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Phosphorylation of MAP2c and MAP4 by MARK Kinases Leads to the Destabilization of Microtubules in Cells

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Microtubules serve as transport tracks in molecular mechanisms governing cellular shape and polarity. Rapid transitions between stable and dynamic microtubules are regulated by several factors, including microtubule-associated proteins (MAPs). We have shown that MAP/microtubule affinity regulating kinases (MARK) can phosphorylate the microtubule-associated-proteins MAP4, MAP2c, and tau on their microtubule-binding domain in vitro. This leads to their detachment from microtubules (MT) and an increased dynamic instability of MT. Here we show that MARK protein kinases phosphorylate MAP2 and MAP4 on their microtubule-binding domain in transfected CHO cells. In CHO cells expressing MARK1 or MARK2 under control of an inducible promoter, MARK2 phosphorylates an endogenous MAP4-related protein. Prolonged expression of MARK2 results in microtubule-disruption, detachment of cells from the substratum, and cell death. Concomitant with microtubule disruption, we also observed a breakdown of the vimentin network, whereas actin fibers remained unaffected. Thus, MARK seems to play an important role in controlling cytoskeletal dynamics.
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Key words: MARK; MAP4; MAP2c; tau; inducible expression; microtubule; vimentin

INTRODUCTION

Microtubules play an important role in cell division, establishment of cellular morphology and polarity, as well as in intracellular trafficking [for reviews see Drubin and Nelson, 1996; Rickard and Kreis, 1996]. They are dynamic both in interphase and in mitosis, which is achieved by rapid transitions between growth and shrinkage [Hyman and Karsenti, 1996; Desai and Mitchison, 1997; Jordan and Wilson, 1998]. The dynamic behavior must be modulated to allow changes in cellular morphology to occur, e.g., during the establishment of axonal and dendritic polarity in differentiating neurons [Lim et al., 1989; Baass and Black, 1990; Li and Black, 1996]. Thus, the switch between dynamic and stable microtubules has to be tightly controlled. A variety of mechanisms controls the plasticity of the microtubule array, including posttranslational modifications of tubulin (e.g., detyrosination, glutamylation, or acetylation) [reviewed by Luduena, 1998], sequestering of tubulin subunits by stathmin [Belmont and Mitchison, 1996; Antonsson et al., 1998], and factors that sever microtubules (katanin) [Hartman et al., 1998] or stabilize or destabilize them at their ends [Xkcm1: Walczak et al., 1996; STOP: Bosc et al., 1996].

Microtubule-associated proteins (MAPs) modulate dynamics by attaching to microtubules and stabilizing them along their length [Hirokawa, 1994; Schoenfeld and Obar, 1994; Mandelkow and Mandelkow, 1995; Joshi,

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Abbreviations used: MARK = microtubule affinity regulating kinase; MT = microtubule(s); dox = doxycylin; MAP = microtubule associated protein.

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1998]. The best studied family of MAPs includes the proteins MAP4, MAP2, and tau, which contain a conserved region in the C-terminal microtubule-binding domain with three or four imperfectly repeated motifs of ~31 amino acids each [Chapin and Bulinski, 1992] (Fig. 1). Their N-terminal domains form projections from the microtubule surface and serve as microtubule spacers or anchors for enzymes [Chen et al., 1992]. The developmental and phosphorylation-dependent regulation of MAP2 and tau, e.g., during neuronal differentiation, suggests an important role in organizing the microtubule cytoskeleton [Matus, 1991]. By contrast, MAP4 is more prominent in non-neuronal cells where it contributes to the regulation of microtubule dynamics in interphase and mitosis [Olson et al., 1995; Ookata et al., 1997; Shiina and Tsukita, 1999].

Another prominent feature of MAPs is their ability to interfere with microtubule-dependent transport. The movement of the molecular motors kinesin and dynein is inhibited by the binding of MAPs to microtubules in vitro and in vivo [Paschal et al., 1989; von Massow et al., 1989; Lopez and Sheetz, 1993; Hagiwara et al., 1994; Bulinski et al., 1997]. Furthermore, organelle transport along microtubules in vivo is facilitated by kinase activators, concomitant with an increased phosphorylation of MAPs [Sato-Harada et al., 1996]. We have shown recently that a moderate expression of tau suffices to inhibit preferentially the plus-end directed (kinesin-dependent) transport of vesicles and organelles along microtubules, leading to the retraction of mitochondria and endoplasmic reticulum towards the cell interior [Ebneth et al., 1998; Trinczek et al., 1999].

Phosphorylation of MAPs is an important factor regulating the balance between plasticity and stability of the microtubule-network [Lopez and Sheetz, 1995; Preuss et al., 1995; Illenberger et al., 1998; Johnson and Jenkins, 1996]. MAPs isolated from cells are phosphorylated and it has been shown that this phosphorylation interferes with binding to microtubules both in vitro and in vivo [Illenberger et al., 1996; Drechsel et al., 1992; Utton et al., 1997]. During mitosis where microtubule turnover increases about 20-fold, MAPs show a higher degree of phosphorylation [Vandré et al., 1991; Preuss et al., 1995; Vincent et al., 1996; Ookata et al., 1997; Illenberger et al., 1998; Shiina and Tsukita, 1999]. In axonal growth cones, where a dynamic microtubule array is indispensable for differentiation, tau-phosphorylation seems to be spatially regulated, suggesting a tightly controlled balance between kinases and phosphatases [Mandell and Banker, 1996; DiTella et al., 1996; Rocha and Avila, 1995].

Since MAPs are substrates for several protein kinases, it has been difficult to correlate specific phosphorylation sites and kinases with microtubule binding and with the regulation of dynamic instability. The phosphorylation of several Ser-Pro or Thr-Pro motifs outside tau's microtubule binding repeats has a moderate influence on microtubule binding [Drechsel et al., 1992; Biernat et al., 1993; Trinczek et al., 1995], compared with phosphorylation of Ser214 in the proline rich region by PKA which detaches tau from microtubules in vitro and during mitosis [Brandt et al., 1994; Illenberger et al., 1998]. Another prominent site important for the regulation of the affinity of tau, MAP2c, and MAP4 to microtubules is the serine residue within the KXGS-motifs located in the repeat domains (particularly Ser262 for tau). Phosphorylation of these residues dramatically reduces microtubule affinity [Biernat et al., 1993; Illenberger et al. 1996]. The KXGS motifs are phosphorylated by the MARK kinases (MAP/microtubule affinity regulating kinases), which were discovered by their ability to phosphorylate the tubulin binding domain of tau [Drewes et al., 1997]. Molecular cloning revealed a family of kinases consisting of at least four members derived from different genes [Drewes et al., 1998] that share homologies to kinases from lower eukaryotes like yeast or *C. elegans*, which are thought to play a role in establishing cell polarity [Levin and Bishop, 1990; Guo and Kemphues, 1996].

In the present study, we show that MARK1 and MARK2 phosporylate transfected MAP2 and MAP4 in living cells. Likewise, the endogenous MAPs of CHO cells are phosphorylated on the KXGS motifs of their microtubule-binding region, concomitant with the disruption of the microtubule network. These results suggest that MARK kinases can regulate microtubule stability by controlling the binding of MAPs to microtubules.

MATERIALS AND METHODS

Plasmids and Transfection

The MARK cDNAs were inserted into a derivative of pRc/CMV (Invitrogen, Leek, The Netherlands) using synthetic linkers, which introduced an N-terminal hemagglutinin-epitope tag, to yield pEUHATagMARK1 and pEUHATagMARK2 [Drewes et al., 1997]. Site-directed mutagenesis was performed with the QuickChange kit (Stratagene, La Jolla CA). A cDNA coding for MAP2c (a juvenile MAP2 isoform from mouse) [Kindler et al., 1990] was a gift from C. Garner (University of Alabama, Birmingham, AL). MAP4-BDC comprising the MT binding domain of murine MAP4 starting from Ser640 [West et al., 1991] was a gift of J. Olmsted (University of Rochester, Rochester, NY). For expression in CHO cells, MAP cDNAs were cloned into pCDNA3 (Invitrogen).

