The phosphorylation of microtubule-associated proteins (MAPs) is thought to be a key factor in the regulation of microtubule stability. We have shown recently that a novel protein kinase, termed p110 microtubule-affinity regulating kinase ("MARK"), phosphorylates microtubule-associated protein tau at the KXGS motifs in the region of internal repeats and causes the detachment of tau from microtubules (Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H. E., Mandelkow, E. M., and Mandelkow, E. (1993). J. Biol. Chem. 270, 7679–7688). Here we show that p110mark phosphorylates analogous KXGS sites in the microtubule binding domains of the neuronal MAP2 and the ubiquitous MAP4. Phosphorylation in vitro leads to the dissociation of MAP2 and MAP4 from microtubules and to a pronounced increase in dynamic instability. Thus the phosphorylation of the repeated motifs in the microtubule binding domains of MAPs by p110mark might provide a mechanism for the regulation of microtubule dynamics in cells.

In living cells, microtubules undergo transitions between stable and dynamic states. They are organized into stable cytoskeletal structures such as the processes of neuronal cells or the axonemes of cilia and flagella, but are also key players in dynamic events during cell morphogenesis or chromosome partitioning at mitosis. Microtubule stability is thought to be modulated by a variety of post-translational modifications of both tubulin and MAPs. Structural MAPs are filamentous proteins which bind to microtubules in a nucleotide-insensitive way, forming elongated projections from the microtubule surface (for reviews, see Olmsted (1991), Hirokawa (1994), Schoenfeld and Obar (1994), and Mandelkow and Mandelkow (1995)). MAPs can control microtubule dynamics in vitro and in vivo (Drechsel et al., 1992; Pryer et al., 1992; Umemura et al., 1993; Gustke et al., 1994; Brandt et al., 1994; Dhamodharan and Wadsworth, 1995; Trinczek et al., 1995). Tau and MAP2 are the most studied MAPs in the vertebrate nervous system; tau is abundant in the axon, whereas MAP2 is localized predominantly in dendrites (Binder et al., 1985; Riederer and Matus, 1985). MAP4 is not limited to the nervous system and is the predominant MAP in many types of cells and tissues (Bulinski and Borisy, 1980; Parysek et al., 1984; Aizawa et al., 1990). MAP2, tau, and MAP4 are grossly similar in domain structure, having N-terminal projection domains and C-terminal microtubule binding domains (Lee et al., 1988; Lewis et al., 1988; Aizawa et al., 1991; West et al., 1991; Chapin and Bulinski, 1991). The C-terminal part of these proteins displays considerable homology in a repeated sequence motif. The sequences in the C-terminal region are rich in basic amino acids which probably interact with the acidic sequence in the C terminus of tubulin (Littauer et al., 1986).

Several lines of evidence suggest that the binding of MAPs to microtubules is regulated by phosphorylation. MAPs isolated from tissue or cells are phosphoproteins (Sloboda et al., 1975; Vallee, 1980; Burns et al., 1984; Tsuyma et al., 1986; Brugg and Matus, 1991; Watanabe et al., 1993), MAPs are good substrates for many protein kinases in vitro (Theurkauf and Vallee, 1983; Lindwall and Cole, 1984; Mori et al., 1991; Drewes et al., 1992), and phosphorylation interferes with their microtubule stabilizing capacity (Brugg and Matus, 1991; Shinya et al., 1992; Drechsel et al., 1992; Biernat et al., 1993; Brandt et al., 1994; Ookata et al., 1995; Trinczek et al., 1995). In the case of tau protein, phosphorylation has been extensively studied, because aberrantly phosphorylated tau is involved in the neurofibrillary pathology of Alzheimer’s disease (reviewed by Goedert (1993), Mandelkow and Mandelkow (1993), and Trojanowski and Lee (1994)). However, it has been difficult to establish the relationship between protein kinases, phosphorylation sites, and their effect on microtubule affinity, nucleation, and dynamic instability. Recently, we have used an approach which combined site-directed mutagenesis of recombinant tau and in vitro phosphorylation by a brain tissue extract to identify sites that are crucial for microtubule binding (Gustke et al., 1992). We found that phosphorylation of tau at a single serine residue, located within the sequence KXGS(900) in the first repeat of the binding domain, strongly suppresses microtubule binding (Biernat et al., 1991). The phosphorylation of sites outside the microtubule binding domain, which occurred mostly on Ser/Thr-Pro motifs, had a relatively weak effect. Subsequently, we characterized and purified from brain tissue a novel kinase of molecular mass 110 kDa, which effectively phosphorylated Ser962 and displayed a pronounced specificity for all four KXGS motifs in the repeat domain of tau (Drewes et al., 1995). This kinase efficiently caused the loss of
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MATERIALS AND METHODS

