**MARK4 Is a Novel Microtubule-associated Proteins/Microtubule Affinity-regulating Kinase That Binds to the Cellular Microtubule Network and to Centrosomes**

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The MARK protein kinases were originally identified by their ability to phosphorylate a serine motif in the microtubule-binding domain of tau that is critical for microtubule binding. Here, we report the cloning and expression of a novel human paralog, MARK4, which shares 75\% overall homology with MARK1–3 and is predominantly expressed in brain. Homology in brain is most pronounced in the catalytic domain (90\%), and MARK4 readily phosphorylates tau and the related microtubule-associated protein 2 (MAP2) and MAP4. In contrast to the three paralogs that all exhibit uniform cytoplasmic localization, MARK4 colocalizes with the centrosome and with microtubules in cultured cells. Overexpression of MARK4 causes thinning out of the microtubule network, concomitant with a reorganization of microtubules into bundles. In line with these findings, we show that a tandem affinity-purified MARK4 protein complex contains α-, β-, and γ-tubulin. In differentiated neuroblastoma cells, MARK4 is localized prominently at the tips of neurite-like processes. We suggest that although the four MARK/PAR-1 kinases might play multiple cellular roles in concert with different targets, MARK4 is likely to be directly involved in microtubule organization in neuronal cells and may contribute to the pathological phosphorylation of tau in Alzheimer’s disease.

Microtubules and their associated proteins (MAPs) provide a dynamic network that is critical for chromosome segregation in mitosis and for the establishment of cellular polarity in interphase cells, enabling asymmetric cell division and morphogenesis (1–3). During these processes, microtubules serve as tracks for regulated movement and positioning of organelles and other membrane microdomains. Such processes require a dynamic microtubule array and are regulated by motor proteins and structural MAPs (4–7). An intriguing case of cellular polarization is the outgrowth of neurites in neuroblasts or neuroblastoma cells. This process is not completely understood; however, it has been shown to involve the axonal microtubule-stabilizing proteins tau and MAP1B (8, 9) and the dendritic protein MAP2 (10, 11). The binding of tau and MAP2 to microtubules is controlled by phosphorylation in a spatial and temporal fashion (12). The purification of the activity that was most effective in phosphorylating the microtubule-binding domain of the MAPs tau, MAP2, and MAP4 led to the identification of three kinases that were termed MAP/microtubule affinity-regulating kinases (MARKs) (13, 14). MARKs phosphorylate tau and related MAPs on their tubulin binding repeats and consequently catalyze their detachment from microtubules in vitro and in cultured cells (13, 15, 16). Overexpression of high levels of MARKs in cells causes the disruption of the cellular microtubule network (15, 17). Intriguingly, the major serine in tau specifically phosphorylated by MARK, serine 262, is hyperphosphorylated in tau from the neurofibrillary deposits found in Alzheimer’s disease brains (18). This finding is in agreement with the observation that tau purified from such neurofibrillary deposits has lost the ability to bind to microtubules, which can be restored by dephosphorylation (19).

Candidate orthologs of the mammalian MARKs have been described in fission yeast (20), nematodes (21), frogs (22), and flies (23, 24). Interestingly, in these organisms, the MARK-like kinases are involved in processes related to the establishment of cell polarity. The MARK ortholog in Schizosaccharomyces pombe, kin1p, is necessary for proper growth initiation from the cell tips (25). In Caenorhabditis elegans, defective PAR-1 prevents the early asymmetric cell divisions of the germ line lineage (21), and in Drosophila melanogaster, PAR-1 is necessary for microtubule-driven processes both early in the germ line for establishing cyst polarity and oocyte specification (26, 27) and later for the formation of the embryonic axe (23, 24). Recently, it was reported that Drosophila PAR-1 also functions as a molecular switch by phosphorylating Dishevelled, thus enabling it to transduce the Wnt signal to β-catenin (28), linking polarity regulation by MARK kinases to molecular pathways that involve β-catenin in cancer (29), neurodegeneration, and apoptosis (30).