For doxycyclin-inducible expression, epitope-tagged MARK2 was cloned into pUHD10–3 to yield pINDMARK2 [Drewes et al., 1997]. pUHD172–1 and pUHD10–3 were kindly provided by Dr. H. Bujard

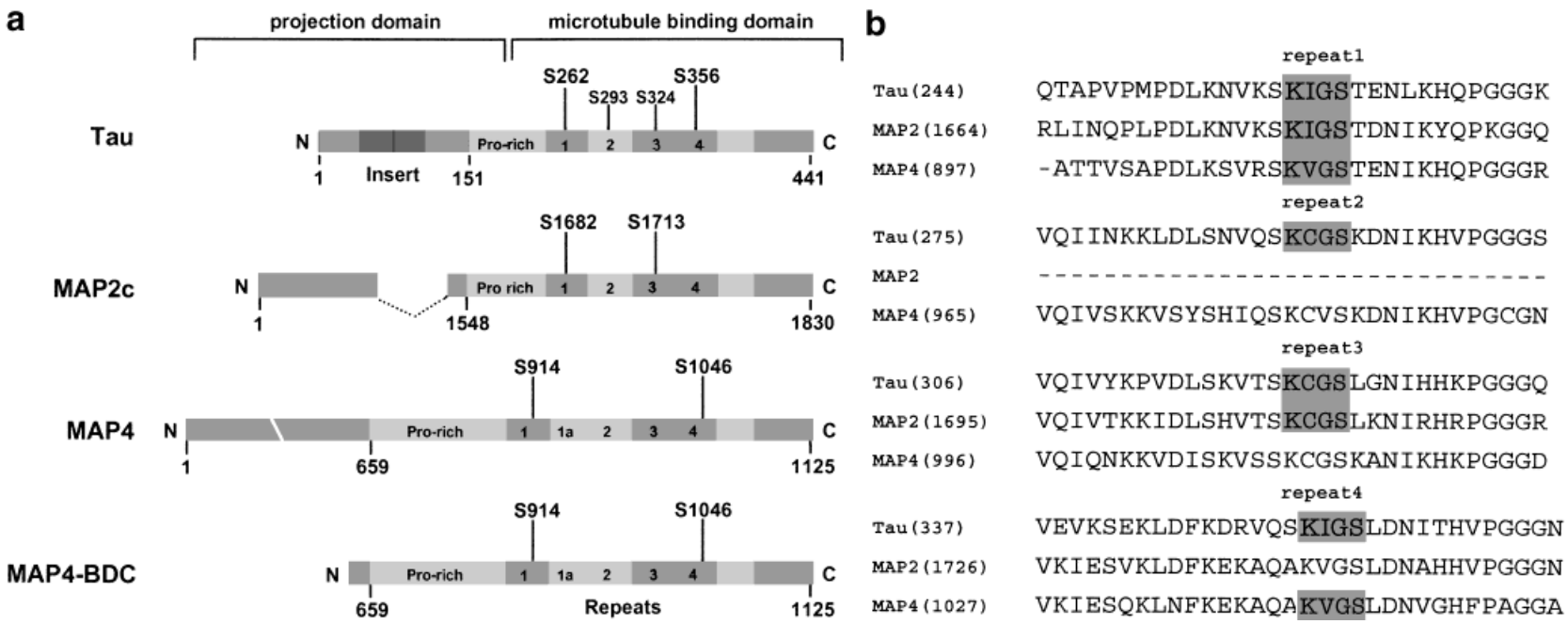


Fig. 1. Domain structure of tau, MAP2c, MAP4, and MAP4-BDC. **a:** Bar diagram of the human tau isoform tau40 [Goedert et al., 1989], rat juvenile MAP2c used in this study, which differs from adult MAP2 by a 1,364 residue deletion after residue 151 (dotted line) [Kindler et al., 1990] and murine MAP4, which comprises the construct MAP4-BDC (starting at residue 640) [West et al., 1991] containing the microtubule-binding domain of MAP4. The MAPs can be divided into an N-terminal projection domain and a C-terminal microtubule binding domain in which the homologous repeats are located. The repeat region is flanked by proline rich regions. Residues phosphorylated by MARK in vitro [Illenberger et al., 1996] are indicated. **b:** Sequence of the homologous repeat regions found in tau, MAP2c, and MAP4. MAP2c lacks the second repeat due to alternative splicing. The consensus motif KXGS that has been shown to be phosphorylated by MARK in vitro is highlighted.

(ZMBH, Heidelberg, Germany). The inducible expression system under the control of the tetracyline responsive promoter [Gossen et al., 1995] was established since transient expression of MARK1 and MARK2 is toxic to CHO-cells [Drewes et al., 1997]. Upon induction with 1 μg/ml doxycyclin MARK2 becomes detectable on blots (see Fig. 5). However, although the system works properly with regard to the inducibility of MARK we also observed some promoter leakiness (~2% of non-induced cells without doxycylin in the medium show expression of MARK in immunofluorescence analysis, rising to ~100% in the induced state). We ruled out that this might be due to a cross-reactivity with endogenous MARK by using an antibody raised against the HA epitope-tag fused to the N-terminus of MARK. Presumably because of promoter leakage it was necessary to reclone the MARK-inducible cell line every 2–3 months, since the inducibility and the expression level dropped continuously. In the presence of doxycylin, when MARK-expression is switched on, the cells grew considerably slower (about 5–10-fold) compared to cells without induction. Cells kept in culture in the presence of doxycyclin lost inducibility with time, presumably due to the fact that MARKoverexpression exerts toxic effects.

In Vitro Phosphorylation

MAP2c, MAP4-BDC, and tau proteins were obtained by expression in *Escherichia coli* using pET-vectors (Novagene) [for details see Illenberger et al., 1996]. MARK was isolated from porcine brain [Drewes et al., 1995] and 0.1 μ Units of the kinase were incubated with 5 μg of each MAP in 40 mM Hepes, pH 7.2, 1 mM ATP, 5 mM $MgCl_2$, 2 mM EGTA, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% Brij-35 for 4 h at 37°C. As a control, MAPs were incubated in the absence of MARK. Reactions were terminated by heating to 95°C for 5 min and phosphorylated and non-phosphorylated proteins assayed by SDS PAGE and Western blotting.

Cell Culture

CHO-cells were grown in HAM's F12 medium, supplemented with 10% FCS and 2 mM glutamine (Biochrom, Berlin, Germany) at 37°C and 5%CO_2 in a humidified chamber. For transient transfection, cells were seeded at approximately 70% confluency on coverslips and transfected with 1 μg plasmid DNA in the presence of DOTAP (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. After 24 h, cells were fixed in paraformaldehyde and subjected to immunofluorescence analysis essentially as described [Preuss et al., 1995]. Stably transfected MARK2-inducible CHO cells (CHO I3) were cloned as described [Drewes et al., 1997]. Induction of MARK2 was achieved by addition of 1 μg/ml doxycylin (Sigma, Deisenhofen, Germany) into the medium and incubation for 24–48 h.

Antibodies and Dyes

The following antibodies were used: Mouse monoclonal anti-HA (12CA5, Boehringer), 12E8 (phosphorylation dependent mouse anti tau-KXGS, a gift from D. Schenk, Athena Neuroscience, San Francisco, CA), rat anti-tubulin (YL1/2, Serotec, Oxford, UK), rabbit polyclonal anti-HA antibody (MBL, Watertown, MA) and rabbit CHO-MAP antibody (a gift from F. Cabral, University of Texas Medical School, Houston, TX). Rabbit anti MARK-antisera were raised against MARK N- and C-terminal synthetic peptides [Drewes et al., 1997]. Fluorescently labeled (FITC, TRITC, AMCA) and peroxidase-conjugated secondary antibodies were from Dianova (Hamburg, Germany). TRITC-labeled phalloidin was purchased from Sigma (Deisenhofen, Germany).

Western Blotting and Quantitation of Phosphorylated Endogenous CHO MAP

CHO I3 cells were seeded at 70% confluency onto dishes and MARK2-expression was induced the following day with 1 μg/ml doxycylin for 48 h. Cells were harvested, washed in PBS, and resuspendend in ice-cold PBS, supplemented with the phosphatase inhibitors okadaic acid (100 nM) and microcystin (1 μM) (Biomol, Hamburg, Germany). After incubation at 95°C for 10 min and centrifugation at 4°C for 30 min (14,000 rpm), the heat-stable supernatant was TCA-precipitated, resuspended in SDS-loading buffer and analysed by PAGE. The same amounts of protein were loaded onto the SDS-gel as judged from Bradford-protein quantitation to ensure equal amounts of endogenous CHO-MAP in induced and non-induced samples (approximately 10^7 cells per lane corresponding to approximately 1 μg total protein). The gel was blotted onto PVDF-membrane, incubated either with an antibody against CHO-MAP or the phospho-KXGS epitope-specific antibody 12E8 and visualized with ECL (Amersham, Braunschweig, Germany). Blots were then stripped by incubation at 55°C in 2% SDS/100 mM 2-mercaptoethanol in 62.5 mM Tris/HCl, pH 6.7, for 30 min, washed in PBS, reprobed with 12E8 or CHO-MAP antibody, respectively, and again visualized with ECL. Four independent experiments were performed and quantitated densitometrically with TINA software (Raytest, Straubenhardt, Germany).

