Regulation of the kinase MARK from *Rattus norvegicus (Berkenhout, 1769)*: activation by MARKK and inhibition by PAK5

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Abstract

MARK/Par1, a kinase involved in establishing cell polarity, phosphorylates microtubule-associated protein (tau/MAP2/MAP4) at KXGS motifs, and causes their detachment from microtubules. This leads to the destabilization of the microtubules, the tracks of axonal transport. The phosphorylation of the target sites on tau by MARK occurs at an early stage of Alzheimer neurodegeneration. This study addresses the regulation of MARK. It is activated by phosphorylation of an upstream kinase MARKK. It is inhibited by binding of PAK5, a kinase which normally regulates the actin cytoskeleton.

The upstream kinase, MARKK, is a member of the Ste20 kinase family. It phosphorylates MARK within the activation loop (T208 in MARK2). In cells the activity of MARKK enhances the dynamics of the microtubule network through the activation of MARK and leads to the phosphorylation and detachment of tau or equivalent MAPs from microtubules. Overexpression of MARKK eventually leads to microtubule breakdown and cell death, but in neuronal cells the primary effect of endogenous MARKK is to allow the development of neurites during differentiation.

A brain specific kinase PAK5, a member of the p21-activated kinase family, is found to inhibit MARK activity by direct binding, not by phosphorylation. The binding occurs through the catalytic domains of the two kinases. In cells this leads to the protection of the microtubule network. However, PAK5 itself destabilizes the F-actin network so that stress fibers and focal adhesions disappear and cells develop filopodia. In transfected Chinese hamster ovary cells both PAK5 and MARK show a vesicular distribution. Vesicles of inactive PAK5 are also transported to the microtubule organizing center.

Yeast two hybrid data also reveal the interaction between PAK5 and MARKK. This interaction occurs through the binding of the non-catalytic domains of the two kinases. In CHO cells overexpression of PAK5 inhibits the destructive effect of MARKK on the microtubule network.

1. Introduction

1.1 Alzheimer's Disease (AD) and Tauopathies

AD is named after a German doctor Dr. Alois Alzheimer, who in 1906 described a case of a 51-year old woman who died in a completely demented state. The detailed publication in the following year pointed out that the woman had experienced the first symptoms around 5 years previously. She became sequentially unable to care for herself. Her symptoms were impaired memory, disorientation and troubles in reading and writing. The symptoms deteriorated into changes in personality, hallucinations and an irreversible loss of advanced mental functions (Alzheimer, 1907).

Alzheimer's disease is the most common cause of pre-senile dementia. It is a sporadic and hereditary heterogeneous neurodegenerative disease (Trojanowski et al., 1997). It is a chronic, progressive organic, mental disease due to the atrophy of several regions in the brain including hippocampus, temporal lobe, medial parietal lobe and frontal lobe (Scahill et al., 2002). The exact causes of AD are not yet fully understood, however, age is the most important known risk factor. It is thought to affect 5 percent of people over age 65 and 35 percent of people over age 80. Patients younger than 45 years are seldomly found and belong to the families of hereditary AD (familial AD, FAD) which is linked to mutations in three genes: Amyloid Precursor Protein (APP), Presenilin-1 (PS1) and Presenilin-2 respectively (Selkoe, 1994; Wisniewski et al., 1994; Levy-Lahad et al., 1995; Sherrington et al., 1995, Tanzi et al., 1996). On average, patients survive for 8 to 10 years after first experiencing symptoms, though the duration of the illness may vary from 3 to 20 years. Patients usually die of pneumonia, heart attack, or stroke.

Autopsy reveals two hallmarks of abnormal protein deposits in the brains of AD patients (Braak and Braak, 1997; Rosenwald et al., 1993): the extracellular amyloid plaques in the cortex consisting mainly of β-amyloid peptide (Aβ) (Glenner and Wong, 1984; Masters et al., 1985), and the intraneuronal neurofibrillary tangles (NFT) containing mainly hyperphosphorylated tau protein in the form of aggregated paired helical filaments (PHF)

(Grundke-Iqbal et al., 1986; Goedert et al., 1996; Mandelkow and Mandelkow, 1998; Mandelkow, 1999).

It is controversial whether the NFT or the amyloid plaques are the primary cause of the disease. The discovery of mutations in genes that affect the production of Aβ in FAD (Tanzi et al., 1987) and the findings demonstrating the toxicity and pathogenic properties of Aβ (Atwood et al., 2003) bring the amyloid hypothesis into focus (Selkoe 1994; Selkoe 1994). However, the observation of diffuse Aβ deposits without senile plaques or symptoms of dementia (Berr et al., 1994) and the presence of NFTs in very old patients without amyloid deposits (Bancher and Jellinger, 1994) suggest that Aβ deposits may not be sufficient to cause AD (Shastry and Giblin, 1999). In 1998, multiple mutations in the tau gene were discovered in frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). This provides the evidence that tau abnormalities alone are sufficient to cause neurodegenerative disease. The growing list of neurodegenerative diseases that are linked to the progressive intracellular accumulation of phosphorylated filamentous tau inclusions and the absence of other disease-specific neuropathological abnormalities suggest a common mechanism of tau pathology in these diseases, they are termed 'Tauopathies' (Lee et al., 2001; Yancopoulou and Spillantini, 2003) (Table 1).

Table 1: Tauopathies characterized by abnormal hyperphosphorylation of tau

• Alzheimer disease, including tangle-only form of the disease	• Fronto-temporal dementia
• Down syndrome	• Cortico-basal degeneration
• Guam Parkinsonism dementia complex	• Pallido-ponto-nigral degeneration
• Dementia pugilistica	• Progressive supranuclear palsy
• Gerstmann–Sträussler–Scheinker disease with tangles	• Pick disease
• Dementia with argyrophilic grains	

1.2 Tau protein

Tau protein belongs to the microtubule-associated protein (MAP) family. It is abundant in neurons (Schoenfeld et al., 1994), although trace amounts can be found in several peripheral tissues such as heart, kidney, lung, muscle, testis, and pancreas as well as in fibroblasts (Gu et al., 1996; Ingelson et al., 1996; Vanier et al., 1998). Tau protein can also be expressed in glial cells, mainly in pathological conditions (Chin and Goldman, 1996). Tau protein is encoded by one gene on chromosome 17. In the human central nervous system (CNS) there are six tau isoforms which are generated by alternative splicing.

Tau shows an interaction with the actin cytoskeleton (Cunningham et al., 1997), with the plasma membrane (Brandt et al., 1995; Lee et al., 1998). It is involved in promoting neurite outgrowth (Biernat and Mandelkow, 1999; Esmaeli-Azad et al., 1994), regulating vesicle transport (Ebneth et al., 1998) and anchoring signaling molecules such as kinases and phosphatases (Liao et al., 1998; Reszka et al., 1995; Sontag et al., 1996). Despite the diverse roles that tau plays in the neuron, the most well-known function of tau is to stabilize the microtubules in the axon as transport tracks and control their dynamic behaviors (Drubin and Kirschner, 1986; Brandt and Lee, 1993; Cleveland et al., 1977; Nixon and Sihag, 1991). This function is regulated by phosphorylation which results from the activity of specific kinases and phosphatases. It has been shown that phosphorylated tau is less effective than non-phosphorylated tau in promoting microtubule polymerization (Biernat et al., 1993; Bramblett et al., 1993). Hyperphosphorylation of tau abolishes its ability to bind to the microtubules and increases the tendency of tau to aggregate into paired helical filaments.

The structure of the longest isoform, human tau40 is shown as an example in Figure 1. Tau has an N-terminal projection domain and a C-terminal microtubule binding domain (Gustke et al., 1994). The projection domain extends away from the microtubule surface where it may interact with other cytoskeletal elements and the plasma membrane (Brandt et al., 1995; Hirokawa et al., 1988; Steiner et al., 1990). The three or four repeat regions in the microtubule binding domain, which depend on alternative splicing of exon 10 (Figure 1), are mediating the binding of tau proteins to the microtubules. Phosphorylation or dephosphorylation in KXGS motifs

within this repeat region are crucial for the regulation of this interaction (Biernat et al., 1993; Drewes et al., 1995; Drewes et al., 1997; Preuss et al., 1997). Besides, multiple phosphorylation sites by different kinases have been identified in the regions flanking the repeats. Most of them are Ser-Pro and Thr-Pro motifs (Gustke et al., 1994; Lichtenberg-Kraag et al., 1992). The functions of these phosphorylation sites are less understood, they moderately influence the binding of tau to the microtubules. Tau can also be phosphorylated at Tyr residue by the tyrosine kinase Fyn (Lee et al., 2004; Lee et al., 1998).

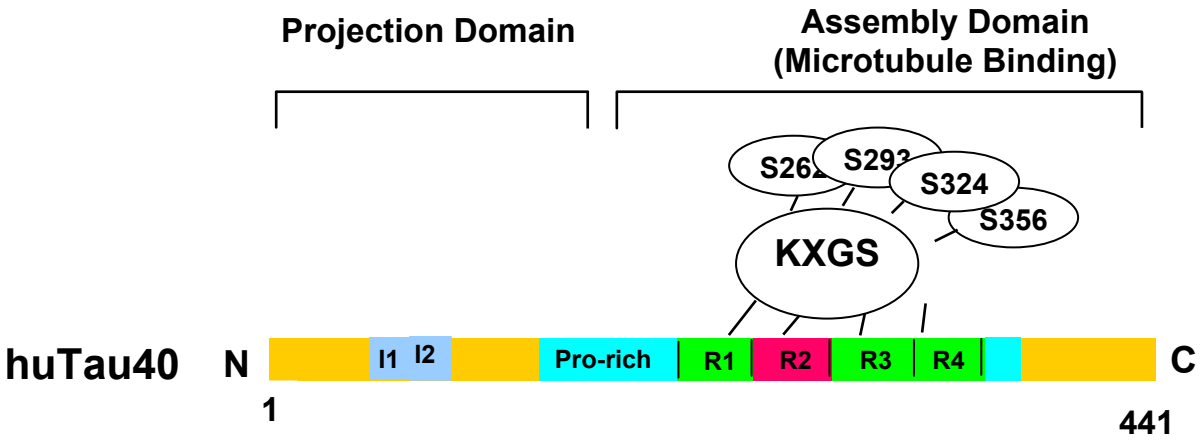

Figure 1: Domain Structure of human tau40.

Tau has an N-terminal projection domain and a C-terminal microtubule binding domain. There are four repeat regions (R1-R4) in the longest human tau isoform, each contains a KXGS motif. The phosphorylation at serine residues of these motifs regulates the binding of tau to the microtubules. The regions flanking the repeats (cyan) are proline rich. There are numerous sites in these regions which have the potential for being phosphorylated by multiple kinases, particularly by proline directed kinases. Human tau40 has two N-terminal inserts (I1 and I2 in light blue) and one additional repeat (R2 in red) due to alternative splicing.

Both proline-directed kinases and non-proline-directed kinases are involved in the phosphorylation of tau proteins. Proline-directed kinases include glycogen synthase kinase 3β (GSK3β) (Hanger et al., 1992; Mandelkow et al., 1992), extracellular signal regulated kinase (ERK) (Drewes et al., 1992; Goedert et al., 1997), stress-activated protein kinase (SAP kinase) (Goedert et al., 1997; Reynolds et al., 1997), cyclin-dependent kinase 2 and 5 (cdk2, cdk5) (Baumann et al., 1993). Non-proline-directed kinases include microtubule-affinity regulating

kinase (MARK) (Drewes et al., 1997), Ca^{2+}/calmodulin-dependent protein kinase II (Johnson, 1992; Steiner et al., 1990), cyclic-AMP-dependent kinase (PKA) (Drewes et al., 1995; Jicha et al., 1999b), protein kinase C (Ekinci and Shea, 1999) and casein kinase I and II (Pierre and Nunez, 1983; Greenwood et al., 1994). The expression of the key kinases that phosphorylate tau changes developmentally in cultured hippocampal neurons (Ferreira et al., 1997). Phosphorylation of tau is higher in the fetal than in the adult state (Bramblett et al., 1993; Brion et al., 1999).

Tau protein can be rapidly dephosphorylated by phosphatases. Phosphatases have many physiological effects, and counter-balance the action of kinases. The unbalance of kinases and phosphatases results in the hyperphosphorylation of tau, a hallmark of AD tau. Phosphatase protein 1 (PP1), PP2A, PP2B as well as a novel protein phosphatase PP5 are able to dephosphorylate tau proteins both *in vitro* and in cells (Drewes et al., 1993; Goedert et al., 1995; Yamamoto et al., 1988; Yamamoto et al., 1995; Saito et al., 1995; Gong et al., 2004). PP2A and PP1 are the major phosphatases of the phosphorylated tau (Tian and Wang, 2002). PP2A is abundant in brain and is associated with the microtubules (Price et al., 1999). The activities of PP1 and PP2A reduced ~20-30% in AD brain (Gong et al, 1993; Gong et al., 1995). Inhibition of PP1 and PP2A induces tau hyperphosphorylation and memory impairment in rats (Sun et al., 2003). Inhibition of PP2A results in an increased level of phospho-tau in the axons of rat bran slices (Gong et al., 2003).

1.3 Microtubule affinity regulating kinase (MARK)

GSK3β and cdk5 are the major kinases that phosphorylate numerous Ser-Pro and Thr-Pro motifs in the regions flanking the microtubule binding domain of tau. But the effects on tau-microtubule binding of these phosphorylation sites are much less pronounced than those in the repeat regions of the microtubule binding domain. In each repeat there is a sequence Lys-(Ile/Cys)-Gly-Ser (KXGS motif), and the phosphorylation of the Serine residues in these motifs greatly affect the tau-microtubule binding. Phosphorylation of tau at KXGS motifs is increased in brain tissue taken from individuals with Alzheimer's disease and this KXGS-phosphorylation markedly increases the phosphorylation of tau by GSK3β and cdk5 at the Ser-

Pro and Thr-Pro sites (Wang et al., 1998; Sengupta et al., 1998; Cho and Johnson, 2003; Nishimura et al., 2004).

CaM Kinase II and PKA have been shown to phosphorylate the KXGS motifs with low efficiency (Steiner et al., 1990; Scott et al., 1993; Sironi et al., 1998). Some years ago a kinase was purified from brain in our lab that strongly reduces the binding of tau to the microtubules and destabilizes them *in vitro* and in cells by phosphorylating KXGS motifs. This kinase is called MAP/Microtubule Affinity Regulating Kinase (MARK) (Biernat et al., 1993; Drewes et al., 1995, 1997 and 1998). In addition to the axonal tau, MARK can also phosphorylate the dendritic MAP2 and the ubiquitously expressed MAP4 at KXGS motifs (Illenberger et al., 1996, Ebneth et al., 1999).

MARK belongs to the AMPK/Kin1/Par1/Snf1-Kinase family of CaM Kinase group II (Hanks and Hunter, 1995; Manning et al., 2002). Homologous kinases include four MARK isoforms in human, Par1 (Partition defective mutation) (Guo and Kemphues, 1995) in *C. elegans* and *D. melanogaster*, Kin1 and Kin2 in *S. cerevisiae*, Kin1+ from S. pombe (Levin et al., 1987; Levin and Bishop. 1990), EMK (ELKL motif kinase) (Inglis et al., 1993) and mPar (Mammalian Par-1) (Böhm et al., 1997) in mouse (Figure 2).

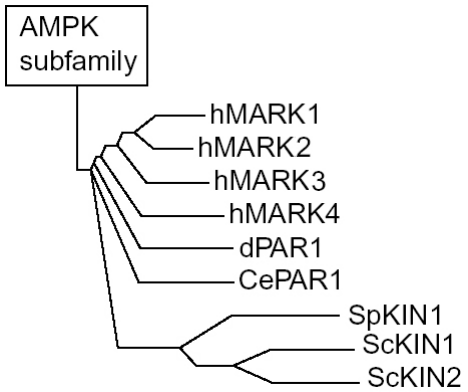

Figure 2: MARK/Par1 kinases form a subfamily of the AMP-dependent protein kinase (AMPK) family.

(Adapted from Drewes, 2004)

MARKs share a similar structure (Figure 3). They have a diverse N-terminal header domain followed by a highly conserved catalytic domain. The activation of MARK can be achieved by the phosphorylation of a conserved threonine in the activation loop (T215 in MARK1 and T208 in MARK2) within the catalytic domain (Drewes et al., 1997; Timm et al., 2003). Part of the native kinase purified form brain showed phosphorylation at a second residue in the activation loop (S219 in MARK1 and S212 in MARK2), which aligns with a Ser/Thr residue highly conserved among kinases (Drewes et al., 1997). The full activity of MARK requires the presence of this residue in an unphosphorylated state. The phosphorylation of this residue is inhibitory for MARKK (Timm et al., 2003). The ubiquitin-associated domain (UBA) might be involved in the ubiquitin-dependent interaction with other proteins (Hoffmann and Bucher, 1996; Brajenovic et al., 2004; Bertolaet et al., 2001). The most diverse spacer domain may play a role in the different regulatory pathways (Drewes et al., 1998). At the C-terminal tail there is a kinase-associated domain of unknown function which is unique for this kinase family (KA1) (Hoffmann and Bucher, 1996).

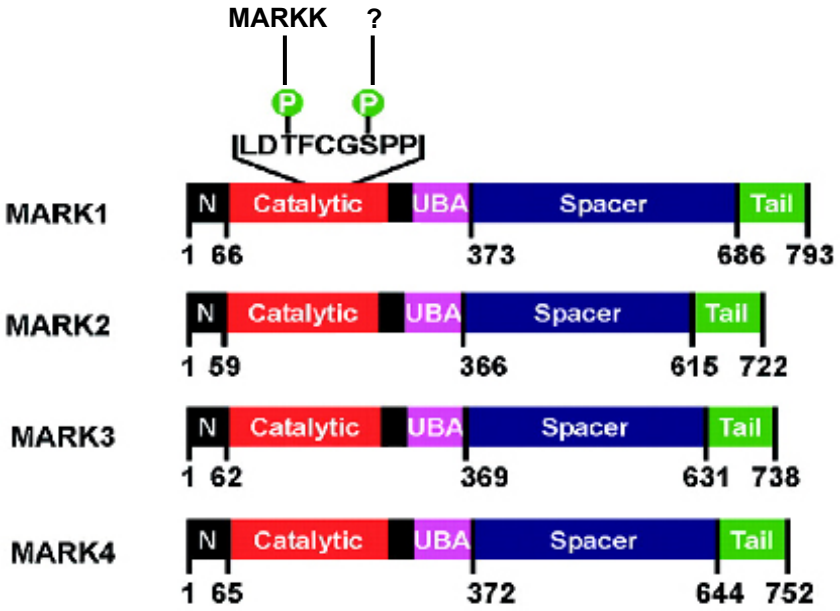

Figure 3: Diagram of MARK 1-4 (DDBJ/EMBL/GenBank accession Nos Z83868, Z83869, AF240782 and AY057448). (Adapted from Timm et al., 2003)

Conserved domain structure of MARK kinases. N=header, UBA=ubiquitin-associated. A fraction of MARK in brain tissue is double phosphorylated in the activation loop (T208 and S212 in MARK2) (Drewes et al., 1997). These two sites are important for activity and are conserved among kinases. The upstream kinase of MARK,

MARKK, phosphorylates MARK at T208, resulting in full kinase activity when S212 is present and unphosphorylated. The kinase which phosphorylates S212 is unknown.

One important function of MARK/Par1 is to regulate the microtubule dynamics by phosphorylating MAPs at KXGS motifs (Drewes et al., 1997; Timm et al., 2003). Involvement of the regulation of tau-microtubule interaction has been suggested to play a role in the polarity establishment and axonal transport (Mandelkow et al., 2004). Members of MARK/Par1 family are involved in generating cell polarity during development, for instance, in yeast budding, partitioning of the *C. elegans* zygote, embryonic axis formation in *Drosophila*, and Wnt pathway regulation by phosphorylation of *dishevelled* (Guo and Kemphues, 1995; Shulman et al., 2000; Tomancak et al., 2000; Sun et al., 2001). In mammalian polarized cells such as epithelia, the localization of MARK/Par1 is important for the cells to maintain asymmetric distribution of cell components (Böhm et al., 1997). In neuronal cells, MARK/Par-1 is required for the neurite outgrowth and the establishment of polarity (Biernat et al., 2002). In addition, mice lacking EMK (Par1) kinase show an immune system dysfunction and autoimmune disease (Hurov et al., 2001). MARK/Par1 is found in a complex with 14-3-3 family proteins in flies and humans (Brajenovic et al., 2004; Benton et al., 2002). 14-3-3ζ and 14-3-3β are found as the interaction partners of MARK2 in yeast-two hybrid screening of human fetal brain cDNA library (Matenia et al., 2005).

1.4 MARK Kinase (MARKK) – a Ste20-like kinase

Recently, two kinases were found in our laboratory that regulate MARK/Par1 kinase. A kinase purified from brain was shown to activate MARK, thus it was termed MARKK (MARK Kinase) (Timm et al., 2003). Another brain specific kinase, PAK5, was found to inhibit MARK by binding (Matenia et al., 2005). Both MARKK and PAK5 belong to the Sterile 20 (Ste20) Ser/Thr protein kinase family. There are also reports in the literature, that the tumor suppressor protein LKB1 is able to activate the kinases of the AMPK subfamily, including MARK/Par1 (Lizcano et al., 2004).

The Ste20 kinase family was first identified in *S. cerevisiae* as part of the mating and pheromone response signaling (Ramer and Davis, 1993; Leberer et al., 1992; Kyriakis and

Avruch, 1996). There are about 30 Ste20-related kinases in mammals in addition to homologues in *Drosophila, C. elegans* and other organisms. Ste20 family kinases have various intracellular regulatory effects. Except the homology between the mammals and the yeast in the catalytic domain, these kinases differ noticeably in domain structure and sequence. Based on the structural organization and functional analogy, members of Ste20 family can be classified into two groups. The first group is p21-Activated Kinases (PAKs). Members of this family have a kinase domain at the C-terminus and a Cdc42/Rac-Interactive Binding domain (CRIB) in the N-terminal noncatalytic region. The second group is the Germinal Center Kinases (GCKs), which have a conserved kinase domain at the N-terminus (Figure 4).

MARKK is highly homologous to TAO1 (Thousand And One-amino acid protein kinase 1) (Hutchison et al., 1998). Together with PSK (Prostate-derived Sterile20-like Kinase)/TAO2 (Chen et al., 1999) and JIK (JNK/SAPK-Inhibitory Kinase)/TAO3 (Tassi et al., 1999), they form the GCK-VIII subfamily of mammalian Ste20 kinase family. MARKK/TAO1 and TAO2 are highly expressed in brain. MARKK/TAO1 can phosphorylate MKK3 (Mitogen-Activated Protein Kinase Kinase 3), which in turn specifically activates p38 MAPK (Hutchison et al., 1998). KFC (Kinase From Chicken), which has an 88% identity within the kinase domain and 71% overall to MARKK, is a specific activator of SAPK/JNK (Stress-Activated Protein Kinase/c-Jun N-terminal Kinase), and the intrinsic kinase activity seems to be negatively regulated by its coiled-coil domain. In addition, the full length KFC differs from its spliced form in structure and biological properties (Yustein et al., 2000). TAO2 is shown to phosphorylate MKK3 and MKK6, which are the specific activators of p38 MAPK pathway (Chen et al., 1999; Chen and Cobb, 2001); and this pathway is suggested to mediate the signals from carbachol through heterotrimeric G proteins to ternary complex transcription factors in the nucleus (Chen et al., 2003). PSK, a human homolog of TAO2, exclusively activates the JNK pathway by phosphorylation of MKK4 and MKK7. When microinjected into Swiss 3T3 cells, PSK localizes to a vesicular compartment and reduces actin stress fibers by the presence of its C-terminus and kinase activity (Moore et al., 2000). By contrast, JIK acts as a negative regulator in the JNK/SAPK pathway rather than an activator; its activity is decreased by the activation of tyrosine kinase receptors (Tassi et al., 1999).

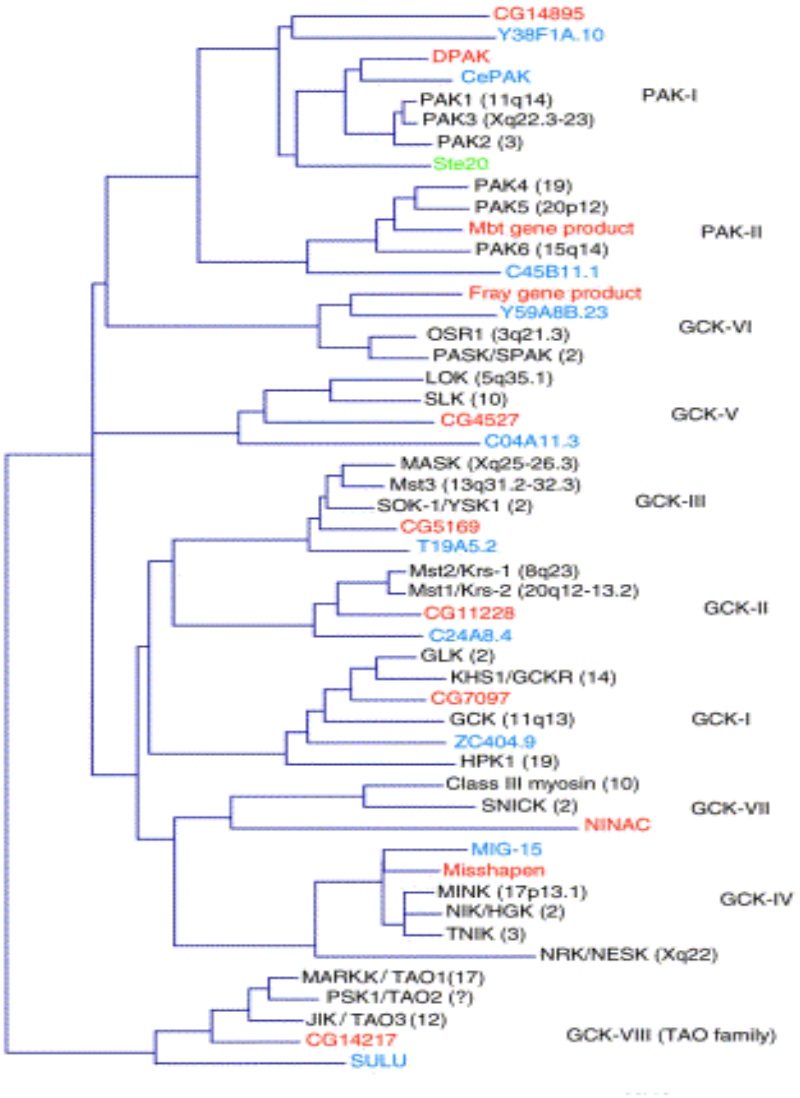

Figure 4: Phylogenetic relations among Ste20 group kinases.
(Adapted from Dan et al., 2001)

The sequence of each Ste20 group kinase was obtained from human (black), *Drosophila* (red) and *C. elegans* (blue) databases. The budding yeast Ste20 protein is included to provide references (green). The chromosomal location is indicated in parentheses except for PSK1/TAO2.

The PAK family has a kinase domain at the C-terminus and a CRIB domain in the noncatalytic region. GCK-family has a conserved N-terminal kinase domain (in contrast to PAK-family kinases, which have a C-terminal kinase domain), but their noncatalytic regions exhibit a wide variety of structures.

MARKK/TAO1 belongs to the GCK-VIII subfamily; members of this subfamily contain a short less-conserved intermediate region downstream the catalytic domain, a long conserved region in the middle of the noncatalytic region, and a C-terminal less-conserved stretch. They are integrated into the GCK family because of the location of the kinase domain, but might form a distinct family.

1.5 p21-Activated Kinases (PAKs)

To date, six isoforms of PAK have been identified in humans. PAK1-3 make up the conventional PAK-I subfamily; PAK4-6 form the non-conventional PAK-II subfamily of Ste20 Ser/Thr kinase family. All PAKs share two common structural features: a kinase domain at the C-terminal end and a CRIB domain at the N-terminus.

1.5.1 PAK-I subfamily

PAK1, 2, 3 were discovered using an overlay assay to screen for Rho GTPase binding partners in rat brain cytosol (Manser et al., 1994). The kinase activities can be simulated by binding of GTP-bound forms of Rac or Cdc42, but not Rho. PAK1 is expressed in brain, muscle, and spleen; PAK2 is ubiquitously expressed; and PAK3 is expressed only in the brain. They serve as important regulators of actin cytoskeletal dynamics and cell motility. They are also implicated in MAPK mediated transcription, apoptosis, cell-cycle progression, and in pathological conditions and cell transformation (Bokoch, 2003; Wolf et al., 2001).

The PAK-I subfamily members share common structures (Figure 5). The autoinhibitory domain (KI, Kinase Inhibitor) locates downstream of the CRIB domain, slightly overlapping it. PAKs are inactivated by the interaction of the KI domain with the kinase domain. The binding of Rac or Cdc42 to the CRIB domain disturbs this interaction, resulting in the activation of the kinase (Bagrodia and Cerione, 1999). Flanking the CRIB domain there are several proline-rich motifs that bind to SH3 (Src-homology 3) domain containing proteins, such as adaptor protein Nck (Bokoch et al., 1996), Grb2 (Puto et al., 2003), and Cool (Cloned Out Of Library)/PIX (Pak-Interacting eXchange factor; also known as Rac/Cdc42 GEF6) (Manser et al., 1998), suggesting that all PAK-I family members can be recruited in a similar manner to a variety of signal transduction pathways.

Figure 5: Schematic diagram indicating conserved features of PAK-I family members. (Adapted from Zhao and Manser, 2005)

The conventional PAK family contains a conserved CRIB domain (red) that overlaps a KI domain (yellow). Cdc42/Rac1 binding to the CRIB rearranges the KI domain and releases its binding to the catalytic domain (blue), resulting in the activation of the kinase. The three purple boxes correspond to conserved proline-rich motifs that bind SH3 domain-containing proteins Nck, Grb2 and PIX (left to right respectively).

The partial crystal structure of PAK1 brings light to the regulation of PAK at the molecular level (Lei et al., 2000; Parrini et al., 2002). The kinase has a dimerized auto-inhibited conformation in head-to-tail fashion (Figure 6); the KI domain of one molecule is placed across the kinase cleft of the other molecule and is supported by PIX dimers. The binding of GTP-Cdc42 or Rac disrupts this inhibitory interaction and releases the KI domain from the catalytic domain. The kinase undergoes a conformational change that allows autophosphorylation, which is required for full kinase activity (Lei et al., 2000). The autophosphorylation at Ser144 within the KI domain of PAK1 is thought to prevent the reattempt of KI-catalytic domain interaction (Chong et al., 2001). Autophosphorylation of Ser198 and Ser203 down-regulate the PIX-PAK interaction (Mott et al., 2005). Phosphorylation of Thr423 in the activation loop of PAK1 is crucial for the kinase to maintain release from auto-inhibition state and to achieve its full activity (Gatti et al., 1999; Zenke et al., 1999). This phosphorylation may occur through autophosphorylation or a third kinase, such as PDK1 (3-Phosphoinositide-Dependent Kinase-1) in the presence of sphingosine (King et al., 2000).