Proteins—A cDNA clone of the rat juvenile MAP2 isoform, MAP2c, was a gift of C. Garner (Kindler et al., 1990). Three point mutants of MAP2c were constructed: MAP2cA319, MAP2cA350, and MAP2cA319 to 350, in which serines at position 319 and/or 350 were mutated to alanine (corresponding to positions 1682 and 1713 in the full MAP2 numbering, Table I). Human tau cDNA clones were a gift of M. Goedert (Goedert et al., 1989). The numbering used here refers to the rat sequence of full-length MAP2 (1830 residues) and the biggest human tau isoform (clone httau40, 441 residues). The MAP4 construct used was that obtained from a murine MAP4 clone (Couchie et al., 1989) and comprises the microtubule binding region, from residue 640 to the C terminus, and carries an N-terminal hemagglutinin tag sequence (Field et al., 1988). The numbering used here refers to the full-length murine MAP4 sequence (1125 residues). Proteins were obtained either by expressing constructs in Escherichia coli using variants of the PET expression vector (Studier et al., 1990), or by purification from tissues. Brain MAP2 was prepared from porcine brain microtubule protein by heat treatment, Mono S FPLC (Pharmacia Biotech Inc.), and gel filtration as described by Willis et al. (1992). MAP4 was prepared from a mouse heart and lung tissue extract by ammonium sulfate precipitation, heat treatment, Mono S FPLC, and hydrophobic interaction chromatography on Phenyl-Sepharose and, proteins were purified by MALDI-TOF mass spectrometry (modified after Aizawa et al., 1989)). Phosphocellulose-purified tubulin was purified from porcine brain following Mandelkow et al. (1985). The protein kinase P110\textsuperscript{\textregistered} was prepared from porcine brain as described recently (Drewes et al., 1995). Using 50 μM MAP2c as substrate and 1 mM ATP at 37 °C, the preparation was determined to have an activity of 33 milliunits/μl by the phosphocellulose paper assay (1 unit is defined by the transfer of 1 μg of phosphate/min at 1 mM ATP at 37 °C).

Phosphorylation Reactions—Phosphorylation reactions were carried out essentially as described (Drewes et al., 1995). Briefly, the buffer was 40 mM Heps, pH 7.2, containing 1 mM ATP, 5 mM MgCl\textsubscript{2}, 2 mM EGTA, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% Brij-35. Reactions were terminated by brief heating to 95 °C, and phosphorylation was assayed in SDS gels stained with Coomassie Brilliant Blue.

Phosphopeptide Mapping—Following phosphorylation reactions, the kinase was removed by boiling the samples in 0.5 M NaCl, 10 mM dithiothreitol and centrifugation. The heat-stable MAPs were precipitated with 15% trichloroacetic acid, cysteine residues were modified by reaction with 15% trichloroacetic acid, cysteine residues were modified by reaction with 0.2 M dithiothreitol, 0.1 M phenylmethylsulfonyl fluoride, and 0.01% Brij-35. Reactions were terminated by brief heating to 95 °C, and phosphorylation was assayed in SDS gels stained with Coomassie Brilliant Blue.