Immunofluorescence

For immunofluorescence analysis, cells were fixed approximately 24 h after transfection in paraformaldehyde as described [Preuss et al., 1995]. Briefly, cells were washed in stabilizing buffer (80 mM Pipes, pH 6.9, 4% (w/v) polyethylene glycol 6,000, 1 mM $MgCl_2$, 1 mM

EGTA) for 5 min at 37°C, fixed for 20 min at 37°C in 2% (w/v) paraformaldehyde in PBS, permeabilized in 0.2% Triton X-100 in PBS, and blocked with 5% BSA/0,1% Triton X-100 in PBS. Primary antibody incubation was performed in PBS, 3% goat serum for 1 h at 37°C, cells were washed in PBS and incubated for 1 h at 37°C with fluorescently labeled secondary antibodies at 1:200 dilution. Coverslips were then mounted in permafluor (Dianova, Hamburg, Germany) and analyzed the following day. Immunofluorescence-microscopy was performed with a Zeiss Axioplan microscope equipped with an 100x oil immersion objective (Zeiss, Jena, Germany) using filters optimized for triple label experiments. Pictures were taken with a cooled CCD-camera (Visicam, Visitron, Puchheim, Germany) using MetaMorph software (Visitron, Puchheim, Germany).

RESULTS

MARK Phosphorylates MAP2c-, MAP4-BDC, and Tau on KXGS-Motifs and Induces a Phosphorylation-Dependent Antibody Epitope

We have previously shown that MARK phosphorylates the structural MAPs tau, MAP2c, and MAP4 in vitro [Drewes et al., 1995; Illenberger et al., 1996], thereby abolishing MT-binding and enhancing dynamic instability. Subsequently, it was shown that transiently transfected tau is phosphorylated upon MARK2 expression in CHO cells [Drewes et al., 1997]. We wanted to know if MARK is also able to phosphorylate MAP2 and MAP4 in cells. For the study of tau phosphorylation by MARK in cells, we had used a phosphorylation sensitive antibody, 12E8 [Seubert et al., 1995]. To look for phosphorylation of MAP2c and MAP4 on the homologous KXGS-motif by MARK with 12E8 as a marker, the analysis of the specificity of this antibody toward phosphorylated MAP2 and MAP4 was a prerequisite. Therefore, we performed an in vitro phosphorylation experiment in which purified MARK was incubated in the presence of ATP with a MAP4 construct comprising the microtubule-binding domain (MAP4-BDC), MAP2c, and the six different isoforms of tau found in human brain (Fig. 1) [Goedert et al., 1989]. As shown in Figure 2, in the absence of MARK the antibody 12E8 does not recognize MAP4-BDC or MAP2c, whereas in the presence of MARK an immunoreactive band corresponding to the molecular weight of the different MAPs is detectable (MAP+MARK, Fig. 2). In the case of tau-protein, at the concentrations used here, this antibody also weakly recognizes non-phosphorylated proteins (tau, Fig. 2) although after phosphorylation the affinity of 12E8 to tau-protein is increased severalfold (tau+MARK, Fig. 2). From densitometric analysis, we estimate the difference in affinity constants of phosphorylated compared to non-phosphorylated tau-protein to be about two orders of magnitude (data not shown).

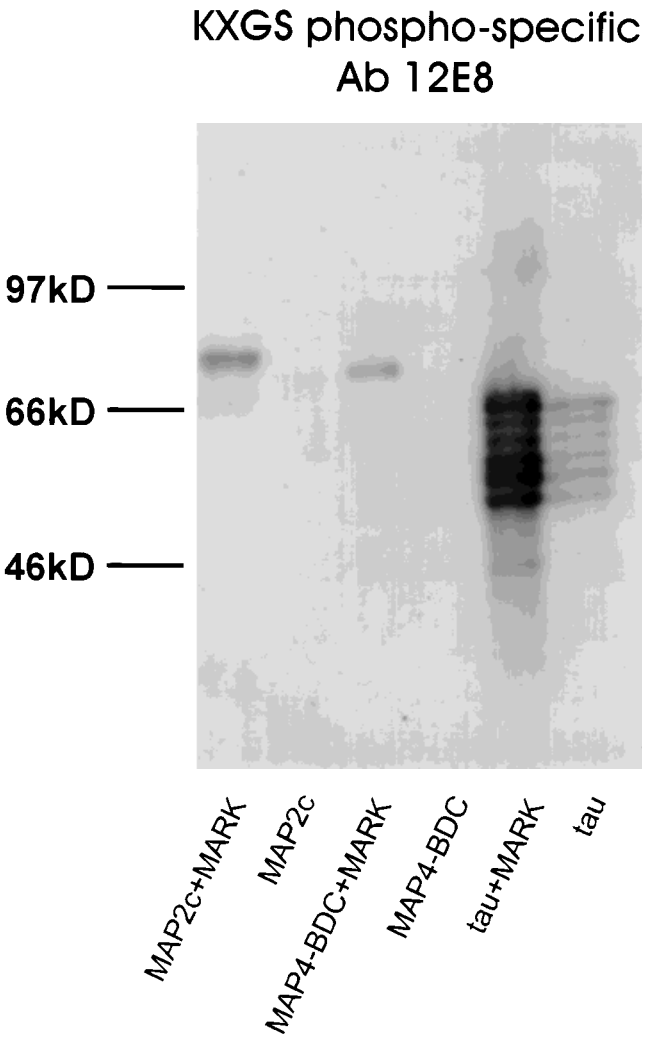


Fig. 2. MARK phosphorylation generates the epitope of the antibody 12E8 on MAP4-BDC and MAP2c. MAPs were incubated in the absence and in the presence of MARK purified from porcine brain. Proteins were then analyzed by immunoblotting with the monoclonal antibody 12E8, which has been raised against a phospho-KIGS peptide from the first repeat of tau [Seubert et al., 1995]. In the case of MAP4-BDC and MAP2c, the 12E8 antibody exclusively recognizes the phosphorylated MAPs (MAP + MARK). In contrast, at the concentration used here non-phosphorylated tau displays a certain affinity to the phosphospecific antibody (tau). The bands represent the six different isoforms found in human brain. However, in the presence of the kinase (tau + MARK) there is a severalfold stronger immunoreactivity towards 12E8, indicating the presence of the phosphorylated KXGS-motif. Densitometric analysis yields an approximately 100-fold higher affinity of 12E8 towards phosphorylated compared to non-phosphorylated tau-protein (data not shown).

In the case of MAP4 and MAP2c, the 12E8 antibody clearly discriminates between phosphorylated and non-phosphorylated proteins, despite the fact that 12E8 was raised against a synthetic phosphopeptide

corresponding to amino acids 257–270 of tau-protein (according to the numbering of human tau40), which is partially conserved in MAP2c or MAP4 (see Fig. 1b). Thus, MARK phosphorylates the microtubule-associated proteins on their conserved KXGS motifs in the repeat region in vitro and introduces the 12E8 epitope.

MARK1 and MARK2 Phosphorylate MAP2c and MAP4-BDC on Their Microtubule-Binding Site in Living Cells

To investigate whether MAP2 and MAP4 are also cellular substrates for MARK, we cotransfected MARK1 or MARK2 together with MAP2c and MAP4-BDC (Fig. 1a) [Illenberger et al., 1996]. Cells were then fixed in paraformaldehyde, and analyzed by triple immunofluorescence using antibodies against epitope-tagged MARK, tubulin and the phosphospecific antibody 12E8 (Figs. 3, 4).