The few studies performed in mammalian cells have supported the notion that the role of MARK/PAR-1 kinases in the maintenance and establishment of cell polarity is conserved. Overexpression of MARK2 abrogates epithelial polarity in...
Madin-Darby canine kidney cells (31), and MARK1 depletion by an antisense probe in PC12 cells or the expression of a catalytically inactive mutant in neuroblastoma cells inhibits process outgrowth in these systems (32, 33).

A comprehensive sequence analysis of the protein kinases of the human genome (34) shows that the human MARK/PAR-1 kinase family consists of four paralogous genes, of which one has not yet been characterized. In this study, we describe the cloning and characterization of the MARK4 protein kinase and demonstrate that MARK4 is distinct from the other members of the MARK kinase family, in that it associates with microtubules, centrosomes, and neurite-like processes of neuroblastoma cells.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning—Cosmid R31237 from chromosome 19 contained part of the open reading frame of a novel MARK family member, which we termed MARK4, and was used for the design of primer 5'-GGCTCAGAAGCTGAGGGCCCGACGAC-3' to amplify the complete open reading frame from human fetal brain Marathon cDNA (BD Biosciences, Clontech) by RACE-PCR using rTthXL polymerase (PerkinElmer Life Sciences) and the adaptor-specific primer AP1 (BD Clontech). For reasons of sequence fidelity, the 3' portion of the amplified open reading frame was then replaced by the Scal-XhoI fragment from an EST from human neuroepithelium, IMAGE:531830 (RZPD, Germany). The open reading frame was fully sequenced on both strands, and the sequence was deposited into GenBankTM (accession number AF057448). For expression, the MARK4 open reading frame was fused N-terminally with a HA tag (15) or with GFP (BD Biosciences, Clontech) and subcloned into pZeo1 (Cellzome AG, Heidelberg, Germany) or pcDNA3 (Invitrogen) via BamHI and KpnI restriction sites. Between the tag sequences and the ATG of the open reading frame, we inserted an oligonucleotide cassette encoding a tobacco etch virus protease cleavage site as described previously (14). To test whether the expression vector itself or the site where GFP is fused to MARK4 has any effect on the expression and intracellular location of the fluorescent signal, MARK4 was also subcloned via GatewayTM cloning into pcDNA-DEST47 and pcDNA-DEST33 providing a C- or N-terminal GFP tag, respectively (Invitrogen). Human MARK3 was amplified by RT-PCR from human fetal brain mRNA (BD Clontech) using rTthXL polymerase and primers 5'-CAGGGCTCCTTCTCCGGGCACC-3' and 5'-ACCCTGAGAAAGGGATGTATTTTTA-3', cloned into pcDNA3, and sequenced on both strands, and the sequence was deposited into GenBankTM (accession number AF465413). Human MARK2 and γ-tubulin were subcloned by PCR from cDNA clones IMAGE:161417 and IMAGE:3345973, respectively (RZPD) and subcloned into pcDNA3, pcDNA3/Zeo1, respectively. MARK4 was expressed from a pcDNA3 plasmid as described previously (15). A catalytically inactive mutant of MARK4 was generated by mutating the last 150 nucleotides of the open reading frame from fetal brain mRNA. The encoded protein contains 2% (v/v) paraformaldehyde for 20 min at 37 °C (Fig. 3). After fixation, cells were unlocked with 5% bovine serum albumin in 0.1% Triton X-100 in phosphate-buffered saline. Primary antibody incubation was performed in phosphate-buffered saline, 3% goat serum for 1 h at 37 °C. Monoclonal mouse anti-γ-tubulin antibody (Sigma) and polyclonal rabbit anti-GFP antibody (BD Biosciences Clontech) were used. Cells were washed in phosphate-buffered saline and incubated for 1 h at 37 °C with secondary antibodies (fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG and Cy3-conjugated donkey anti-mouse IgG, both from Jackson Immunoresearch, West Grove, PA). For paraformaldehyde fixation, cells were washed in microtubule-stabilizing buffer (80 mM Pipes, pH 6.9, 4% (w/v) polyethylene glycol 6,000, 1 mM MgCl2, 1 mM EGTA) for 5 min at 37 °C, fixed for 20 min at 37 °C in 2% (w/v) paraformaldehyde in phosphate-buffered saline, and stored in 0.2% Triton X-100. Antibody treatment was performed using polyclonal rabbit anti-HA antibody and monoclonal mouse anti-α-tubulin antibody followed by incubation with TRITC-conjugated anti-rabbit antibody and fluorescein isothiocyanate-conjugated anti-mouse antibody (Sigma). Coverslips were mounted in Permafluor (Dianova, Hamburg, Germany) and analyzed the following day. For life observation (Figs. 4 and 5), cells were directly examined 16–18 h after transfection. Images were captured with an Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a ×63 oil immersion objective, a CCD-camera using filters optimized for double-labeled experiments, and with Photoshop software (Adobe Systems, San José, CA) implemented for image processing.

