

# Signaling from MARK to Tau: Regulation, Cytoskeletal Crosstalk, and Pathological Phosphorylation

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## Key Words

Actin · Microtubules · p21-activated kinase · Phosphorylation of tau

## Abstract

The hyperphosphorylation of tau is an early step in the degeneration of neurons in Alzheimer's disease and other tauopathies. Of particular importance is the phosphorylation of tau in the repeat domain which detaches tau from microtubules. This makes microtubules dynamic for their role in differentiation and neurite outgrowth, and it controls the level of tau on the microtubule surface which keeps the tracks clear for axonal transport. However, the detachment of tau from microtubules can also initiate the reactions that lead to pathological aggregation into neurofibrillary tangles. Phosphorylation of tau in the repeat domain is achieved by the kinase MARK/Par-1, a member of the calcium/calmodulin-dependent protein kinase group of kinases. In this report, we focus on the modes of MARK regulation. MARK contains several domains which offer multiple ways of regulation by posttranslational modification (e.g. phosphorylation), interactions with scaffolding proteins and subcellular targeting (e.g. 14-3-3), and interactions with other proteins. We consider in particular the interactions between MARK and other kinases, notably MARKK/TAO-1 and PAK5. MARKK (a member of the Ste20 family of kinases) activates MARK by phosphorylating it at a critical threonine residue within the activation loop. Activated MARK in turn phosphorylates tau, causes its detachment from microtubules and renders them

labile. PAK5 inactivates MARK, not by phosphorylation, but by binding to the catalytic domain. PAK5 contributes to microtubule stability by preventing the MARK-induced phosphorylation of tau; conversely, PAK5 contributes to actin dynamics, presumably through the activation of cofilin, an F-actin severing protein. Thus, MARK and its regulators MARKK and PAK5 appear to mediate the crosstalk between the actin and microtubule cytoskeleton in an antagonistic fashion.

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## Introduction

The function of tau in neurons is to stabilize microtubules and to ensure axonal transport along microtubules. In degenerating neurons, tau is hyperphosphorylated, detaches from microtubules, and aggregates into pathological filaments (paired helical and straight filaments). The detachment from microtubules is achieved most efficiently by phosphorylating the KXGS motifs in the microtubule-binding domain of tau. Enhanced phosphorylation at these sites occurs early in Alzheimer's disease (AD) [1]. A search for the responsible kinase led to the identification of microtubule-associated protein (MAP)/microtubule affinity-regulating kinase (MARK) kinases, a subfamily within the calcium/calmodulin-dependent protein kinase group of kinases [2]. These kinases are related to the partitioning defective mutant 1 (Par-1) kinases in *Caenorhabditis elegans* and *Drosophila melano-*

*gaster* which are involved in the determination of embryonic polarity [for reviews, see 3, 4], and indeed the activity of MARK/Par-1 kinases is important for neuronal polarity as well [5].

MARK kinases consist of an N-terminal catalytic domain, followed by the ubiquitin-associated (UBA) domain, spacer, and tail domains (kinase-associated, KA, domain). The size of MARK and its multidomain composition suggests that regulation could take place on several levels [6]. Indeed, our recent elucidation of the X-ray structure of several domains of MARK2 [7] suggests at least four possibilities: phosphorylation of the activation loop in the catalytic domain, binding of regulatory proteins to the 'common docking' (CD) domain, regulation by ubiquitin on the UBA domain, and dimerization. Further regulatory options, derived from studies of *Drosophila* Par-1, include the interaction with 14-3-3 (alias Par-5, a scaffolding protein) [8], phosphorylation in the spacer domain by atypical protein kinase C (aPKC) and subsequent binding of 14-3-3 [9, 10], and the interaction between the N- and C-terminal tails [11]. To explore these possibilities, we embarked on a program to identify regulatory partners of MARK. Here, we focus on two interaction partners: (1) the activation of MARK by the upstream kinase MARKK which phosphorylates MARK in the regulatory loop of the catalytic domain, (2) the inhibition of MARK by p21-activated kinase 5 (PAK5) and the ensuing effects on the actin/microtubule cytoskeleton.

MARKK belongs to the Ste20 family within the STE group of kinases which can be divided into the PAKs (with an N-terminal p21-binding domain and a C-terminal kinase domain) and the germinal center kinases (GCKs; N-terminal kinase domain, no p21-binding domain). About 31 Ste20-related kinases are known (2 PAK, 8 GCK subfamilies, [12]). The kinases have various effects, including the regulation of apoptosis and the rearrangement of the cytoskeleton, and many play a role in the activation of MAP kinases. Regulation of Ste20 kinases is achieved through the Ras family of GTPases, additional protein kinases, adapter proteins coupled to cytokine receptors, and via dimerization or association with inhibitors combined with autophosphorylation after stimulation [13]. MARKK/thousand-and-one-aminoacid kinase 1 (TAO-1) is part of the kinase subfamily GCK-VIII, together with the Prostate-derived ste20-like kinase (PSK, alias TAO-2) [14] and c-Jun activating kinase (JNK)-inhibiting kinase (JIK, alias TAO-3) [15]. The sequence of MARKK and its two relatives in humans [12] is unusually long, suggesting several functions besides the kinase activity. The active complex from the brain has a mass of ~330 kDa which would

be compatible with a complex of 2–3 MARKK molecules, or with a complex between MARKK, scaffolding proteins, and other cofactors. MARKK contains predicted amphipathic helices in the spacer and tail domains which would favor protein-protein interactions.

The kinase PSK/TAO-2 has been reported to be a regulator of the stress-activated MAP kinase pathway [16], but this is not the case for MARKK/TAO-1, although it has the ability to phosphorylate mitogen-activated protein kinase kinase 3 (MKK3) and MKK6, the activators of p38 stress-activated kinases in vitro [17]. Endogenous MKK3 can be copurified with transfected TAO-1 and TAO-2 from Sf9 cells [14, 17]. This involves the binding of the substrate-binding domain of TAO to the N-terminal header domain of its substrate MKK3 [14]. At present, the relationship between MAP kinase signaling and MARK signaling is unclear, but it is interesting to note that cellular stress can lead to the phosphorylation of KXGS motifs in tau through the activation of MARK [18]. Furthermore, the transcripts of MARK1 and MKK3 are upregulated after differentiation of PC12 cells [19, and our results]. Thus far, upstream effectors or scaffolding proteins for MARKK are unknown.