Transfection of MARK1 and MARK2 could easily be demonstrated by the presence of the hemagglutinin epitope-tag. The transient expression of MAP2c and MAP4-BDC was visible through their bundling effect on the microtubule cytoskeleton. It is known that overexpression of MAP4 and MAP2c leads to alterations of the MT cytoskeleton [Weisshaar et al., 1992; Olson et al., 1995; Yoshida et al., 1996]. MT bundles were induced by overexpression of MAP2c (Fig. 3). Transfection of the MAP4-BDC construct resulted in a different phenotype (Fig. 4): the bundles appeared to be more flexible and of a wavy appearance and were significantly longer. Figure 3 shows that cells co-transfected with MARK2 and MAP2c stained brightly with the antibody 12E8 (Fig. 3a–c). Non-transfected cells whose microtubule network is not altered are not visible in the 12E8 stain. The same result was observed when a catalytically inactive MARK2-mutant was cotransfected, where target residues for kinase activation (Thr208 and Ser212) [Drewes et al., 1997] had been mutated (Fig. 3d–f), or when MAPs were expressed in the absence of MARK (data not shown). Similar results were obtained when MARK2 was cotransfected with MAP4-BDC. Again, cells which stained positive for MARK2 (Fig. 4a) and the MAP (note the bundling of microtubules through MAP4-BDC overexpression, Fig. 4b) were recognized by the phospho-KXGS specific antibody 12E8 (Fig. 4c). The catalytically inactive mutant of MARK2 was not able to phosphorylate MAP4-BDC. Cotransfection of another MARK isoform, MARK1, with MAP2c and MAP4-BDC yielded similar results (data not shown). Cells transfected with epitope-tagged kinase and the MAPs were stained with 12E8. Thus, MARK1 and MARK2 are capable to phosphorylate MAP2c and MAP4 in cells on KXGS-motifs within the microtubule binding region.

Doxycyclin-Inducible Expression of MARK2 in CHO-Cells

Since constitutive expression of transfected MARK is lethal to cells by disrupting the microtubule network [Drewes et al., 1997], we established a doxycyclin-inducible expression system [Gossen et al., 1995]. This approach allowed us to establish a cell line stably transfected with a toxic gene-product, although the clones were losing their inducibility during several months of propagation and the level of MARK expression decreased with time (data not shown). Thus, the tet-responsible promoter still seemed to allow for a low-level expression in the absence of doxycyclin. Since MARK2 overexpression is toxic to cells [Drewes et al., 1997], a constitutive expression even at very low levels can be expected to affect the viability of the cells and accounts for the decreasing expression levels as well as the partial loss of inducibility.

Figure 5 shows the analysis of the MARK2-inducible CHO cell-line I3. Upon addition of 1 μg/ml doxycylin into the medium for 24 h, MARK2 expression can be detected by two antibodies raised against synthetic peptides (Fig. 5, I3 +dox), derived from the C-terminal and the N-terminal region of the protein. Without induction, no expression is detectable in Western blot analysis (Fig. 5, I3 -dox). The kinase runs at approximately 80 kDa, which corresponds to the calculated molecular weight (81 kD) but is lower than the apparent molecular weight of MARK purified from porcine brain (110 kD) [Drewes et al., 1995]. Since the electrophoretic mobility of brain MARK is significantly increased by dephosphorylation with PP2A [Drewes et al., 1997], it is likely that the kinase expressed in CHO cells is not phosphorylated at all, or only phosphorylated to a minor extent. Also, it is possible that brain MARK carries additional posttranslational modifications, or that it constitutes a different MARK isoform. Since kinase activity depends on phosphorylation of Thr215 and Ser219 (numbering according to MARK1), it is likely that only a minor fraction of the overexpressed MARK2 is catalytically active.

MARK2 Induction Disrupts Microtubules and Interferes With Cell Growth

We have shown recently that transient overexpression of MARK1 or MARK2 in CHO cells severely affects the MT network and leads to cell death [Drewes et al., 1997]. The inducible cell line allowed us to study the effect of MARK kinase in more detail since lower expression levels could be achieved. We employed paraformaldehyde as a fixative, which retains soluble proteins in the cells. Figure 6 illustrates that induction of MARK2 in CHO cells fragments the microtubule network (compare the MARK-induced cell in Fig. 6a with non-induced

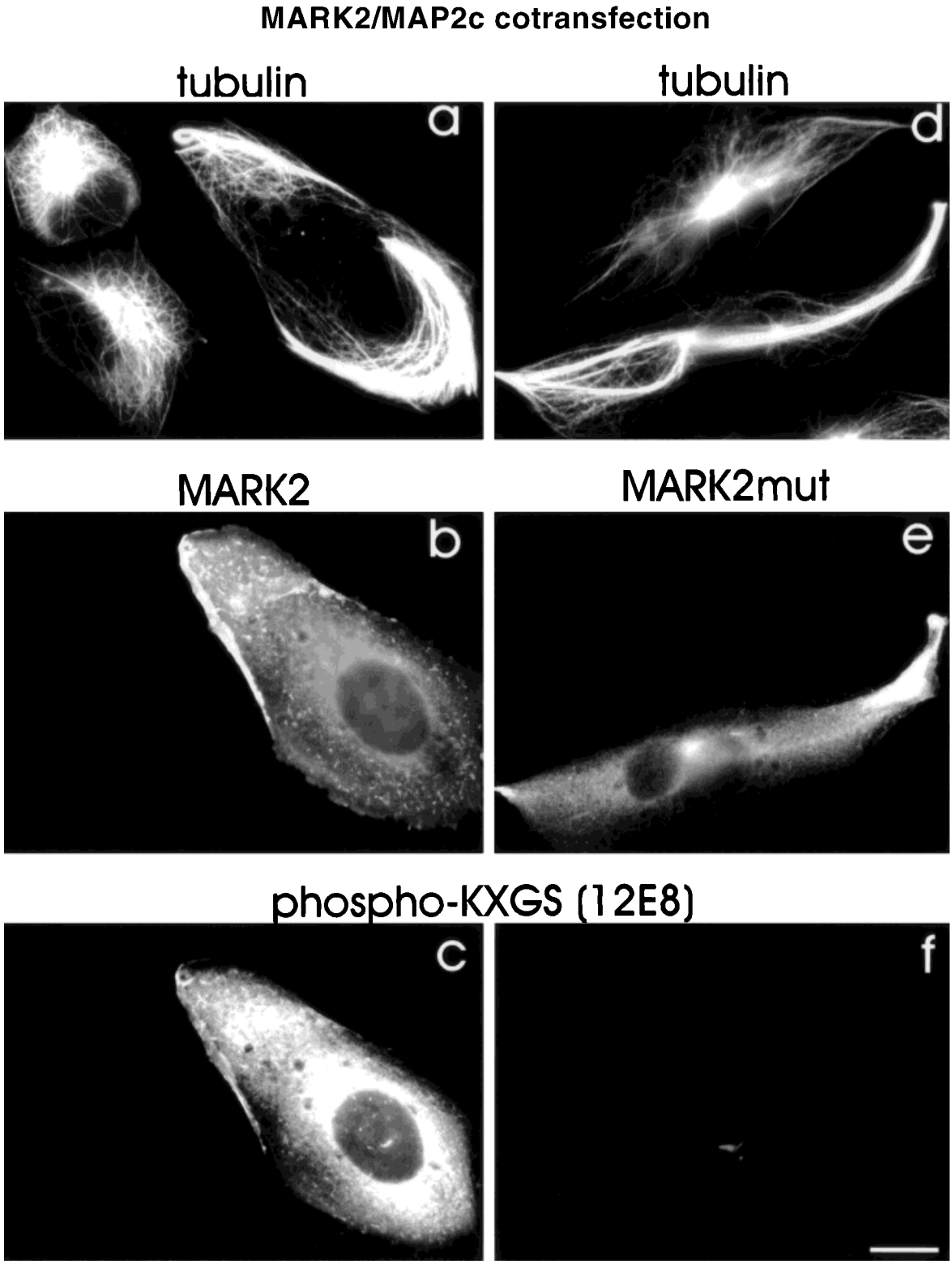


Fig. 3. Phosphorylation of MAP2c by MARK2 in CHO cells. MARK2 (**A–C**) or a catalytically inactive mutant of MARK2 (**D–F**), where the activating residues Thr208 and Ser212 have been mutated to alanine, were cotransfected with MAP2c in CHO-cells. The cells were stained with antibodies against tubulin (YL1/2; A–D), epitope-tagged MARK2 (B and E) and the phosphospecific antibody 12E8 (C–F). Expression of MAP2c is noticeable because of its bundling-effect on the microtubule network in the tubulin stain (compare transfected cells with non-transfected cells, e.g. D, bottom and top). In the presence of wt MARK2, 12E8 brightly stains cotransfected cells indicating phosphorylated MAP2c, whereas no staining is detectable in non-transfected cells. However, when cotransfecting the catalytically inactive MARK2 mutant (D-F), 12E8 does not stain the cotransfected cells. The same is true, if only MAP2c or MARK2 are transfected (data not shown). These data show that MARK2 phosphorylates MAP2c on the conserved KXGS-motif within the repeat region in vivo. Bar = 10 μm.