**Immunoprecipitation and Kinase Assays**—After transfection, HEK293 cells were collected from one 10-cm dish in 400 μl of lysis buffer (250 mM Tris pH 7.5, 25% glycerol, 7.5 mM MgCl2, 0.5% (v/v) Triton X-100, 500 mM NaCl, 125 mM NaF, 5 mM Na2VO4, 1 mM diithiothreitol, Roche protease inhibitor tablets). Lysates were cleared by centrifugation at 14,000 × g at 4 °C. A 10-μl sample of the supernatant was removed for immunoblotting. The remainder was used for immunoprecipitation with anti-Myc (BD Biosciences Clontech) or anti-GFP antibody (BD Biosciences Clontech) were used. Cells were washed three times with lysis buffer and once with lysis buffer without Triton X-100. Beads were resuspended in SDS-PAGE sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and detected using either horseradish peroxidase-conjugated 3F10 at 1/200,000 for horseradish peroxidase-conjugated 9E10 at 1/10,000 (BD Biosciences Clontech). Tandem affinity purification of MARK4 was performed from HEK293 cells stably transduced with pZeo1-MARK4, and copurifying proteins were sequenced by LC-MS/MS essentially as described previously (36). For kinase assays, immunoprecipitates were washed three times with lysis buffer as above and once with kinase buffer (50 mM Hepes, pH 7.4, 10 mM MgCl2, 200 μM ATP, 100 mM NaCl, 1 mM diithiothreitol). Beads were incubated with 10 μl of kinase buffer supplemented with 2 μCi of [γ-32P]ATP and 1 μg of protein substrate for 50 min at 30 °C. To stop the reaction, 5 μl of boiling 4% SDS-PAGE sample buffer was added. In vitro phosphorylation studies were performed with bacterially expressed substrate proteins: human tau isoforms, MAP2e, and the tau-MTBD and MAP4-MTBD fragments (comprising the repeats of the microtubule-binding domain) as described previously (14). Northern blots were performed using 2 μg of poly(A)+ mRNA from different tissues, fractionated by denaturing agarose gel electrophoresis, and transferred to nylon membranes (BD Biosciences Clontech). Oligonucleotides 5'-CTGCCCCCGGGCTCCCAG-3' and 5'-GCA-ACCTGTGACCCGGCCTGTC-3' (MWG Biotech, Munich, Germany) were mixed and labeled to 106 cpm/μmol using 1γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and used for hybridization using QuickHyb solution according to the manufacturer (Stratagene, Heidelberg, Germany). Western blotting was performed as described previously (17).