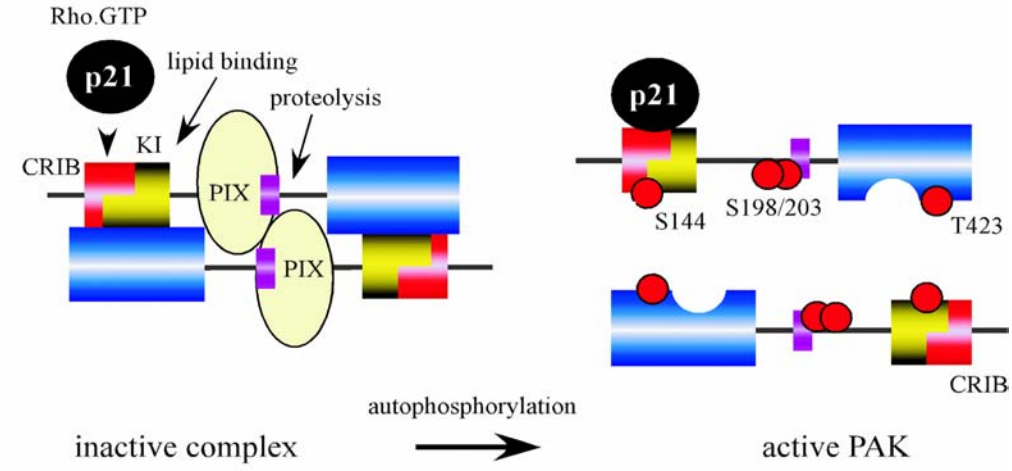

Figure 6: A model for PAK1 activation. (Zhao and Manser, 2005)

The auto-inhibited PAK1 kinase is arranged in head-to-tail fashion as homodimers, in which the catalytic domain (blue) binds the KI domain (yellow) and is supported by associated PIX dimers. Upon Cdc42 or Rac binding to

the CRIB domain (red), proteolysis or lipid binding (arrows), the kinase undergoes a conformational change that allows autophosphorylation (red circles). Phosphorylation of Ser144 serves to disable the KI-catalytic domain interaction. Phosphorylation of Ser198/203 reduces the affinity for PIX. Phosphorylation of Thr423 in the activation-loop finally activates the kinase.

The activation of conventional PAKs can also be GTPase-independent. An alternative spliced isoform of PAK3 termed PAK3b, which is expressed in various areas of the adult mouse brain, has an insertion in the CRIB/KI sequence, thus the KI domain of PAK3b no longer inhibits the kinase activity. The insertion impedes the binding of GTP-Cdc42 or Rac, resulting in constitutive activation of the kinase in the absence of GTPase binding (Rousseau et al., 2002). Proteolysis of PAK1 produces a complex consisting of a regulatory fragment (residues 57 to 200) and a catalytic fragment (residues 201 to 491), which is active in the absence of Cdc42. (Buchwald et al., 2001). PAK2 is found to be a substrate for caspase 3, generating a catalytically activated kinase during apoptosis (Rudel and Bokoch, 1997). Lipid such as sphingosine can stimulate the autophosphorylation and activity of PAK2 associated with the membrane-containing particulate fraction in a Cdc42-independent manner (Roig et al., 2001).

1.5.2 PAK-II subfamily

Compared to the PAK-I subfamily, the function and the regulation of the non-conventional PAK-II subfamily are poorly understood. Members of this subfamily (PAK4-6) have a C-terminal catalytic domain, which are >75% identical to each other, but are only about 54% similar to those of PAK-I subfamily. They also have an N-terminal CRIB domain, which share at least 60% identity to each other, but is less than 40% identical to the CRIB domains of PAK1-3. Although the CRIB domain mediates the binding of Cdc42 or Rac to PAK4, 5, 6, this does not stimulate the activity of these kinases (Abo et al., 1998; Pandey et al., 2002; Lee et al., 2002). There are no recognizable KI domains resembling that in PAK1. Recently an autoinhibitory domain of PAK5 is reported and the autophosphorylation of PAK5 can be achieved by GTP-Cdc42 (Ching et al., 2003).

PAK4 is the first reported member of non-conventional PAKs. It is ubiquitously expressed, with highest level in the prostate, testis and colon (Abo et al., 1998; Callow et al., 2002). PAK4

has many functions. Binding of Cdc42Hs results in the redistribution of PAK4 to the Golgi apparatus and the subsequent induction of filopodia (Abo et al., 1998), which might be mediated by LIM kinase1 and cofilin (Dan et al., 2001). It is involved in oncogenic transformation by dissolving stress fibers and focal adhesions, promoting anchorage-independent growth and suppression of caspase signaling to protect cells from apoptosis (Gnesutta et al., 2001; Qu et al., 2001; Callow et al., 2002; Gnesutta and Minden, 2003). PAK4 is essential for embryonic neuronal development in mice, since the absence of PAK4 is lethal (Qu et al., 2003). Several Rho-family GEFs (Guanine Nucleotide exchange Factors) have been reported to interact with PAK4. For instance, direct interaction between PAK4 and PDZ RhoGEF results in the phosphorylation of PDZ RhoGEF, abolishing its ability to mediate accumulation of Rho-GTP and stress fiber formation (Barac et al., 2004). PAK4 associates with GEF-H1 through GID (GEF Interaction Domain). Endogenous PAK4-GEF H1 complex associates with microtubules. Phosphorylation of GEF H1 by PAK4 releases it into the cytoplasm and inhibits GEF H1 dependent stress fiber formation while promoting the formation of lamellipodia (Callow et al., 2005).

The next member of PAK-II subfamily, PAK6, is expressed most highly in brain and testis, with lower levels in multiple tissues including prostate and breast (Yang et al., 2001; Lee et al., 2002). It specifically interacts with androgen receptor and represses the transcription mediated by androgen receptor in a kinase dependent and Rho GTPase independent manner (Yang et al., 2001; Lee et al., 2002; Schrantz et al., 2004). Recent research shows that MKK6 and its downstream p38 MAPK is able to activate PAK6 by phosphorylation, in addition, MKK6 can also activate PAK4 and PAK5 by phosphorylation at the same tyrosine residue (Y608 in PAK5) within a conserved threonine-proline-tyrosine motif (Kaur et al., 2005).

The last member, PAK5, is predominantly expressed in brain and not in most other tissues. Its kinase activity, which does not require the binding of Cdc42, is required to promote the formation of filopodia and neurite outgrowth (Dan et al., 2002; Pandey et al., 2002). PAK5 can activate the JNK pathway but not the p38 and the ERK pathway, but the activation of this pathway is not involved in the induction of neurite outgrowth (Dan et al., 2002; Pandey et al., 2002). Both PAK5 and PAK1 are involved in neurite outgrowth and dendritic spine formation

induced by GEF T in neuroblastoma cells, in which PAK5 plays the major role (Bryan et al., 2004). However, transgenic PAK5 knockout mice are viable, presumably because of functional redundancy with other PAKs (Li and Minden, 2003). PAK5 can prevent apoptosis by phosphorylating pro-apoptotic protein BAD and preventing the localization of BAD to mitochondria (Cotteret et al., 2003). Latest research shows that PAK5 inhibits MARK/Par1 by binding, which leads to stable microtubules and dynamic actin (Matenia et al., 2005).

1.6 Aim of this work

MARK/Par1 phosphorylates tau at KXGS motifs (recognized by the 12E8 antibody). This causes the detachment of tau from microtubules and destabilization of microtubules. Strong 12E8 staining is found in the pre-neurofibrillary tangles in Alzheimer's disease (Augustinack et al., 2002), suggesting the priority of this type of phosphorylation of tau proteins over other sequential hyperphosphorylation in Alzheimer's diseases. Thus investigating the regulation of the MARK-Tau pathway is important to understand the pathology of AD and related neurodegenerative diseases.

Recently, the upstream kinase of MARK was purified from brain, termed MARKK (Timm et al., 2003). Therefore, a goal of this study was to show the MARKK-MARK-Tau cascade and its destructive effect on the microtubule network in cells. A further goal was the use of RNAi to study the physiological function of the endogenous MARKK-MARK-Tau cascade in a neuronal cell line.

Another brain specific kinase PAK5, a novel member of PAK-II kinase subfamily, was recently found as an interaction partner of MARK2 in the yeast two hybrid screen of human fetal brain cDNA library (Matenia et al., 2005). Kinase assays showed the inhibition of MARK by PAK5, without affecting the activity of PAK5. The goal of this work was to investigate whether the upstream kinase MARKK was also associated with PAK5. The inhibitory effect of PAK5 on the MARKK-MARK-Tau cascade and how it is affecting the two cytoskeletal networks, actin and microtubules, was characterized in detail.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased at analytical grade from following companies:

Amersham Pharmacia Biotech	Invitrogen
AppliChem	Merck
BD Biosciences	New England Biolabs
Boehringer-Mannheim	Qiagen
Calbiochem	ROTH
Clontech	SERVA
Fluka	Sigma-Aldrich
GERBU	Stratagene
GiBCO BRL	

2.1.2 Bacteria

All plasmids were cloned in the *E.coli* strains XL2-Blue ultracompetent cells (Bullock, 1987) from Stratagene or TOP10 chemical competent cells (Grant, 1990) from Invitrogen.

XL2-Blue Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F´ proAB lacI^{q}ZΔM15 Tn10 (Tet^{r}) Amy Cam^{r}]*

TOP10 Genotype: *F^{-} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139Δ(ara-leu)7697 galU galK rpsL (Str^{R}) endA1 nupG*

2.1.3 Plasmids

MARKK cDNA was provided by Dr. T. Timm (MPG-ASMB, Hamburg). PAK5 cDNA was provided by Dr. D. Matenia (MPG-ASMB, Hamburg). All other plasmid and cDNAs were provided by Dr. J. Biernat (MPG-ASMB, Hamburg).

2.1.4 Oligomers

All sub-cloning primers and sequencing primers were obtained from MWG-Biotech AG.
The siRNA-oligonucleotides were purchased from Qiagen-Xeragon.

2.1.5 Enzymes

Restriction endonucleases	New England Biolabs
Quick T4 DAN Ligase	New England Biolabs
Pfu Polymerase	Stratagene
Pwo Master Polymerase	Roche

2.1.6 Antibodies

Anti-TAO1	BD Biosciences
12E8	from Dr. P. Seubert, Elan Pharmaceuticals
SA6941	Eurogentec
SA2118	Eurogentec
SA4632	Eurogentec
SA4635	Eurogentec
YL1/2	Serotec Ltd.
Anti-α-Tubulin clone Dm1A	Sigma
Anti-β-Actin clone AC-15	Sigma
Anti-γ-Tubulin clone GTU-88	Sigma
Anti-human-vinculin clone hVIN-1	Sigma

Oregon Green Phalloidin	Molecular Probes
Rhodamin Phalloidin	Molecular Probes
Goat anti mouse Cy5	ImmunoResearch
Goat anti mouse TRITC	ImmunoResearch
Goat anti rat Cy5	ImmunoResearch
Goat anti rat TRITC	ImmunoResearch
Goat anti rabbit Cy5	ImmunoResearch
Goat anti rabbit TRITC	ImmunoResearch

2.1.7 Equipment and Accessories

Centrifuges:

Cold centrifuge J2-21 M/E with corresponding rotors	Beckman
Bench top centrifuge 5402	Eppendorf
Bench top centrifuge 5415D	Eppendorf
Centrifuge 5810R	Eppendorf

Cell Culture Equipments and Reagent:

SterilGARD Hood class II Type A/B3	Labtect
HERA safe	Heraeus
HERA cell 240	Heraeus
Flasks, Dishes, Plates and Pipets	Corning or NUNC
HAM's F12, DMEM, MEM, DMEM/F12	GiBCO BRL
Trypsin	GiBCO BRL
Penicillin/Streptomycin	Sigma
Geneticin 418	Sigma
Fetal calf serum	Sigma
Horse serum	Sigma
Effectene transfection kit	Qiagen
Oligofectamine reagent for siRNA	Invitrogen

Microscopy:

Confocal LSM510 Meta System	Zeiss
Axiovert 100M fluorescence microscope	Zeiss
Axiovert 200 MAT fluorescence microscope	Zeiss
Olympus CK2 inverted microscope	Olympus

Software:

VectorNTI 9.0	InfoMax & Invitrogen
LSM5 Image Processing Software	Zeiss

Other:

Speed Vac Concentrator	Bachofer
Incubator shaker Innova 4300	New Brunswick Scientific
U/V Visible spectrophotometer Ultrospec 1000	Amersham Pharmacia Biotech
Gel Photo Processor System Photo PR SID	Mitsubishi
LAS3000 Chemiluminescence Detector	Fuji

2.2 Methods

2.2.1 Molecular Biological Methods

Restriction Endonuclease Digestion of DNA:

1-2 µg DNA was mixed with restriction enzymes (1-2 U; NEB) and enzyme buffer in a 0.5 ml reaction tube, BSA was optional according to the enzyme used. The total volume of the reaction mixture was 10 µl. The mixture was incubated at the recommended temperature for the enzyme (usually 37 °C) for 1 hour. 6× DNA loading buffer (0.2% Bromphenolblue, 0.2% Xylene cyanol FF, 60% Glycerol, 60 mM EDTA) was added directly to the reaction mixture after incubation. The analysis of the digestion was carried out by agarose gel electrophoresis.

DNA Agarose Gel Electrophoresis (Sambrook et al., 1989) and Staining:

DNA Marker (Smart Ladder, Eurogentec) and DNA samples with loading buffer were applied to 0.8-1% agarose gel (according to the size of the DNA fragments). The electrophoresis was performed horizontally in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) at 100 mA for 30-90 minutes. After electrophoresis the agarose gel was stained in ethidiumbromide solution (4 μg/ml in H_2O) for 15 minutes, then destained in H_2O for 10 minutes. Photos of DNA bands were taken with the gel photo processing system.

Isolation of DNA Fragments from Agarose Gels:

Correct DNA fragments were excised under a UV light and DNA fragments were extracted with UltraClean 15 DNA Purification Kit (MO BIO Laboratories Inc.) according to the manufacturer's instruction.

Ligation:

Ligation of DNA fragments was performed with Quick Ligase (NEB). Briefly 50-100 ng of vector DNA fragment was mixed with 3-fold molar excess of insert DNA fragment together with 10 μl 2×Quick Ligase buffer and 1 μl Quick Ligase in 20 μl volume. The ligation mixture was incubated at room temperature for 5 minutes and then chilled on ice.

Transformation:

1 μl of the ligation mixture was added to 20 μl chemical ultracompetent cells XL2-Blue (Stratagene). After 30 minutes of incubation on ice cells were treated with heat-shock for 30 seconds in a 42°C water bath, and then incubated on ice for 2 minutes. Afterwards 200 μl SOC medium at room temperature was added. Cells were incubated at 37 °C and 200 rpm for 1 hour and spread onto LB-medium dishes containing selective antibiotic.

Screening the Positive Clone:

LB-medium dishes were incubated at 37°C for 16-20 hours. 5-10 single colonies were picked and incubated overnight separately in 5ml LB medium containing selective antibiotic. Mini plasmid DNA preparation was done according to the manufacturer's instructions (Invitek). Analysis of the clones was carried out by restriction endonuclease digestion and DNA

sequencing. One positive clone was inoculated to 100ml LB medium containing selective antibiotic, and cultured at 37°C and 200 rpm overnight. Midi plasmid DNA preparation was done according to the manufacturer's instructions with the Qiagen Endofree plasmid kit.

DNA Sequencing:

DNA sequencing reactions were performed following the Sanger-Didesoxy-Method (Sanger et al., 1977) using the fluorescent dye labeling After PCR reaction, the mixture was supplemented with 100% ethanol to a final concentration of 70% to precipitate the DNA. After 10 minutes incubation on ice, the sample was centrifuged at 14000 rpm at 4°C for 30 minutes. The supernatant was discarded and the pellet was air-dried and resuspended in 75µl HPLC water, heated at 80°C for 2 minutes and applied to the sequencing tube. Sequencing was done by ABI PRISM 310 Genetic Analyser (PE Applied Biosystems).

Polymerase Chain Reaction:

Mutagenesis

The mutants used in this research were created using the Quick Change Site-Directed Mutagenesis Kit (Stratagene):

dsDNA template	25 ng
Forward primer	10 pmol
Reverse primer	10 pmol
10× Pfu buffer	2 µl
dNTPs mixture (2.5 mM each)	2 µl
Pfu DNA polymerase (2.5U/µl)	0.5 µl
ddH_2O to final volume of	20 µl

PCR program:

1. Denaturing	95 °C	30 sec
2. Denaturing	95 °C	30 sec

3. Annealing	Tm-5 °C	1 min
4. Elongation	68 °C	~2 kb/min
Repeat step 2-4 16×		
5. Elongation	68 °C	20 min
6. Hold at 4 °C		

Adaptor Introduction and DNA Fragment Amplification

The design of the primers containg adaptors corresponding to each sub-cloned constructs were carried out with VectorNTI 9.0 (InfoMax & Invitrogen):

With Pfu polymerase:

dsDNA template	100 ng
Forward primer	20 pmol
Reverse primer	20 pmol
10× Pfu buffer	5 µl
dNTPs mixture (2.5 mM each)	2 µl
Pfu DNA polymerase (2.5 U/µl)	1 µl
ddH_2O to final volume of	50µl

PCR program:

1. Denaturing	96 °C	90 sec
2. Denaturing	96 °C	15 sec
3. Annealing	Tm-5 °C	40 sec
4. Elongation	72 °C	~2 kb/min
Repeat step 2-4 30×		
5. Elongation	72 °C	20 min
6. Hold at 4 °C		

Or with Pwo Master Polymerase (for fragments shorter than 3 kb):

Step1:

dsDNA template	10 ng
Forward primer	100 pmol
Reverse primer	100 pmol
PCR H_2O to	25 µl

Step2:

add Pwo Master	25 µl
Total volume	50 µl

PCR program:

1. Denaturing	94 °C	2 min
2. Denaturing	94 °C	30 sec
3. Annealing	Tm-5 °C	30 sec
4. Elongation	72 °C	~1 kb/min
Repeat step 2-4 30×		
5. Elongation	72 °C	5 min
6. Hold at 4 °C		

Sequencing PCR

Purified DNA template	1 µg
Sequencing primer	5 pmol
Big-Dye Mix (Applied Biosystems)	2 µl
Reaction buffer (Applied Biosystems)	6 µl
HPLC H_2O to final volume of	20 µl

PCR program:

1. Denaturing	96 °C	10 sec
2. Annealing	50 °C	5 sec
3. Elongation	60 °C	4 min
Repeat step 1-3 26×		
4. Hold at 4 °C		

2.2.2 Biochemical Methods

SDS-Polyarcrylamide Gel Electrophoresis (SDS-PAGE):

A modified protocol (Laemmli, 1970; Mandelkow et al., 1985) was used for SDS-PAGE. The 0.5 mm thick gel consisted of a 4% acrylamide stacking part and a 10% acrylamide separation part. Protein samples were mixed with 2× sample buffer (160 mM Tris, pH 6.8, 10% (w/v) SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% (w/v) bromphenolblue) and heated to 95 °C for 5 minutes to denature. Molecular weight protein markers were β-galactosidase (116 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), lactate dehydrogenase (35 kD), restriction endonuclease Bsp981 (25 kD), β-lactoglobulin (18.4 kD), lysozyme (14.4 kD). The electroporesis was carried out at 250 Volts in SDS-PAGE running buffer (25 mM Tris-HCL, 190 mM glycin, 0.1% SDS).

Western Blotting:

Western blotting was carried out following the method of Towbin (Towbin et al., 1979). After electrophoresis, the SDS–polyacrylamide gel was placed in Western blotting buffer (48 mM Tris, 39 mM glycin, 5 % methanol, 0.1% (w/v) SDS) for 10 minutes, then transferred electrophoretically to a methanol activated PVDF membrane at a current of 1mA/cm^2 for 60 minutes. The membrane was blocked in TBST (Tris-Buffered Saline containing Tween, 10 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.05 % (v/v) Tween20) containing 5% (w/v) milk powder. After incubation with the primary antibody (diluted in 5% milk/TBST) at 37°C for 1 h, the PVDF membrane was washed 3 times with TBST and incubated with a peroxidase

conjugated secondary antibody (diluted in 5% milk/TBST) at 37°C for 1 h. The membrane was again washed 3 times with TBST. The ECL (Enhanced Chemiluminescence Light) based detection system was used to detect the fluorescent product of the secondary antibody.

Kinase assay:

Kinase activities were assayed in 50 mM Tris-HCL buffer pH_{RT} 7.4 containing 5 mM $MgCl_2$, 2 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, 2 mM benzamidine at 30°C for 2 hours. 150µM ATP (γ-[^{32}P] ATP, 7.4 MBq/mol, Amersham Pharmacia Biotech) and 150µM substrate peptide, which is derived from the first repeat of tau protein containing S262 in the KXGS motif (TR 1-peptide $_{255}$NVKSKIGSTENLK$_{267}$, Drewes et al., 1997) were used. 50 ng (0.55 pmol) recombinant MARK2 and 25ng (0.21 pmol) recombinant MARKK were used for each reaction in a final volume of 10 µl. Reactions were stopped by addition of half the volume of 30% (w/v) TCA. After 30 minutes incubation on ice and 15 minutes centrifugation (RT, 14000×g) the supernatant was applied to phosphocellulose-paperdiscs, washed with phosphoric acid (0.1 M), air-dried and radioactivity was measured in a scintillation counter (Tricarb 1900 CA, Packard).

Immunoprecipitation:

8×10^6 Sf9 cells were plated in a T25 flask, infected with recombinant balculoviruses encoding His-tagged MARKK and HA-tagged PAK5 kinase at a MOI (multiplicity of infection) of 1, and incubated at 27°C for 72 h. The cells were collected and resuspended in 250 µl ice cold lysis buffer (50mM Tris-HCL pH 7.4, 100 mM NaCl, 3 mM EGTA, 3 mM $MgCl_2$, 0.1% NP-40, 5 mM Chapso, 1 mM DTT, 2 mM benzamidin, 1 mM Na_3VO_4 and Sigma mammalian protease inhibitor cocktail) for 30 minutes, then centrifuged at 14000 rpm at 4 °C. Agarose beads conjugated with anti-HA-antibody (Santa Cruz) were added to the supernatant (50 µg/ml) and incubated at 4 °C for 4 hours, then spun at 12000 rpm for 20 seconds. The supernatant was colleted and the pellet was washed with lysis buffer without Chapso. Both pellet and supernatant were used for Western blotting.

2.2.3 Cell Biological Methods

Cell Lines:

Chinese Hamster Ovary Wild Type (CHO wt)

Chinese Hamster Ovary human tau40 Stably Transfected (CHO htau40)

Rat pheochromocytoma PC12

Culture Medium:

For *CHO wt* cells: F-12 (HAM) Nutrient Mixture + L-Glutamine, supplied with 10% Fetal Calf Serum (FCS) and penicillin streptomycin mixture.

For *CHO htau40* cells: F-12 (HAM) Nutrient Mixture + L-Glutamine, supplied with 10% FCS and 600µg/ml Geneticin 418.

For *PC12* cells: DMEM (Dulbecco's Modified Eagle Medium) + 4500mg/L Glucose + L-Glutamine, supplied with 15% heat inactivated horse serum, 5% FCS and penicillin streptomycin mixture.

Cell Culture:

All cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Supply with medium was done simply by removing old culture medium and adding fresh medium. To split cells the old culture medium was removed and discarded. Cells were washed once with pre-warmed PBS and 0.5 – 1 ml Trypsin-EDTA solution was added to the flask. Cells were placed at 37 °C to facilitate trypsinization. After the cell layer was dispersed, fresh complete growth medium was added to stop the reaction and cells were resuspended by gentle pipetting. Aliquots of the cell suspension were added to new culture flasks.

Differentiation of PC12 cells:

Cells were grown in DMEM:F12 1:1 medium containing 0.1% horse serum and FCS mixture, supplied with 100 ng/ml NGF and differentiated for 48 to 72 hours. Differentiation medium was changed every day.

Transfection:

Plasmid DNA

Effectene (Qiagen) was used to transfect CHO wt and CHO htau40 cells. Cells were seeded at 1×10^4 cells/coverslip in 24-well plates in 0.5 ml culture medium and grown for 24 hours. For each coverslip 0.2 µg plasmid DNA was combined with 1.6 µl Enhancer in 25µl EC Buffer. After 5 minutes incubation at room temperature, 3 µl Effectene was added and mixed gently. After another incubation of 10 minutes at room temperature, 150 µl of fresh culture medium was added and mixed gently. The whole transfection mixture was titrated to cells, which had been washed once with PBS and supplied with 400 µl fresh culture medium.

Lipofectamine2000 (Invitrogen) was used to transfect PC12 cells. Cells were seeded at 5×10^4 cells/coverslip in 24-well plates in 0.5 ml culture medium and grown overnight. For each coverslip, 0.8 µg plasmid DNA was diluted into 50 µl Opti MEM I medium and 2 µl Lipofectamine2000 was diluted into another 50 µl Opti MEM I medium. These two parts were gently mixed and incubated at room temperature for 20 minutes. Cells were washed once with PBS and supplied with 0.5 ml Opti MEM I medium. All of the transfection mixture was added to the cells. 4-6 hours post-transfection, cells were washed once with PBS and supplied with growth medium. 24 hours post-transfection, the growth medium was replaced by differentiation medium if differentiation was planned for the experiment.

siRNA

21-nucleotide RNAs were designed as recommended (Elbashir et al., 2001) and chemically synthesized by Qiagen-Science. The siRNA targeting sequences corresponded to the coding regions 182-202 (siRNA Oligo1: 5-AAAGAAGATCCGGAAAAACTC-3) and 1699-1719 (siRNA Oligo2: 5-AAAGTGATGGCCAACGAGGAG-3) of MARKK. Both siRNA Oligo1 and 2 can silence the endogenous MARKK in PC12 cells efficiently, while Oligo1 works better than Oligo2. In this work we used the mixture of Oligo1 and Oligo2 in the siRNA experiment against the endogenous MARKK in PC12 cells.

Oligofectamine (Invitrogen) was used to transfect the siRNA of MARKK into PC12 cells. Cells were seeded at 4×10^4 cells/coverslip in 24-well plates in 0.5 ml culture medium and

grown overnight. For each coverslip 3 µl siRNA duplexes stock (20 µM) was diluted into 50 µl MEM medium and 3 µl Oligofectamine was diluted into 12 µl MEM medium. Both solutions were incubated at room temperature for 10 minutes, mixed gently and incubated at room temperature for another 20 minutes. Cells were washed once with PBS and supplied with 0.5 ml DMEM medium containing 0.1% FCS. The transfection mixture was brought to a volume of 100 µl with MEM medium and added to the cells. 24 hours post-transfection, cells were washed once with PBS and supplied with differentiation medium. Neurite extension was quantified by analyzing three experiments with 200 cells each.

Immunoflorescence:

Cells were taken out of the incubator and directly fixed with 3.7% Formaldehyde (Sigma) at room temperature for 15 minutes. After washing three times with PBS, cells were permeabilized with pre-cooled 80% methanol at -20 °C for 5 minutes, then washed three times with PBS and blocked with 10% goat serum at 37 °C for 1 hour. After incubation with primary antibody at 37 °C for 1 hour, cells were washed three times with PBS and incubated with labeled secondary antibody at 37°C for 1 hour. After washing three times with PBS, coverslips were mounted onto SuperFrost glass (VWR) with Permafluor aqueous mounting medium (Beckman Coulter) and dried overnight at 4 °C. Confocal microscopy was done with a Zeiss LSM510 Meta System using a 63× objective.

Yeast Two-Hybrid Analysis (Clontech Matchmaker Gal4 Two-Hybrid System 3):

In order to test the interaction between MARKK and PAK5 proteins, the yeast strain AH109 was cotransformed with different pGBKT7-MARKK and pGADT7-PAK5 constructs encoding full length protein or truncated mutants and plated on selective plates lacking leucine and tryptophan to screen for the transformed clones. After 3 days of incubation at 30°C, three colonies from each plate were picked and plated on the most stringent selective plates lacking leucine, tryptophan, histidine and adenine. The growth of the yeast colonies was examined after 3 days incubation at 30°C.

3. Results

Tau protein is a hallmark of Alzheimer's disease in its abnormal phosphorylation and aggregation state. The kinase MARK (MAP/Microtubule Affinity Regulating Kinase) is able to phosphorylate the KXGS motifs of tau and equivalent MAPs. This phosphorylation causes detachment of tau from the microtubules, and destabilization of microtubules, which serve as the tracks of axonal transport (Drewes et al., 1997).

Recently an upstream kinase of MARK, termed MARKK (MARK Kinase), was purified from porcine brain (Timm et al., 2003). MARKK is highly homologous to TAO1 (Hutchison et al., 1998) and belongs to the Ste20 kinase family. In the first part of this study, the recombinant MARKK was used to characterise its ability to activate MARK and to analyse the effect on the microtubule dynamics *in vitro* and in cells. To address the function of the MARKK-MARK-Tau cascade in neuronal cells, PC12 cells were used and the endogenous MARKK and MARK were examined. These results make up the second part. The third part illustrates the inhibitory regulation of the MARKK-MARK-Tau cascade by PAK5, which was found as an interaction partner of MARKK and MARK in the yeast two hybrid tests.

3.1 Characterisation of MARKK activity through the activation of MARK

3.1.1 Effects of overexpressed MARKs on the microtubules in Chinese Hamster Ovary (CHO) wild type cells

The phosphorylation of MAPs by MARK at the KXGS motifs leads to their detachment from microtubules, and the increase in microtubule dynamics (Ebneth et al., 1998; Illenberger et al., 1998). In CHO cells, overexpression of MARK results in microtubule dynamics, breakdown and eventually cell death (Drewes et al., 1997). As an example Figure 7 shows the different expression levels of MARK4 (as indicated by different expression time) and its dramatic morphological effect on the cells. Prolonged expression of MARK4 leads to the increase in dynamic microtubules and final disruption of the microtubule array; the cells round up and die.

MARK4 colocalizes to the centrosome at low expression level and shows punctate distribution all over the cell.