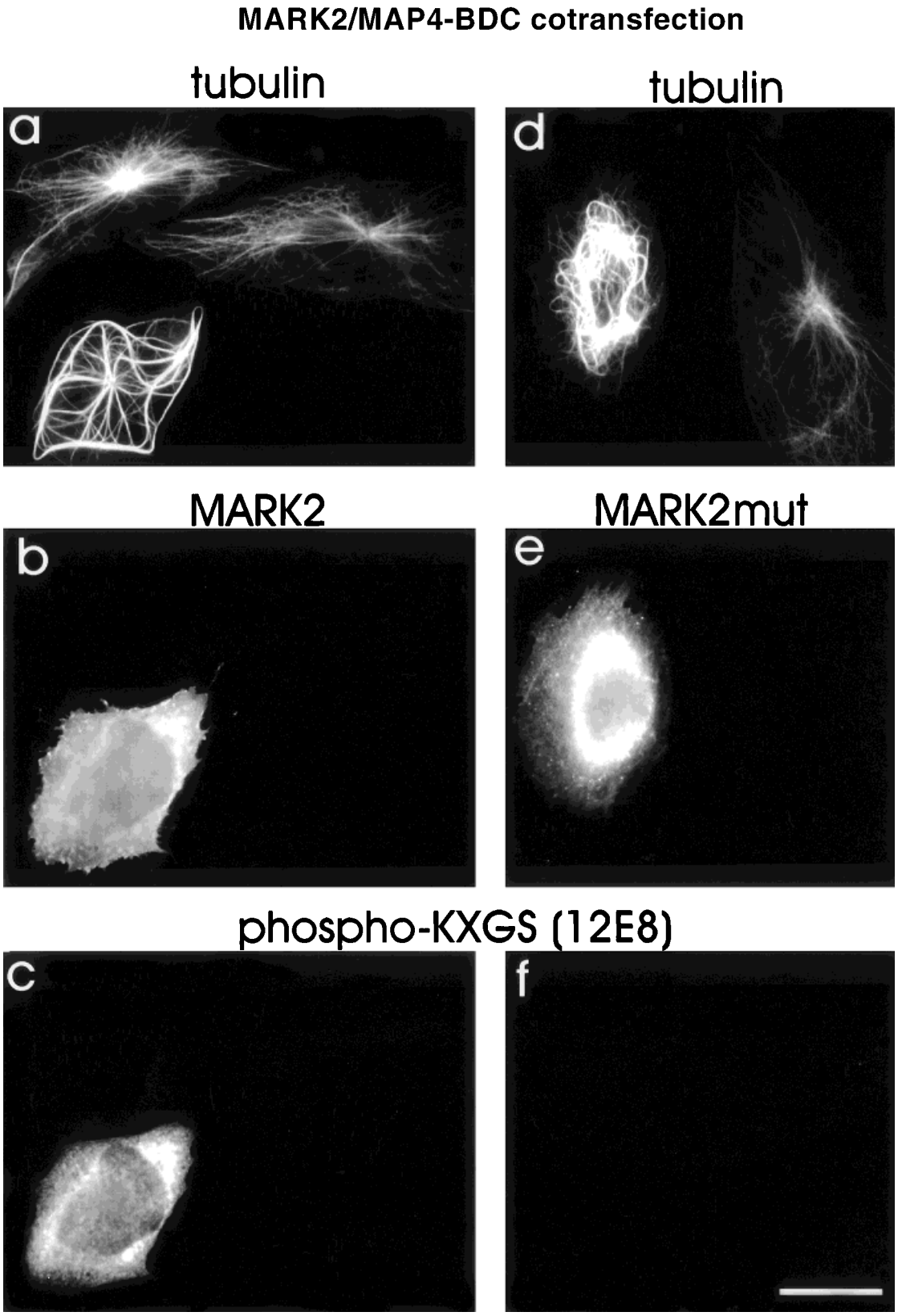


Fig. 4. Phosphorylation of MAP4-BDC by MARK2 in CHO cells. MARK2 (**A–C**) or the catalytically inactive mutant of MARK2 (**D–F**) were cotransfected with MAP4-BDC, a construct that comprises the microtubule binding domain of MAP4. Cells were stained with antibodies against tubulin (YL1/2; A and D), epitope-tagged MARK (B and E), and the phosphospecific antibody 12E8 (A-D). The expression of MAP4-BDC is detectable through reorganization of the microtubule-cytoskeleton. Note that the effect of MAP4-BDC differs significantly from MAP2c. The microtubule bundles are more flexible and wavy than in the case of MAP2c transfection. Cells that are positive for both MARK2 and MAP4-BDC are stained with the 12E8 antibody, whereas non-transfected cells are not visible, indicating the absence of cross reactivity towards endogenous proteins. Cotransfection of a catalytically inactive mutant of MARK2 (D–F) or no transfection (data not shown) does not generate the phospho-KXGS motif recognized by 12E8 on the MAP4 construct, indicating that MARK2 phosphorylates MAP4-BDC in vivo. Bar = 10 μm.

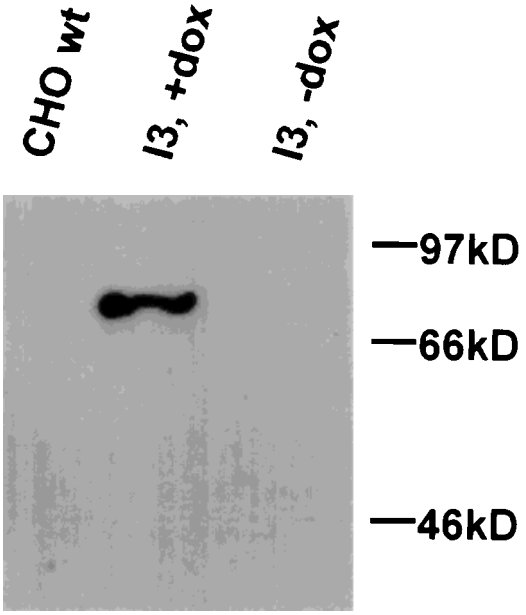


Fig. 5. MARK2 is expressed upon addition of doxycylin in the inducible CHO-cell line I3. Cells were grown overnight and MARK2 was induced in the presence of 1 µg/ml doxycylin for 24 h. Cells were harvested and resuspended in SDS-loading buffer. After SDS-gel separation and Western blotting, the blot was probed with polyclonal antibodies raised against peptides derived from the N- and C-terminal region of MARK (N-terminal Ab and C-terminal Ab, respectively). In the presence of doxycylin, an immunoreactive band of approximately 80 kD is stained by both antibodies (I3, +dox). MARK2 is not detectable in non-induced cells (I3, -dox). The difference in the molecular weight of induced or recombinant MARK2 compared to the kinase purified from porcine brain (~110 kDa) [Drewes et al., 1995] is presumably due to a difference in posttranslational modifications (e.g., phosphorylation).

cells). Small microtubules (fragments of normal cellular microtubules) can be observed in the cytoplasm (arrows) that are not connected to the microtubule organizing center (MTOC, arrowhead), which also is severely affected by MARK2 expression. This gives rise to an increased tubulin background stain in the cell, which is probably caused by the presence of disassembled cytosolic tubulin oligomers or dimers. The disruption of microtubules then leads to the rounding up of cells, their detachment from the substratum and cell death. The loss of cell viability is also demonstrated by the fact that we were unable to establish stable MARK1 and MARK2 cell lines under the control of the constitutively active CMV-promoter (data not shown). The complete disruption of microtubules was most apparent in cells that expressed MARK to a high level, suggesting that a lower amount of induced kinase could be tolerated. This can be seen in Figure 6c, where we analyzed the growth behavior of the MARK inducible cell line in the presence and absence of doxycylin. Without induction, the cell number increased from $1*10^4$ to $3*10^5$ within 5 days, but in the induced state the cell number was ~5-fold lower. Thus, at lower expression levels MARK retarded growth but was not lethal to all cells.