**RESULTS**

Cloning and Sequence Analysis—A search for sequences encoding additional members of the human MARK/PAR-1 protein kinase family yielded two cosmids from chromosome 19 with pronounced sequence homology to MARK1, MARK2, and MARK3 (37), representing a fourth paralog, which we termed MARK4. Using 5'-RACE, we cloned the complete open reading frame from fetal brain mRNA. The encoded protein contains the characteristic sequence domains of the MARK/PAR-1 kinase domain (Fig. 1A). The overall sequence homology within the MARK family is 55% with a higher degree of homology among MARK1, MARK2, and MARK3 (70%). The kinase do-
Fig. 1. MARK4 is a member of a conserved protein kinase family. A, sequence alignment of human MARK4 (GenBank™ accession number AAL23683) with its three paralogs MARK1 (GenBank™ accession number AAF72103), MARK2 (GenBank™ accession number CAA66229), and MARK3 (GenBank™ accession number AAL69982). Conserved domains are indicated in the consensus sequence as follows: red, catalytic domain, conserved activating phosphorylation sites underscored; green, ubiquitin-associated domain; and blue, KA domain, predicted amphipathic helices underscored. B, phylogenetic tree showing the relationship between the four human MARK gene products and their putative orthologs from D. melanogaster, C. elegans, S. pombe, and S. cerevisiae. C, Northern blot. Upper part, hybridization with a MARK4-specific probe; lower part, same blot hybridized with an actin probe as a loading control. MARK4 is ubiquitously expressed as a message of 4.4 kb (arrow), most prominently in brain and testis.
Fig. 2. MARK4 phosphorylates KKGS motifs in the microtubule-binding domains of tau, MAP2c, and MAP4. A–C, HA-tagged MARK4 was immunoprecipitated from transfected HEK293 cells. The following samples were incubated with (+) or without MARK4 (−) as indicated: human 4-repeat tau in the presence of peptide TR1, which acts as inhibitor (lanes 1 and 2); human 4-repeat tau (lanes 3 and 4); a mixture of all six human tau isoforms (lanes 5–6); a tau-MTBD fragment (lanes 7–8); MAP2c (lanes 9–10); and MAP4-MTBD fragment (lanes 11–12). Samples were analyzed on SDS-PAGE (A), autoradiography (B), and Western blotting (C, note that lanes 1–2 as in A and B are not present). In all of the samples, phosphorylation as detected by autoradiography (B) or by immunoblotting (C) is only detected when the HA-MARK4 immunoprecipitate was added to the reaction. D, transfected MARK4 binds to and phosphorylates tau, inducing reactivity toward the P-tau (Ser-262) antibody. MARK4 was transfected into HEK293 cells stably expressing Myc-tau. Lysates were assayed by Western blot with antibody 12E8. Lane 1, HEK293 control lysate; lane 2, cells expressing tau transfected with MARK4; lane 3, mock-transfected tau expressing cells; lanes 4 and 5; Tau was immunoprecipitated from the lysate shown in lane 2, and the precipitate was analyzed by immunoblotting against Myc-tau (lane 4) and HA-MARK4 (lane 5).

main at the N terminus of the protein exhibits the highest degree of homology, slightly over 90%. Notably, the LDTP/F-FCG8/P/P motif containing the two activating phosphorylation sites in the regulatory loop is conserved (15). The catalytic domain is followed by the small ubiquitin-associated domain (38), which is thought to mediate interaction with ubiquitin (39). Adjacent to the ubiquitin-associated domain is a large less conserved "spacer" region and a conserved C-terminal ubiquitin-associated (KA) domain (38), which is an exclusive feature of this protein family (Fig. 1A). In MARK4, the large spacer region is the least conserved, the overall homology with MARK1, MARK2, and MARK3 being <90%, thus setting MARK4 apart from MARK1–3 in a phylogenetic diagram (Fig. 1B). The human MARK4 locus is 19q13.3, which is close to a known susceptibility locus for Alzheimer's disease ascribed to the ApoE gene. The 4.4-kb MARK4 mRNA is ubiquitously expressed as analyzed by Northern blot with the highest levels in brain and testis. In testis, a larger mRNA, which may represent a splice variant, is also detected (Fig. 1C).