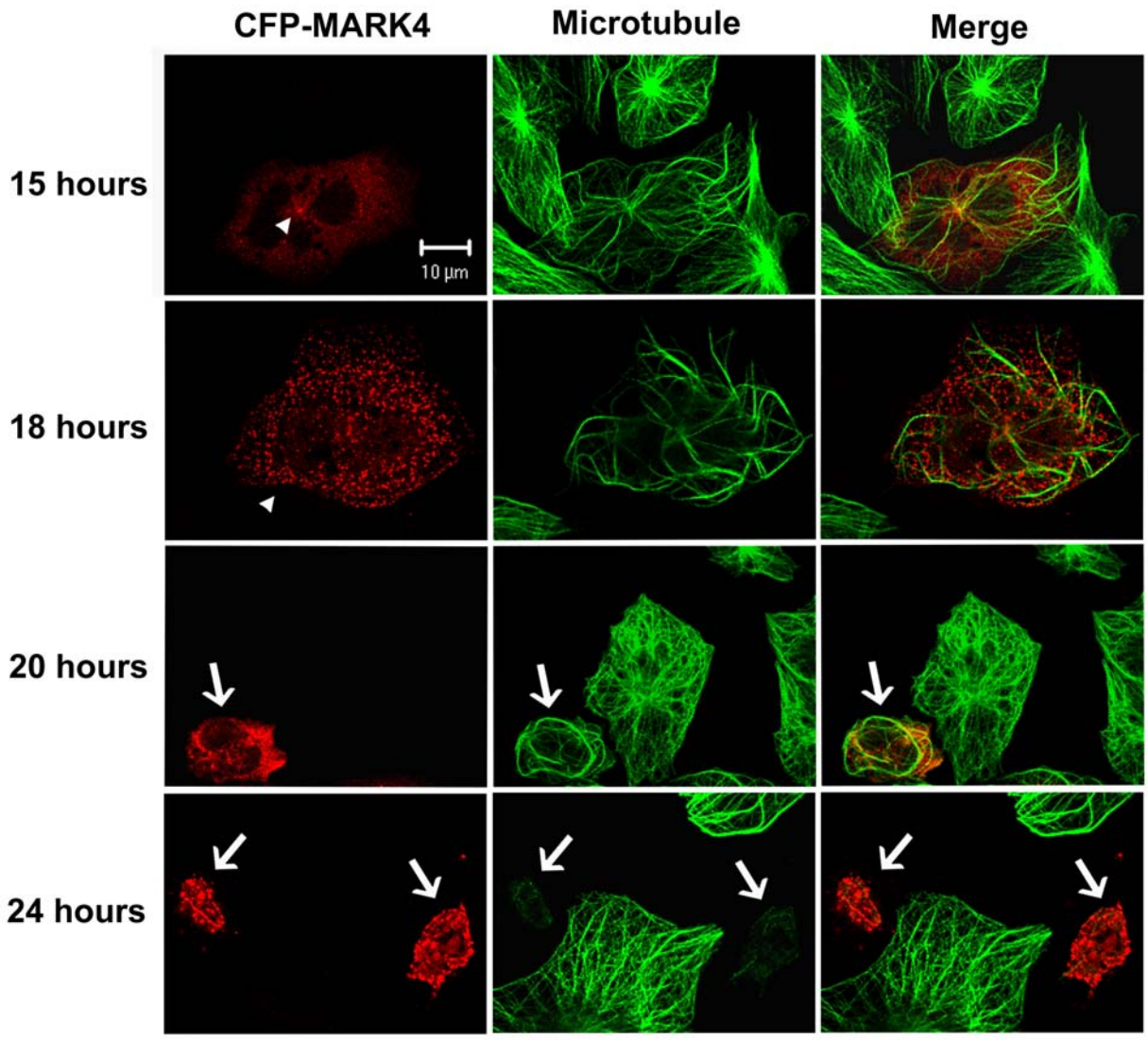

Figure 7: Time course of MARK4 expression and its effect on the microtubules in CHO cells.

CHO wild type cells were transfected with CFP-MARK4. Cells were fixed at different time post-transfection (15hr, 18hr, 20hr and 24hr), and stained with Anti-α-Tubulin Dm1A antibody to visualize the microtubules (Cy5). Cells lose microtubules and finally round up and die. At low expression level, MARK4 concentrates at the centrosome (arrow). At higher expression level MARK4 has a vesicular distribution (arrow head).

The three isoforms of the mammalian MARK family, MARK1, MARK2 and MARK3, have a similar effect on microtubules in CHO cells when they are expressed for a prolonged time (over 20 hours, Figure 8). These results indicate the common feature of MARKs to destroy the microtubule network by phosphorylation of the tau-related endogenous MAP (MAP4) in CHO cells.

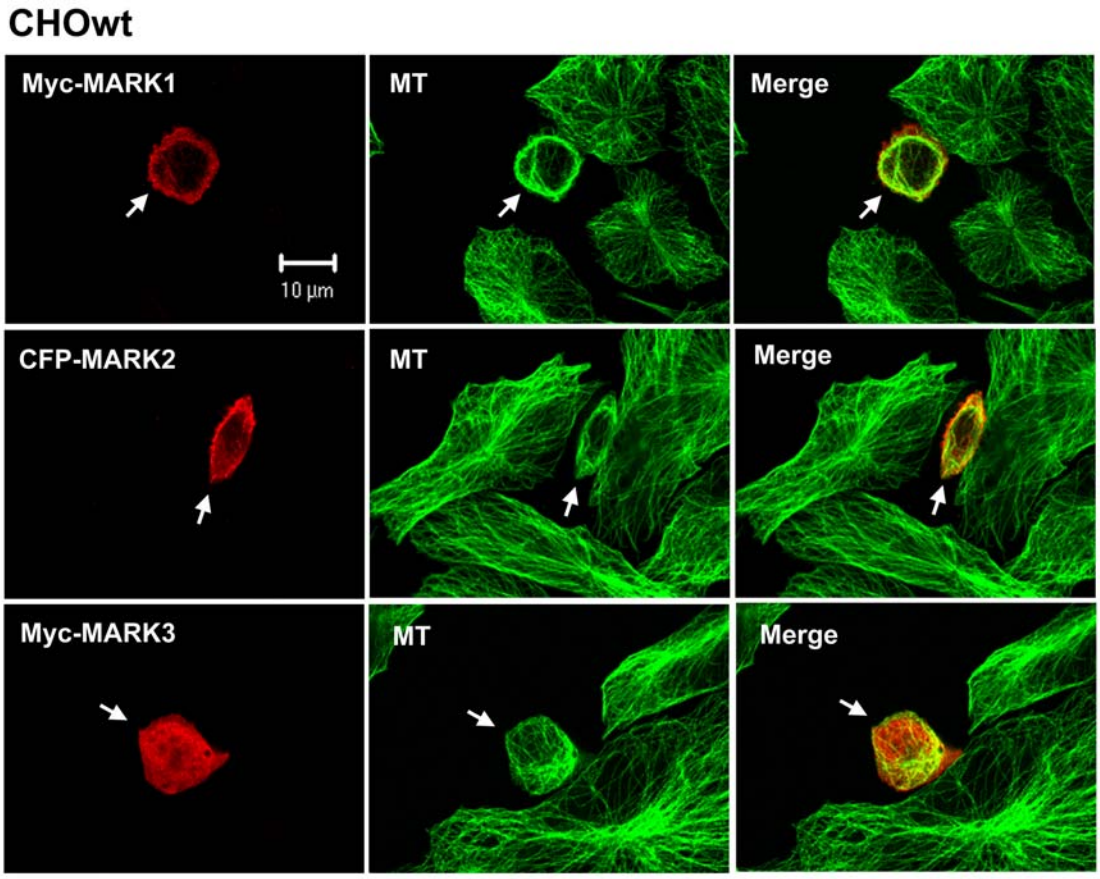

Figure 8: Overexpression of MARK1, MARK2 and MARK3 break down the microtubule network in CHO cells.

CHO cells were transfected with CFP-MARK2, Myc tagged MARK1, or MARK3 respectively. 20 hours post-transfection, cells were fixed and stained with Anti-α-Tubulin Dm1A antibody to visualize the microtubules (Cy5), and anti-Myc 9E10 to detect MARK1 or MARK3 (TRITC). Similar to MARK4, overexpression of MARK1, MARK2 or MARK3 leads to the destruction of the microtubule network in CHO wild type cells (arrow).

3.1.2 Characterization of the activity of recombinant MARKK by in vitro kinase assay

Recombinant MARKK and MARK are used for the kinase assay. The tau peptide TR1 ($_{255}$NVKSKIGSTENLK$_{267}$) is used as a substrate. This peptide contains S262, the major target of native MARK and the major site controlling tau-microtubule interactions. The experiment was performed with the MARK2 isoform, as there was no major difference between the MARKs with regard to tau. As shown in Figure 9, recombinant MARK2 alone phosphorylates the TR1 peptide at a constant low rate, because it is not phosphorylated thus is not activated in E.coli. With recombinant MARKK, the activity of MARK2 is increased ~16-fold after 2 hours. MARKK itself does not phosphorylate TR1 peptide. These *in vitro* experimental results clearly show that MARKK can phosphorylate and activate MARK, which further phosphorylates MAPs at the KXGS motifs.

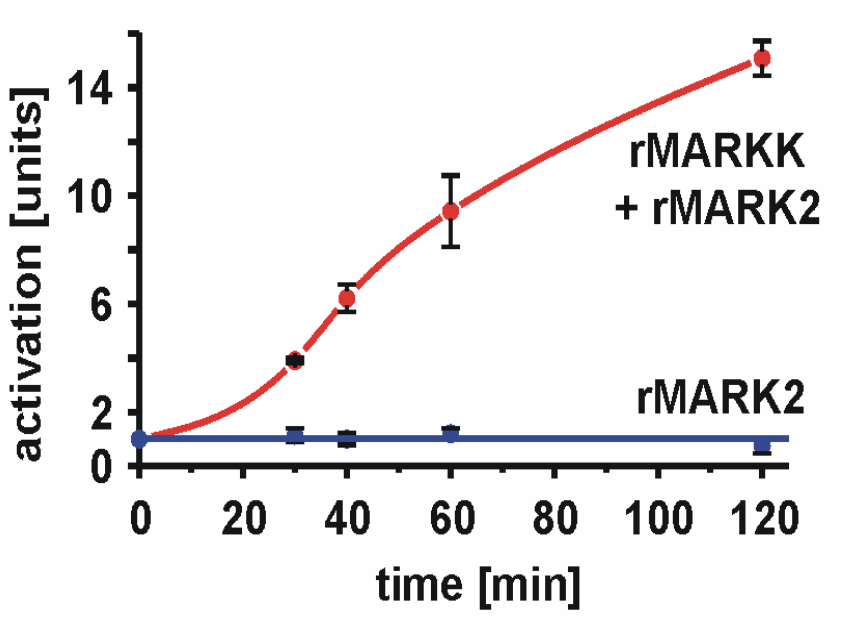

Figure 9: Activation of the recombinant MARK2 by MARKK.

MARK2 activity was measured by the phosphorylation of the TR1 peptide in the kinase assay. Bottom curve (squares), recombinant MARK2 alone phosphorylates the TR1 peptide at a constant low rate, wheras

MARKK does not phosphorylate this peptide (data not shown). MARKK activates MARK2 ~16-fold after 2 hours incubation (top curve, circles).

3.1.3 Transfection of MARKK into CHO cells leads to the loss of microtubules

To study the MARKK-MARK cascade in cells, CHO cells were transfected with MARKK. CHO cells contain endogenous MAP4 (phosphorylatable at KXGS motifs in the repeat domain). While normal cells have an extended shape with a elaborate microtubule network, MARKK transfected cells show the phenotype of microtubule breakdown, shrinkage, and eventually cell death (Figure 10:1-3). As a control, transfection of inactive mutant of MARKK (MARKK K57A, ATP binding defect) does not perturb microtubules (Figure 10:7-9), demonstrating that the kinase activity is required for the effect. Microtubule breakdown is more drastic when MARKK and MARK2 are co-transfected into CHO cells (Figure 10:4-6).

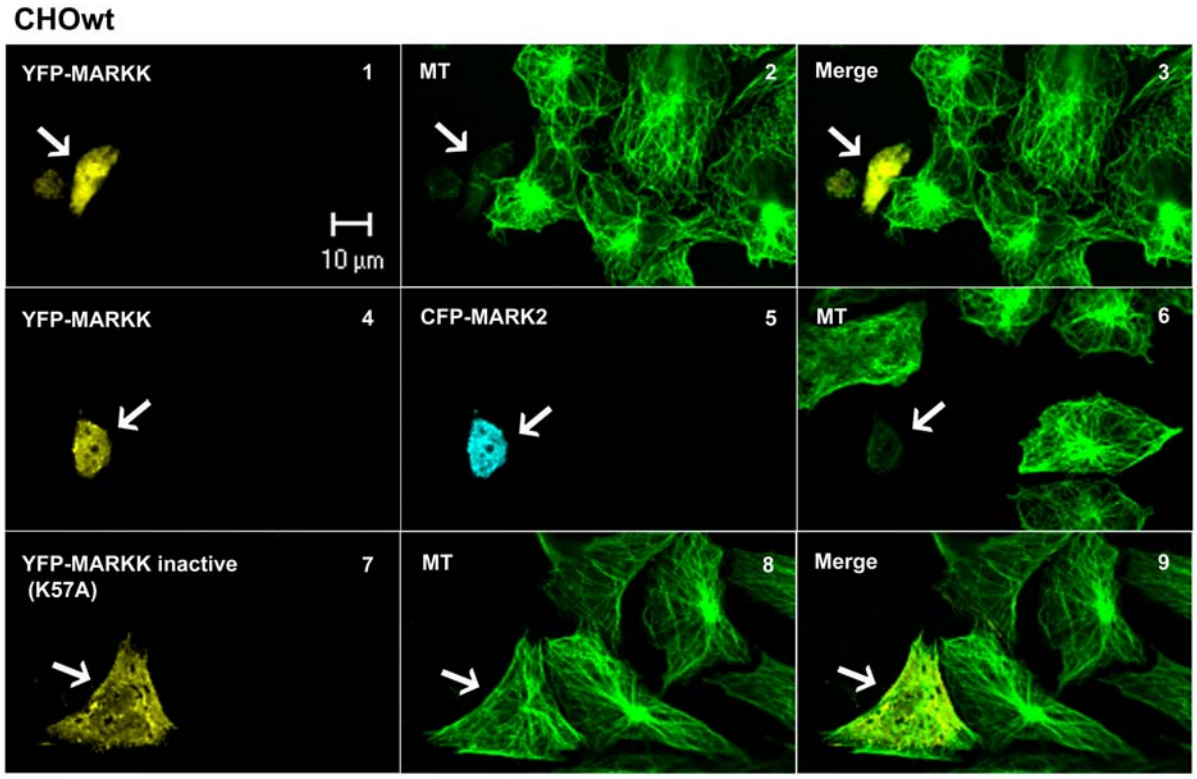

Figure 10: MARKK destroyes microtubules in CHO cells.

CHO cells were transfected with YFP-MARKK (1), YFP-MARKK inactive mutant (K57A, 7) or co-transfected with YFP-MARKK and CFP-MARK2 (4 and 5). After 24 hours, cells were fixed and stained for microtubules

with antibody YL1/2 and Cy5-secondary antibody (2, 6 and 8). Note the cell transfected with MARKK (1) loses its microtubule network (2), rounds up, and appears smaller (3). Similar results were observed in MARKK and MARK2 co-transfected cells (4, 5 and 6). CHO cells transfected with inactive YFP-MARKK K57A (7) retain their microtubule network (8, 9). Arrows indicate the transfected cells.

3.1.4 Stabilized microtubules can prevent the catastrophic effect of MARKK in transfected CHO cells

If it is true that MARKK activates MARK, and activated MARK phosphorylates MAPs and causes their detachment from microtubules and eventually breakdown of microtubules in cells, stabilizing the microtubules should prevent the catastrophic effect of MARKK.

We used two ways of stabilizing microtubules. The first way was using Taxol. Taxol is a drug that stabilizes microtubules *in vitro* and in cells. Taxol binds to β tubulin on the microtubule's inner surface. This binding counteracts the effects of GTP hydrolysis in microtubule dynamics (Amos et al., 1999). Overnight treatment of CHO cells with 10 μM Taxol prevents microtubule destruction and cell death in MARKK transfected CHO cells. These cells have normal size and an intact microtubule network even in the presence of well-expressed MARKK (Figure 11:1 and 2).

Another way of stabilizing microtubules is using microtubule-associated proteins like tau. A CHO cell line, stably transfected with human tau40 (htau40, the longest human tau isoform in the brain), was used to study the MARKK-MARK-MAP signaling cascade and its effect on microtubules. As a MAP, htau40 can stabilize the microtubules; therefore transfected MARKK cannot destroy the microtubules (Figure 11:3 and 4). Apparently, the endogenous MARK activity is not sufficient to counteract the increased stability of microtubules by excess tau.

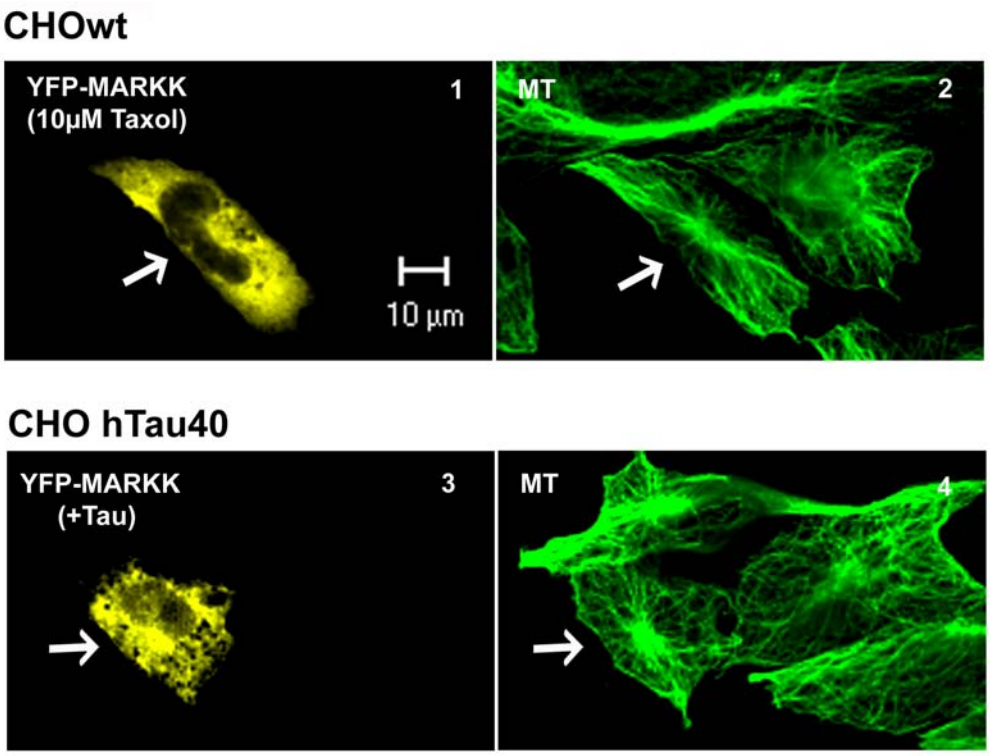

Figure 11: Stabilizing microtubules can counteract the catastrophic effect of MARKK.

Top: CHO cells were transfected with YFP-MARKK (1) and incubated with 10µM Taxol overnight. 24 hours post-transfection, cells were fixed and stained for microtubules with antibody YL1/2 and Cy5-secondary antibody (2). Note that taxol prevents the destabilization of microtubules by MARKK (1and 2).

Bottom: CHO cells stably transfected with human tau40 were transiently transfected with YFP-MARKK for 24 hours (3). Cells were stained with YL12 (Cy5) (4) to visualize the microtubule network. Note that elevated tau stabilizes the microtubules (3 and 4).

Arrows indicate the transfected cells.

3.1.5 Active MARK2 T208E is able to break down the microtubule network in CHO htau40 cells

Transient transfection of MARK2 into CHO htau40 cells causes a higher activity of MARK as seen by the staining of 12E8 antibody (Figure 12:2), but this still is not enough to work against the stabilization by tau, and the cell nearly retains its microtubule network. However, when the constitutively active mutant of MARK2 (MARK2 T208E) is introduced into CHO htau40

cells, the presence of tau cannot protect the microtubule network anymore. Tau is phosphorylated at S262 (Figure 12:5) and as a consequence the microtubule network breaks down (Figure 12:6).

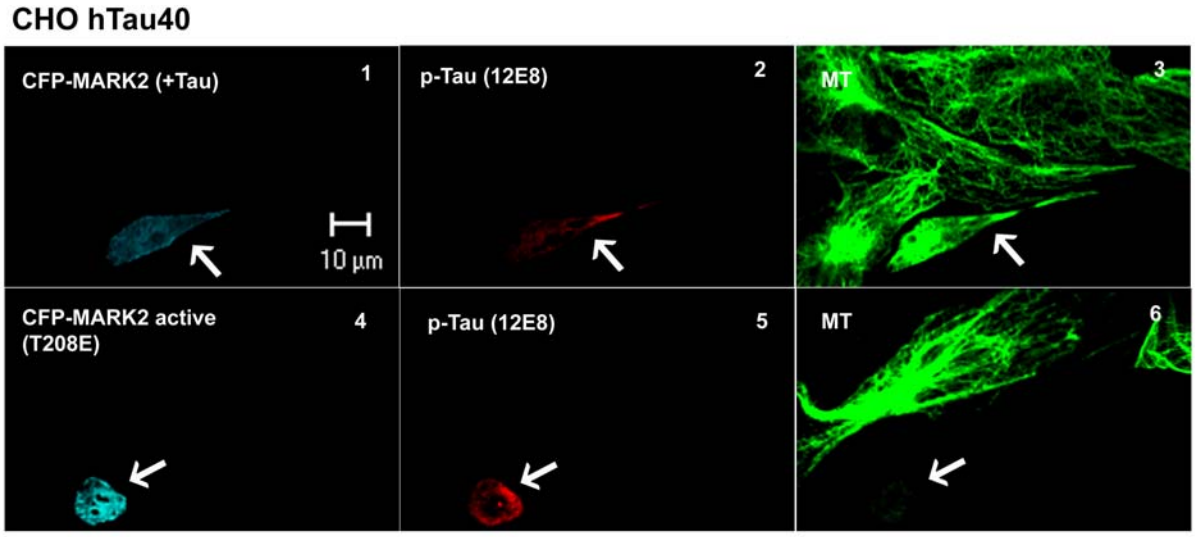

Figure 12: Active MARK2 T208E rather than MARK2 wt is able to destroy the microtubules in CHOhtau40 cells.

CHO cells were stably transfected with human tau40 and transiently transfected with CFP-MARK2 wild type (1) or active CFP-MARK2 T208E (4). 24 hours post-transfection, cells were fixed and stained for phospho-tau (KXGS motifs) by antibody 12E8 (TRITC) (2 and 5), microtubules with antibody YL1/2 (Cy5) (3 and 6). Note that elevated tau stabilizes microtubules against the effect of MARK2 (1, 2 and 3). However, constitutively active MARK2 T208E (4) phosphorylates tau at KXGS motifs (5) and destroys microtubules (6). Arrows indicate the transfected cells.

3.1.6 Co-transfection of MARKK and MARK2 leads to the phosphorylation of tau and the destruction of the microtubule network stabilized by htau40 in CHO cells

MARKK phosphorylates MARK2 at a conserved threonine residue T208 within the activation loop and activates it in this way (the corresponding residue is T215 in MARK1). Since MARKK can phosphorylate and activate MARK2 *in vitro* (see 3.1.2, Figure 9), and neither MARKK nor MARK2 alone could destroy microtubules in CHO cells stably transfected with

htau40 (3.1.4), we were wondering whether co-expression of MARKK and MARK2 in CHO htau40 cells could eliminate the stability of microtubules conferred by tau or not. When MARKK was co-transfected with MARK2 into CHO htau40 cells, it activated MARK2, as seen by the strong staining of an antibody diagnostic for active MARK (Figure 13:7). This demonstrates that the microtubule-stabilizing effect of tau can be overcome by the phosphorylation of tau at the KXGS motifs (strong staining of 12E8 antibody, Figure 13:3), which leads to the collapse of microtubules and the rounding up of cells (Figure 13:1-8).

To verify that MARK plays an immediate role in the effect seen above, MARK2 inactive mutant (T208A/S212A) was transfected together with MARKK into CHO htau40 cells. Though MARKK is well expressed (Figure 13:9 and 13), however, in the presence of MARK2 inactive mutant, it cannot exert its catastrophic effect on microtubules, and there is no detectable activated MARK (Figure 13:15) and S262 phosphorylated tau (Figure 13:11).

These results elucidate the signaling pathway of MARKK-MARK-MAP in the cells. MARKK can phosphorylate and activate MARK both *in vitro* and in cells; activated MARK can phosphorylate the MAP in this case human tau40 at KXGS motifs. This phosphorylation causes the detachment of tau from microtubules, which increases microtubule dynamics, and leads to breakdown of the microtubule network and eventually cell death.

3.1.7 MARK1, MARK3 and MARK4 have a similar effect as MARK2 when co-transfected with MARKK into CHO htau40 cells

Analogous experiments to 3.1.5 were done in CHO htau40 cells with MARKK and MARK1, MARK3, MARK4 respectively. Similar to MARK2, expression of MARK1, MARK3 or MARK4 alone is not able to break down the microtubules in CHO cells that are stabilized by htau40 (Figure 14:1-3, 7-9, 13-15). Together with MARKK, all three MARK isoforms MARK1, 3, 4 show the destruction of microtubules and cause the cells to round up (Figure 14:4-6, 10-12, 16-18). This indicates a similar role of all MARK isoforms in the activation cascade mentioned above.

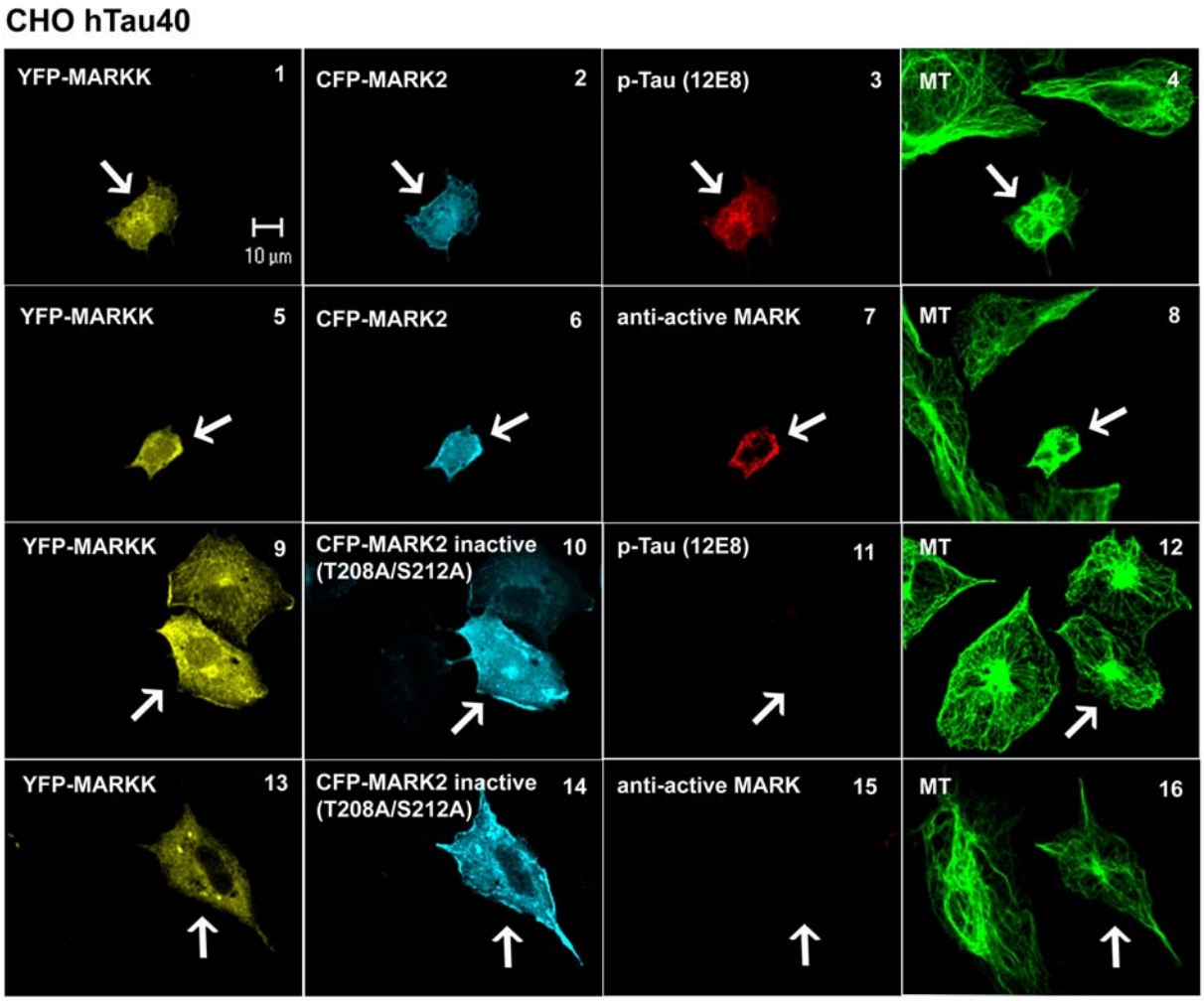

Figure 13: MARKK destroys the microtubules via the activation of MARK2 and the phosphorylation of tau in CHO htau40 cells.

CHO cells were stably transfected with human tau40 and transiently co-transfected with YFP-MARKK (1, 5, 9 and 13) and CFP-MARK2 (2, 6) or its inactive mutant CFP-MARK2 T208A/S212A (10, 14). 24 hours post-transfection, cells were fixed and stained for phospho-tau (KXGS motifs) by antibody 12E8 (TRITC) (3, 11), the active MARK2 by antibody SA6941 against the phosphorylation of the activation loop, T208 (TRITC) (7, 15) and microtubules by YL1/2 (Cy5) (4, 8, 12 and 16). The combined effect of MARKK and MARK2 (1 and 2, 5 and 6) destabilizes microtubules (4, 8) in spite of excess tau; MARK2 is activated as shown by the anti-active MARK antibody (7) and tau is phosphorylated at KXGS motifs as seen by the 12E8 antibody (3).

Microtubules remained intact (12, 16) when cells are transiently co-transfected with YFP-MARKK and inactive mutant of MARK2 (9 and 10, 13 and 14), because MARK mutant cannot be phosphorylated and activated (15), and there is no phospho-S262-staining of tau (11).