MARK2 Induction Leads to Phosphorylation of the Endogenous MAP4 Of CHO Cells

Phosphorylation of MAP4, MAP2, and tau by MARK detaches these MAPs from microtubules in vitro and leads to disruption of microtubules [Drewes et al., 1995; Illenberger et al., 1996]. To find out whether the breakdown of the microtubule-cytoskeleton after MARK-induction is mediated through phosphorylation of endogenous CHO cell MAPs, we looked for phosphorylation of the conserved KXGS-motif in the repeat region of endogenous, heat stable MAPs in induced and non-induced cells. It has been shown previously that CHO cells are expressing a MAP that is similar to the ubiquitous MAP4 [Brady and Cabral, 1985]. Its phosphorylation can be detected by the phosphorylation dependent antibody 12E8 since this antibody recognizes both MAP2 and MAP4 after phosphorylation of their conserved repeat regions (Fig. 2). After MARK2-induction and isolation of the heat stable fraction of MAPs, a 12E8-immunoreactive protein of ~200 kDa appeared (Fig. 7a, phospho KXGS), corresponding to the endogenous MAP4 from CHO cells [Brady and Cabral, 1985]. In non-induced control cells, the intensity of the immunoreactive band was very low. In addition, we verified the identity of this band by reprobing the blot with a phosphorylation-independent antibody raised against CHO-MAP4 [Brady and Cabral, 1985]. Figure 7a shows that this antibody stains the same band, demonstrating that the 12E8 antibody recognizes MARK-phosphorylated CHO-MAP4. By matching the amounts of protein loaded onto the SDS-gels, we estimated the relative amounts of phosphorylated CHO-MAP4 in induced and non-induced cells by densitometric quantitation of the relative intensities of 12E8 vs. anti CHO-MAP immunoreactivity in four independent experiments. The result shows that induction of MARK2 leads to the formation of the epitope recognized by 12E8: In Figure 7b, the relative signals of 12E8 to CHO-MAP4 immunoreactivity without induction are significantly lower (~4-fold) than in the induced samples. This indicates that MARK2 phosphorylates the endogenous MAP4 in CHO-cells, leading to the typical microtubule destabilization effects discussed above.

MARK2 Disrupts Microtubules and Intermediate Filaments But Does Not Affect Actin Filaments

To investigate whether the effects of MARK-expression in CHO cells, such as rounding up and detachment from the substratum, resulted from the disruption of microtubules we looked for the integrity of other

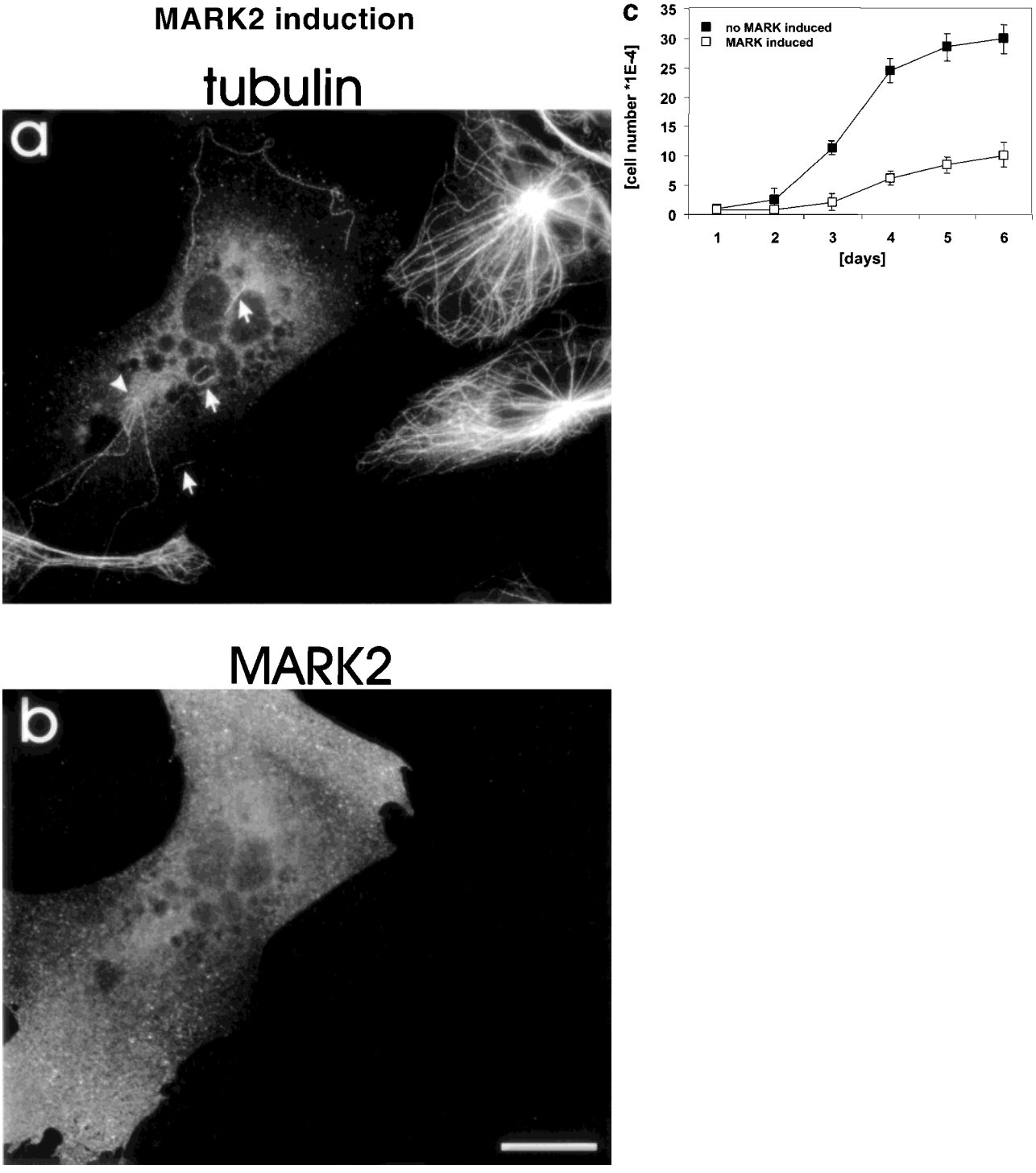


Fig. 6. MARK2-expression in the inducible cell line disrupts the microtubule cytoskeleton and interferes with cell viability. **a,b:** MARK2 was induced for 48 hours, cells were fixed in paraformaldehyde and stained for tubulin (a) and induced MARK2 (b). Whereas in noninduced cells, microtubules and the microtubule organizing center (MTOC) are clearly visible, in the induced state only residual microtubules can be seen (*arrows*), while the MTOC is nearly completely destroyed (*arrowhead*). The increased cytosolic stain in Figure 7a is presumably due to the presence of disassembled tubulin dimers or oligomers. **c:** The induction of MARK expression causes the CHO cells to grow about 5-fold slower than noninduced cells. A high percentage of cells surviving prolonged exposure to doxycyclin lost the ability to overexpress MARK.

cytoskeletal systems. The actin stress fiber network of MARK2 transfected cells remains intact, even when the microtubule cytoskeleton is severely affected (data not shown). On the other hand, the integrity of the intermediate filament depends on intact microtubules [Klymkowski, et al., 1989]. This is in agreement with our results (Fig. 8): In cells where MARK2 was induced for ~48 h so that microtubules were destroyed, the integrity of the vimentin network was also affected. Therefore, the effect of MARK2-expression on cell shape and viability is mediated mainly through breakdown of the microtubule cytoskeleton concomitant with disruption of the intermediate filaments, independently of the actin network. Although we cannot rule out that MARK2 might disrupt the intermediate filament system through some other mechanism, IF-disruption is likely a direct consequence of microtubule disruption [Prahlad et al., 1998].