MARK4 Phosphorylates tau Family MAPs on Their Microtubule-binding Domain—Given the extensive homology of the catalytic domain, we asked whether MARK4 specifically phosphorylates tau and related MAPs similar to MARK1 and MARK2 (15). We fused the MARK4 open reading frame to an N-terminal HA tag and immunoprecipitated the fusion protein from transfected HEK293 cells. It readily phosphorylated recombinant tau (Fig. 2B), a fragment of tau comprising only the microtubule-binding domain, and a synthetic peptide, TR1 (NVKSKIGSTENLK) derived from the first repeat of the tau microtubule-binding domain. When a 10-fold excess of the TR1 peptide was added in addition to full-length tau, the phosphorylation of tau was almost completely inhibited, indicating that the immunoprecipitated activity is specific (Fig. 2, A and B, lanes 1 and 2). After phosphorylation, the tau protein was detected by the phospho-tau antibody 12E8 (Fig. 2C), which recognizes tau phosphorylated at serine residues 262 and/or 356, the first and fourth KKGS motif within the tubulin-binding domain (40). As described previously for MARK1 and MARK2 (14), MARK4 also readily phosphorylates the related MAP2 and MAP4 (Fig. 2, A–C). In this in vitro phosphorylation assay, there was no significant difference between the four MARKs, which all generate the 12E8 phosphoepitope on tau (data not shown). MARK4 also associates with and phosphorylates tau in transfected cells. In lysates of HEK293 cells stably expressing tau, a 12E8-immunoreactive band of the size of tau appeared after MARK4 transfection and tau protein immunoprecipitated from these cells contains MARK4 (Fig. 2D).

MARK4 Is Associated with the Microtubule Cytoskeleton—We have previously reported that overexpression of MARK1 or MARK2 causes the disruption of the cellular microtubule network (15). To study the effect of MARK4 on the microtubule network, we transfected CHO cells with epitope-tagged MARK4 and stained the fixed cells with antibodies directed against the tagged protein and against tubulin (Fig. 3, B and C). For comparison, similarly HA-tagged versions of the other three MARKs were transfected and stained in an analogous fashion (Fig. 3, F–H). MARK1 was expressed from a rat cDNA as described previously (15), and MARK2 and MARK3 were expressed from human cDNAs as described under "Experimental Procedures." We have shown previously that a prolonged post-transfection interval in concert with high levels of MARK1 or MARK2 expression leads to the disruption of microtubules, detachment of the cells from the support, and cell death (15). We observed that, at high expression levels, all four MARKs exhibited these effects to a comparable degree (data not shown). However, when cells were fixed at shorter time
intervals post-transfection, typically 6–16 h, we noticed that in many cells the integrity of the microtubule cytoskeleton was largely preserved. Interestingly, in these cells the MARK4 signal significantly differed from that of the other three MARKs (Fig. 3, compare B–C with F–H). We observed a pronounced colocalization of MARK4 with microtubules. This colocalization represents a specific feature of MARK4 that is not observed with the other MARKs (see also Refs. 15 and 17). At 16 h post-transfection, the microtubule array of cells expressing MARK4 is often less dense as compared with nontransfected cells as a result of the formation of microtubule bundles (pronounced in Fig. 3C).

We next asked whether the localization of MARK4 at microtubular structures and its effect on microtubular organization might be a consequence of the phosphorylation of microtubule-associated proteins and hence should be dependent on its kinase activity. We performed an analogous transfection experiment, now using an inactive MARK4 mutant, in which threonine 214 and serine 218 in the kinase regulatory loop were changed into alanine. Interestingly, the catalytically inactive MARK4 mutant does not label microtubules nor does it induce microtubule bundling or any significant thinning of the microtubule network. Instead, it is found in the proximity of the nucleus, possibly associated to the ER or ER-like structures (Fig. 3, D and E).