Arrows indicate the transfected cells.

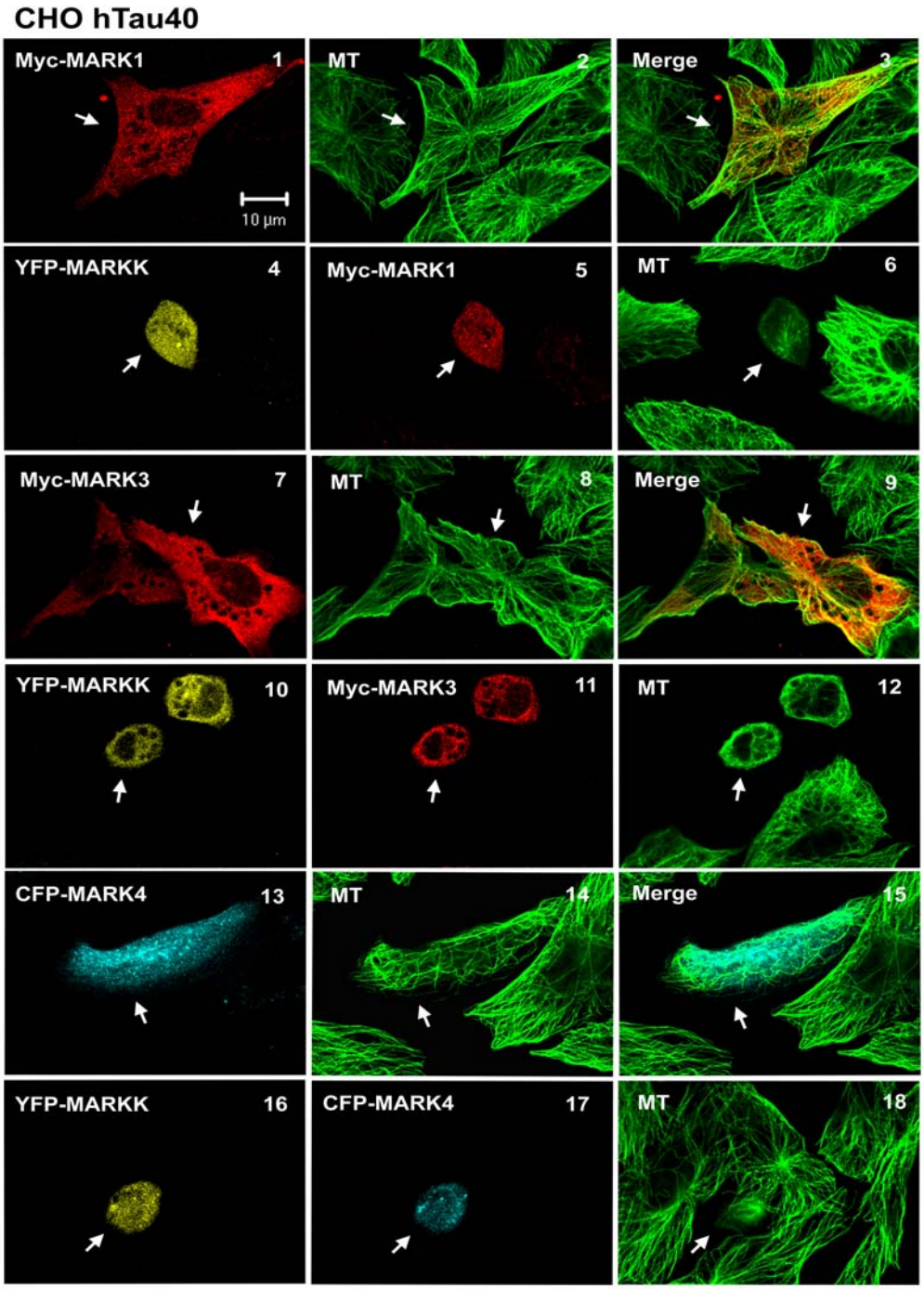

Figure 14: MARK isoforms destroy the microtubules when co-transfected with MARKK in CHO htau40 cells.

Myc tagged MARK1, MARK3 or CFP-MARK4 was transfected alone (1, 7, and 13) or jointly with YFP-MARKK (4 and 5, 10 and 11, 16 and 17) into CHO cells stably transfected with human tau40. 20 hours post-transfection, cells were fixed and stained for MARK1 or MARK3 by antibody SA4632 (against N terminal aa1-377 of MARK1, TRITC), and microtubules by Anti-α-Tubulin Dm1A (Cy5) (2, 6, 8, 12, 14, and 18), merged images (3, 9, and 15).

Top panels (1-6) show that MARK1 alone (1) is not sufficient to destabilize the microtubules (2) in CHO htau40 cells. When MARK1 is co-transfected with MARKK, the microtubule network is broken down (6) and the cell becomes smaller and round.

Middle panels (7-12) show similar results of analogous experiments with MARK3.

Bottom panels (13-18) show similar results of analogous experiments with MARK4.

Arrows indicate the transfected cells.

3.2 The role of endogenous MARKK and MARK in differentiated neuronal PC12 cells

To further study MARKK-MARK-Tau cascade at the physiological level, endogenous MARKK and MARK are examined in neuronal PC12 cells. PC12 is a cell line derived from pheochromocytoma cells of the rat adrenal medulla (Greene et al., 1976), which originates from a primitive stem cell of neural crest of embryonic ectoderm. PC12 cells stop dividing and develop neuronal phenotype when treated with nerve growth factor (NGF), making the cell line a useful model system for neuronal differentiation studies.

3.2.1 The expression pattern of endogenous MARK in differentiated PC12 cells

Figure 15 shows the endogenous MARK staining with SA4635 polyclonal antibody from rabbit (raised against N-terminal amino acid 1-377 of MARK1) at different time points after NGF treatment. There is a significant up-regulation of endogenous MARK upon differentiation, indicated by the increasing intensity of the antibody staining. Endogenous MARK is mainly present in the cytoplasm and infrequently found in the short sprouts in untreated cells (Figure 15A, 0hr, arrows). After NGF treatment there is an increase in the number and length of processes and MARK is found enhanced in the periphery of the cell and at the termini of the processes (Figure 15A, arrows), suggesting a role in initiating neurite outgrowth. There is no detectable nuclear localisation of MARK in untreated or NGF treated PC12 cells. Western blotting confirms the up-regulation of endogenous MARK after NGF treatment in PC12 cells. The endogenous MARK restores to basal level after 24 hours treatment with NGF (Figure 15B).

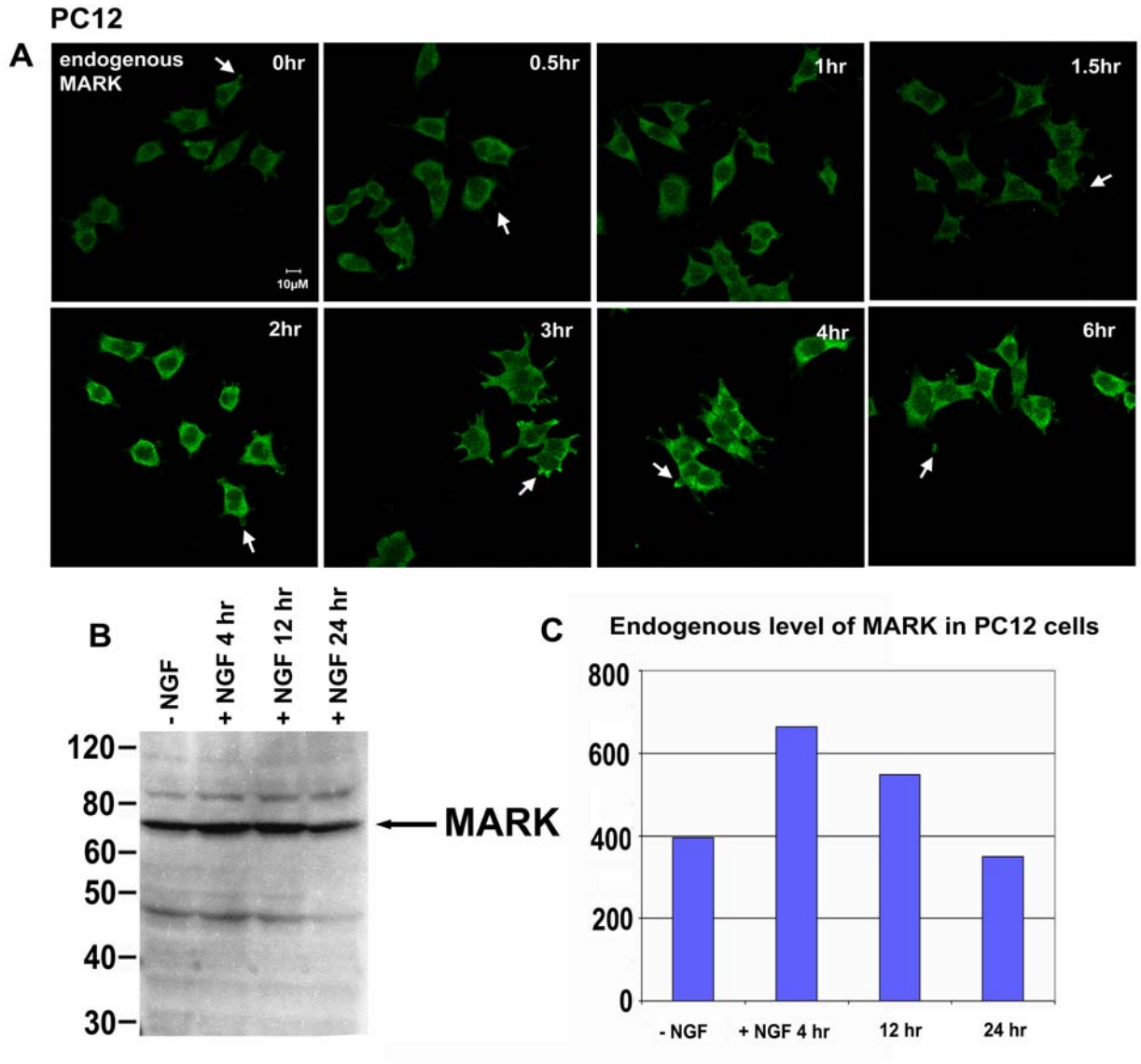

Figure 15: Endogenous MARK in PC12 cells after the induction of differentiation with NGF.

PC12 cells were differentiated with 100ng/ml NGF for different time points (as indicated in the figure).

A: Cells were stained with the antibody SA4632 (against N-terminal aa1-377 of MARK1) and the secondary antibody TRITC to visualize the endogenous MARK. Note that there is an up-regulation of endogenous MARK shortly after the induction of differentiation. Endogenous MARK is present in the cytosol and enriched in the cell protrusions (arrows). There is no nuclear localization of endogenous MARK.

B: Western blotting with antibody SA4632 shows the change of the endogenous level of MARK in PC12 cells at different time points after differentiation with NGF. There is an up-regulation of endogenous MARK in PC12 cells (4 hour and 12 hour). In 24 hour the endogenous MARK restores to basal level.

C: Diagram of the endogenous level of MARK in PC12 cells upon NGF treatment (see B). Quantitative data is measured by the computer programm AIDA.

3.2.2 Co-localization between endogenous MARKK and MARK in differentiated PC12 cells

PC12 cells were differentiated with 100 ng/ml NGF for 48-72 hours. Since neuronal cells have higher levels of endogenous MARKK and MARK, immunofluorescence was performed to detect MARKK (anti-TAO1 antibody, BD Biosciences) and MARK (SA2118, antibody against N-terminal peptide of MARK). As shown in Figure 16, under the experimental conditions, PC12 cells can be well differentiated, exhibiting neuronal phenotype with long neurites and growth cones. Endogenous MARKK co-localizes partly to MARK (Figure 16:1-3), to phospho-KXGS-tau (12E8 antibody, Figure 16:4-6) and the actin network (Oregon green phalloidin, Figure 16:7-9) near the plasma membrane and at the growth cones (Figure 16, arrows) where a highly dynamic microtubule and actin network is required. These results suggest a role of MARKK-MARK-Tau cascade in regulating the dynamic behavior of microtubules and actin filaments, which is important for neuronal cells to explore the environment, establish polarity and connections with other cells.

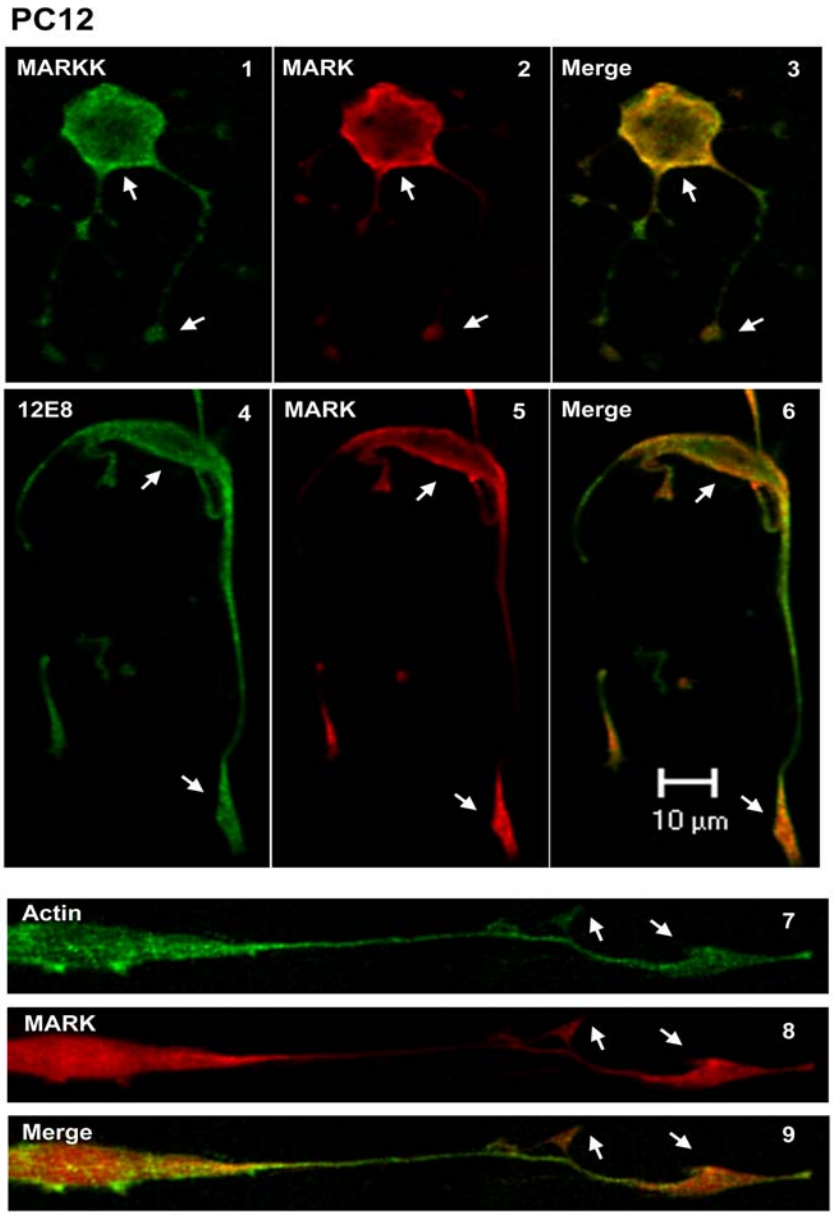

Figure 16: Endogenous MARKK co-localizes with the endogenous MARK as well as phospho-tau and actin in differentiated PC12 cells.

PC12 cells were differentiated with 100ng/ml NGF for 48-72 hours and immunostained for endogenous MARKK (1, antibody TAO1 and Cy5), MARK (2, 5, and 8, antibody SA2118 and TRITC), phospho-KXGS-tau (4, antibody 12E8 and TRITC) and actin (7, Oregon-green phalloidin), merged images (3, 6, and 9). Endogenous MARKK co-localizes with the endogenous MARK, notably beneath the plasma membrane and at the growth cones (1-3, arrows). Endogenous phospho-KXGS-tau co-localizes with endogenous MARK, especially near the plasma membrane and at the growth cones (4-6, arrows). Endogenous MARK co-localizes with actin at the growth cones (7-9, arrows).

3.2.3 RNA interference (RNAi) of endogenous MARKK in PC12 cells leads to the inhibition of differentiation

RNA interference is recently emerged as a specific and efficient method to silence gene expression in mammalian cells by transfection of short interfering RNAs (siRNAs). Some small double strands of RNA (sdRNA) can block the expression of a target gene by degradation of related mRNA in a highly efficient and specific way (Elbashir et al., 2001; Yu et al., 2002). This mechanism of post-transcriptional gene silencing is an important pathway to prevent cells from exogenous infection (Cogoni et al., 2000; Guru, 2000; Hammond et al., 2001). RNAi is an efficient and simple genetic tool to execute functional studies of genes by gene silencing.

As demonstrated in 3.2.2, endogenous MARKK-MARK-Tau signaling is important for PC12 cells to differentiate in response to NGF treatment. The question arises what would happen if endogenous MARKK was knocked out. Therefore, RNAi experiment was carried out to suppress the endogenous MARKK in PC12 cells.

The RNAi targeting sequences of MARKK correspond to the coding regions 182-202 (Oligo1) and 1699-1719 (Oligo2). Both siRNA oligonucleotides can efficiently silence the endogenous MARKK in PC12 cells, while Oligo1 works better than Oligo2. In this work we used the mixture of Oligo1 and Oligo2 in the siRNA experiment against the endogenous MARKK in PC12 cells. Mock-transfected PC12 cells can be differentiated with high efficiency (Figure 17A:1 and 2) without disturbing the expression of endogenous MARKK. Similar to Figure 16, endogenous MARKK is found enriched at the growth cone (Figure 17A:1 and 2, arrow). However, MARKK-siRNA (small-interfering RNA) successfully silences endogenous MARKK in transfected PC12 cells, and as a consequence there is no differentiation of these cells upon NGF treatment (Figure 17A:3 and 4). Western blotting of cell extracts confirms the suppression of MARKK in siRNA transfected cells (Figure 17B). Quantification shows that with MARKK around 80% of the cells develop neurites after NGF treatment, while the number drops to 20% with MARKK being knocked out in the cells (siRNA) (Figure 17C). These

experiments demonstrate the important role of the MARKK-MARK signalling pathway to induce the dynamics of microtubules necessary for neurite outgrowth and neuronal polarity.

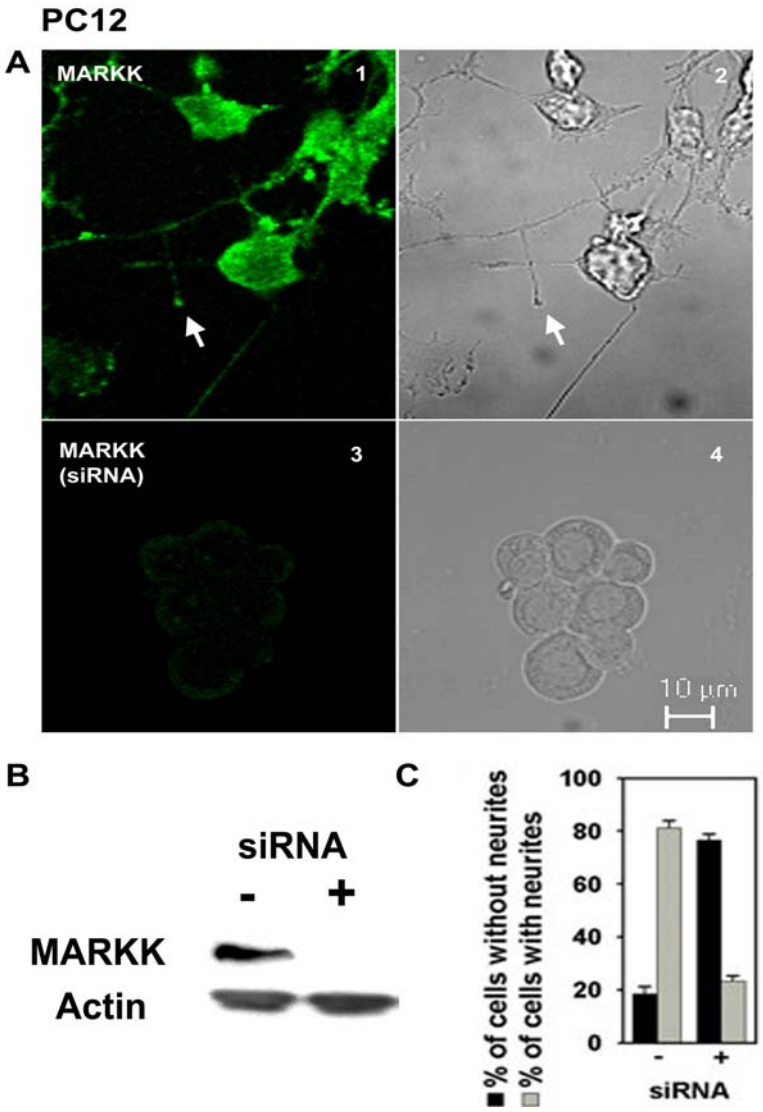

Figure 17: Suppression of the endogenous MARKK by RNAi represses differentiation of PC12 cells.

A: PC12 cells were transfected with mock (A1, A2) or siRNA against MARKK (A3, A4) and differentiated with 100ng/ml NGF for 48 hours. Cells were stained for endogenous MARKK (A1, A3, antibody TAO1and Cy5), phase images (A2, A4). Mock transfected cells can be well differentiated (A2) and there is strong expression of endogenous MARKK (A1). MARKK also localizes to the growth cone (A1, A2, arrow). In siRNA transfected cells, MARKK is largely suppressed (A3) and the cells could not be differentiated (A4).

B: Western blotting of PC12 cells after NGF-differentiation, mock-transfected (left) or with siRNA against MARKK (right). Note that expression of MARKK is suppressed.

C: Quantification of the effects of siRNA against MARKK (see A1-A4). The fraction of cells with neurites decreases from ~80% to 20% upon treatment with siRNA against MARKK.

3.3 Characterization of the inhibitory effect of PAK5 on MARKK-MARK-MAP cascade

PAK5 belongs to the Group II of the p21-Activated Ser/Thr Kinase family (Jaffer and Chernoff, 2002). Members of this kinase family can bind to and, in some cases, are stimulated by active forms of the small GTPases, such as Cdc42 and Rac (Zhao and Manser, 2005). While the diverse roles of Group I PAKs (PAK1, PAK2, PAK3) in stress response, gene transcription, cytoskeleton regulation, transformation and apoptosis have been studied in detail, the function and the regulation of Group II PAKs (PAK4, PAK5, PAK6) remain unclear. PAK5, the most recently characterized member, is enriched in the brain and can induce filopodia, neurite outgrowth and dendritic spines (Pandey et al., 2002; Dan et al., 2002; Bryan et al., 2004). PAK5 was found as a MARK2-interaction partner when a human fetal brain cDNA library was screened using the yeast two-hybrid system to identify regulators of MARK and possible substrates of MARK (Matenia et al., 2005).

3.3.1 Subcellular distribution of PAK5 and their morphological effects in transfected CHO cells

Human PAK5 wt cDNA as well as constitutively active mutant (S573N/S602E, in the catalytic loop) or kinase-dead mutant (S602M/T606M) was fused to yellow or cyan fluorescence protein (YFP, CFP, BD Biosciences, Clontech) and transfected into CHO cells. The analysis of the cytoskeleton was performed by immunostaining of microtubule (anti-total α-tubulin clone DM1A, Sigma), actin (anti-β-actin clone AC-15, Sigma) and focal adhesions (anti-vinculin clone hVIN-1, Sigma).

As shown in Figure 18, wild type PAK5 has a dotted appearance in the cell. This suggests a localisation on vesicles, which were identified by Matenia and colleagues as early endosomes by staining with an antibody against the AP-1 adaptor protein (Matenia et al., 2005). Kinase-dead mutant PAK5 (S602M/T606M) has a similar vesicular distribution; in addition, it is more concentrated around the centrosome (Figure 20, arrows), suggesting microtubule minus end directed transport is involved (see also 3.3.2).

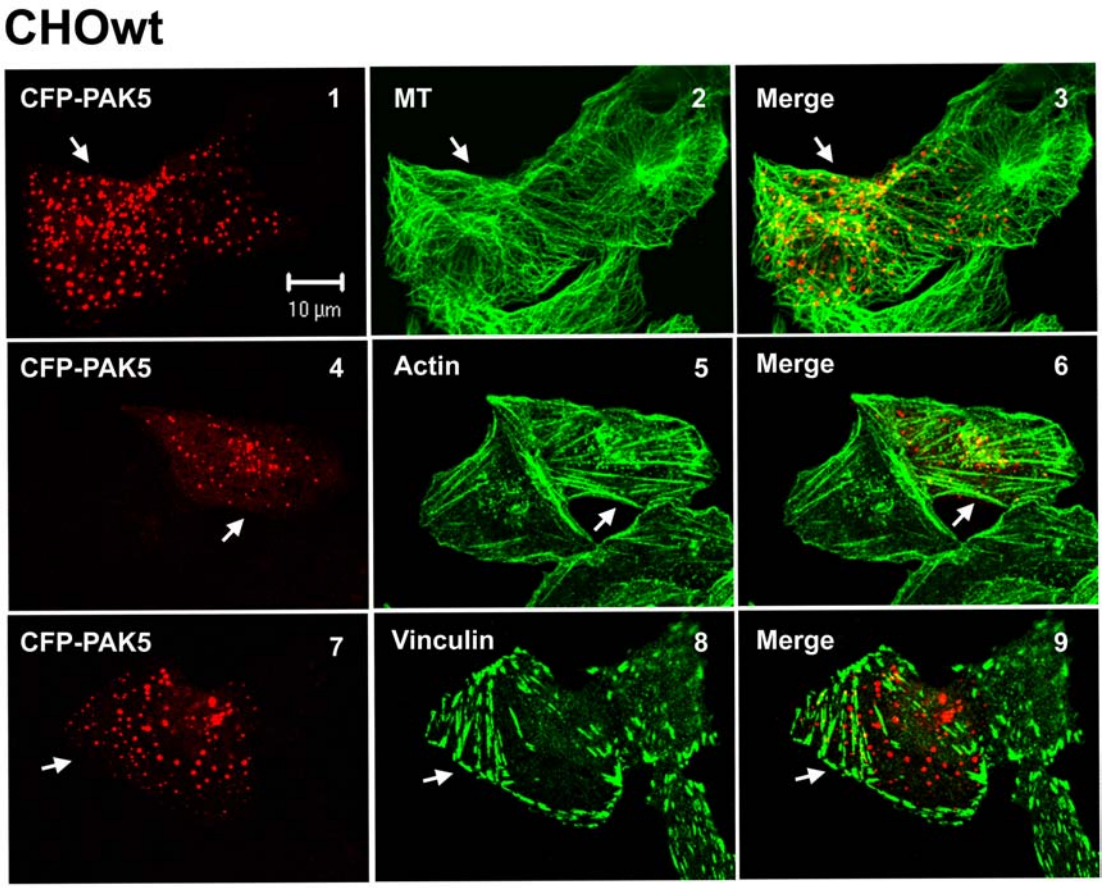

Figure 18: Subcellular localization of transfected wild type PAK5 and its effect on the cytoskeleton in CHO cells.

CHO cells transfected with wild type CFP-PAK5 were cultured for 18 hours, fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).
Wild type CFP-PAK5 is distributed in vesicles (1, 4 and 7). It has no significant effect on the microtubule network (2), actin network (5) and focal adhesions (7). Merged images (3, 6 and 9). Arrows indicate the transfected cells.

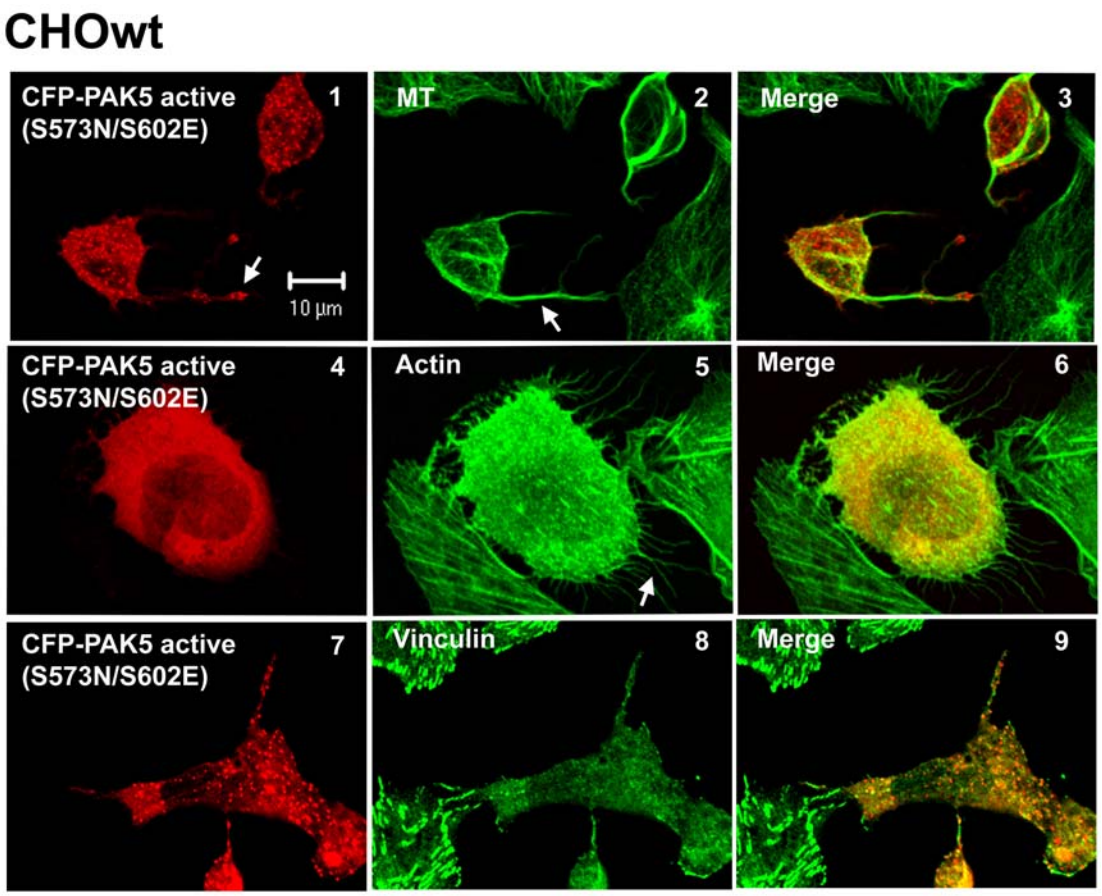

Figure 19: Subcellular localization of transfected active PAK5 and its effect on the cytoskeleton in CHO cells.