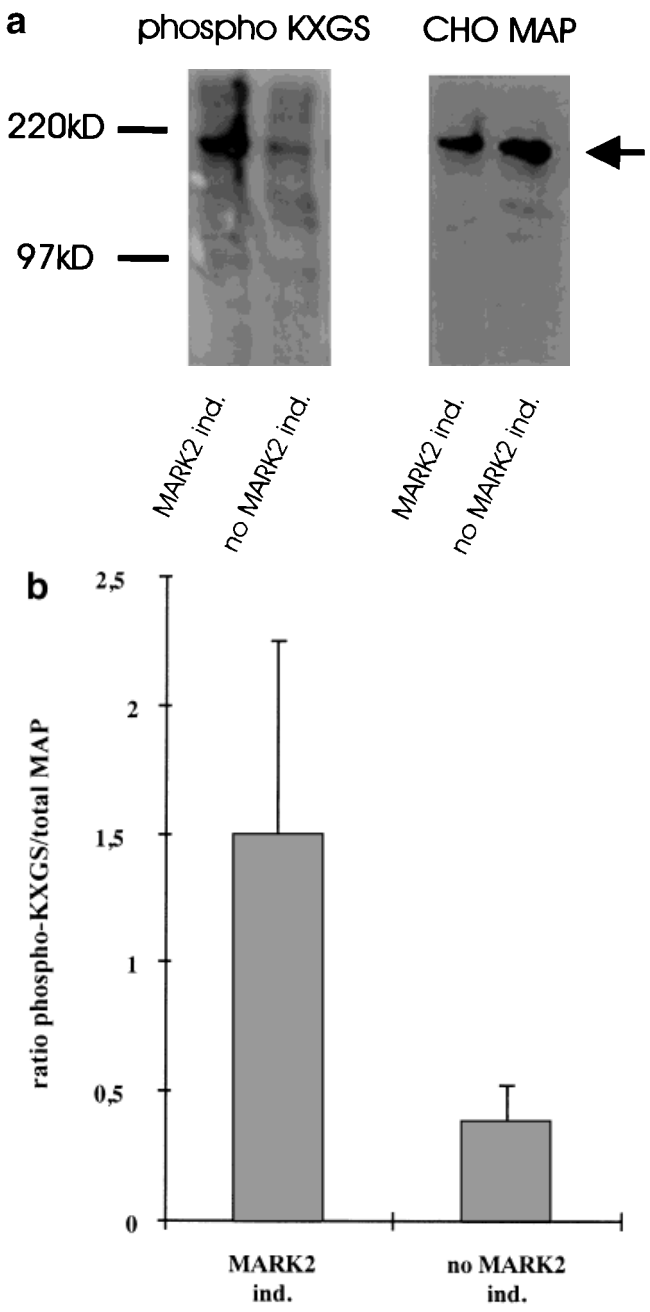
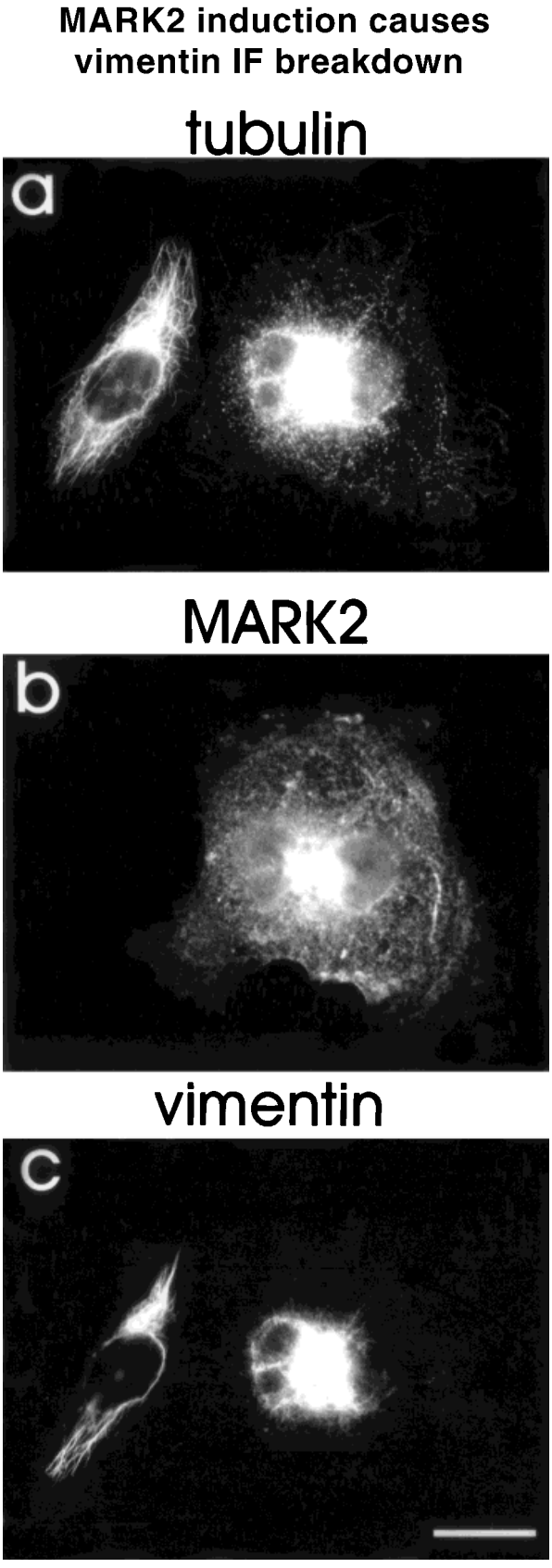


Fig. 7. MARK2 induction leads to phosphorylation of endogenous CHO-MAP. MARK2-inducible cells were grown for 24 hours in the presence or absence of doxycylin. The endogenous CHO-MAP was then purified from heat-stable supernatants and subjected to gel-electrophorisis and immunoblotting with the phosphospecific antibody 12E8. Later, the blot was reprobed with a polyclonal antibody raised against CHO-MAP (CHO MAP). **a:** In the induced state (''MARK2 ind.''), 12E8 recognizes the endogenous CHO-MAP more strongly than in the non-induced state (''no MARK2 ind.''). After reprobing the blot with an anti CHO-MAP antibody, bands at a corresponding molecular weight were recognized, indicating that 12E8 stains endogenous phosphorylated MAP. The immunoreactive band (*arrow*) runs at approximately 200 kD, which is in agreement with the previously published molecular weight of the endogenous CHO-MAP of 210kDa [Brady and Cabral, 1985] and to the molecular weight of the ubiquitous MAP4 (about 200 kDa in different species) [West et al., 1991]. Note that although there is a higher amount of CHO-MAP in the non-induced sample loaded onto the gel (compare no MARK2 ind. with MARK2 ind. in the CHO MAP blot), there is only a faint band recognized by 12E8 at the corresponding area (phospho-KXGS, no MARK2 ind.). **b:** Four independent experiments were performed, the blots were incubated with 12E8, stripped, reprobed with the phosphorylation independent antibody, and quantitated densitometrically. The ratios of the intensities on the films generated by 12E8 vs. CHO-MAP-antibody were then plotted. In the induced state (MARK2 ind.), this ratio is 4-fold higher ($P < 0.08$, two-tailed Student's *t*-test) than in the non-induced state (no MARK2 ind.). This result confirms that MARK2-induction phosphorylates endogenous CHO-MAP. Error bars indicate standard deviation.

DISCUSSION

The function of the microtubule cytoskeleton is regulated by a variety of associated proteins that stabilize microtubules and interact with other cell components. The ability of MAPs to bind to microtubules is largely governed by the phosphorylation of MAPs. In most cases known so far, phosphorylation leads to a lower affinity, which can cause the detachment of MAPs from microtubules and their destabilization. However, since MAPs may contain many phosphorylation sites that are targetted by different protein kinases, it is difficult to know which sites or which kinases are responsible for the MAP-microtubule interaction. One of the best-studied families of MAPs comprises the proteins MAP2, tau, and MAP4; MAP2 and tau are mostly neuronal where they stabilize microtubules in dendrites and axons, and MAP4 is ubiquitous. These MAPs share a homologous region of 3–5 internal repeats in their C-terminal domains and other characteristics, notably a basic domain rich in prolines flanking the repeats. Since these domains contain several Ser-Pro or Thr-Pro motifs, they can be phosphorylated by



several proline-directed kinases. This type of phosphorylation appears to have only a moderate influence on their binding to microtubules. By contrast, tau protein contains two sites whose phosphorylation by non-proline-directed kinases causes its detachment from microtubules: Ser 214, a target of PKA, and Ser262, a target of MARK, which phosphorylates at the KXGS motifs within the repeats.