The PAKs are members of Rac/Cdc42-associated Ser/Thr protein kinases, characterized by a conserved amino-terminal p21-binding domain and a carboxyl-terminal kinase domain. Six human PAKs, which can be classified into two distinct subfamilies, have been identified. Group I PAKs (PAK 1–3) differ significantly in their structural organization and regulation from the more recently discovered group II PAKs (PAK 4–6) [20; reviewed in 21–24]. They regulate death and survival signaling, cell-cycle progression, and furthermore they seem to play a key role in coordinating the dynamics of the actin and microtubule cytoskeletons. PAK5 contains 719 residues, binds Cdc42 and occurs mainly in the brain [25, 26]. It contains a p21-binding domain (PBD) around residues 9–30, and an inhibitory KI motif around residues 120–133 [27]. The kinase domain extends from about ~453 to 700. Ser602 in the activation loop must be phosphorylated for activity. PAK5 can induce filopodia, neurite outgrowth and dendritic spines [25, 28]. It has a mainly cytosolic distribution where it can activate the JNK kinase pathway [25, 26].

## Materials and Methods

The experimental procedures have been described in previous studies [5, 29–31]. Briefly, plasmids encoding MARK2, PAK5, and active or inactive mutants were generated by standard cloning techniques [5, 31]. Yeast two-hybrid screening and assays were

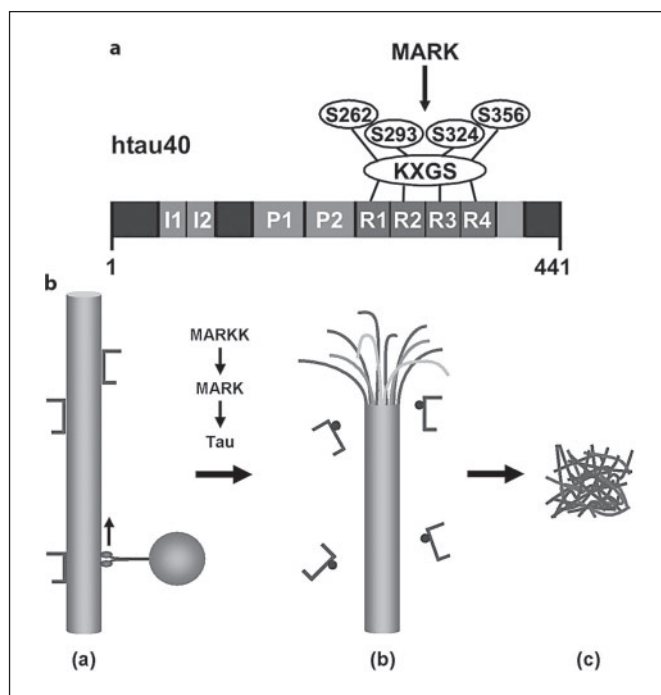
performed according to the manufacturer's instructions (Clontech, Yeast Protocols Handbook). Cell culture and transfection were performed with HEK293, Chinese hamster ovary cells (CHO), LAN5 and Sf9 cells following standard protocols. The preparations of kinases and the kinase activity assays have been described in previous studies [2, 29, 31].

## Results and Discussion

The initial motivation for our investigations stems from the involvement of tau protein in AD. The abnormal aggregation of this protein correlates well with the clinical stages of AD, Braak stages 1–6 [32], and an analogous correlation is found in other brain diseases with tauopathy, notably frontotemporal dementia and parkinsonism linked to chromosome 17 [for review, see 33]. The major changes of tau in AD include not only abnormal aggregation, but also abnormal hyperphosphorylation and detachment from microtubules. Microtubules are the physiological partners of tau in neuronal axons. Microtubules are necessary for axonal stability, growth cone advance, and as tracks for axonal transport of vesicles and organelles, mediated by motor proteins. In principle, the physiological regulation of the tau-microtubule interaction (by phosphorylation) can affect microtubule dynamics and microtubule-based traffic. While bound tau stabilizes microtubules, detached tau allows them to become dynamic and disassemble. The dynamics is necessary for restructuring the cytoskeleton during changes of cell shape; for example, microtubules must be dynamic, at least temporarily, for neurite outgrowth and differentiation. On the other hand, bound tau not only stabilizes microtubules but also has the potential of inhibiting motor proteins because tau can occlude binding sites on the microtubule surface. In this situation, the removal of excess tau from microtubules can clear the way for axonal traffic. In both contexts, the MARKK-MARK cascade is operational [5, 29, 30, 34]. If it becomes overactive, the detached cytosolic tau protein is free for other interactions, including those that lead to abnormal tau aggregates (paired helical filaments) which in turn also obstruct the cell interior.

### MARK

Since the interaction between tau and microtubules is regulated by phosphorylation, a great deal of research in the field has been devoted to the identification of kinases that phosphorylate tau. This problem is complicated by the fact that tau is a natively unfolded protein with a large number of phosphorylatable residues (mostly Ser or Thr)



**Fig. 1.** Tau, MARK target sites, and effects of phosphorylation. **a** The bar diagram illustrates the domains of tau (2N4R isoform, the largest in human CNS, 441 residues). The inserts near the N-terminus (N1, N2) and repeat R2 may be absent due to alternative splicing, creating the 6 main isoforms in the human CNS. The repeat domain (R1–R4, containing 3 or 4 repeats) and the flanking regions constitute the microtubule (MT)-binding domain. Each repeat contains a KXGS motif (serines 262, 293, 324, 356), which is a target site of MARK kinases. This type of phosphorylation efficiently detaches tau (or other related MAPs) from microtubules, which results in microtubule destabilization. **b** Diagram of signaling pathway from MARKK and MARK through tau to microtubules, resulting in microtubule breakdown, tau detachment and abnormal aggregation. The microtubules serve as tracks for the transport of vesicles and are stabilized by tau protein (a). Upon phosphorylation of the tau protein by the MARKK/MARK cascade, tau detaches from the microtubule resulting in its disassembly (b). The unbound tau protein then tends to aggregate into paired helical filaments of AD (c).