CHO cells transfected with active CFP-PAK5 S573N/S602E were cultured for 18 hours, fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).
Constitutively active CFP-PAK5 S573N/S602E shows vesicular and cytosolic distribution (1, 4 and 7). It stabilizes microtubules (bundles, 2, arrow), but dissolves actin stress fibers (5) and focal adhesion (8). Merged images (3, 6 and 9). Active PAK5 transfected cells form neurite-like and growth cone-like structures (1, arrow) or filopodia (5, arrow) indicating a highly dynamic actin cytoskeleton.

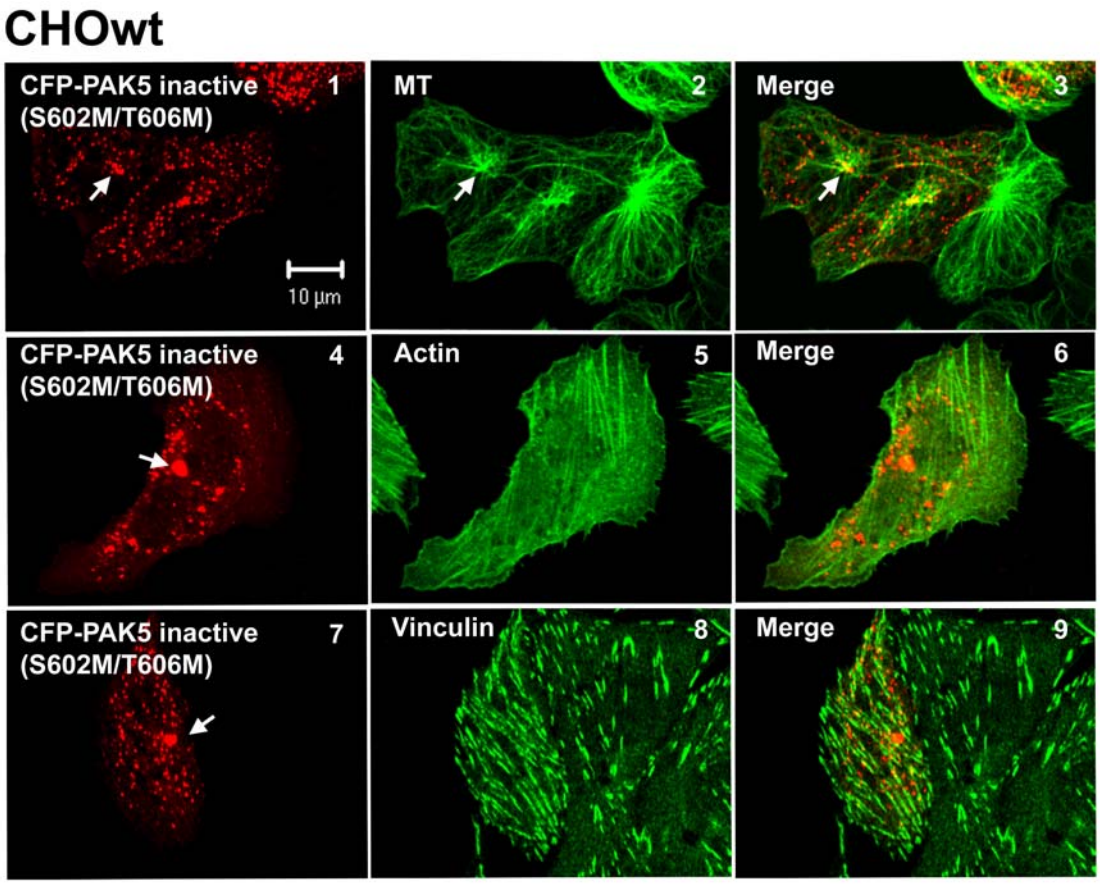

Figure 20: Subcellular localization of transfected inactive PAK5 and its effect on the cytoskeleton in CHO cells.

CHO cells transfected with inactive CFP-PAK5 S602M/T606M were cultured for 18 hours, fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5). Inactive CFP-PAK5 S602M/T606M has a similar vesiclular distribution to PAK5 wild type (1, 4 and 7), besides, it concentrates at MTOC (1-3, 4 and 7 arrows). It has no effect on the stability of microtubules, actin network and focal adhesion (2, 5 and 8, merged in 3, 6 and 9).

The constitutively active mutant of PAK5 (S573N/T602E) is also found in vesicles, besides a diffuse distribution in the cytoplasm (Figure 19:1, 4, and 7). Overexpression of PAK5 wt and PAK5 S602M/T606M cause no detectable changes in cell morphology. Immunofluorescence staining confirms the presence of a well-spread microtubule network with a pronounced MTOC (Microtubule Organizing Center) and normal actin staining with stress fibers (Figure 18 and 20). However, CHO cells show remarkable morphological changes when transfected with constitutively active PAK5 S573N/S602E (Figure 19). Actin staining shows that PAK5 S573N/S602E induces dramatic filopodia formation and dissolution of stress fibers, indicating

highly dynamic actin (Figure 19). In the extreme case, CHO cells form neuronal-like processes and growth cone-like structures (Figure 19, arrows). In these cells microtubules are bundled, thus stabilized. This allows the dissolution of the actin stress fibers and the generation of dynamic filopodia made of actin.

Focal adhesions are membrane-associated complexes that serve as anchoring sites for actin stress fiber ends and as cross-linkers between the extracellular matrix and actin cytoskeleton in response to the adhesion signaling (Yamada and Geiger, 1997). They consist of trans-membrane integrin receptors and intracellular protein complexes containing vinculin, talin, α-actinin, paxillin, tensin, zyxin and focal adhesion kinase (FAK) (Turner and Burridge, 1991). The assembly and disassembly of focal adhesions reflect the dynamic behavior of the actin cytoskeleton. Vinculin is a universal focal adhesion marker; Figure 18:8, Figure 19:8 and Figure 20:8 show CHO cells staining with vinculin after transfection of PAK5 wt, active mutant, or inactive mutant respectively. Wild type and inactive PAK5 S602M/T606M transfected cells display a typical pattern of vinculin staining correlated to the orientation of stress fibers. This indicates that mature focal adhesions exist between the cell surface and the substrate and that we are dealing with a relative stable actin cytoskeleton. Conversely, there is only diffuse background instead of focal adhesion staining in active PAK5 S573N/S602E transfected cells (Figure 19: 7-9). Theses cells show remarkable formation of filopodia and neurites-like cell processes. The absence of focal adhesion supports the observation that in these cells the actin network is highly dynamic to be able to rearrange the cell morphology as necessary.

3.3.2 The transport of PAK5 kinase dead mutant towards the centrosome in live cells

As shown in 3.3.1, PAK5 wt and mutants have a punctuate distribution in CHO cells, furthermore, PAK5 wt and inactive mutant also accumulate at the centrosome (especially in the case of PAK5 inactive mutant). To study this phenotype in detail, inactive YFP-PAK5 S602M/T606M plasmid was transfected into CHO cells. Live cell images were taken by confocal microscopy. Figure 21 shows a series of images continuously taken for ~5 minutes. At 0 time point there are vesicles of different size distributed in the whole cell, while after 280

seconds, there is remarkable clustering of vesicles at the centrosome (Figure 21, 0s and 280s, arrow head). Most vesicles are highly motile; some of them are motionless (Figure 21, arrow).

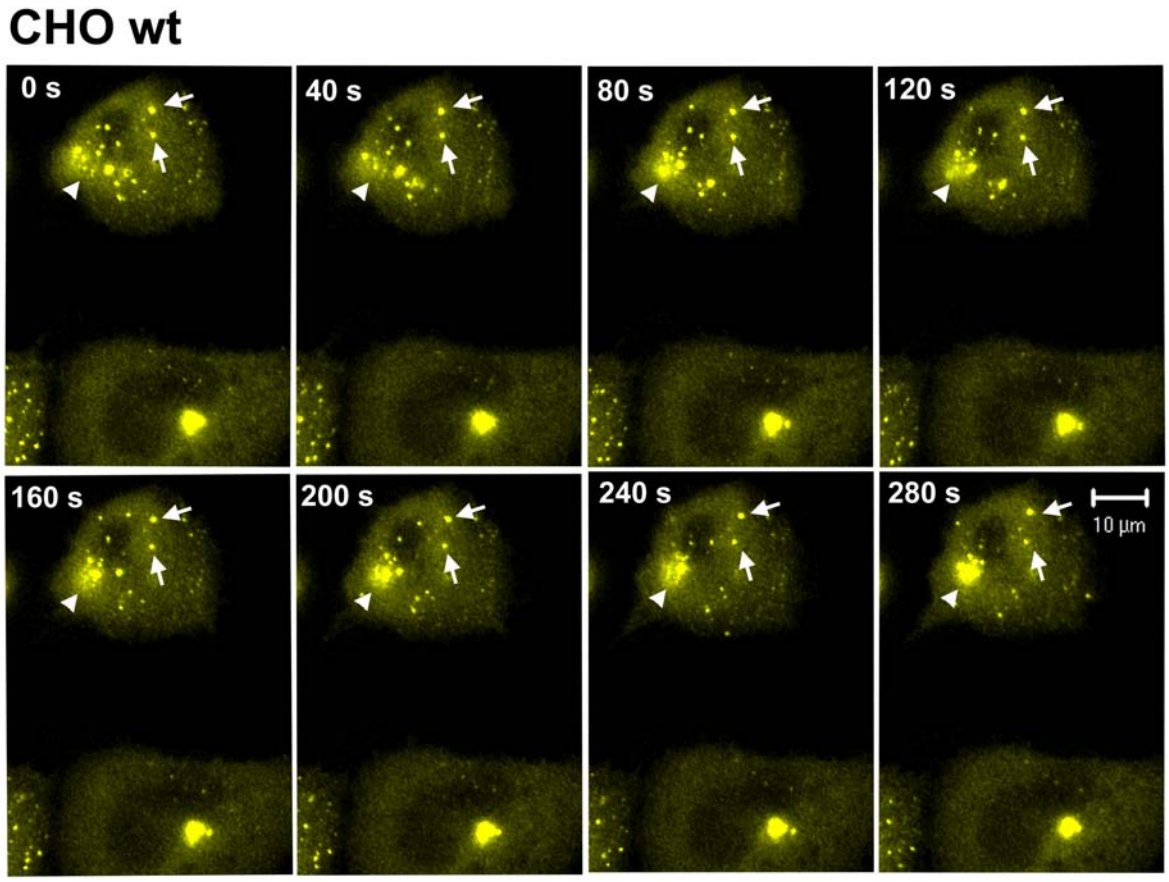

Figure 21: Vesicles with PAK5 inactive mutant are transported to the centrosome in live CHO cells (1).

CHO cells were transfected with inactive YFP-PAK5 S602M/T606M. 15 hours post-transfection live cells were imaged continuously. At 0 sec, there are vesicles of different size in the cytoplasm (top cell, arrow head); while at 280 sec, clear centrosome localization can be detected (arrow head). Note that most of the vesicles are highly motile; some of them are immobile during imaging (arrows).

In Figure 22 the movement of a single vesicle of YFP-PAK5 S602M/T606M is imaged in detail. The movement of the vesicle is variable. It initially moves to the centrosome (0-30 second), then stops and moves back to the original place (second 30-80), afterwards it moves again in the direction towards the centrosome and finally fuses with the area around the centrosome (80-150 second). These bidirectional movements imply the involvement of both microtubule minus end (dynein as the motor protein) and plus end transport (kinesin as the

motor protein). In this case, the minus end directed transport is dominant as the net outcome is the concentration of inactive PAK5 vesicles at the centrosome.

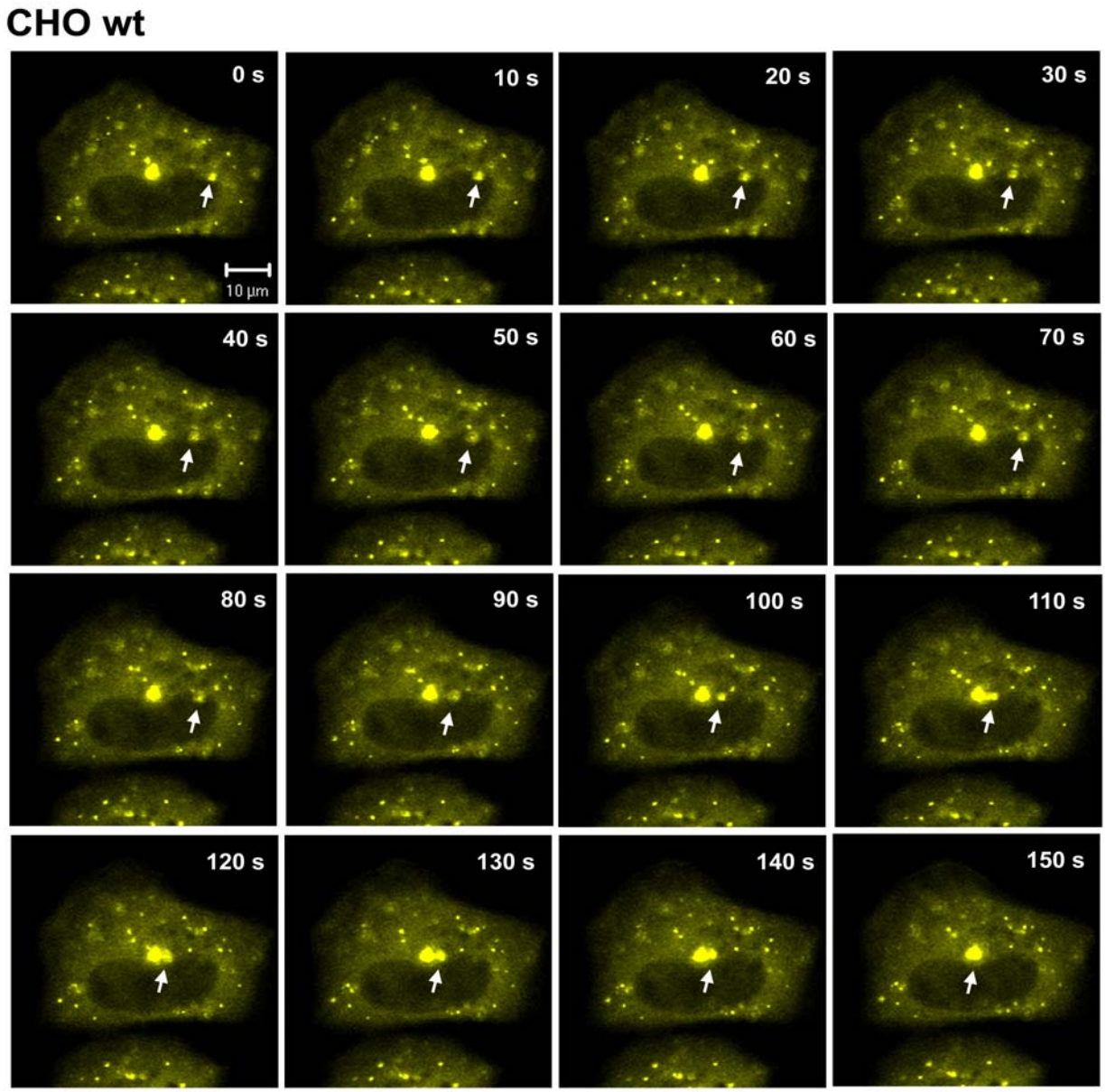

Figure 22: Vesicles with PAK5 inactive mutant are transported to the centrosome in live CHO cells (2).

CHO cells were transfected with inactive YFP-PAK5 S602M/T606M. 15 hours post-transfection live cells were imaged continuously. Vesicles are found through out the cell and most of them are moving to the centrosome. Arrows point at a single vesicle that exhibits variable movements during its progress to the centrosome (see detailed description in 3.3.2).

3.3.3 PAK5 co-localizes with MARK2 in CHO cells and inhibits its ability to destroy the microtubules

Recently, Matenia and colleagues identified PAK5 as a MARK2 interaction partner by yeast two hybrid screen of a human fetal brain cDNA library (Matenia et al., 2005). Detailed studies revealed that the catalytic domain of PAK5 and the catalytic domain of MARK2 interact with each other. To find out how this interaction influences the properties of the kinases, activity assays were performed by measuring the phosphorylation of the tau peptide TR1 in the presence of active MARK2. As shown in figure 23, wild type PAK5, active PAK5 and inactive PAK5 can inhibit the activity of active MARK2. Further studies reveal that MARK2 cannot inhibit the activity of PAK5 (Matenia et al., 2005).

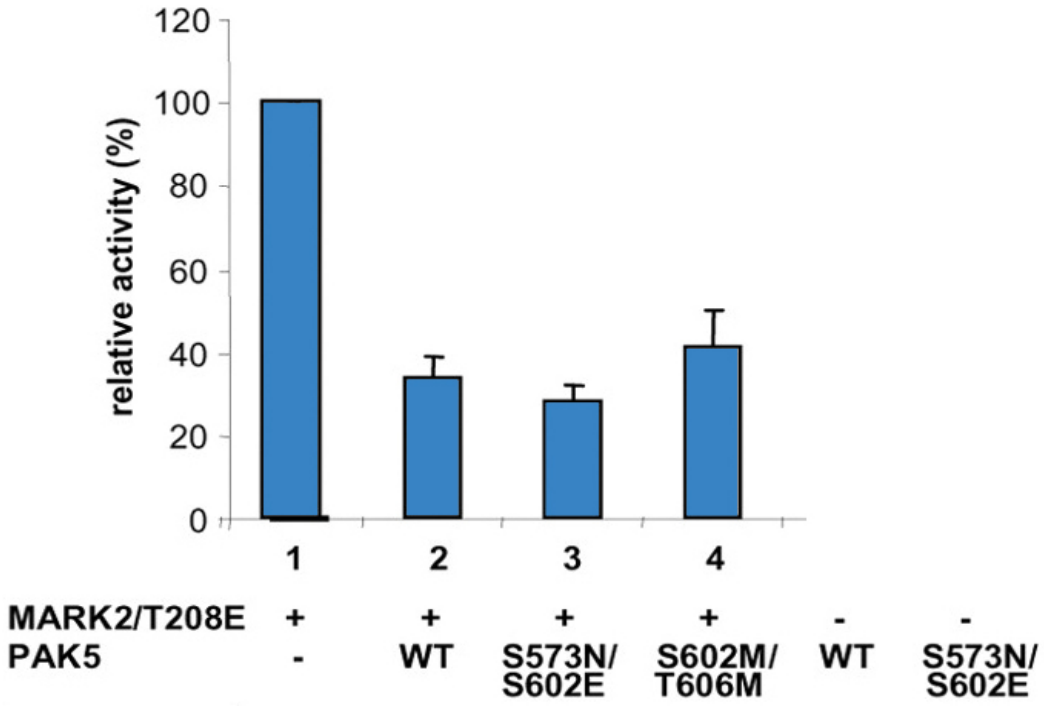

Figure 23: PAK5 inhibits the kinase activity of MARK2 *in vitro*.

The inhibition of constitutively active MARK2 T208E by recombinant PAK5 was measured via the phosphorylation of the tau peptide TR1 by MARK2. The kinase activity of MARK2 T208E alone is normalized to 100% (lane 1). PAK5 wild-type and different mutants reduce the kinase activity of MARK2 about threefold (lanes 2-4). Triplicate experiments showing mean ± SE.

To confirm these results in cells, CHO cells were co-transfected with plasmids of the active form of MARK2 (T208E) with wild type PAK5, active PAK5 (S573N/S602E) and inactive PAK5 (S602M/T606M) respectively, and analyzed by confocal microscopy.

The co-localization of PAK5 wild type and active MARK2 are observed on vesicular structures as well as at membrane protrusions (Figure 24). There is also a diffuse cytolsolic background of both kinases. A similar distribution is detected when inactive PAK5 and active MARK2 are co-expressed (Figure 26). When active PAK5 and active MARK2 are co-transfected, there is an enhanced cytosolic background of both kinases; and co-localization is also observed at membrane (Figure 25).

Active MARK2 has a significant capability to destroy the microtubule network and to cause cell shrinkage and death (see 3.1). Nevertheless, when active MARK2 is coexpressed with wild type PAK5 or inactive PAK5, MARK2 is not able to depolymerize the microtubules. The cells retain their normal size and shape. The regular staining of microtubules, actin, and vinculin proves this inhibition of active MARK2 T208E activity (Figure 24 and 26). Coexpression of active PAK5 with active MARK2 also protects the microtubule network (Figure 25:3), whereas actin stress fibers (Figure 25:6) and focal adhesions (Figure 25:9) are dissolved, which correlate with a morphological change of the cells, implying that actin is very dynamic. These results show two independent effects of active PAK5: first, similar to PAK5 wt and the inactive form it can stabilize microtubules by binding and inhibiting MARK2; secondly, constitutively active PAK5 S573N/S602E can make actin dynamic by dissolving the stress fibers and focal adhesion and inducing filopodia. The kinase activity of PAK5 is not required in the inhibition of MARK2, but it is definitely required for dissolving stress fibers and focal adhesion and inducing filopodia.

Taken together with the fact that the two proteins closely colocalize in cells, these results suggest that PAK5 indeed binds to MARK2 and is able to inhibit MARK2 (even the active form of MARK2) in cells and the kinase activity of PAK5 is not required for this inhibition.

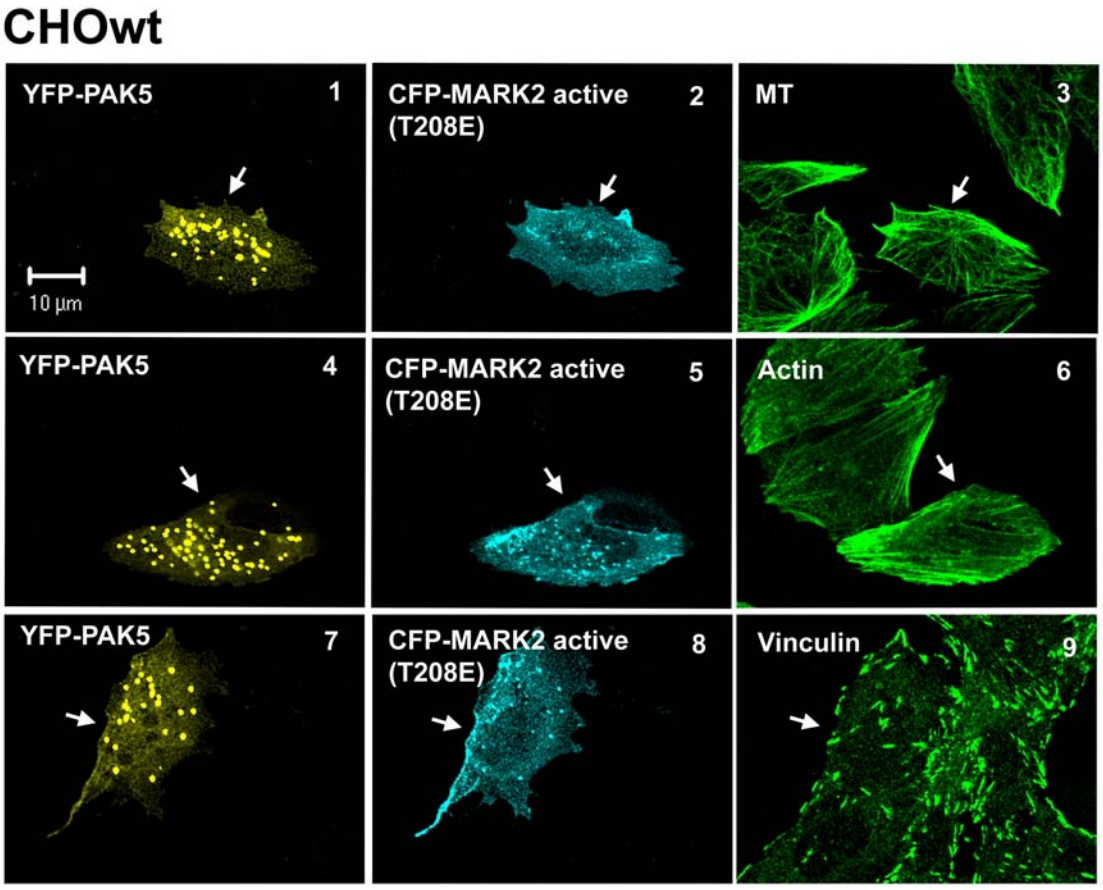

Figure 24: Wild type PAK5 inhibits the effect of active MARK2 on the cytoskeleton in CHO cells.

CHO cells were co-transfected with wild type YFP-PAK5 and active CFP-MARK2 T208E for 20 hours, fixed and stained for microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).

YFP-PAK5wt co-localizes with active CFP-MARK2 T208E in vesicles and membrane protrusion (1 and 2, 4 and 5, 7 and 8). Cells co-expressed with YFP-PAK5 wt and CFP-MARK2 T208E show a stabilized microtubule network (3), actin network (6) and focal adhesion (9) indicating the inhibition of active MARK2.

Arrows indicate the transfected cells.

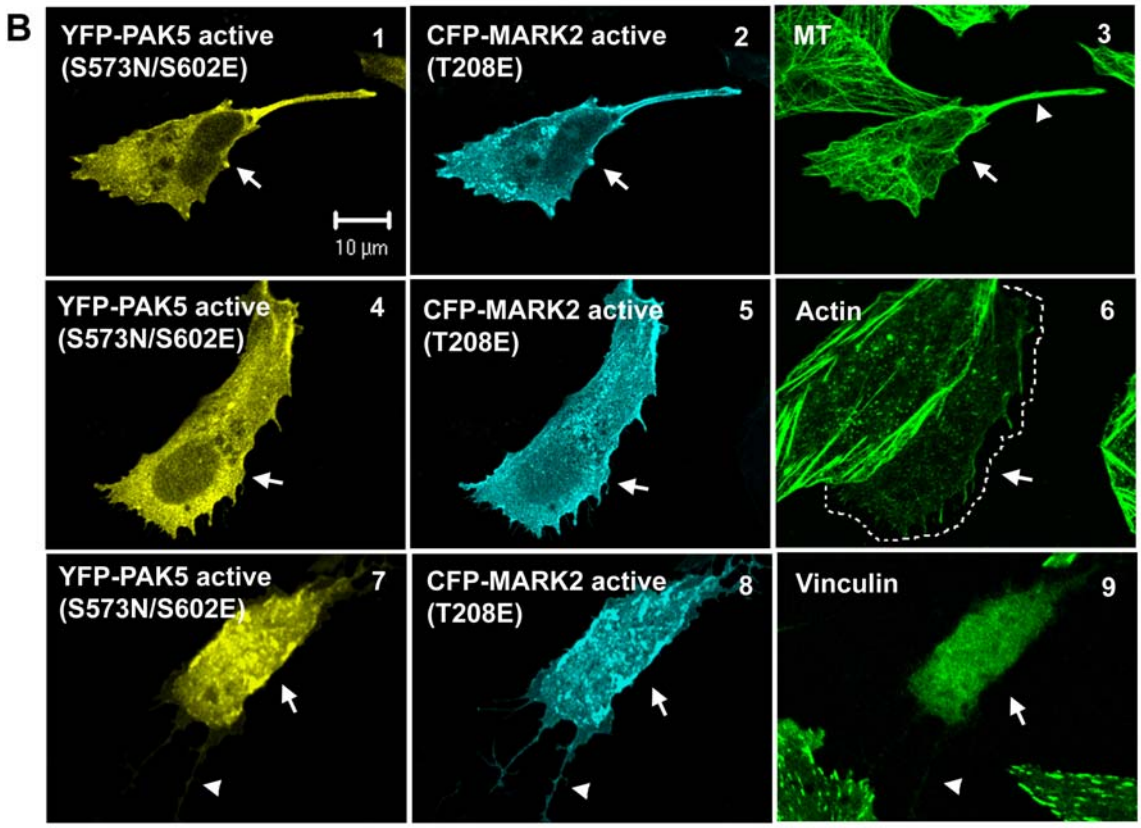

Figure 25: Active PAK5 inhibits the effect of active MARK2 on the cytoskeleton in CHO cells.

CHO cells were co-transfected with active YFP-PAK5 S573N/S602E and active CFP-MARK2 T208E for 20 hours, fixed and stained for microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).

Constitutively active PAK5 S573N/S602E and MARK2 T208E co-expressed cells show an enhanced cytosolic distribution of the two kinases, and they co-localize at membrane protrusions (1 and 2, 4 and 5, 7 and 8). Cells now show the stabilized microtubules (bundles, 3, arrow head) and the formation of filopodia (7-9, arrow head), but dissolved actin stress fibers (6) and focal adhesion (9), indicating the dynamic behavior of the actin cytoskeleton.

Arrows indicate the transfected cells.

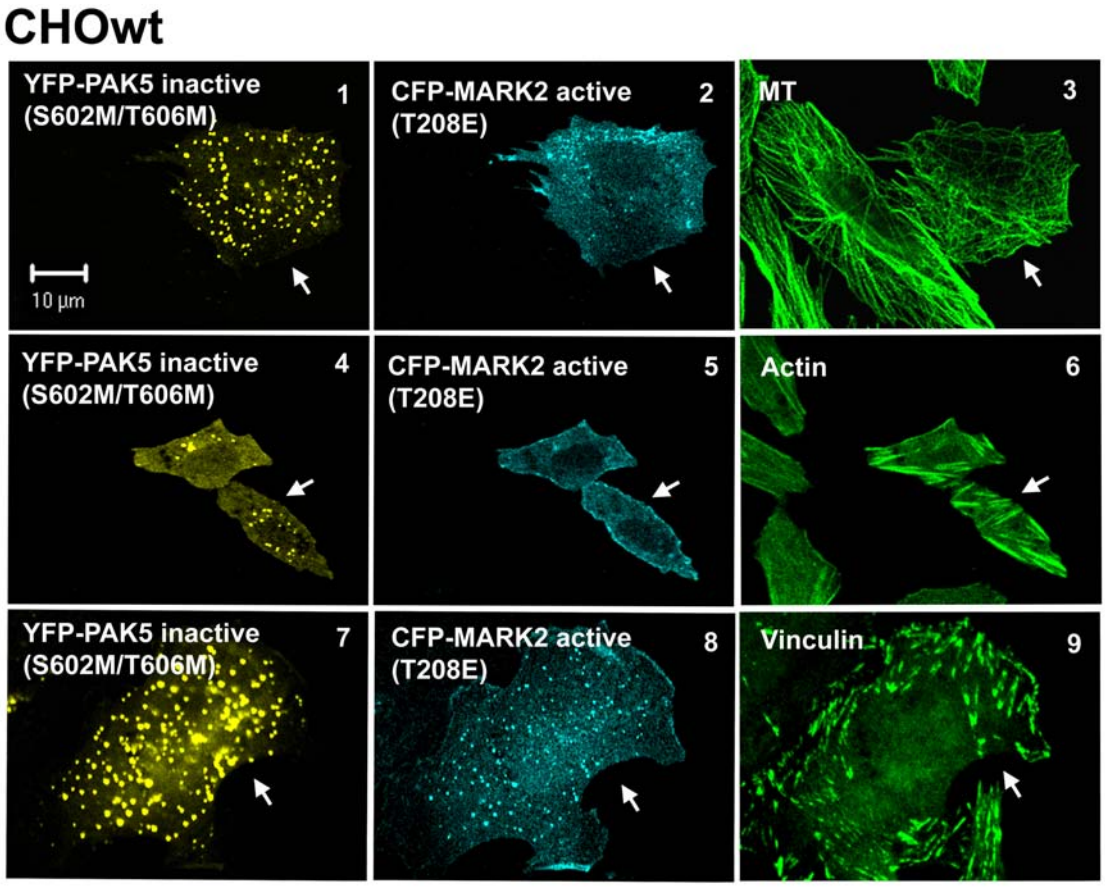

Figure 26: Inactive PAK5 inhibits the effect of active MARK2 on the cytoskeleton in CHO cells.