MARK4 Binds to the Microtubule-organizing Center in Living Cells—To investigate the localization in living cells, we tagged MARK4 and the catalytically inactive mutant with GFP, transfected the constructs into CHO cells, and analyzed the cells by epifluorescence microscopy at different time intervals after transfection (Fig. 4). Around 8 h post-transfection, both MARK4 and the mutant labeled dotlike structures close to the nucleus (Fig. 4, A and D). At later time points from 16 h post-transfection, we observed labeling of filaments emanating from these structures by active MARK4 but not by the mutant (Fig. 4, B and E), in agreement with the observed effects in immunofluorescence (Fig. 3, B and C). At 48 h post-transfection, most cells round up, detach from the support, and die (Fig. 4, C and F).

These data suggest that, at low expression levels, MARK4 specifically binds to the MTOC and to microtubules nucleated by it. As noted above, in many cells transfected with active MARK4, we observed labeling of thick filaments indicating microtubule bundles, concomitant with a decrease in the overall number of microtubules in the cell. In such cells, typically only a few microtubules originate from the centrosome (Fig. 4, G–L). The thinning and final disappearance of the microtubule array, but not the bundling effect, has been previously described for CHO cells overexpressing active MARK1 or MARK2 (17).

The low number of MTs and the fluorescent staining of the MTOC and of the remaining microtubules or microtubule bundles was also observed for C-terminally GFP-tagged MARK4 (data not shown). Likewise, we observed similar effects in fibroblasts (CHO cells in Figs. 3 and 4), neuroblastoma cells (Neuro2A in Fig. 5), and epithelial cells (HEK293, data not shown). GFP fluorescence is detected at centrosomes (Fig. 5), and amplified centrosomes are also labeled (Fig. 5, A and B), sometimes with no or few filaments attached. Hence, the origin of the cells studied does not reveal obvious functional differences.

MARK4 Localizes to Growth Conelike Structures in Differentiated Neuroblastoma Cells—Recently, it has been suggested that functional MARK1 or MARK2 kinases are necessary for process outgrowth in human neuroblastoma cells differentiated in culture (32, 33). Hence, we analyzed whether MARK4 might be localized along these processes in differentiated Neuro2A cells (Fig. 5, G and J). Interestingly, we found GFP-MARK4 localized along microtubules, most prominently in the cell body, but also at the tip of the typical neurite-like processes formed by cells under serum starvation. The strong GFP signal at the tips was found in almost all of the cells investigated.

Fig. 3. MARK kinases exhibit distinct cytoplasmic localization patterns, but only MARK4 colocalizes with microtubules. HA tag plasmid alone (A) or HA tag containing different tagged MARK constructs as indicated were transiently expressed in CHO cells, and cells were stained with antibodies against the HA epitope (left panels) and against tubulin (right panels). B and C, MARK4 exhibits colocalization with microtubules and induces thinning of the microtubule array and rearrangement into bundles. D and E, MARK4-T214A/S218A, a catalytically inactive MARK4 point mutant, does not colocalize with microtubules or induce bundling. F–H, MARK1, MARK2, or MARK3 does not exhibit colocalization with microtubules. Scale bar, 10 μm.
MARK4 decreases the number of microtubules within the cells by inducing bundling. In and filamentous structures emanating from the centrosomes, which likely represent bundles of microtubules. The centrosomes become clearly visible even with epifluorescent microscopy because MARK4 decreases the number of microtubules within the cells by inducing bundling. In some cells, only faint labeling is observed along the elongated processes (left, arrows). Scale bar, 10 μm.

However, its occurrence is independent of whether the labeling of the processes themselves is strong, indicating the presence of microtubules (Fig. 5F), or the labeling is weak, probably because of the disappearance of microtubules caused by the GFP-MARK4 activity (Fig. 5, G–I).