which can be targets of many kinases [for reviews, see 35, 36]. It is therefore difficult to judge which combination of kinases and phosphorylation sites is responsible for the critical changes in tau activity in neurons. We have sought to simplify the issue by concentrating on phosphorylation sites and kinases that have the most pronounced effect on the tau-microtubule interaction. A search for the important phosphorylation sites revealed the 'KXGS' motifs in the repeat domain of tau, containing serines 262, 293, 324, and 356 (fig. 1a). In fact, phosphorylation

of Ser262 alone already accounts for a large decrease in the affinity [37]. This finding prompted a search for the responsible kinases which resulted in the identification of the MARK family of kinases, MARK1–4 [2]. Members of this kinase are ubiquitous but enriched in the brain. Activation or expression of the kinase in cells indeed leads to the phosphorylation of tau at the KXGS motifs, detachment from microtubules, and microtubule breakdown (fig. 1b, 2b) [5]. This type of phosphorylation can be detected by specific phosphorylation-dependent antibodies such as 12E8 [38]. The biochemical analysis of phospho-tau from AD brain shows that the KXGS motifs are indeed among the detectable phosphorylation sites [39], and the histological analysis shows that MARK is present in neurofibrillary tangles, and that the MARK-type phosphorylation of tau occurs early in the disease process [1, 40].

The identification of MARK as a tau kinase raised the question of its regulation. Judging by its kinase domain, MARK is part of the calcium/calmodulin-dependent protein kinase group of kinases in the human kinome [12]. The various MARK isoforms and splice variants appear to have multiple functions; in *C. elegans* and *D. melanogaster* its homologues (termed Par-1) [3, 41] are responsible for embryonic polarity, and the establishment and maintenance of polarity may be one of their functions in humans as well [42]. As shown in figure 2, MARK is a large multidomain protein which suggests several modes of regulation. The initial identification of MARK2 revealed the presence of phosphorylated residues in the ‘activation loop’ of the kinase catalytic domain. This is a common feature of many kinases and strongly suggests that there must be upstream activating kinases [for reviews, see 6, 43].

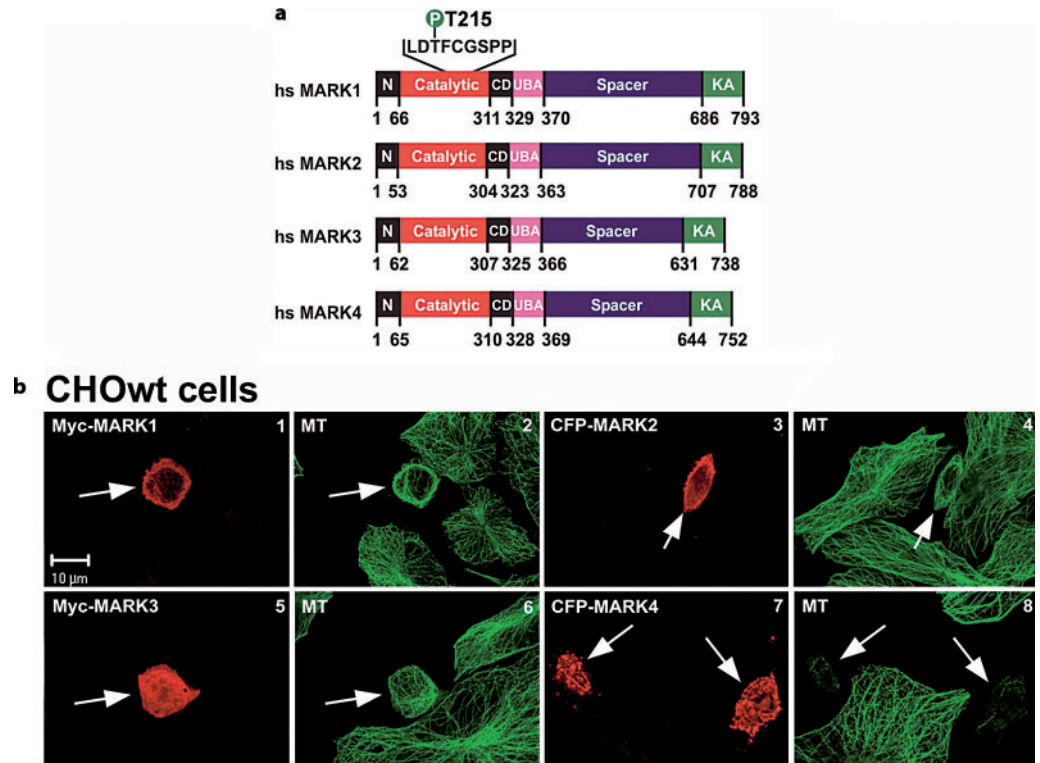
#### MARKK

We therefore embarked on a search for kinases that would phosphorylate the activation loop of MARK2 (as a representative of all MARKs since their catalytic domains are highly homologous, >98%). This search turned out to be elusive because the activating kinase is embedded in a 330-kDa protein complex that is easily disrupted during purification. However, the kinase (termed MARKK for MARK-activating kinase) turned out to be a member of the GCK-VIII subfamily of the Ste20 family within the STE group of kinases [32] (fig. 3). This, too, is a large multidomain kinase, suggestive of multiple pathways of regulation. It is highly related to the kinase TAO-1 which is involved in the MKK3/6-p38-signaling cascade [17, 44]. Other members of this subfamily include

PSK/TAO2 and JIK which have similar kinase domains and spacers (99 and 97% homology) but differ in their other domains [14, 15, 45]. An intriguing aspect was the possibility of dual regulation at the activation loop of MARK. With most kinases, activation by phosphorylation requires only one phosphorylated residue, but MARK2 peptides isolated from brain were phosphorylated at two sites, Thr208 and Ser212. These positions are reminiscent of the two sites required for the activity of the MAP kinase family. A further search showed that this analogy does not hold for MARK. In this case, only one site in the activation loop (Thr208) is phosphorylated by MARKK and leads to activation. The other site must remain unphosphorylated for activity but may possibly be phosphorylated by some as yet unknown inhibitory kinase. Mutations of Ser212 into Ala or Glu both lead to an inactive kinase.