CHO cells were co-transfected with inactive YFP-PAK5 S602M/T606M and active CFP-MARK2 T208E for 20 hours, fixed and stained for microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).

Inactive PAK5 S602M/T606M and active MARK2 T208E have a similar localisation and cytoskeleton organization as seen in Figure 24.

Arrows indicate the transfected cells.

3.3.4 Characterization of the interaction between PAK5 and MARK

PAK5 has a C-terminal kinase domain and an N-terminal CRIB domain. The CRIB domain is able to interact with Cdc42 in the presence of GTP, but the kinase activity of PAK5 does not require the binding of cdc42 (Pandey, 2002). According to this fact, one would expect that the inhibition of MARK2 by PAK5 is mediated through other mechanisms, such as PAK5 interfering with Rho GTPase-effectors that are needed for MARK2 signaling. To answer this question, the inactive catalytic domain of PAK5 (N-terminus truncated and S602M/T606M mutated to eliminate the activity) was constructed and transfected alone or together with active MARK2 T208E into CHO cells.

As the results demonstrate, single transfection of active MARK2 leads to microtubule breakdown and contraction of the cell (Figure 27:1-3). These cells still preserve some stress fibers and focal adhesion (Figure 28:1-3, Figure 29:1-3). Single transfection of inactive PAK5 catalytic domain S602M/T606M has no significant effect on the microtubule network, the actin network, or on the focal adhesions (Figure 27:4-6, Figure 28:4-6 and Figure 29:4-6). Note that inactive PAK5 catalytic domain S602M/T606M is as before localized on vesicles, at the centrosome and diffusely distributed in the background. When co-transfected, inactive catalytic domain of PAK5 mostly co-localizes with active MARK2 and successfully eliminates the effect of active MARK2 on the cytoskeleton as indicated by the staining of intact microtubule network (Figure 27:7-9). The cells also exhibit normal actin stress fibers (Figure 28:7-9) and focal adhesion staining (Figure 29:7-9). These results indicate that the direct binding of PAK5 catalytic domain is sufficient and efficient to inhibit the kinase activity of MARK2.

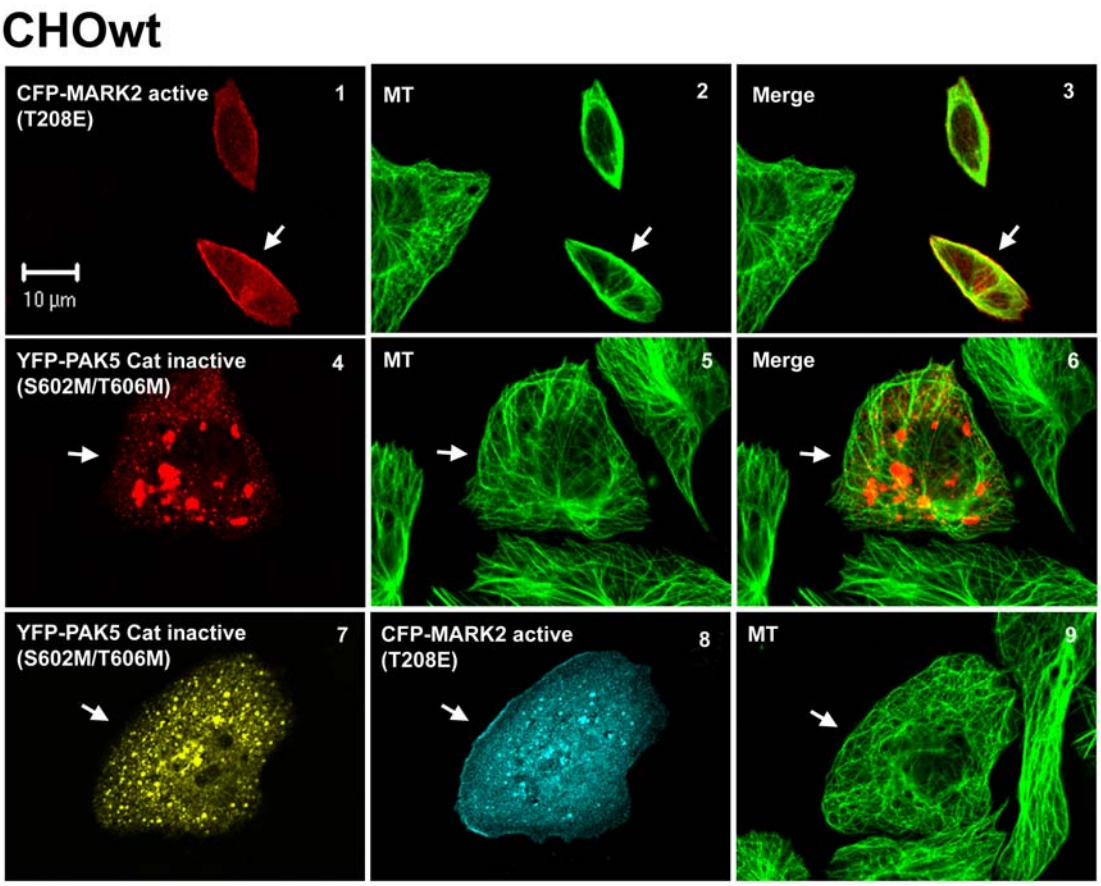

Figure 27: Inactive catalytic domain of PAK5 inhibits the effect of active MARK2 on the microtubule network in CHO cells.

CHO cells were transfected with inactive YFP-PAK5 catalytic domain and constitutively active CFP-MARK2 T208E individually or jointly. After 20 hours, cells were fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5).

Constitutively active MARK2 (1) destroys the microtubules (2), merged in 3. Inactive catalytic domain of PAK5 (4) has no effect on the microtubule network (5), merged in 6. Co-expression of inactive catalytic domain of PAK5 and active MARK2 in CHO cells shows co-localization of the two kinases (7 and 8) and the inhibition of MARK2 activity, as seen by the intact microtubule network (9). Arrows indicate the transfected cells.

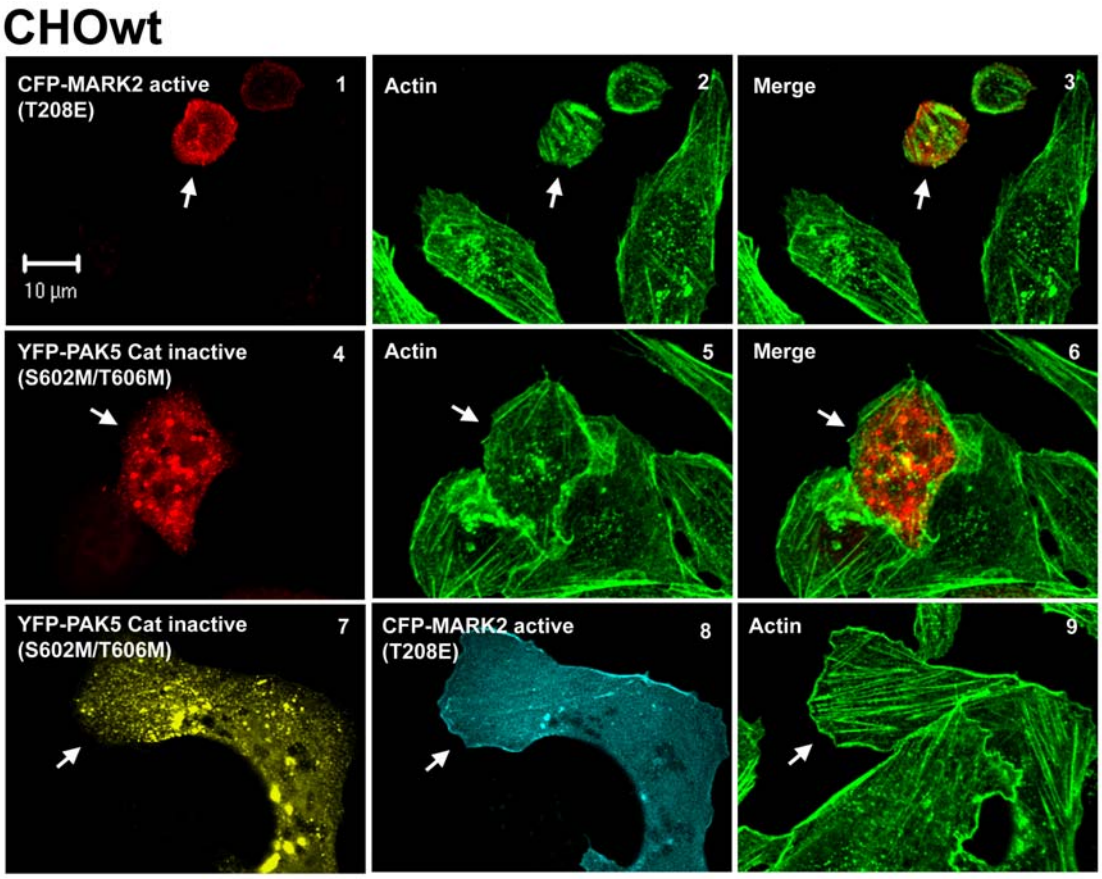

Figure 28: Inactive catalytic domain of PAK5 inhibits the effect of active MARK2 on the cytoskeleton in CHO cells (stained for actin).

CHO cells were transfected with inactive YFP-PAK5 catalytic domain and constitutively active CFP-MARK2 T208E individually or jointly. After 20 hours, cells were fixed and stained for the actin (anti-β-actin, Cy5).

Active MARK2 T208E (1) leads to the shrinkage of the cell, although partial stress fibers are preserved (2), merged in 3. Inactive catalytic domain of PAK5 (4) has no effect on the actin network (5), merged in 6. Co-expression of inactive catalytic domain of PAK5 and active MARK2 (7 and 8) leads to the inhibition of MARK2 activity; cells are normal in size with stress fibers (9). Arrows indicate the transfected cells.

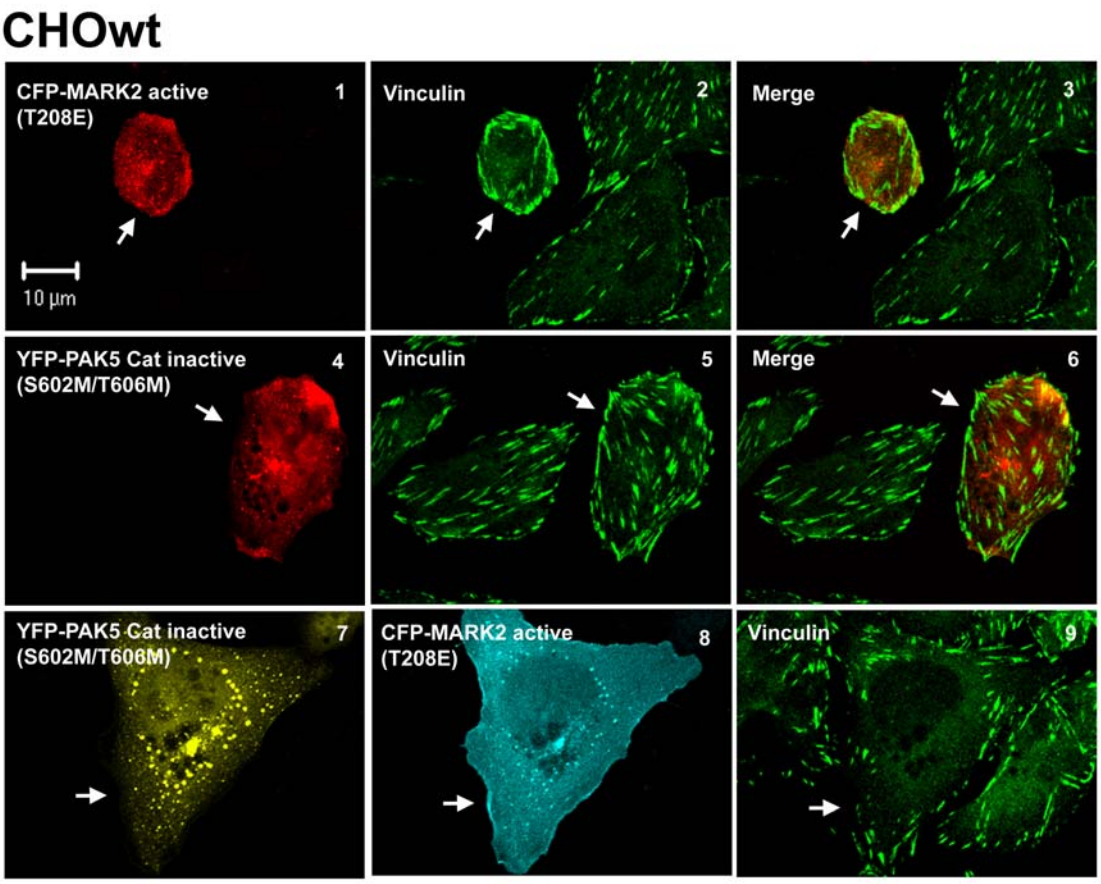

Figure 29: Inactive catalytic domain of PAK5 inhibits the effect of active MARK2 on the cytoskeleton in CHO cells (stained for vinculin).

CHO cells were transfected with inactive YFP-PAK5 catalytic domain and constitutively active CFP-MARK2 T208E individually or jointly. After 20 hours, cells were fixed and stained for the vinculin (anti-vinculin, Cy5). Active MARK2 T208E transfected cells round up (1); there are some focal adhesions mainly at the cell periphery (2), merged in 3. Inactive catalytic domain of PAK5 (4) has no effect on the focal adhesion formation (5), merged in 6. Cells co-expressed with inactive catalytic domain of PAK5 and active MARK2 T208E show partial co-localization of these two enzymes (7 and 8) and the inhibition of MARK2 activity, as seen by the extended shape of the cell and normal focal adhesion staining (9). Arrows indicate the transfected cells.

As described in the introduction, the PAK kinase family has many different members. We were therefore wondering whether other isoforms, for instance PAK1, had a similar inhibition effect like PAK5 on the MARKK-MARK-MAP signaling cascade. PAK1, the member of the first group of the PAK family, was co-transfected with wild type YFP-MARK2 in CHO cells. Though expressed at a high level, neither does PAK1 co-localize to MARK2, nor can it inhibit

the activity of wild type MARK2, because the microtubule network is damaged and the cell has rounded up and will go into apoptosis (Figure 30). This confirms the specific interaction and inhibition of MARK2 by PAK5.

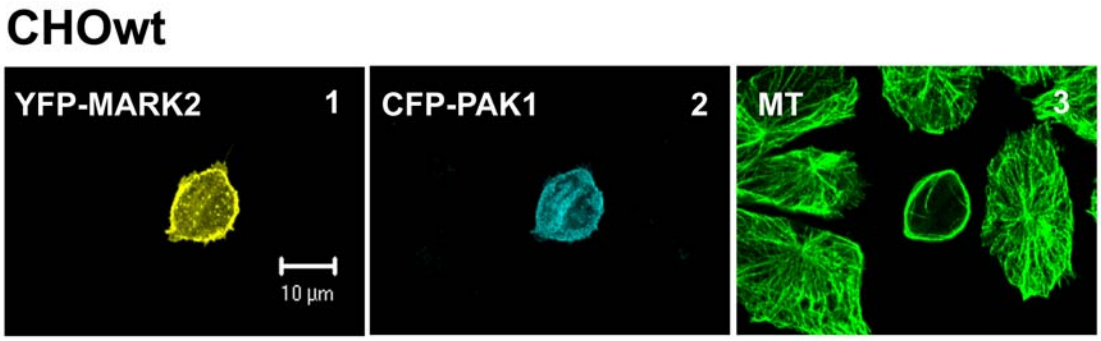

Figure 30: PAK1 is not able to inhibit the effect of MARK2 on the microtubules in CHO cells.

CHO cells were co-transfected with YFP-MARK2 wild type (1) and CFP-PAK1 (2) for 20 hours, and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5, 3). Although expressed at a high level (2), PAK1 cannot inhibit the activity of MARK2, as shown by the collapse of the microtubules (3).

Since the catalytic domains of MARK1, 2, 3 and 4 are highly homologous; the next question is whether PAK5 can also inhibit other MARK isoforms. To address this question, wild type CFP-MARK4 was co-transfected with wild type YFP-PAK5, constitutively active mutant, and kinase dead mutant respectively. Similar to MARK2, MARK4 co-localizes with PAK5 in vesicles as well as in the cytosol (Figure 31). MARK4 alone is able to destroy the microtubules (Figure 7); when coexpressed with PAK5 wild type or its mutants, MARK4 gets inhibited and the microtubule network is protected (Figure 31: 3, 6 and 9). Parallel experiments were performed with MARK1 or MARK3 and equivalent results were observed (data not shown). Therefore, PAK5 can inhibit the activity of all four MARK isoforms by binding to them, independently of its own kinase activity. This reaction is specific since PAK1 does not show this effect.

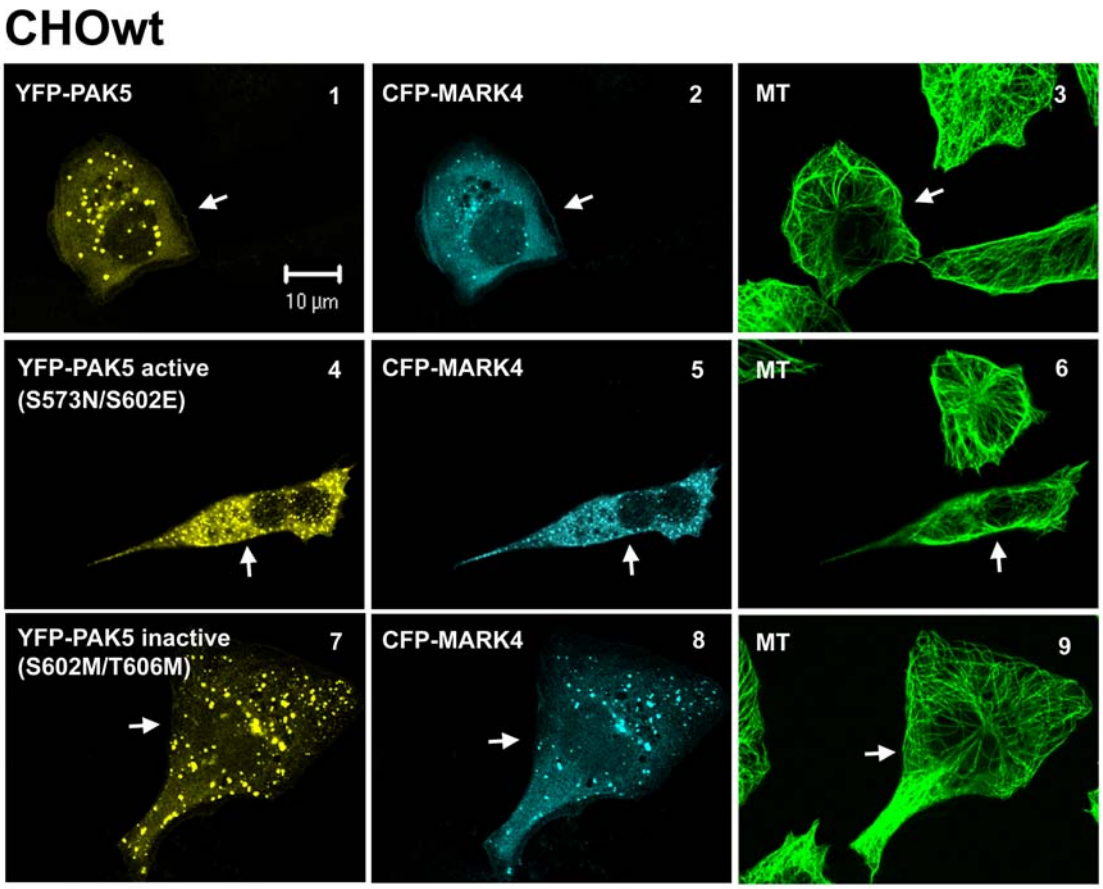

Figure 31: PAK5 inhibits MARK4 and protects the microtubules in CHO cells.

CHO cells were co-transfected with CFP-MARK4 (2, 5, and 8) and YFP-PAK5 (1), active YFP-PAK5 (4), inactive YFP-PAK5 (7) respectively. After 20 hours, cells were fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5, 3, 6 and 9). Note that both kinases co-localize in vesicles and in addition are diffusely distributed in the cytosol (1 and 2, 4 and 5, 7 and 8). PAK5 wt and its mutants inhibit MARK4 activity, as shown by the intact microtubule network (3, 6 and 9). Arrows indicate the transfected cells.

3.3.5 The co-localization of exogenous PAK5 with endogenous MARK in differentiated PC12 cells

Endogenous PAK5 and endogenous MARK show the same distribution at the cell membrane and in neurites in human neuroblastoma cells (LAN5) (Matenia et al., 2005). To check their roles in neuronal differentiation, differentiated PC12 cells were used as a cell model. However, none of the available commercial PAK5 antibodies recognize the endogenous PAK5 in PC12

cells, thus experiments were carried out with the staining of transfected PAK5 and endogenous MARK as a compromise.

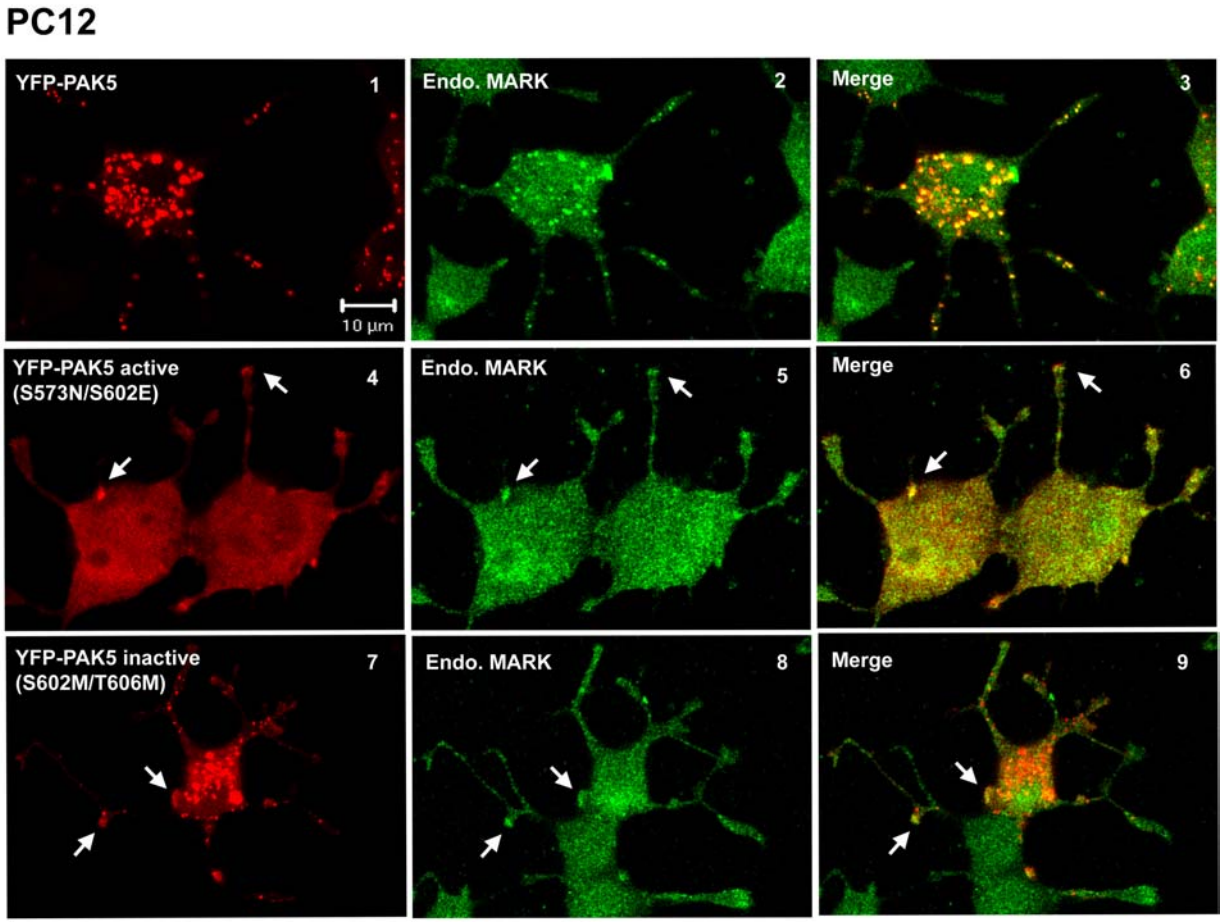

Figure 32: Transfected PAK5 co-localizes to the endogenous MARK in differentiated PC12 cells.

PC12 cells were transfected with wild type YFP-PAK5 (1), active YFP-PAK5 (4) and inactive YFP-PAK5 (7), and differentiated with 100ng/ml NGF for 48 hours. Cells were fixed and stained for endogenous MARK (antibody SA4635, Cy5) (2, 5, 8), merged in 3, 6, 9. Wild type PAK5 has a vesicular distribution (1) and co-localizes with endogenous MARK (2, 3). Active PAK5 is mainly cytosolic (4) and co-localizes with endogenous MARK (5) at the growth cones and membrane protrusions (4-6, arrows). Inactive PAK5 is mainly vesicular (7), but also partly co-localizes with endogenous MARK (8) at the membrane protrusions (7-9, arrows).

PC12 cells were transfected with wild type PAK5, active PAK5, and inactive PAK5 respectively, differentiated with 100ng/ml NGF for 48-72 hours and stained with endogenous MARK (SA4635, against amino acid 1~377 of MARK1). Similar to the observations in CHO cells, wild type PAK5 shows vesicular distribution in differentiated neuronal PC12 cells (Figure 32:1). Larger vesicles are mainly in the cell body and relative smaller vesicles in the neurites and in the growth cones. Interestingly, besides diffused distribution in the cytoplasm, endogenous MARK co-localizes to wild type PAK5 in these vesicles (Figure 32: 2 and 3). A much more enhanced cytosolic distribution is detected when active PAK5 is transfected into PC12 cells (Figure 32:4). It is enriched and co-localizes to endogenous MARK at the growth cones and at some membrane protrusions (Figure 32:4-6, arrows). The inactive PAK5 shows similar distribution to wild type PAK5, but there is less endogenous MARK co-localizing to these vesicles. However, there is also enrichment and co-localization between inactive PAK5 and endogenous MARK at some cell processes (Figure 32:7-9, arrows). The expression of PAK5 and its mutants does not seem to disturb the differentiation of PC12 cells. These data suggest that PAK5 might precisely regulate the MARK signaling at those key structures (i.e. growth cones) of neuronal cells.

3.3.6 Characterization of MARKK as a PAK5 interaction partner

PAK5 interacts with MARK2; it is also interesting to ask whether PAK5 can interact with MARKK, the activator of MARK. Clontech Matchmaker Gal4 Two-Hybrid System 3 was used to test the interaction between MARKK and PAK5. The constructs used are: full length wild type MARKK, N-terminal domain of MARKK containing the catalytic domain (aa1-431), C-terminal domain of MARKK (aa432-1001), full length wild type PAK5, N-terminal domain of PAK5 (aa1-447) and C-terminal domain of PAK5 containing the catalytic domain (aa410-719). Surprisingly, the conclusion from the yeast two hybrid test is: under the most stringent screen conditions PAK5 is able to interact with MARKK C-terminal regulatory domain with its N-terminal regulatory domain (Figure 33). This interaction is further confirmed by co-immunoprecipitation of His-tagged MARKK with HA-tagged PAK5 in Sf9 cells (Figure 34).