**MARK4 Physically Interacts with α-, β-, and γ-Tubulin in Cells**—We performed double-labeled immunofluorescence experiments of GFP-MARK4 expressing CHO cells using antibodies against γ-tubulin, an integral component of the centrosome. The structures labeled with GFP-MARK4 colocalize with γ-tubulin (Fig. 6, A–C). To study whether MARK4 physically interacts in a complex with tubulins and other associated proteins, we purified the MARK4 protein complex under close to physiological conditions using tandem affinity purification (35, 41), submitted the purified complex to SDS-PAGE, and sequenced the bands by liquid chromatography-coupled tandem mass spectrometry. Prominent bands were identified as the microtubule subunits α- and β-tubulin as well as γ-tubulin and myosin (Fig. 6D). A number of other proteins were identified from less prominent bands (data not shown). To further confirm the association of MARK4 with γ-tubulin, we cotransfected HA-tagged MARK4 and Myc-tagged γ-tubulin and performed immunoprecipitation with anti-HA or anti-Myc antibodies. MARK4 and γ-tubulin were found to be associated, regardless of which protein was subjected to precipitation (Fig. 6E).

**DISCUSSION**

The human genome contains four functional MARK genes plus 28 pseudogenes (34, 42). In this study, we describe the characterization of the fourth paralog in the MARKPAR-1 kinase family, MARK4. The domain structure of MARK gene products is highly conserved. The catalytic domains at the N terminus of all of the MARKs are highly homologous, suggesting that consensus phosphorylation sites are likely to be similar. The adjacent ubiquitin-associated domain may be involved in the interaction with other proteins in a ubiquitin-dependent fashion (39). The C-terminal KA domain, a domain of unknown function found only in this gene family, is likely to be involved in protein-protein interactions as well because it is predicted to contain amphipathic helices. In MARK4, the KA domain differs from the other family members at several positions (Fig. 1A). However, we find that the fusion of GFP to the C terminus of MARK4 does not interfere with the localization of the protein.

Phylogenetic analysis indicates that MARK4 is evolutionary, most closely related to the putative orthologs in lower eu-
karyotes (Fig. 1). These orthologs have been described in *S. pombe* (kin1), *C. elegans* (PAR-1), and *D. melanogaster* (dPAR-1) where they play crucial roles in cell polarity. In *S. pombe*, cells deficient in kin1p fail to activate polarized growth (25). In worms and flies, PAR-1 is essential for establishing anterior-posterior polarity in the zygote and the oocyte, respectively. The mammalian PAR-1 homologs MARK1 and MARK2 were discovered as kinases that regulate the binding of tau family MAPs to microtubules and thus termed MAP/microtubule affinity-regulating kinases (13). Since MARK2 has been shown to asymmetrically localize in Madin-Darby canine epithelial kidney cells and expression of dominant-negative forms disrupts apical-basal polarity in this system (31), it seems likely that a principal function in cell polarity is conserved from lower eukaryotes to mammals.

The overexpression of MARK1 and MARK2 was shown previously to cause depolymerization of cellular microtubules as a consequence of the dissociation of hyperphosphorylated MAPs (15, 17). We show here that MARK4 also phosphorylates the microtubule-binding domains of tau and the related MAP2 and MAP4, which is not surprising because of the homology of the catalytic domains. In contrast to the other three paralogs, MARK4 exhibits colocalization with microtubules and the MTOC in transfected cells. In cells expressing low levels of GFP-MARK4, we observed GFP-MARK4-labeled microtubule network observed in cells expressing GFP-tubulin or GFP-tau in which the fluorescence of the labeled microtubules typically outshines the spot where the MTOC is located (43). The thick GFP-MARK4-labeled filaments are likely to represent bundles rather than individual microtubules. The bundling effect was not observed with the other three MARKs and is dependent on the kinase activity of MARK4. Hence, the bundling may be a consequence of the phosphorylation of microtubule-associated proteins, e.g. MAP4, on domains projecting from the microtubule surface that function as microtubule spacers (14, 44). At high levels of or prolonged exposure to MARK4 expression, the microtubule network eventually disintegrates, presumably because of phosphorylation of microtubule-associated proteins on the microtubule-binding domain as described previously for MARK1 and MARK2 (15).