An important question about the proposed MARKK-MARK-tau cascade was whether it is operational in cells, and whether it is specific. This was demonstrated by testing neuronal and non-neuronal cell models with constitutively active or inactive variants of the kinases. Overexpression of MARKK in CHOwt cells leads to microtubule breakdown and shrinkage of the cells (fig. 3b 1–3). This phenotype is similar to that of overexpressed MARK itself (fig. 2b) and can be suppressed by stabilization of the microtubules by taxol (fig. 3b 4–6) or overexpression of tau. However, when MARKK and MARK are expressed together, the stabilizing effect of the additional tau can be overcome (fig. 3c). The results showed that indeed the activation of MARKK leads to the activation of MARK and hence to the phosphorylation of tau at the KXGS motifs. But the KXGS motifs in tau could not be phosphorylated when MARK was inactivated (even when MARKK was active), and consequently no destabilization of microtubules was observed [5, 29]. The function of the cascade is important for differentiation and neurite outgrowth because this requires dynamic microtubules. Conversely, if one inactivates the cascade, for example by transfecting with a kinase-dead mutant of MARK, by changing the KXGS into KXGA motifs in tau, or by silencing MARKK with siRNA, then differentiation is no longer possible. This is demonstrated in figure 3d where PC12 cells differentiate readily upon nerve growth factor (NGF) treatment; however, when MARKK is knocked down by siRNA the cells can no longer differentiate [5, 29].

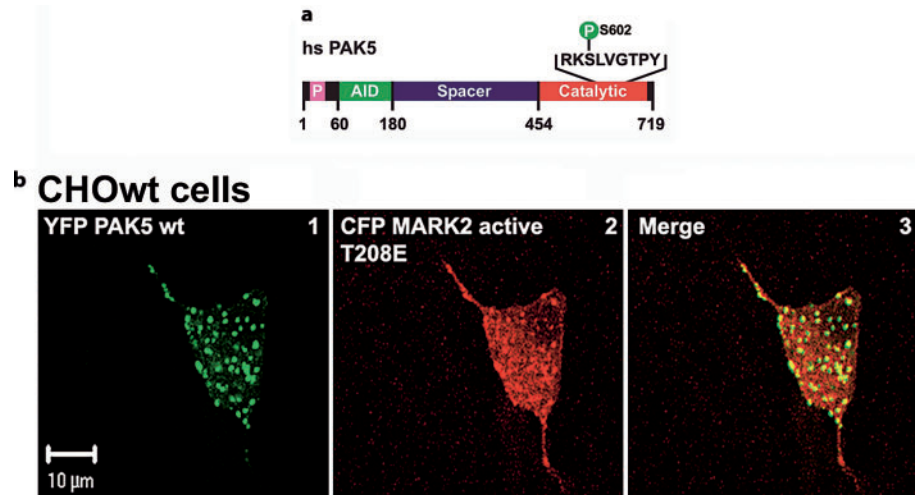
The above results corroborate a kinase cascade pathway that leads to the phosphorylation of tau in the repeat domain, but this is not sufficient to explain why the cascade might be overactive in AD. In particular, we note