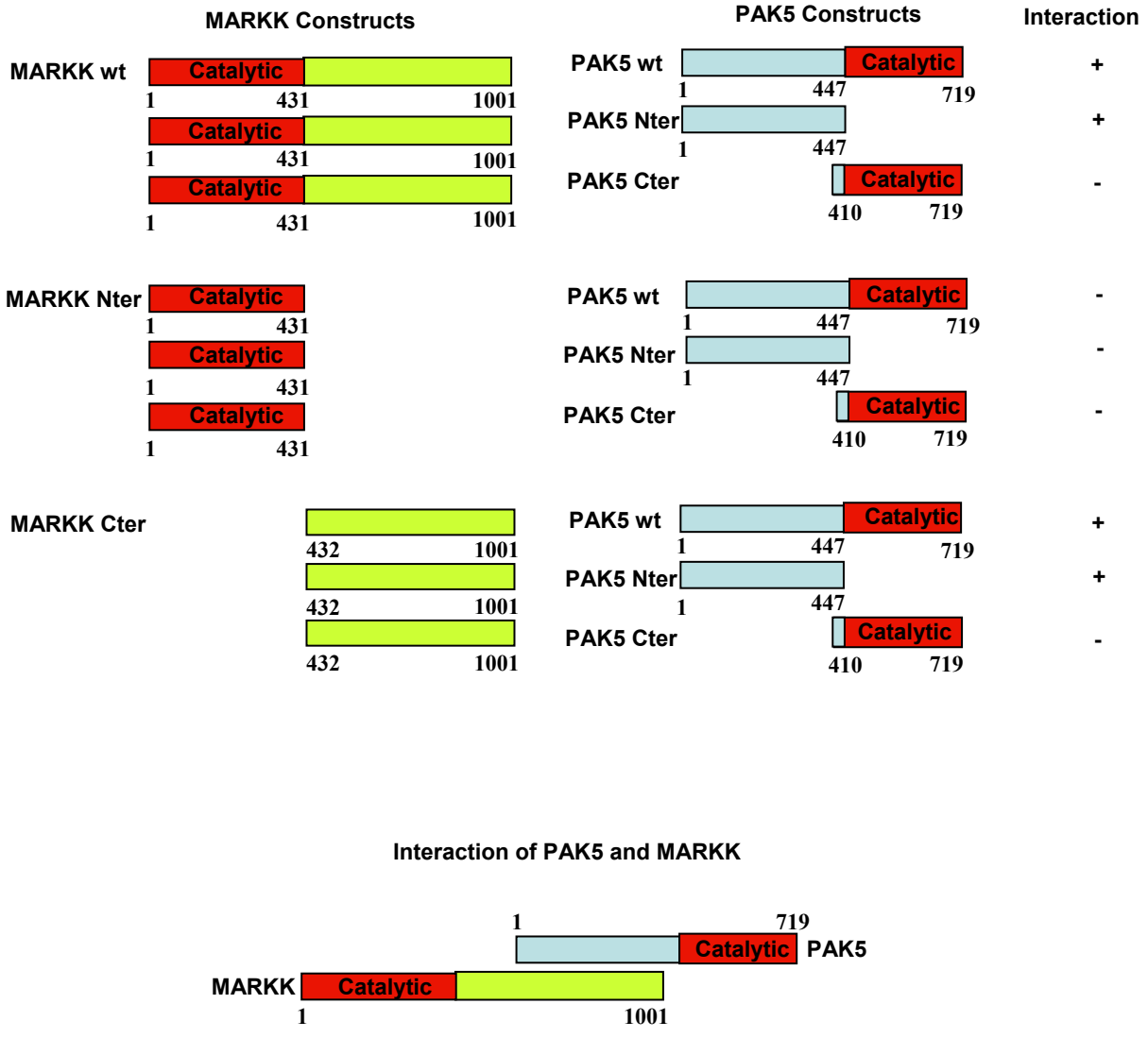

Figure 33: Interaction of PAK5 and MARKK by yeast two hybrid.

MARKK wt, N-terminal and C-terminal constructs were tested with PAK5 wt, or its N- or C-terminal constructs respectively in a yeast two hybrid screen. Data were collected under the most stringent growing conditions for the yeast. Results show the interaction between PAK5 N-terminus and MARKK C-terminus.

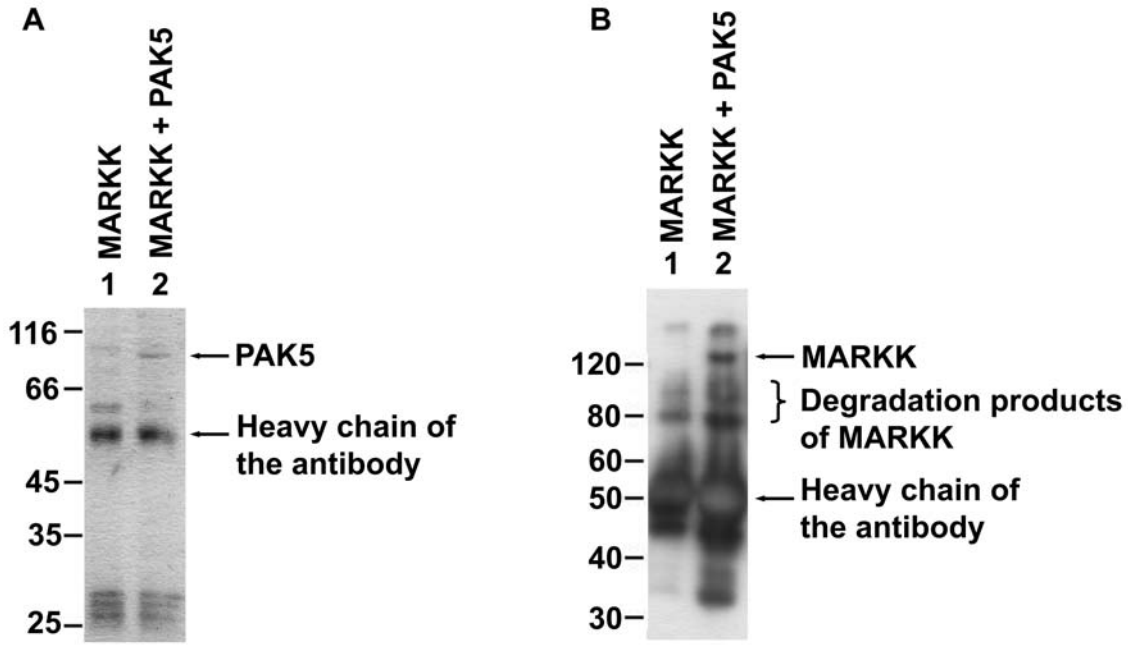

Figure 34: Co-immunoprecipitation of HA-PAK5 and His-MARKK in Sf9 cells.

Sf9 cells were transfected with His-MARKK alone or together with HA-PAK5. After 72 hours, cell lysates were precipitated with anti-HA-beads and stained in a Western blot with anti-PAK5 antibody (A) or anti-MARKK antibody (B).

(A) After incubation with the cell lysates HA-beads were examed by Western blotting with anti-PAK5 antibody. PAK5 is expressed in the Sf9 cells that were co-transfected with HA-PAK5 and His-MARKK (lane 2), but not expressed in the cells only transfected with His-MARKK (lane 1).

(B) The same samples used in (A) were examed by Western blotting with anti-MARKK antibody. When MARKK is co-transfected with HA-PAK5, it is immunoprecipitated with HA-tagged PAK5 in the cell lysate (lane 2), but not immunoprecipitated with the cells only transfected with MARKK (lane 1). This indicates a specific interaction of PAK5 and MARKK in Sf9 cells and not an unspecific binding of MARKK to HA-tagged beads.

3.3.7 Identification of the inhibitory effect of PAK5 on MARKK in CHO cells

Previous data show that MARKK can activate MARK through the phosphorylation at T208 (MARK2) in the regulatory loop; PAK5 can inhibit MARK by binding. Since MARKK is also classified as a PAK5 interaction partner, one would ask about the functional relationship between MARKK and PAK5. To answer this question, constructs of CFP-PAK5 wild type, constitutively active or inactive mutant were transfected together with wild type YFP-MARKK in CHO cells. The cells were stained for microtubules, actin, and focal adhesion.

Wild type PAK5 has a typical vesicular distribution throughout the cell (Figure 35: 2, 5 and 8). However, MARKK wild type, which normally has a cytosolic distribution (see 3.1), is found co-localizing with PAK5 wild type in vesicles (Figure 35: 1, 4 and 7), moreover, its ability to destroy microtubules is inhibited. The staining of intact microtubules is observed, as well as the normal actin or focal adhesion staining (Figure 35: 3, 6 and 9). There is also diffuse cytosolic distribution and co-localization of MARKK and PAK5 wild type. When coexpressed with MARKK wild type, inactive PAK5 has a similar distribution and effect for the cytoskeleton (Figure 37); in addition, both MARKK and inactive PAK5 concentrate and co-localize at the centrosome (Figure 37, arrow head). When the experiment was performed with MARKK and active PAK5, CHO cells did not round up; they developed neurite-like outgrowth and filopodia in agreement with the absence of stress fiber staining and focal adhesion staining (Figure 36: 3, 6 and 9). The microtubule network shows bundles (Figure 36:3, arrow head) in agreement to the morphological changes of the cell to form long processes and filopodia (Figure 36: 7 and 8, arrow head), indicating a stable microtubule network and a labile actin network. These results suggest that PAK5, regardless of its activity, can block the catastrophic effect of MARKK on microtubules. Its kinase activity is only required to trigger the dynamic reorganization of the actin cytoskeleton.

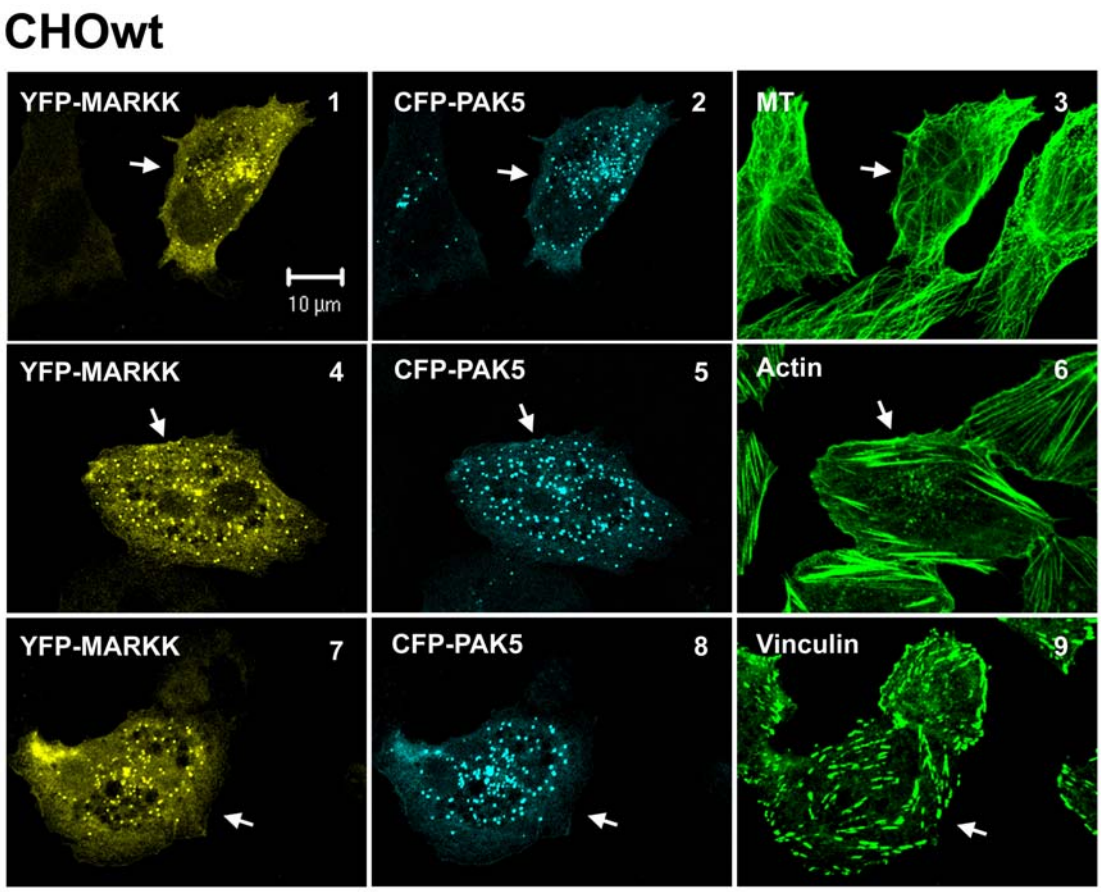

Figure 35: PAK5 co-localizes with MARKK and inhibits its effect on the cytoskeleton in CHO cells.

CHO cells were co-transfected with wild type CFP-PAK5 and wild type YFP-MARKK for 20 hours, fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).

YFP-MARKK and CFP-PAK5 co-localize in vesicles and in addition are diffusely distributed in the cytosol (1 and 2, 4 and 5, 7 and 8). Cells co-expressed with YFP-MARKK and CFP-PAK5 show a stabilized microtubule network (3), a normal actin network with stress fibers (6) and focal adhesions (9), indicating the inhibition of the catastrophic effect of MARKK on the cytoskeleton. Arrows indicate the transfected cells.

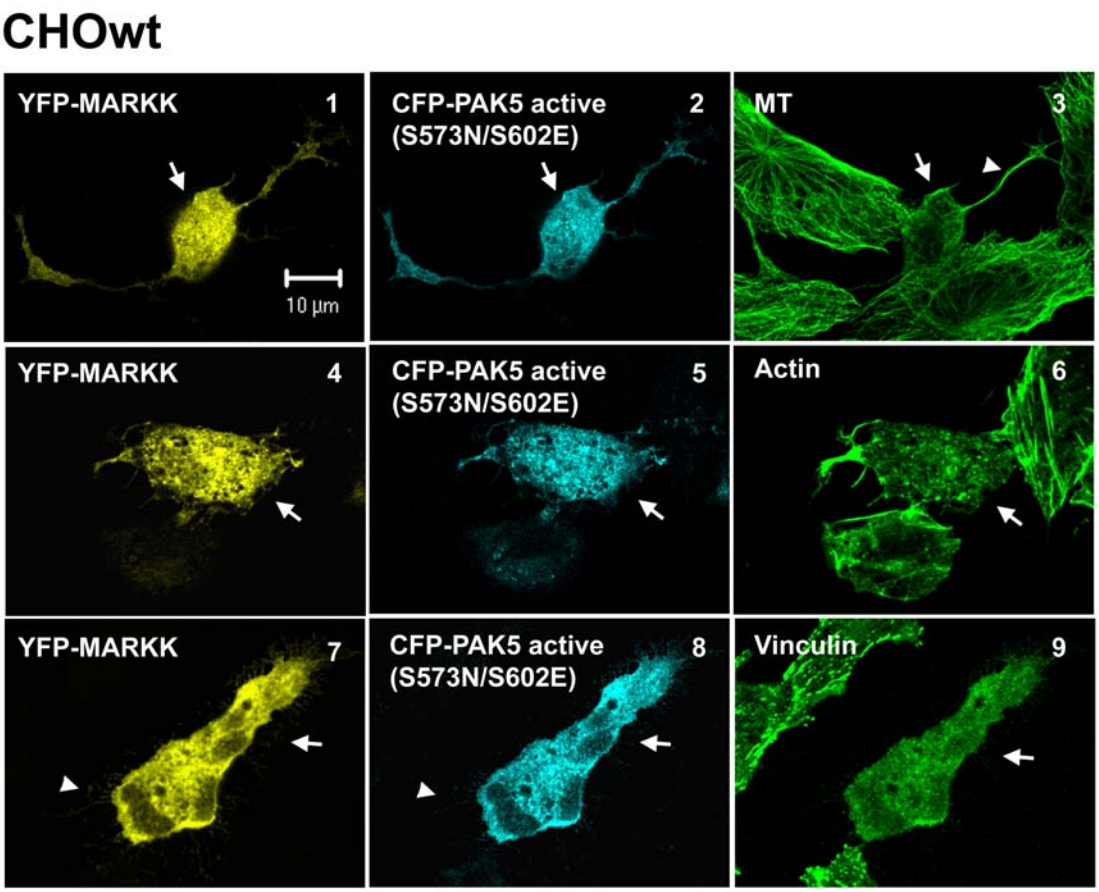

Figure 36: Active PAK5 co-localizes with MARKK and inhibits its effect on the cytoskeleton in CHO cells.

CHO cells were co-transfected with active CFP-PAK5 S573N/S602E and wild type YFP-MARKK for 20 hours, fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).

Transfected YFP-MARKK and active CFP-PAK5 S573N/S602E co-localize and have an enhanced cytosolic distribution (1 and 2, 4 and 5, 7 and 8). Co-expression of YFP-MARKK and active CFP-PAK5 S573N/S602E leads to the protection of the microtubule network (3) and the dissolution of actin stress fibers (6) and focal adhesions (9). Transfected cells form bundles of microtubules (3, arrow head) and filopodia (7 and 8, arrow head), indicating a stable microtubule network and a labile actin network. Arrows indicate the transfected cells.

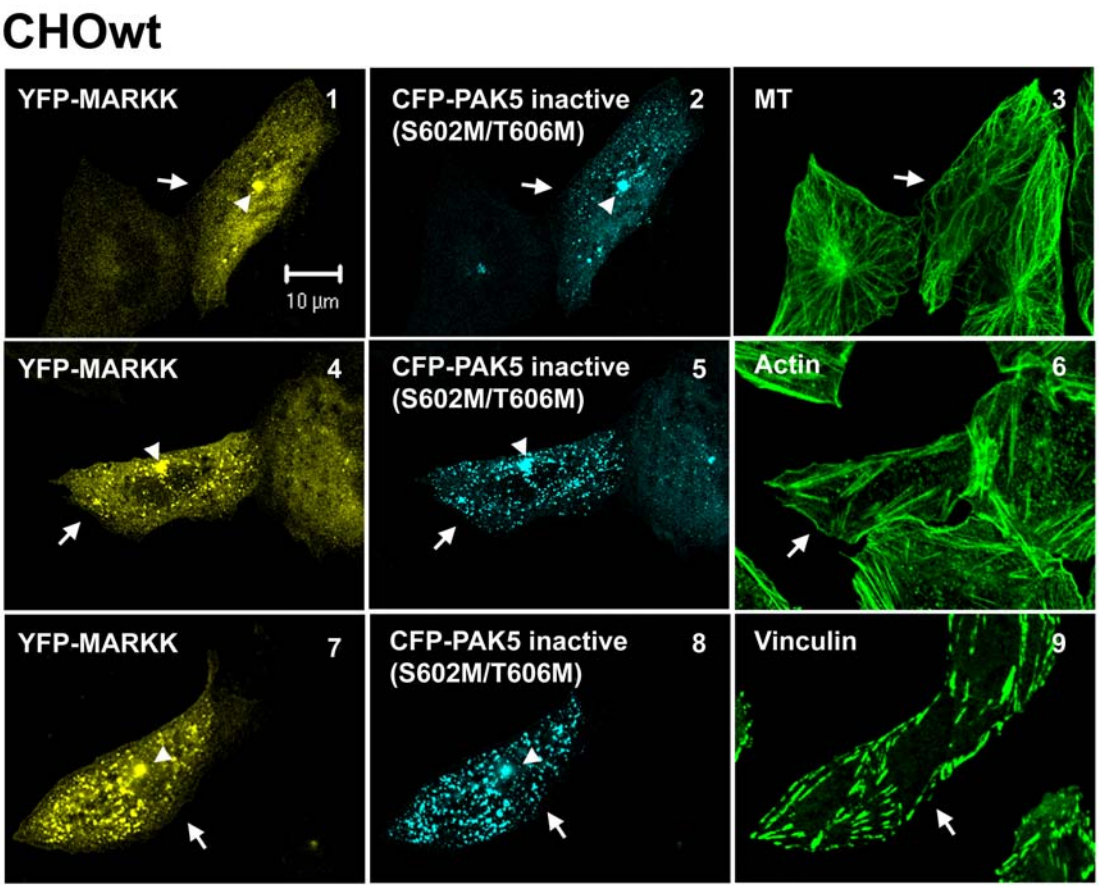

Figure 37: Inactive PAK5 co-localizes with MARKK and inhibits its effect on the cytoskeleton in CHO cells.

CHO cells were co-transfected with inactive CFP-PAK5 S602M/T606M and wild type YFP-MARKK for 20 hours, fixed and stained for the microtubules (Anti-α-Tubulin Dm1A, Cy5), actin (anti-β-actin, Cy5), and vinculin (anti-vinculin, Cy5).

Cells co-transfected with wt YFP-MARKK and inactive CFP-PAK5 S602M/T606M also show co-localization of the two kinases on vesicles, and a diffuse background; both kinases concentrate at the centrosome (1 and 2, 4 and 5, 7 and 8, arrow heads). Effect of MARKK is inhibited by inactive PAK5, as shown by the staining of an intact microtubule network (3), actin stress fibers (6) and focal adhesion (9). Arrows indicate the transfected cells.

4. Discussion

A common feature shared by many neurodegenerative diseases is the accumulation of proteins with abnormal conformation. Tau is an axonal microtubule-associated protein that is thought to stabilize axonal microtubules (Binder et al., 1985). In tauopathies, tau is abnormally phosphorylated in dying neurons, leading to its detachment from microtubules; this in turn is presumably related to microtubule breakdown, interruption of axonal transport and tau aggregation into intracellular neurofibrillary tangles (Stamer et al, 2002, Feany and Dickson, 1996; Goedert, 1998; Lee et al., 2001; Ebneth et al., 1998). Tau is a highly soluble protein and belongs to the class of natively unfolded proteins with no apparent ordered secondary structure (Schweers et al., 1994), but it becomes abnormally hyperphosphorylated and insoluble in NFTs. Many kinases have been reported to phosphorylate tau at numerous sites *in vitro* and *in vivo*, leading to the question which ones contribute to the pathological process. Previous work in our lab reported that phosphorylation of tau at a single residue, S262 in the KXGS motif within the first of three or four repeats in the microtubule binding domain, dramatically reduces microtubule binding (Biernat et al., 1993). The degree of phosphorylation at KXGS motifs is normally low (Seubert et al., 1995). It has been shown that phosphorylation of S262 is elevated in tau protein isolated from the neurofibrillary tangles of AD (Hasegawa et al., 1992), concomitant with a loss of microtubule binding and aggregation into paired helical filaments (Mandelkow et al., 1995). Fractionation of a porcine brain extract resulted in the discovery of a kinase activity, which would phosphorylate S262 in tau and detach it from microtubules. This kinase activity was later on identified as MARK, an enzyme with a molecular mass of 110 kD. MARK specifically phosphorylates tau at S262 and the other KXGS motifs in the repeats, and causes the disruption of the microtubule array in cells (Drewes et al., 1995; Drewes et al., 1997; Ebneth et al, 1999). Recent studies in a drosophila model system showed that the direct phosphorylation of tau at S262 and S356 within the KXGS motifs by MARK led to neurodegeneration. This phosphorylation is a prerequisite for other kinases, including GSK3β and Cdk5, to phosphorylate several other sites in tau and generate disease-associated phospho-epitopes. Phosphorylation at S262 by MARK probably dislodges tau from the microtubules thereby allowing the access of other kinases (Nishimura et al., 2004; Augustinack et al., 2002; Fortini 2004). These findings place MARK in a key position in the signaling cascade to the

onset of disease. Hence, characterizing the regulators of MARK is of great importance to understand Alzheimer's process and related diseases.

4.1 MARKK-MARK-MAP cascade in CHO cells and its effect on the microtubule network

Our previous work showed that in CHO cells, overexpression of MARK1 or MARK2 resulted in a smaller or more rounded cell shape. However, there were also cells normal in shape and size but with obvious loss of microtubules (Drewes et al., 1997). Similar effects can be achieved with MARK3 and MARK4 (Figure 7 and 8). This morphological difference may be due to the differences in the expression level of MARK. Figure 7 shows the progressive destruction of microtubules in CHO cells when MARK4 is overexpressed for different times. Actually when expressed long enough, all four MARK isoforms cause the breakdown of microtubules in CHO cells, resulting in similar morphological changes (Figure 7 and 8). This is not surprising because of the homology of the MARK catalytic domains and the similar structure shared by tau and related MAP2 and MAP4 in their microtubule-binding domains (Drewes et al., 1995; Ebneth et al., 1999; Trinczek et al., 2004).

In view of the fact that native MARK purified from brain was phosphorylated in its activation loop and in this way activated (Drewes et al., 1997), we searched for the upstream kinase of MARK. A kinase was purified from brain extract by chromatographic fractionation and identified and termed MARKK (Timm et al., 2003). A kinase assay shows that MARKK increases the activity of MARK and enhances the phosphorylation of the KXGS motifs in tau (Figure 9). To test whether the MARKK-MARK-MAP cascade is functional in cells, CHO wild type cells and htau40 stably transfected cells were used as model systems. Transfection of MARKK into CHO cells results in an even more dramatic loss of microtubules and subsequent rounding up of the cells and cell death as seen with the similar experiments with MARK (Figure 10:1-3). This effect is dependent on the kinase activity and is not due to the overexpression of the protein itself, because cells transfected with inactive mutant of MARKK retain their normal size and shape with an intact microtubule network (Figure 10:7-9). Northern blot shows that MARK isoforms are highly expressed in brain, but are also expressed

in several other tissues (Drewes et al., 1997; Drewes 2004). MAP4 is ubiquitously expressed, though a neuronal cell specific variant of MAP4 is identified recently (Matsushima et al., 2005). Thus we draw the conclusion that in CHO cells the effect of MARKK is mediated through endogenous MARK and MAP4 to destroy microtubules. To further confirm that the morphological changes described above are due to the direct destabilization of microtubules, we stabilized microtubules with Taxol or by tau in CHO cells. As expected in the case of tau, MARKK is not able to exert its depolymerization effect on microtubules and there are no detectable differences in the microtubule network and cell morphology between the transfected and non-transfected cells (Figure 11:3 and 4). This could be explained by the low level of enough endogenous MARK to cope with the high concentration of tau on the microtubules. This can be proven by transfection of constitutively active MARK, which counteracts the protective effect of transfected tau in CHO cells, resulting in the destruction of microtubules and shrinkage of the cells (Figure 12:4-6). Similar results were observed when MARKK and MARK were co-transfected into the tau-stable CHO cells. This indicates that the microtubule-stabilizing effect of tau can be overcome by the phosphorylation of tau by MARK at the KXGS motifs (strong staining by 12E8 antibody); this causes its detachment from microtubules, which makes microtubules labile. To verify that MARK plays this crucial role in the signaling cascade MARKK and inactive MARK mutant are co-transfected into CHO htau40 cells. As anticipated, tau shows no phosphorylation, the cells remain healthy, and there is no detectable activated MARK (Figure 13:9-16). A recent genetic screen of modifiers of tauopathy in Drosophila reveals MARKK as an enhancer of tau toxicity (Shulman and Feany, 2003).

All four MARK isoforms have a highly conserved catalytic domain. This provides the structural basis of being phosphorylated by the same upstream kinase. It is not surprising to find that MARKK can activate all four isoforms of MARK, leading to the loss of the microtubule network in tau-stabilized CHO cells; while the isoforms alone are not sufficient to cause the same effect (Figure 14). However, based on the divergence in their spacer domain (Figure 3), MARK isoforms might have different cellular distribution and be involved in different signaling pathways.

Recently, LKB1 has been reported to activate the kinases of the AMPK subfamily, including MARK/Par1 (Lizcano et al., 2004). LKB1 is a tumor suppressor protein whose gene is mutated in the inherited human Peutz-Jehgers syndrome (Hemminki et al., 1998). It has also been implicated in cell-cycle arrest, apoptosis, Wnt signaling, cell transformation, energy metabolism and regulating cell polarity in C. elegans and Drosophila (Baas et al., 2004). Lizcano and colleagues argued that in contrast to MARKK, which activates MARK2 10-fold (Timm et al., 2003), the LKB1 complex increased MARK2 activation 200-fold (Lizcano et al., 2004). However, we notice (i) the difference in the substrate peptides used in each experiment; (ii) in our case, recombinant MARK2 has a basal activity of ~55 nmol/min/mg; while the basal activity of MARK2 is low (< 1 nmol/min/mg) in Lizcano's report. Thus the final activity of MARK after activation by MARKK is even higher than by LKB1 complex in our study. Although LKB1 is reported to regulate the Wnt signaling component Par1A (Spicer et al., 2003) and LKB1 immunoprecipitated from cell lysates can phosphorylate and activate MARK4 (Brajenovic et al., 2003), so far no data suggest whether LKB1 affects microtubule network through activation of MARK. In addition, the activation of LKB1 requires the formation of a complex with STRAD, an inactive pseudokinase, and MO25, a scaffolding-like protein. Binding of LKB1 to STRAD-MO25 activates LKB1 and re-localizes it from the nucleus to the cytoplasm (Boudeau et al., 2003; Boudeau et al, 2004). However, it is questionable whether this activation mechanism of LKB1 is suitable to regulate the dynamics of microtubules when needed. MARKK, MARK are normally present in the cytoplasm and enriched at highly dynamic cytoskeletal structures like the membrane and filopodia i.e. to change their morphology when needed.

4.2 MARKK-MARK-MAP cascade in neuronal polarity establishment

C. elegans Par1, highly homologous to mammalian MARK, is involved in establishing cell polarity during development (Guo and Kemphues, 1995; Böhm et al., 1997; Tomancak et al., 2000; Sun et al., 2001). Cell polarization requires an asymmetric distribution of cell components that are normally transported along microtubules. This suggests a link between microtubules and cell polarity. Actually microtubules play key roles in the generation of polarity, for instance, in the rotational alignment of the centrosome-nucleus complex in *C.*

elegans and the orientation of the spindle pole body in *S. pombe* (Chang and Nurse, 1996; White and Strome, 1996), more remarkably, in neuronal differentiation (Caceres and Kosik, 1990; Dinsmore and Solomon, 1991). We showed previously that transfection of MARK in N2a cells induced tau phosphorylation at KXGS sites and increased microtubules dynamics, a property necessary for neurite outgrowth (Biernat et al., 2002).

NGF-induced differentiation of PC12 cells presents a model system for the study of the physiological functions of the MARKK-MARK-Tau, because of the detectable endogenous levels of these proteins. Endogenous MARK is present at the cell periphery, especially enriched in the sprouts and in the growth cones in differentiated PC12 cells (Figure 15A, 16:2, 5 and 8). Moreover, differentiation causes an obvious upregulation of MARK (Figure 15) which is consistent with the observation of MARK mRNA upregulation following NGF treatment in PC12 cells (Brown et al., 1999). In differentiated PC12 cells, endogenous MARKK, MARK and phospho-KXGS-tau colocalize, particularly near the plasma membrane and in growth cones where actin is enriched (Figure 16). Conversely, suppression of endogenous MARKK by RNAi abolishes the cell's ability to develop neurites in spite of an NGF differentiation signal (Figure 17). These results support the notion that neurite outgrowth requires active MARK, tau phosphorylatable at KXGS motifs and dynamic microtubules (Biernat and Mandelkow, 1999; Biernat et al., 2000).

The microtubule dynamics differs spatially in the axon (Ahmad et al., 1993). The proximal section of microtubules is rather stable while the distal region is relatively more dynamic; particularly dynamic microtubules are present in the growth cone. This kind of distribution corresponds to the different functions needed in the axon. Stable microtubules provide mechanical strength and tracks for the intracellular transport of components essential for axonal outgrowth and maintenance of synapses. Dynamic microtubules in the distal region of the axon are regulated for new assembly of microtubules necessary for growth and elongation of the axon. The dynamic instability of microtubules in the growth cone is especially important for this process (Rochlin et al., 1996; Liao et al., 1995). The inhibition of microtubule dynamics without appreciable depolymerization of microtubules inhibits the persistent forward movement of growth cones (Tanaka et al., 1995). Dynamics and reorganization of the actin

cytoskeleton also play an important role in growth cone motility (Kuhn et al., 2000; Brown et al., 2000; Bradke and Dotti, 1999; Bradke and Dotti, 2000; Wu et al., 2005). Members of the Rho family of small GTPases are involved in this regulation, with roles for Cdc42 and Rac1 in regulating filopodia and lamellipodia formation in growth cones, and RhoA in triggering growth cone collapse and neurite retraction (Gallo and Letourneau, 2004).