The association of MARK4 with microtubule subunits and with the centromeric γ-tubulin is sufficiently robust to allow tandem affinity purification from cells of a MARK4 protein complex containing α-, β-, and γ-tubulin (Fig. 6D). However, it...
is not clear whether the interaction of MARK4 with tubulin is direct or whether it is mediated via associated proteins. We have so far not identified tau-type MAPs in this complex, but they may be expressed at too low levels for detection because we observed that tau and MARK4 do co-immunoprecipitate (Fig. 2D). Tau family structural MAPs have not been reported to bind to γ-tubulin or other components of the centrosome; hence, it is possible that the binding to centrosomes occurs via γ-tubulin directly. In addition, we also found actin and nonmuscle myosin to co-purify with MARK4. It has been reported that the C. elegans MARK homolog PAR-1 interacts directly with nonmuscle myosin II (45), and in the nematode, the effects of the PAR genes are dependent on intact actin rather than tubulin filaments (46). This is in contrast to Drosophila where dPAR-1 restriction to the oocyte during oogenesis is microtubule-dependent and where dPAR-1 is required for MTOC positioning during oocyte polarization (47). In this context, the GFP-MARK4 labeling of amplified centrosomes (Fig. 5, A and B) suggests that MARK4 may be involved in the regulation of centrosomal activities, i.e. amplification and positioning of centrosomes or nucleation of microtubules mediated by the centrosomal protein γ-tubulin (48). Hence, in mammals, MARK/ PAR-1 kinases may influence both microtubule- and MTOC-dependent processes as well as actin-dependent processes.

When GFP-MARK4 expressing neuroblastoma cells were subjected to serum starvation, we observed intense labeling of the tips of the neurite-like structures formed by these differentiating cells. It has been reported that, in a growing axon, the microtubule array in the distal axon and growth cone is the most dynamic (49). In contrary to what one would have expected, taking into account the microtubule-stabilizing effect of tau, the tau/tubulin concentration ratio in the distal axon was reported to exhibit a relative increase (50). The presence of MARK4 in growth cone-like structures may explain these findings. Tau in the growth cone may lose its stabilizing effect because of phosphorylation by MARK4. Our findings are in line with a recent report demonstrating that the overexpression of a dominant negative form of MARK2 blocks process outgrowth in Neuro2a cells (33). We do not observe a specific localization of MARK2 in processes (data not shown), but massive overexpression of any MARK paralog may affect most cellular compartments and mimic the effects of MARK4 at the tip. Mice lacking MARK2 do not exhibit central nervous system abnormalities (51, 52), suggesting that MARK4 rather than MARK2 may play a specialized role in neuronal process outgrowth.

The conclusive demonstration of the association of endogenous or untagged MARK4 with the cellular structures described here has to await the availability of MARK4-specific antibodies. It is worth to note however that we have used four different tagged forms of MARK4 in this work (N-terminal HA, GFP, tandem affinity purification, and C-terminal GFP), which all exhibit the same localization, whereas MARK1–3 tagged in the same way are clearly different and hence the microtubule, centrosome, and process-tip staining are unique features of MARK4 as compared with the other MARKs.

The role of MARK kinases in neuronal cells is of particular interest because they have been implied in the formation of the neurofibrillary tangles, pathological intraneuronal aggregates of tau protein that are a hallmark of Alzheimer’s disease. Tau protein purified from neurofibrillary tangles is hyperphosphorylated at several sites, a critical residue being serine 262 in the microtubule-binding domain (53). This residue is specifically phosphorylated by MARKs (15), which results in the detachment of tau from microtubules, in agreement with the fact that tau protein purified from neurofibrillary tangles does only bind to microtubules after phosphatase treatment (19). In line with these findings, a non isoform-specific MARK antibody was shown to label neurofibrillary tangles in Alzheimer’s disease brain (54). Of the four MARK gene products, MARK4 is most likely to play a role in these pathological events because its expression is particularly high in brain, it is localized along microtubular structures, and because it accumulates at the tips of processes where regulation of microtubule dynamics is crucial (45). We suggest that a gain or loss of function of MARK4 in Alzheimer’s disease may result in the loss of axonal-dendritic polarity that is a hallmark of neurodegeneration (55–57).
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