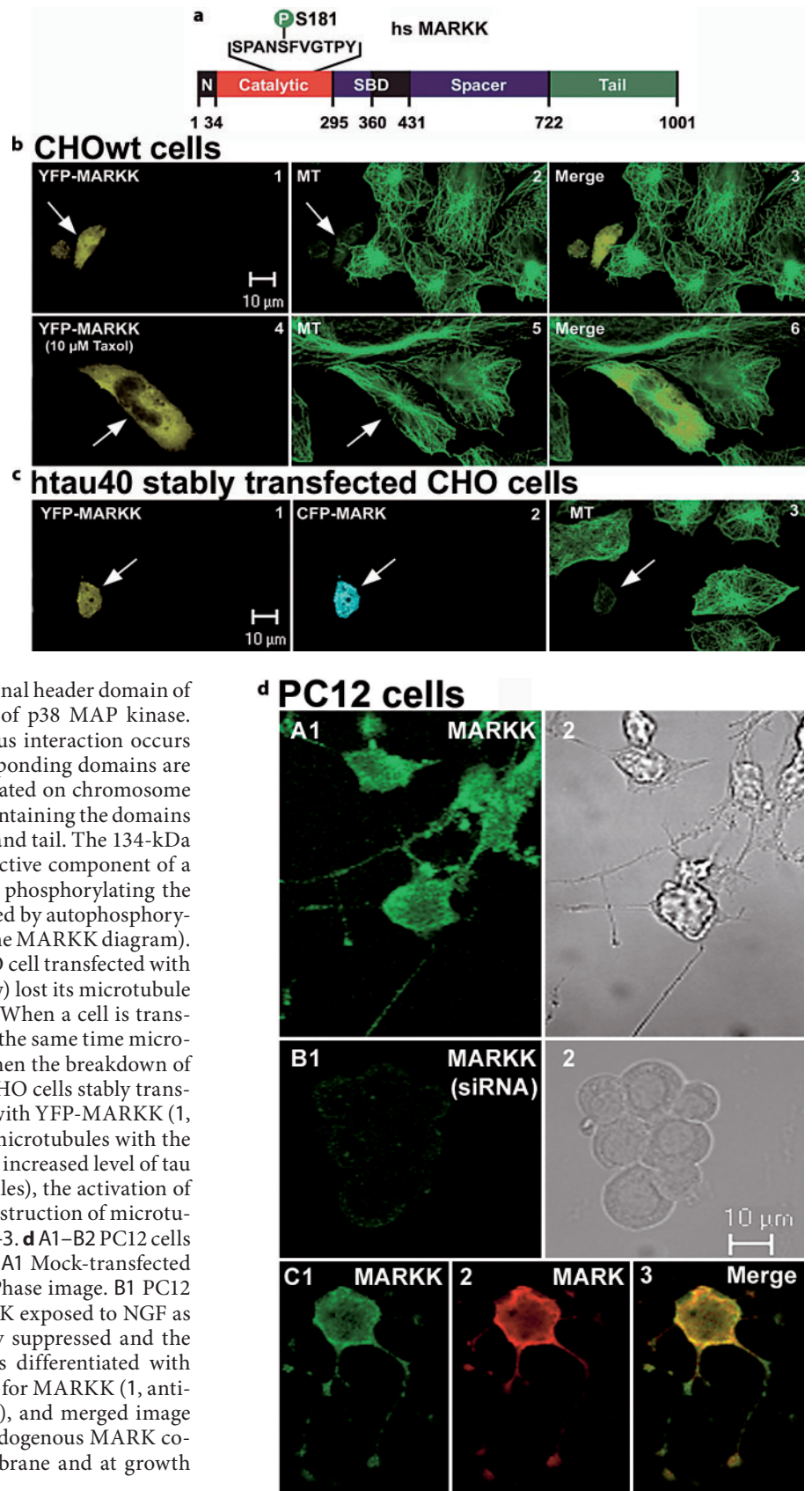


**Fig. 2.** Diagram of MARK/PAR-1 kinases and their effects in cells. **a** The human genome contains 4 MARK genes located on chromosomes 1, 11, 14, and 19. Additional variants are generated by alternative splicing [50, 51]. Sequence analysis reveals 5 basic domains: header, catalytic, UBA, spacer, and tail (also known as KA domain). The kinase is closely related to Par-1 kinases which play a role in the development of embryonic polarity in *C. elegans* and *D. melanogaster* [3]. All MARKs can be phosphorylated by MARKK at a conserved threonine in the catalytic domain (corresponding to T208 in MARK2), leading to activation [29]. The

catalytic domain can bind to PAK5, leading to inhibition [31]. Domains known by structural analysis: catalytic and UBA domain by X-ray crystallography [7], KA domain by NMR spectroscopy [52]. **b** CHO cells expressing the different MARK isoforms MARK1, MARK2, MARK3, and MARK4. The cells were transiently transfected with each of the four MARK isoforms (arrows), leading to the phosphorylation of endogenous MAPs (MAP4) and breakdown of the microtubule network (shown in green, visible best in 8). The severity of this effect is dependent on the expression level of the MARKs.

**Fig. 4.** PAK5. **a** Diagram of PAK5. PAK5 is a member of the Ste20 family of kinases and belongs to the subfamily of PAKs, group II (comprising PAK4–6). It contains the domains header, P = PBD (including a variant of the CRIB motif – but note that this is a matter of debate in the case of PAK5), auto-inhibitory domain (AID), spacer, and catalytic domain. The auto-phosphorylation site at S602 in the activation loop is crucial for activity [25, 27]. **b** Colocalization of YFP-PAK5wt (green) with transfected CFP-MARK2 (red). 1–3 Cotransfection of PAK5wt and active MARK2(T208E) shows colocalization of both kinases on vesicles and a diffuse background of MARK2(T208E).





**Fig. 3.** MARKK/TAO-1. **a** Diagram of MARKK/TAO-1. MARKK is a member of the GCK-VIII subfamily of the Ste20 kinases. Related members of this subfamily include TAO-1, TAO-2, and JIK [14, 15, 17, 45]. These kinases contain an N-terminal header domain, catalytic domain, substrate-binding domain (SBD), spacer domain, and tail domain. Extended coiled-coil sequences are predicted in the spacer and tail domains (S430-A630, K730-F900). The substrate-binding domain of the related PSK/TAO-2 binds to the N-terminal header domain of its substrate MKK3, the kinase upstream of p38 MAP kinase. However, it is unclear whether an analogous interaction occurs with MARKK and MARK since the corresponding domains are poorly conserved. The MARKK gene is located on chromosome 17. MARKK is also a multidomain kinase containing the domains header, catalytic, substrate binding, spacer and tail. The 134-kDa protein was identified as the catalytically active component of a 330-kDa complex that activates MARK by phosphorylating the catalytic loop. MARKK itself can be activated by autophosphorylation in the catalytic loop (residue S181 in the MARKK diagram). **b** Effect of MARKK on CHO cells. 1–6 CHO cell transfected with YFP-MARKK. 2 The transfected cell (arrow) lost its microtubule network, rounded up and appears smaller. When a cell is transfected with YFP-MARKK (4, arrow) and at the same time microtubules are stabilized by 10  $\mu$ M taxol (5), then the breakdown of microtubules by MARKK is prevented. **c** CHO cells stably transfected with tau (htau40) and cotransfected with YFP-MARKK (1, arrow) and CFP-MARK (2) or staining of microtubules with the antibody YL1/2 (3). Note that in spite of the increased level of tau (which would normally stabilize microtubules), the activation of the MARKK-MARK cascade leads to the destruction of microtubules in the cotransfected cell, similar to **b** 1–3. **d** A1–B2 PC12 cells differentiated with NGF (48 h, 100 ng/ml). A1 Mock-transfected cell, stained for endogenous MARKK. A2 Phase image. B1 PC12 cells transfected with RNAi against MARKK exposed to NGF as above. B2 Phase image. MARKK is largely suppressed and the cells cannot differentiate. C1–3 PC12 cells differentiated with NGF (72 h, 100 ng/ml) and immunostained for MARKK (1, antibody TAO-1), MARK (2, antibody SA2118), and merged image (3). Note that endogenous MARKK and endogenous MARK colocalize, notably beneath the plasma membrane and at growth cones.

that MARKK itself must also be activated by phosphorylation in the activation loop, indicating that there must be further upstream kinases. In addition, it is not known which physiological or pathological signals trigger the cascade. The MARK-type phosphorylation of tau is elevated in fetal tau, and indeed MARK is elevated in fetal brain tissue [2, 18, 38]. A possible rationale is that at this stage, prior to massive neuronal outgrowth, tau must be present but only loosely bound to microtubules, ready to stabilize microtubules once the neurites grow out. In this function, the phosphorylation of tau would be in concert with the splicing pattern which keeps the smallest tau isoform (0N3R) predominant before the stage of neurite outgrowth because this isoform has only three repeats and shows the weakest binding and stabilization of microtubules [46]. Conversely, microtubules in adult brain would be stabilized by the higher molecular weight splice isoforms of 4-repeat tau, and by shutting down the activity of MARK.