Recently, functional cooperation between the microtubule and actin cytoskeleton has been observed during vesicle and organelle transport, cellular movement and morphogenesis (Sider et al., 1999; Waterman-Storer and Salmon, 1999; Fuchs and Yang, 1999; Goode et al, 2000; Rodriguez et al., 2003; Hasaka et al., 2004). The interaction between dynamic microtubules and actin filaments is also required for the growth cone function such as advance, retraction, turning, branching, and presynaptic differentiation (Letourneau et al., 1987; Borisy and Svitkina, 2000; Bradke and Dotti, 2000; Dent and Kalil, 2001; Dent and Gertler, 2003; Kalil and Dent, 2005; Kornack and Giger, 2005). Briefly, the actin cytoskeleton in the growth cone reacts to signals and guidance cues to prepare the ground for transient lamellipodia or filopodia formation, for further branching or retraction. This ground is probed by pioneering (highly dynamic) microtubules with plus end tip complexes that make temporary excursions and retractions into the actin meshwork, until a decision is made to determine the behavior of the growth cone and the fate of the axon. All these signaling cross-talk and cytoskeletal reorganization are based on the property of the actin and microtubule cytoskeletons to change their dynamic behaviors. This explains why the suppression of the dynamic instability of either the actin or microtubule cytoskeleton (for example by MARKK RNAi) suffices to block the growth of neurites. The cell seems to use tau or related MAPs for this regulation (Drubin and Kirschner, 1986; Panda et al., 1999). Tau is enriched in the growth cones, where it is largely detached from microtubules (Black et al., 1996). Based on the observation that endogenous MARKK, MARK and phospho-KXGS-tau colocalize in growth cones where actin is enriched (Figure 16, Biernat et al., 2002), we suggest that the role of MARKK-MARK would be to locally and transiently phosphorylate tau at the KXGS motifs, thereby detaching tau from the microtubules and rendering them dynamic. The phosphorylated tau then translocates to the actin network. The function of this relocation is not clear yet. A simple explanation could be that the actin network provides a docking place for the free tau pool. This pool of tau can get

dephosphorylated by phosphatases any time and is than able to switch over to the microtubule network again when the growth cone wants to advance and stable microtubules are now required.

4.3 Cellular distribution and cytoskeletal effects of PAK5

MARK is an unusually large kinase, it contains several domains. Besides its upstream kinase MARKK, it can interact with other regulatory partners. We searched for them by a yeast two-hybrid screen using a fetal brain library and detected 14-3-3 proteins (β and ζ isoforms) and the PAK5 isoform of p21-activated kinases. The p21-activated kinase family is best known for its role in signaling to the actin cytoskeleton after activation by the small GTPase Rac or Cdc42 (Etienne-Manneville and Hall, 2002; Bokoch, 2003).

In CHO cells, the effect of transfected PAK5 is to dissolve the actin stress fibers and focal adhesions and promote filopodia, whereas microtubules are stabilized and form bundles (Figure 19). These cytoskeletal effects depend on the activity of the PAK5 kinase and are not observed with the kinase dead mutant (Figure 20). This is consistent with the observation that PAK5 activity is required to trigger neurite outgrowth in N1E-115 cells (Dan et al., 2002). PAK5 exhibits a punctate distribution throughout the transfected CHO cells; in addition, the inactive mutant shows enhanced labeling at the area around the centrosome (Figure 20 arrows). This accumulation of PAK5 around the centrosome looks morphologically like an aggresome. Aggresomes are newly reported pericentriolar cytoplasmic structures in which aggregated, misfolded proteins are sequestered (Johnston et al., 1998). They are thought to be a third pathway for the cell to cope with misfolded and aggregated proteins in addition to chaperone-mediated refolding or proteasomal degradation (Garcia-Mata et al., 2002). MTOC directed cytoplasmic dynein/dynactin mediates the assembly and transport of aggresomes (Johnston et al., 2002). However, we did not observe the cagelike structures typical of aggresomes by vimentin staining (Kopito, 2000). Recent work from our lab showed the partial overlap of PAK5/MARK with early endosomal vesicles carrying the adaptor complexes AP-1/2; moreover, fractionation of total vesicle proteins from Sf9 cells expressing MARK2 and PAK5 on discontinuous iodixanol gradients revealed PAK5 and MARK2 as part of the trans-Golgi

network (Matenia et al., 2005). Live cell movies also showed the dynamic bidirectional transport of these PAK5 vesicles (Figure 21, 22). In addition, there is a noticeable shift in distribution of PAK5, depending on its activity: the active PAK5 has a higher cytosolic, but lower vesicular fraction than the inactive mutant or wild type PAK5 (Figure 19). This shift can also be detected when cotransfecting PAK5 with MARK or with MARKK in CHO cells, concomitant with the shift in the distribution of MARK or MARKK (Figure 25, 36). This suggests that sequestration of PAK5 together with MARK or MARKK on vesicles is another mechanism of regulation.

4.4 Inhibitory effect of PAK5 on MARKK-MARK pathway and its role in the regulation of the cytoskeleton

Yeast two hybrid screen and coimmunoprecipitation have shown the direct interaction between MARK2 and PAK5 (Matenia et al., 2005). This interaction is mediated by the binding of the catalytic domains of the two kinases. Kinase activity assays reveal an interesting consequence of this interaction: MARK2 is inhibited when bound to PAK5, but PAK5 remains active when bound to MARK2. The inhibition of MARK2 is based on the direct binding to PAK5, not by being phosphorylated by PAK5. This is further proven by the immunofluorescence staining of cotransfected PAK5 and active MARK2 in CHO cells (Figure 24-26): the two proteins largely colocalize in cells; the microtubule network is protected from the destructive effect of active MARK2 in the presence of PAK5. MARK2 cannot inhibit PAK5, that's why active PAK5 still causes the dissolution of stress fibers and focal adhesions and induces filopodia.

The inhibition of PAK5 on MARK2 depends on binding of the PAK5 C-terminal catalytic domain to the MARK2 N-terminal catalytic domain. Although PAK5 activity does not require the binding of Cdc42 to its N-terminal CRIB domain, it can indeed bind preferentially to Cdc42 in the presence of GTP. PAK5 is also reported to operate downstream of Cdc42 and Rac and antagonizes Rho in neurite development (Pandey et al., 2002; Dan et al., 2002). Thus one could suspect that the inhibitory effect of PAK5 on MARK2 might be due to the effects of PAK5 bound Cdc42 and other recruited molecules. To answer this question, inactive PAK5 catalytic domain construct without its N-terminus was cotransfected with active MARK2 into

CHO cells. As figure 27:7-9 shows the effect of active MARK2 destroying the microtubule network is eliminated, which confirms that the direct binding of the inactive catalytic domain of PAK5 is sufficient to inhibit MARK2 activity. This mechanism of inhibition is specific to PAK5, because when PAK1, another member of the PAK family, was tested in place of PAK5, no inhibition of MARK2 activity in CHO cells was found (Figure 30). On the other hand, PAK5 can inhibit the activity of all four isoforms of MARK regardless of its own kinase activity (Figure 31), suggesting the structural similarity among the kinase domains of the MARKs.

Similar results can be observed when cotransfecting MARKK and PAK5 in CHO cells. There is colocalization between MARKK and PAK5 in vesicle structures, which is consistent with the direct interaction data from yeast two hybrid test and coimmunoprecipitation (Figure 33, 34). The effect of MARKK is blocked in the presence of PAK5, since the microtubules are intact, regardless of PAK5's activity. Active PAK5 still dissolves stress fibers and induces filopodia in the presence of MARKK. So far, there is no biochemical data directly showing the inhibition of MARKK by PAK5. Therefore one could not exclude the possibility that the inhibitory effect observed in MARKK and PAK5 cotransfected CHO cells is through the inhibition of endogenous MARK by PAK5. It is also not shown whether phosphorylation is involved in MARKK-PAK5 interaction. Results from the yeast two hybrid system show that the interaction between PAK5 and MARKK is based on the two regulatory domains (Figure 33), namely C-terminal domain of MARKK and N-terminal domain of PAK5. This is different from the interaction between PAK5 and MARK, which is based on the two catalytic domains (Matenia et al., 2005). Therefore one could speculate that the inhibitory effect of PAK5 on the MARKK-MARK-MAP signaling pathway is achieved either by the disruption of MARKK-MARK interaction and/or by the disruption of MARK-MAP interaction. Possible mechanisms could be: (i) PAK5 could use different domains to bind to MARKK and MARK simultaneously. This either inhibits the kinase activity of MARK and MARKK, or prevents MARK from being phosphorylated and activated by MARKK. This complex could be further stabilized by other partners; a possible candidate would be the adaptor protein 14-3-3. An interesting question is whether PAK5, when binding to MARKK and MARK concurrently, could still exert its effect on the actin cytoskeleton or not. (ii) Alternatively, PAK5 could bind

to MARK preferentially. In this case, MARK activity is inhibited by direct binding of PAK5, and MARKK could not activate MARK effectively due to the steric hindrance. (iii) Although lacking of the supporting data, it is possible to suppose, that PAK5 could interact with MARKK prior to MARK. This interaction might suppress MARKK activity and disturb the further phosphorylation of MARK by MARKK. This kind of regulation would take place at the upstream level of the MARKK-MARK-MAP signaling pathway, therefore it would not be as efficient as those at the downstream level (mechanisms i or ii), but it allows the possibilities of other regulations of MARK (Figure 29).

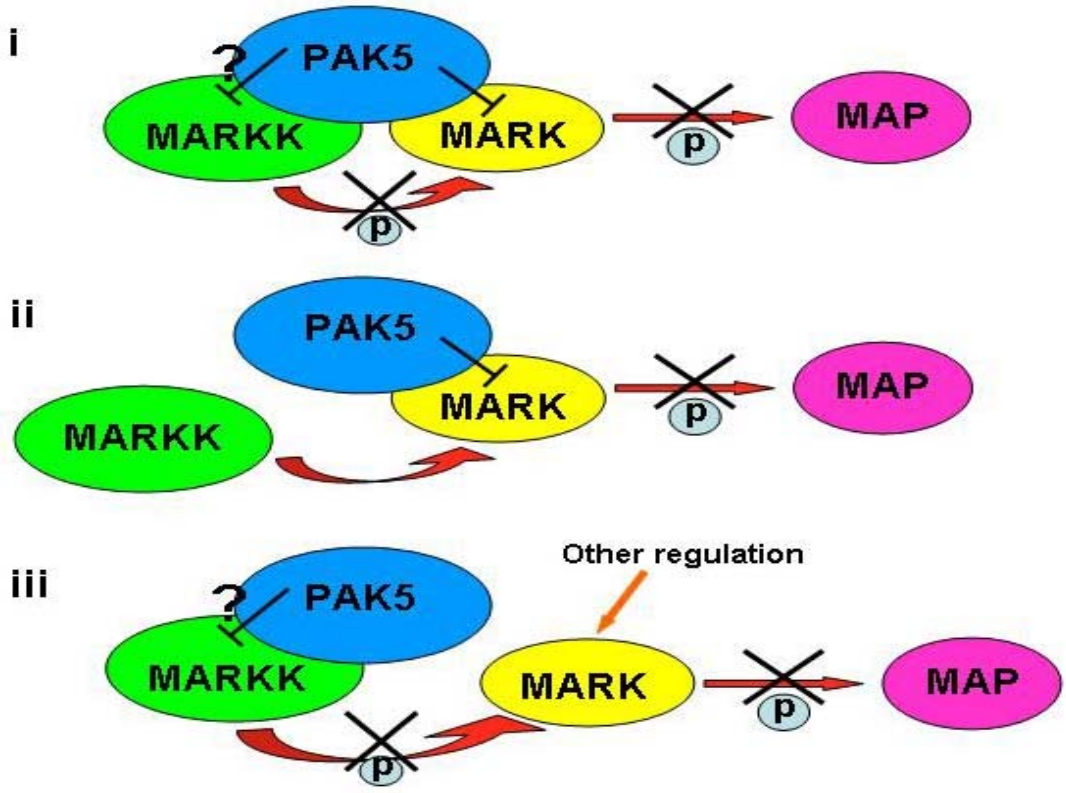

Figure 38: Models of the inhibitory effect of PAK5 on the MARKK-MARK-MAP signaling cascade.

(i) PAK5 binds to MARKK and MARK simultaneously. Binding inhibits the activities of MARKK, MARK and the interaction between MARKK and MARK. Therefore there is no phosphorylation of the MAP. (ii) PAK5 binds to MARK and inhibits its activity; in this case, MARK is not able to phosphorylate the MAP in the presence of the upstream kinase, MARKK. (iii) PAK5 binds to MARKK, inhibits its activity, and disturbs its interaction with MARK, MARK cannot be activated and cannot phosphorylate the MAP. MARK could be regulated by other signaling events.

The interplay between PAK5 and MARKK-MARK pathway exposes a remarkable cross-talk between the actin and microtubule network. On one hand, PAK5 and MARKK-MARK-MAP cascade regulate distinct cytoskeleton systems. PAK5 regulates the actin cytoskeleton, leads to the dissolution of stress fibers and formation of filopodia. MARKK-MARK-MAP cascade regulates microtubule cytoskeleton, leads to the destabilization of microtubules. On the other hand, PAK5 can down-regulate the output of MARKK-MARK signaling, but not vice versa. From the viewpoint of MARK/microtubule signaling it is influenced directly by the PAK5 'competing' actin signaling. This results in an antagonistic regulation of the two cytoskeleton networks, while actin is dynamic microtubules are stable and vice versa. The physiological significance of this kind of regulation is implied by the localization of MARKK, MARK and PAK5. MARKK and PAK5 are brain specific (Timm et al, 2003; Dan et al., 2002) and MARK is mainly expressed in the brain (Drewes et al., 1997). They are colocalized with actin in the growth cone and at the cell membrane. As discussed before (4.2), growth cones consist of highly dynamic actin and microtubules. The two systems have to cooperate to achieve the exploratory function of the growth cone. The actin network is responsible for the initial decisions at the leading edge (formation of lamellipodia, extension of filopodia), but dynamic microtubules must subsequently back up and reinforce the initial gains in territory (Wittmann and Waterman-Storer, 2001; Pollard and Borisy, 2003). When a decision is made, microtubules have to be stabilized, form bundles, and allow for further movement of the growth cone. In this case, the actin network plays a pioneering and dominant role; this explains why PAK5 can regulate MARKK-MARK, but not vice versa. It also requires the transient stability of microtubules for support before growth cone can advance again; this explains why PAK5 inhibits, not activates MARKK-MARK pathway. A simple coordination model in the growth cone is summarized in the diagram of Figure 30.

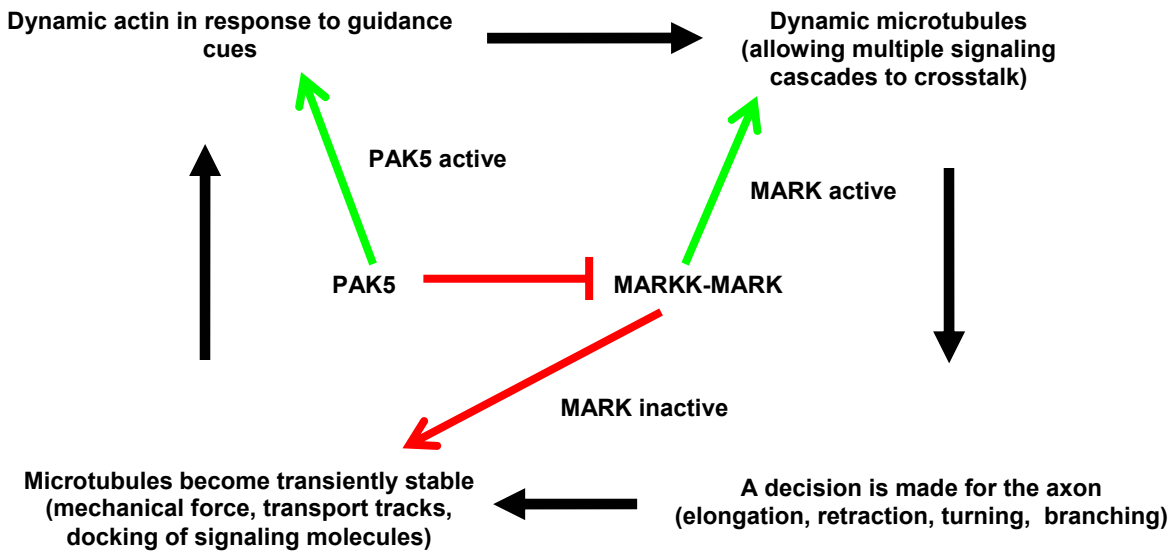

Figure 39: Hypothetical model of the roles of PAK5 and MARKK-MARK cascade in the coordination of the actin and microtubule cytoskeletons in the growth cone.

PAK5 induces dynamic actin and the MARKK-MARK pathway induces dynamic microtubules (green arrows). Microtubule stability is achieved by the inhibitory effect of PAK5 on the MARKK-MARK pathway (red arrows).

In addition to the effects on the cytoskeleton, the complementary effects of PAK5 and MARK isoforms are also connecting several signaling molecules in the growth cones and migrating cells. PAK5 promote F-actin bundles that generate filopodia. This is probably triggered by extracellular cues and involves receptors, G-proteins, and GTP-exchange factors (GEFs) such as PIX. (Buchwald et al., 2001; Chong et al., 2001). Activated Cdc42 can further give rise to the membrane recruitment of polarity-inducing proteins of the Par family (Etienne-Manneville and Hall, 2002; Macara, 2004). This includes Par-3, Par-6, and atypical protein kinase C (αPKC). It involves especially the scaffold protein 14-3-3 (alias Par-5), which also appeared as a MARK2 interaction partner in our yeast two hybrid screen. Furthermore, it is noteworthy that 14-3-3 plays a role in the phosphorylation of tau (Hashiguchi et al., 2000).

An interesting observation is that both MARKK and PAK5 participate in the cellular stress response. MARKK/TAO-1 activates the p38 stress pathway by phosphorylating upstream kinases MEK3 and MEK6 (Hutchison et al., 1998). PAK5 can be activated by MEK6 (Kaur et al., 2005). PAK5 also induces the JNK stress pathway (Dan et al., 2002; Pandey et al., 2002)

and the apoptotic pathway (Cotteret et al., 2003). Consistent with this, the activation of MARK and the phosphorylation of tau are elevated by cellular stress (Jenkins and Johnson, 2000; Schneider et al., 2004). This suggests maybe a link between cellular stress, aging and Azheimer's neurodegeneration.

This work reveals two antagonistic regulations of MARK, a kinase that is hypothetically involved in Alzheimer's disease. One is the activation of MARK by the upstream kinase MARKK to regulate the dynamics of microtubules; the other is the inhibition of MARK by PAK5, a kinase regulating the dynamics of the actin cytoskeleton. Understanding the relationships between these pathways is another challenge in understanding the pathology of Alzheimer's disease and other neurodegenerative diseases.

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6. Appendices

6.1 Abbreviations

Aβ	Amyloid β-peptide
aa	Amino acid
AD	Alzheimer's disease
AID	Autoinhibitory domain
AMP	Adenosine monophosphat
AMPK	AMP-activated protein kinase
aPKC	atypical protein kinase C
APP	Amyloid precursor protein
BAD	Bcl-2/Bcl-XL-Antagonist causing cell death
bp	Base pair
BSA	Bovin serum albumin
CaM Kinase	Ca^{2+}/calmodulin-dependent protein kinase
CDK	Cyclin-dependent kinase
cDNA	Complementary desoxyribonucleic acid
CFP	Cyan fluorescent protein
CHO	Chinese hamster ovary
CNS	Central nervous system
Cool	Cloned out of library
CRIB	Cdc42/Rac-Interactive binding domain
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
ECL	Enhanced Chemiluminescence light
EMK	ELKL motif kinase
ERK	Extracellular signal regulated kinase
FAD	Familial AD
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FTDP-17	Frontotemporal dementia and Parkinsonism linked to chromosome 17
GCK	Germinal center kinase
GEF	Guanine nucleotide exchange factor
GID	GEF interaction domain
G-Protein	GTP/GDP-binding protein
GSK3β	Glycogen synthase kinase 3β
h	hour
HA	Hemagglutinin
Ig	Immunglobulin
JIK	JNK/SAPK-inhibitory kinase
JNK	c-Jun N-terminal kinase
KA1	Kinase-associated domain 1
Kan	Kanamycin
kb	Kilobase
kD	KiloDalton
KFC	Kinase from chicken
KI	Kinase inhibitor

Kin 1/2	Kinase gene product 1/2
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MARK	MAP/ Microtubule-affinity regulating kinase
MARKK	MAP/ Microtubule-affinity regulating kinase kinase
min	Minute
MKK 3/6	Mitogen-activated protein kinase kinase 3/6
mRNA	Messenger ribonucleic acid
MT	Microtubule
MTOC	Microtubule organizing center
NFT	Neurofibrillary tangel
NGF	Nerve growth factor
PAK	p21-activated kinase
Par-1	Partitioning defective-1
PBD	p21-Binding domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK	3-Phosphoinositide dependent kinase
PHF	Paired helical filament
PIX	PAK-interacting exchange factor
PKA	Cyclic AMP-dependent protein kinase A
PP	Phosphatase protein
PS	Presenilin
PSK	Prostate-derived sterile20-like kinase
RNA	Ribonucleic acid
RNAi	RNA interference
s	Second(s)
SAP	Stress-activated protein kinase
sdRNA	Small double stranded RNA
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-polyarcrylamide gel electrophoresis
SH3	Src-homology 3
siRNA	Small interfering RNA
Snf1	Sucrose nonfermenting gene product 1
Ste20	Steril 20
TAE	Tris-Acetate-EDTA buffer
TAO1	Thousand and one-amino acid protein kinase 1
TBST	Tris Buffer Saline containing Tween
TCA	Trichloroacetate
Tm	Basic melting temperature
TRITC	Tetramethylrhodamine isothiocyanate
UBA	Ubiquitin-associated
UV	Ultraviolet
v/v	Volumn pro Volumn
w/v	Weight pro Volumn
wt	Wildtype
YFP	Yellow Fluorescent Protein

6.2 Protein sequence of MARK2

N
1 MSSARTPLPT LNERDTEQPT LGHLDSKPSS KSNMLRGRNS ATSADEQPHI GNYRLLKTIG

61 KGNFAKVKLA RHILTGKEVA VKIIDKTQLN SSSLQKLFRE VRIMKVLNHP NIVKLFEVIE

Catalytic

121 TEKTLYLVME YASGGEVFDY LVAHGRMKEK EARAKFRQIV SAVQYCHQKF IVHRDLKAEN

208

181 LLLDADMNIK IADFGFSNEF TFGNKLDTFC GSPPYAAPEL FQGKKYDGPE VDVWSLGVIL

241 YTLVSGSLPF DGQNLKELRE RVLRGKYRIP FYMSTDCENL LKKFLILNPS KRGTLEQIMK

UBA

301 DRWMNVGHED DELKPYVEPL PDYKDPRRTE LMVSMGYTRE EIQDSLVGQR YNEVMATYLL

361 LGYKSSELEG DTITLKPRPS ADLTNSSAPS PSHKVQRSVS ANPKQRRSSD QAVPAIPTSN

421 SYSKKTQSNN AENKRPEEET GRKASSTAKV PASPLPGLDR KKTTPTPSTN SVLSTSTNRS

Spacer

481 RNSPLLDRAS LGQASIQNGK DSTAPQRVPV ASPSAHNISS SSGAPDRTNF PRGVSSRSTF

541 HAGQLRQVRD QQNLPFGVTP ASPSGHSQGR RGASGSIFSK FTSKFVRRNL NEPESKDRVE

601 TLRPHVVGGG GTDKEKEEFR EAKPRSLRFT WSMKTTSSME PNEMMREIRK VLDANSCQSE

C

661 LHERYMLLCV HGTPGHENFV QWEMEVCKLP RLSLNGVRFK RISGTSMAFK NIASKIANEL

721 KL

Figure A.1: Protein sequence of MARK2 (GenBank accession Nos NP067731) with domain structures.

N is N-terminus, UBA is ubiquitin-associated, C is C-terminus.

6.3 Protein sequence of MARKK

N

1 MPSTNRAGSL KDPEIAELFF KEDPEKLFTD LREIGHGSFG AVYFARDVRT NEVVAIKKMS

61 YSGKQSTEKW QDIIKEVKFL QRIKHPNSIE YKGCYLREHT AWLVMEYCLG SASDLLEVHK

Catalytic

121 KPLQEVEIAA ITHGALQGLA YLHSHTMIHR DIKAGNILLT EPGQVKLADF GSASMASPAN

181 SFVGTPYWMA PEVILAMDEG QYDGKVDVWS LGITCIELAE RKPPLFNMNA MSALYHIAQN

241 ESPTLQSNEW SDYFRNFVDS CLQKIPQDRP TSEELLKHMF VLRERPETVL IDLIQRTKDA

301 VRELDNLQYR KMKKLLFQEA HNGPAVEAQE EEEEQDHGGG RTGTVNSVGS NQSIPSMSIS

SBD

361 ASSQSSSVNS LPDASDDKSE LDMMEGDHTV MSNSSVIHLK PEEENYQEEG DPRTRASAPQ

421 SPPQVSRHKS HYRNREHFAT IRTASLVTRQ MQEHEQDSEL REQMSGYKRM RRQHQKQLMT

481 LENKLKAEMD EHRLRLDKDL ETQRNNFAAE MEKLIKKHQA SMEKEAKVMA NEEKKFQQHI

Spacer

541 QAQQKKELNS FLESQKREYK LRKEQLKEEL NENQSTPKKE KQEWLSKQKE NIQHFQAEEE

601 ANLLRRQRQY LELECRRFKR RMLLGRHNLE QDLVREELNK RQTQKDLEHA MLLRQHESMQ

661 ELEFRHLNTI QKMRCELIRL QHQTELTNQL EYNKRREREL RRKHVMEVRQ QPKSLKSKEL

721 QIKKQFQDTC KIQTRQYKAL RNHLLETTPK SEHKAVLKRL KEEQTRKLAI LAEQYDHSIN

781 EMLSTQALRL DEAQEAECQV LKMQLQQELE LLNAYQSKIK MQAEAQHDRE LRELEQRVSL

Tail

841 RRALLEQKIE EEMLALQNER TERIRSLLER QAREIEAFDS ESMRLGFSNM VLSNLSPEAF

901 SHSYPGASSW SHNPTGGSGP HWGHPMGGTP QAWGHPMQGG PQPWGHPSGP MQGVPRGSSI

961 GVRNSPQALR RTASGGRTEQ GMSRSTSVTS QISNGSHMSY T

Figure A.2: Protein sequence of MARKK (GenBank accession Nos AF084205) with domain structures.

N is N-terminus, SBD is substrate binding domain

6.4 Protein sequence of PAK5

PBD

1 MFGKKKKKIE ISGPSNFEHR VHTGFDPQEQ KFTGLPQQWH SLLADTANRP KPMVDPSCIT

61 PIQLAPMKTI VRGNKPCKET SINGLLEDFD NISVTRSNSL RKESPPTPDQ GASSHGPGHA

AID

121 EENGFITFSQ YSSESDTTAD YTTEKYREKS LYGDDLDPYY RGSHAAKQNG HVMKMKHGEA

181 YYSEVKPLKS DFARFSADYH SHLDSLSKPS EYSDLKWEYQ RASSSSPLDY SFQFTPSRTA

241 GTSGCSKESL AYSESEWGPS LDDYDRRPKS SYLNQTSPQP TMRQRSRSGS GLQEPMMPFG

301 ASAFKTHPQG HSYNSYTYPR LSEPTMCIPK VDYDRAQMVL SPPLSGSDTY PRGPAKLPQS

361 QSKSGYSSSS HQYPSGYHKA TLYHHPSLQS SSQYISTASY LSSLSLSSST YPPPSWGSSS

421 DQQPSRVSHE QFRAALQLVV SPGDPREYLA NFIKIGEGST GIVCIATEKH TGKQVAVKKM

481 DLRKQQRREL LFNEVVIMRD YHHDNVVDMY SSYLVGDELW VVMEFLEGGA LTDIVTHTRM

Catalytic

541 NEEQIATVCL SVLRALSYLH NQGVIHRDIK SDSILLTSDG RIKLSDFGFC AQVSKEVPKR

601 KSLVGTPYWM APEVISRLPY GTEVDIWSLG IMVIEMIDGE PPYFNEPPLQ AMRRIRDSLP

661 PRVKDLHKVS SVLRGFLDLM LVREPSQRAT AQELLGHPFL KLAGPPSCIV PLMRQYRHH

Figure A.3: Protein sequence of PAK5 (GenBank accession Nos AB040812) with domain structures.

PBD is p21-binding domain, AID is autoinhibitory domain

6.5 List of the figures

6.6 Curriculum Vitae

Personal data: Xiaoyu Li
Born on 29.10.1976, in Shanghai
Single

University education:

09.1995 ~ 07.1999 Department of Biological Science and Technology,
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09.1999 ~ 07.2001 Department of Biological Science and Technology,
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Master's Degree of Science in Physiology.
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09.2001~ 02.2006 PhD-student,
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List of Publication:

Matenia, D., Griesshaber, B.(Co-first author), Li, X.Y., Thiessen, A., Johne, C., Jiao, J., Mandelkow, E., and Mandelkow, E.M. (2005) PAK5 Kinase Is an Inhibitor of MARK/Par-1, Which Leads to Stable Microtubules and Dynamic Actin. *Mol. Biol. Cell* **16(9),** 4410-4422.

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Zhou, X.R., Li, X.Y., Qi, W., Xu, X.G. and Zhu, J.Q. (1999) Effects of convulsant drugs on 3H-GABA binding with GABAA receptor in different areas of brain in mice. *J. Suzhou Railway Teachers College* **16(4),** 51-54 (in Chinese).

Zhou, X.R., Li, X.Y., Xin, X.D. and Zhu, J.Q. (1999) Effects of ethanol on 3H-GABA binding with GABAA receptor in different areas of brain in mice. *J. Nanjing University* **35(5),** 532-537 (in Chinese).

6.7 Declaration (Erklärung)

I declare that I have carried out this thesis by myself and have not used external help except where explicitly indicated.

This thesis was not submitted to any other university.

I did not make any earlier attempt to submit this work as a doctoral thesis.

Hamburg, 24, Jan. 2006

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfaßt, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt und die den verwendeten Werken wörtlich order inhaltlich entnommenen Stellen asl solche kenntlich gemacht habe.

Ferner versichere ich, daß ich diese Dissertation noch an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lassen.

Hiermit erkläre ich auch, daß ich keine anderen früheren Versuche gemacht hab, die Arbeit zur Promotion einzureichen.

Hamburg, 24, Jan.2006

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