special thanks to Dr. Thomas Timm for his great help and valuable ideas throughout this project and for his comments in writing the thesis.

I would like to thank Dr. Xiaoyu Li and Edda Thies for helping me with cell culture and confocal microscopy.

I would like to thank Dr. Alexander Marx for providing me the structural model for MARK.

I would like to thank Dr. Jian Jiao and Dr. Claire Goldsbury for their help and support.

I would like to thank Alice, Cindy, Mathias, Ilka, Sabrina, Yipeng and other members in the lab for providing me a stimulating environment to work.

I would like to thank Frau Jacobs, Erna and Henry for their help and support.

Last but not the least I extend my heart felt thanks to my parents for sending me to Germany for my higher education.

European Molecular Biology Laboratory
Europäisches Laboratorium für Molekularbiologie
Laboratoire Européen de Biologie Moléculaire

Dr. Simon Holton

Hamburg, 7. December 2006

To whom it may concern,

This letter is to certify that as a native English speaker I have read the Ph.D. thesis entitled **'Regulation of the kinase MARK from *Rattus norvegicus* by GSK3** ". This thesis is to be submitted to the University of Hamburg by Kiruthiga Balusamy.

Yours sincerely,

S.J. Holton

Dr. Simon Holton