#### PAK5

The activation cascade described above concerns phosphorylation in the strict sense; however, considering the multidomain structure of the interacting components we take the possibility of other types of regulation of MARK into account which would affect the phosphorylation of tau. Thus, yeast two-hybrid analysis was used to search for interaction partners of MARK. We took the full sequence of MARK2 and screened a human fetal cDNA library. Inactivating mutations (T208A/S212A) were introduced because this was known to stabilize the interaction with partners. The search revealed a number of positive clones coding for isoforms of the 14-3-3 protein, and clones coding for PAK5. This kinase was particularly interesting since it is a member of the mammalian PAK II subfamily and is predominantly expressed in the brain [25, 26]. As in the previous cases, PAK5 is a large multidomain kinase (fig. 4). Its catalytic domain is located not near the N-terminus but in the C-terminus and is preceded by a GTPase-binding domain (PBD).

A further interaction assay with different truncation mutants of MARK2 and PAK5 showed that the interaction between MARK2 and PAK5 was based on the catalytic domains. This initially suggested some regulation by phosphorylation. However, surprisingly, PAK5 interacted with MARK2 independently of its state of phosphorylation. The subsequent analysis, using a tau-derived peptide to assess the activity of MARK2, showed that the binding of PAK5 alone is sufficient to inhibit MARK2.

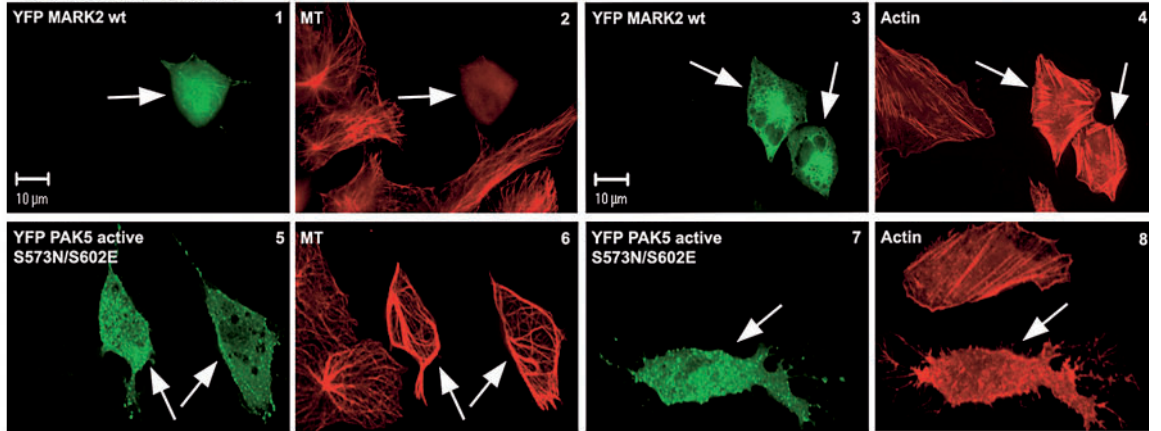
Cotransfection of PAK5 and MARK2 into CHO cells followed by analysis with fluorescence microscopy or immunofluorescence showed that MARK2 and PAK5 colocalize and have a vesicular distribution. To identify the nature of the vesicles carrying PAK5, we labeled the cells with different markers. Partial colocalization of transfected PAK5 and MARK2 was observed with vesicles carrying adaptor protein (AP) complexes of the AP1/2 family. More specifically, in a subcellular fractionation experiment the vesicles carrying MARK2 and PAK5 were identified as part of the trans-Golgi network. PAK5 and MARK2 occurred in the same fractions as  $\beta$ 1/ $\beta$ 2- and  $\gamma$ -adaptin, the subunits of the AP1 complex.

Immunofluorescence experiments using CHO cells show that active or inactive PAK5 eliminates the effect of MARK2 on the cytoskeleton (i.e. the destabilization of microtubules; fig. 5). Figure 5b shows that if constitutively active PAK5 (S573N/S602E) is coexpressed with constitutively active MARK2(T208E) the microtubule network is protected (fig. 5b 1–3) while actin stress fibers and focal adhesions are dissolved, which correlates with the emergence of filopodia (fig. 5b 4–6). A similar effect of MARK2 inhibition and microtubule preservation is obtained by coexpressing inactive PAK5 (S602M/T606M) with active MARK2(T208E). However, in this case the actin stress fibers are not rendered dynamic, and consequently focal adhesions are preserved and filopodia do not evolve (data not shown). This experiment shows that active PAK5(S573N/S602E) has two independent effects on the cytoskeleton. First, it stabilizes microtubules by binding and inhibiting MARK2, and second, it makes actin dynamic by dissolving stress fibers and focal adhesions and inducing the formation of filopodia. Inactive PAK5(S602M/T606M) only shows the first effect (on microtubules), but the second effect is absent because it would require PAK5 activity. To check the inhibition of MARK2 in cells, the ability of PAK5 to suppress the phosphorylation of tau by MARK2 was tested using htau40 stably transfected CHO cells (fig. 5c). The data clearly confirm that the PAK5 acts as a MARK2 inhibitor.