In previous reports, we have shown that overexpression of MARK leads to cell death, that the effect depends on the activation of MARK (by phosphorylation at the regulatory loop in the catalytic domain), and that KXGS motifs in the repeat domain of tau are indeed cellular targets of MARK [Drewes et al., 1995, 1997]. This result was of particular interest in the context of neuronal degeneration in Alzheimer's disease, which is accompanied by hyperphosphorylation of tau at Ser262 in the first KXGS motif, its detachment from microtubules, and subsequent pathological aggregation into paired helical filaments. In addition, several kinases of the MARK family are involved in generating cellular polarity [Drewes et al., 1998], and phosphorylation of the KXGS motifs of tau is important in generating process outgrowth in transfected Sf9 cells [Biernat and Mandelkow, 1999]. Nevertheless, the observations left open several important issues: (1) The results were obtained in CHO cells transfected with tau and/or MARK. It was not clear whether the endogenous MAP4 of CHO cells, or, more generally, the endogenous MAPs of other cells, would be a target of MARK. (2) The expression of active MARK in CHO cells (without exogenous tau) leads to cell death; was this due to the phosphorylation of endogenous MAP4 and subsequent destruction of the microtubule network? (3) Conversely, with CHO cells containing both active MARK and exogenous MAP, it was possible to demonstrate the phosphorylation of Ser262, but in this case the microtubules were preserved and cell death was retarded. The question was, therefore, could the negative effects of MAP phosphorylation on microtubule stability be counteracted by increasing the levels of total MAPs? (4) Finally, how does the activity of MARK affect other components of the cytoskeleton, for example microfilaments and

Fig. 8. The vimentin network is disrupted after MARK2 induction in CHO cells. MARK2 was induced for 48 h, cells were fixed in paraformaldehyde and stained for tubulin (YL1/2, **a**), MARK2 (anti HA-tag, **b**), and vimentin (V9, **c**). MARK expression leads to a breakdown of the microtubule cytoskeleton (compare microtubules of induced with non induced cells). Concomitant with microtubule disruption, the extension of the vimentin intermediate filament system is significantly reduced; it is located at the center of the induced cell where the majority of the residual tubulin fluorescence can be seen. By contrast, in non-induced cells, vimentin is visible as the typical filamentous network extending from the center of the cells to the periphery. Bar = 10 μm.

intermediate filaments? The answer to these questions is summarized below.

1. MARK phosphorylates not only tau, but also the other related proteins MAP2 and MAP4 on the corresponding KXGS motifs in the repeats in CHO cells. To demonstrate this, we first verified that the reporter antibody 12E8, developed originally for phosphorylated Ser262 in tau [Seubert et al., 1995], is diagnostic for phosphorylated MAP2 and MAP4 as well (Figs. 2–4). Secondly, MARK kinases phosphorylate MAP2 and MAP4 not only in vitro, but also in the cellular environment. This was shown by cotransfection of MARK and MAPs (MAP2c or the microtubule-binding fragment MAP4-BDC). In the case of the endogenous CHO-MAP4, we expressed MARK in CHO cells in a doxycyclin-inducible fashion [Gossen et al., 1995]. Using these MARK-inducible cell lines, it became possible to induce MARK and then detect the phosphorylation of endogenous MAPs (CHO-MAP4).
2. MARK-expression affects CHO cells containing only endogenous MAP4, but the cells can be protected by elevating MAPs, e.g., by transfection with MAP2 or MAP4 constructs. This is a strong indication that the damage induced by MARK, indeed, takes place on the level of microtubules: Endogenous MAPs can be effectively depleted by phosphorylation, which leads to the decay of microtubules, but this can be counteracted by replenishing the pool of MAPs competent to bind and stabilize microtubules. By the same token, the destruction induced by MARK is specific for microtubules, whereas microfilaments are not affected. Since the integrity of the intermediate filament network depends on microtubules [Prahlad et al., 1998] they, too, become disrupted along with microtubules (Fig. 8).

The observations tie together complementary functions of MAPs on microtubules, all of which can be regulated by phosphorylation. The traditional view of MAPs has been that of microtubule stabilizing factors. This function is well established in vitro and can be demonstrated in cells as well, i.e., overexpression of MAPs stabilizes microtubules (often in the form of microtubule bundles) [Drubin and Kirschner, 1986; Weisshaar et al., 1992; Chen et al., 1992; Esmaeli-Azad et al., 1994], and depletion of MAPs tends to make them more labile. Phosphorylation that detaches MAPs from microtubules is analogous to depletion and also leads to more labile microtubules (e.g., MAP4 phosphorylation during mitosis) [Ookata et al., 1997; Shiina and Tsukita, 1999]. However, changes in MAP levels or activity cannot explain the full range of microtubule dynamics, and a variety of other factors are now known that influence microtubule stability by different mechanisms, e.g., sequestering of tubulin subunits (via stathmin), endwise stabilization or destabilization (by STOP proteins or uncapping factors) [Bosc et al., 1996; Walczak et al., 1996], or microtubule severing (e.g., katanin) [Hartman et al., 1998]. These observations suggest that MAPs have functions in addition to microtubule stabilization. Examples of such functions are the regulation of spacing between microtubules and other cell components (which depends on the size of the projection domains) [Chen et al., 1992], and the anchoring of cellular enzymes (e.g., kinases, phosphatases) [Obar et al., 1989, Morishima-Kawashima and Kosik, 1996; Liao et al., 1998; Sontag et al., 1995]. One role of MAPs that is currently attracting attention is the regulation of motor-dependent transport of vesicles and organelles. These particles become more mobile when MAP2 or MAP4 are depleted or inactivated by phosphorylation, which removes them from microtubules, or conversely less mobile when MAPs are overexpressed [Lopez and Sheetz, 1995; Sato-Harada et al., 1996; Bulinski et al., 1997]. We showed recently that the inhibition of transport by tau protein affects particularly the plus-end directed, kinesin-dependent movement, such that minus-end directed movements become dominant (as seen by the retraction of mitochondria or ER towards the cell interior) [Ebneth et al., 1998]. These effects of MAPs on motor proteins are rather subtle, which explains why a number of in vitro studies [e.g., von Massow et al. 1989, Lopez and Sheetz, 1993, 1995) have been suggestive but remained somewhat controversial; however, the effects become obvious in the cellular environment.

How could the MAP effect on transport be regulated by phosphorylation? The most obvious mechanism would be the release of MAPs from microtubules. This would require kinases and phosphorylation sites on MAPs, which strongly decrease the MAP-microtubule interaction. In the case of the tau and MAP2, two kinases are of particular interest, PKA and MARK. Activation of PKA appears to facilitate transport processes [Sato-Harada et al., 1996] and can detach tau from microtubules by phosphorylating Ser214 in a region flanking the repeat domain [Brandt et al., 1994; Illenberger et al., 1998]. MARK appears even more potent than PKA; it phosphorylates MAPs at one or several KXGS motifs in the repeat domain [Drewes et al., 1995; Illenberger et al., 1996; Jenkins and Johnson, 1997]. We have shown recently that overexpression of MARK in CHO cells stably transfected with tau leads to the phosphorylation of tau at Ser262 [Drewes et al., 1997]. In the present study, we have shown that MARK can phosphorylate not only tau, but

also the related proteins MAP2 and MAP4 at the corresponding KXGS motifs in the repeat domain, including the endogenous MAP4 of CHO cells. The action of MARK presumably takes place on the level of the MAP-microtubule interaction, since an increased activity of MARK can be counteracted by elevating the level of MAPs. This means that MARK causes microtubule breakdown, presumably by stripping MAPs off the microtubules.

The family of mammalian MARKs includes at least four members [for review see Drewes et al., 1998]. They have recently attracted attention for two reasons: Phosphorylation sites typical for MARK are elevated in pathologically aggregated tau protein in Alzheimer's disease, a neurodegenerative disease [Morishima-Kawashima et al., 1995]. Furthermore, homologues of MARKs have been implicated in the establishment and maintenance of cell polarity, e.g., in the asymmetric division of the *C. elegans* zygote (par-1) [Guo and Kemphues, 1996] or of *S. Pombe* (kin1^{+}) [Levin and Bishop, 1990]. Inactivation or loss of a mammalian homolog of MARK leads to loss of the the polarized structure of epithelial cells (MDCK) [Böhm et al., 1997] or is a negative marker of oncogenic transformation (p78, a relative of MARK3) [Parsa, 1988]. Since loss of cell polarity is also a characteristic feature of neurons in Alzheimer's disease, these observations may point to a related mechanisms of action, involving the stability of microtubules and/or their role in intracellular transport.

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