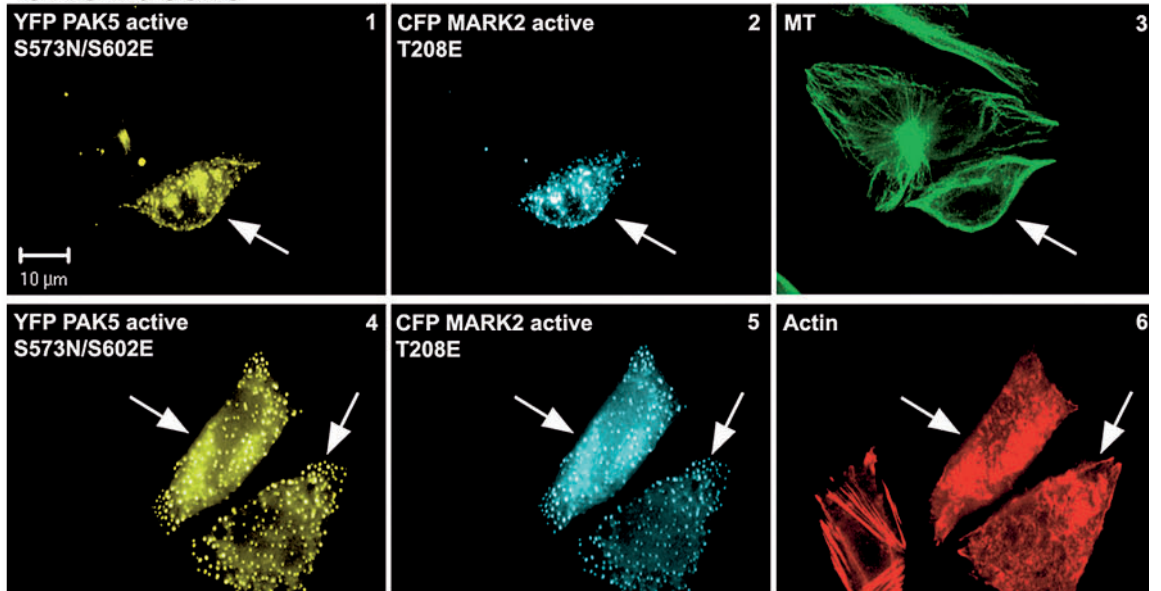
#### *Outlook: Modes of MARK Regulation*

The kinase MARK is regulated by several different mechanisms (fig. 6). First, the activity of the kinase is increased through phosphorylation by MARKK at the activation loop. This is a common regulatory mechanism for serine/threonine kinases [6, 43]. Furthermore, the activity is modulated by interaction with other proteins. The Ste20-kinase PAK5 can bind to the catalytic domain of MARK, which results in inhibition [31]. MARK can

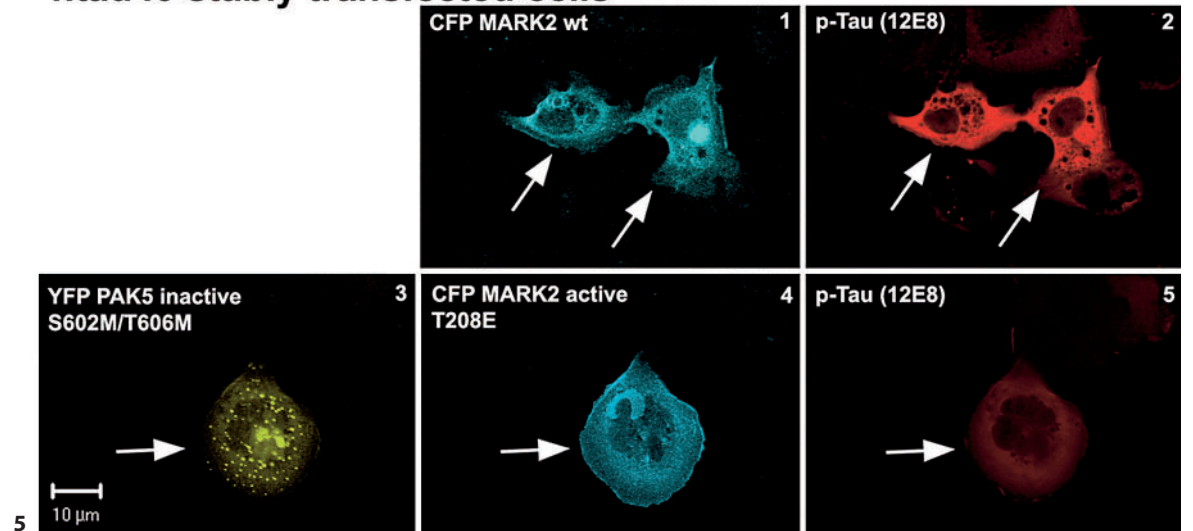
**a CHOwt cells**



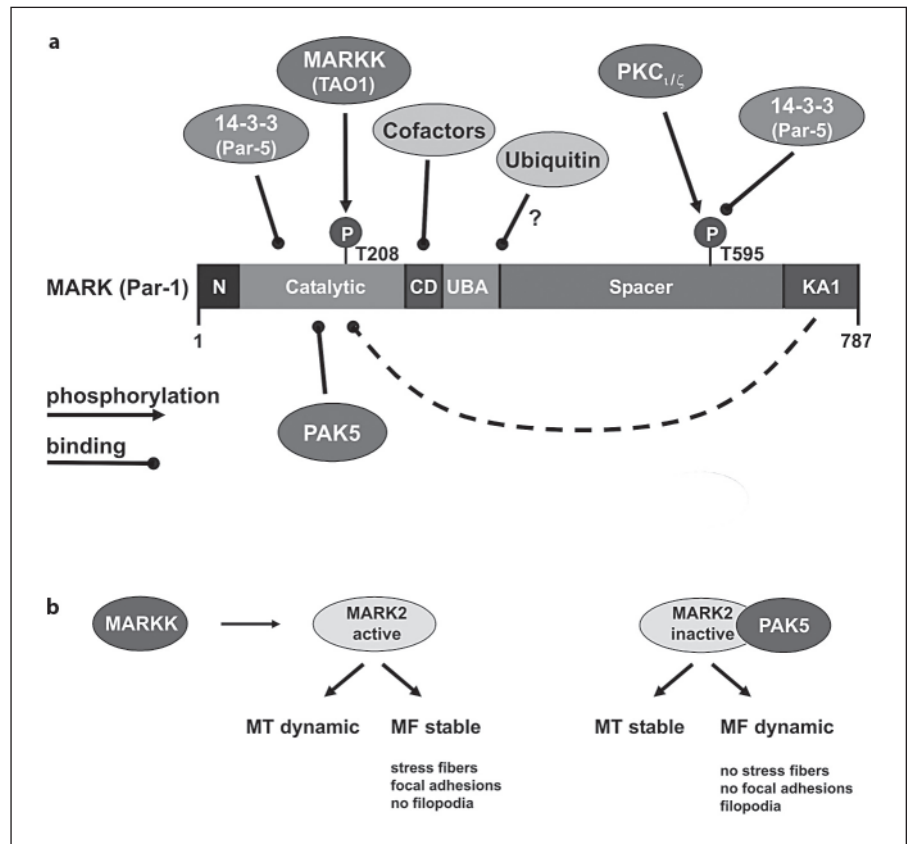
**b CHOwt cells**



**c htau40 stably transfected cells**



**Fig. 5.** Crosstalk between MARK and PAK5 in the regulation of the cytoskeleton. The actin and microtubule cytoskeleton networks in the cell often fulfill complementary functions. Each system is regulated by their set of associated proteins, posttranslational modifications (e.g. by kinases), severing or crosslinking proteins, but there is also a crosstalk which often results in complementary outcomes: When microtubules become unstable, actin fibers become stable (e.g. stress fibers), and vice versa. This can be achieved, for example, by microtubule end-binding proteins which sense the MT dynamics and signal (via GTP-exchange factors or GTPase-activating proteins) to small G-proteins (Rho, Rac) that regulate the actin network [for review, see 53]. A related principle is illustrated here. The activity of MARK tends to make microtubules labile and dynamic which correlates with an increased stability of the actin network. However, MARK can be inhibited by PAK5 (whose normal role is to make the actin network more dynamic), hence microtubules are stabilized. **a** Effects of PAK5 and MARK2 on the stability of microtubules and actin filament networks. CHO cells transfected with YFP-PAK5 and YFP-MARK2 (green) were cultured for 16 h, fixed, and costained with the YL1/2 antibody for tubulin and fluorescently labeled (TRITC) secondary antibody (MT staining, red). Actin was stained using rhodamine-conjugated phalloidin (red). Transfected cells are indicated by arrows. In cells expressing wild-type YFP-MARK2 (1, 3), microtubules disappear (2, arrow) and actin stress fibers are stabilized (4). In contrast, constitutively active PAK5 (5–8) stabilizes MT (6), but the actin stress fibers are dissolved (8). **b** PAK5 inhibits the MARK2 effect on the microtubule and actin networks. CHO cells coexpressing the constitutively active form of PAK5 (1, 4, yellow) and MARK2 (2, 5, cyan) show a stabilized microtubule network (3, green) and a dynamic actin cytoskeleton discernible by loss of actin stress fibers (6, red). **c** Cells stably transfected with tau and transiently transfected with CFP-MARK alone (1, 2) and cotransfected with YFP-PAK5 and CFP-MARK (3–5). Expression of MARK (1, cyan) leads to phosphorylation of overexpressed tau in the KXGS motifs visible with the 12E8 antibody (2, red). Coexpression of inactive



PAK5 (3, yellow) and active MARK2 (4, cyan) results in an inhibition of MARK2 as seen by the low level of phospho-KXGS tau (5, red). Transfected cells labeled by arrows.

**Fig. 6.** Modes of regulating MARK. **a** The diagram summarizes known or plausible modes of MARK regulation which would affect the phosphorylation of tau and hence the stability of microtubules and the aggregation of tau. (a) Activation via phosphorylation by MARKK at the activation loop (T208) [29]. (b) Inhibition by binding of PAK5 to the catalytic domain [31]. (c) Regulation by interaction of the UBA domain with ubiquitin (not proven, but suggested by X-ray structure) [7]. (d) Regulation by interaction of the CD motif with a cofactor, in analogy with MAP kinases where upstream or downstream kinases can be bound [7, 54]. (e) Localization by interaction of the catalytic domain with the AP 14-3-3, in analogy with *Drosophila* Par-1 [8, 29]. This interaction does not depend on prior phosphorylation of MARK. (f) Localization and probably inhibition by interaction of the spacer domain with 14-3-3, after prior phosphorylation by aPKC which creates a 14-3-3 binding motif on MARK [9, 10]. (g) Interaction between the C-terminal tail and the N-terminal header or catalytic domain (dotted line), creating a folded and inhibited MARK molecule (proposed for the yeast homolog Kin-1) [11]. **b** Summary of the antagonistic regulation of MARK by MARKK and PAK5 and the effects on the cytoskeleton. MARK is switched on by an upstream kinase, MARKK, and this leads to increased dynamics of microtubules and stabilization of the actin cytoskeleton. However, switching MARK off can be achieved by PAK5, resulting in increased actin dynamics but stabilization of microtubules.

also interact with 14-3-3. This can happen in two different modes: 14-3-3 can bind in a nonphosphorylation-dependent manner to the N-terminal half of MARK [8], it can also bind to the spacer domain after this has been phosphorylated by atypical PKC [9, 10]. These interactions do not only regulate MARK spatially by altering its localization but also inhibit the catalytic activity of the enzyme, probably by stabilizing the inhibitory interaction of the KA domain with the N-terminal header or the catalytic domain [11 and unpublished data]. From the structural analysis of MARK [7], one can speculate about regulatory interactions of (yet unknown) proteins with the UBA domain and the CD motif. This motif is known in MAP kinases for multiple interactions with upstream and downstream effectors [54], and the CD motif can be found in all MARK family members [7].

Kinases are often involved in more than one signaling cascade. For example, MARK/Par-1 signaling is involved not only in regulating microtubule dynamics (e.g. in neurite outgrowth) and the Par cell polarity determinants but also in Wnt signaling [47]. Furthermore, the upstream kinase MARKK/TAO-1 activates the p38 stress

pathway by phosphorylating MKK3 and MKK6 [17]. On the other hand, PAK5 is not only involved in remodeling the actin cytoskeleton, but also in inducing the JNK stress pathway [25, 26] and in the apoptotic pathway [48]. It is interesting to note that the two kinases whose interaction we have studied in the context of the cytoskeleton also have a relationship to the cell's stress response. Consistent with this, the activation of MARK and the phosphorylation of its downstream target tau is elevated by cellular stress [18, 49]. This might explain the increased phosphorylation of tau at early stages of neurodegeneration in AD and frontotemporal dementias [1]. The impact of PAK5 on MARK during neurodegeneration will be an interesting question to pursue.

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