Sequence Comparisons and Terminology—In order to compare the three MAPs it is useful to describe their domains with a common nomenclature (Table 1, Fig. 1). The MAPs differ in size but contain regions of homologous sequences, and they have similar gross characteristics. Each of these MAPs has an acidic N-terminal region, followed by a basic region containing the repeats, and a short acidic region. The interaction with microtubules lies in the basic region. One can broadly distinguish between an N-terminal “projection” region and a C-terminal “assembly” region. This distinction is based on proteolytic cleavage which leaves the assembly domain attached to the microtubule wall while the projection domain is released (Murphy and Borisy, 1975; Van Hove, 1980). A finer subdivision can be derived from the sequences, as follows.

We delimit tau into an acidic N-terminal domain (Ala, residues Met\textsuperscript{1} to Ala\textsuperscript{115}), containing the two near N-terminal insertions Gly\textsuperscript{85} to Gly\textsuperscript{73} and Asp\textsuperscript{96} to Thr\textsuperscript{102}, exons 2 and 3, which may be absent due to alternative splicing, see Goedert et al. (1989) and Himmler et al. (1989)), the basic region (B, residues Gly\textsuperscript{259} to Ser\textsuperscript{319} and the acidic tail (C), Gly\textsuperscript{460} to Leu\textsuperscript{484}). The basic region contains the proline-rich domain (P, Ile\textsuperscript{468} to Leu\textsuperscript{492}, containing a chymotryptic cleavage site Tyr\textsuperscript{479} which subdivides “P” into “P1” and “P2,” and separates the projection and assembly domains), and the repeats (see below). In the “big tau” isoform of peripheral nerves, there is an insert of about 254 residues between residues Leu\textsuperscript{431} to Leu\textsuperscript{455} (judging from the rat sequence, exon 4a, Coughie et al., 1992); the nature of this insert is acidic so that the size of the acidic region is expanded 3-fold (from 119 to 379 residues).

In MAP2, the acidic N-terminal region extends from Met\textsuperscript{1} to Ala\textsuperscript{142}, the basic region is Arg\textsuperscript{347} to Ser\textsuperscript{597}, and the neutral C-terminal tail is from Glu\textsuperscript{1420} to Ser\textsuperscript{1639}. The acidic repeats contain a proline-rich domain (Leu\textsuperscript{1546} to Leu\textsuperscript{1665}) and the repeats (Arg\textsuperscript{1644} to Ser\textsuperscript{1798}); it also contains cleavage sites for trypsin (behind Lys\textsuperscript{1525} and Arg\textsuperscript{1664}, Wille et al. (1992) and thrombin (behind Arg\textsuperscript{1630}, Ainsztein and Purich (1994)) which roughly separate projection and assembly domains. In the region Asp\textsuperscript{1282} to Thr\textsuperscript{1351} (1363 residues) is spliced out. Most of the insert has acidic character, especially for the last 90 residues (Arg\textsuperscript{1301} to Thr\textsuperscript{1351}), and thus can be regarded as a large extension of the acidic N-terminal region (from 151 residues in MAP2c to 1424 in MAP2).

In MAP4, there is an acidic N-terminal domain (Met\textsuperscript{1} to Thr\textsuperscript{348}) includ-
the acidic "KDM" domain Thr^{243}-Lys^{566}, a basic proline-rich domain (Asn^{659}-Arg^{895}) which can be subdivided into "P" (Asn^{659}-Ala^{730}, proline-rich) and "SP" (Thr^{731}-Arg^{895}, rich in Ser-Pro motifs), the basic repeats (Ala^{897}-Gly^{1090}), and an acidic C-terminal tail (A{^{1091}-I^{1125}}). Repeats "1a" and "2" can be absent due to alternative splicing (Chapin et al., 1995).

The repeats are the most striking aspects of the three MAPs. They are typically 31–32 residues long, and are similar to one another within one MAP and between different MAPs. They can be subdivided into about 13 residues of lower homology (sometimes referred to as the linker region or inter-repeat region), and the 18 C-terminal residues of higher homology, the repeats proper, mostly ending with a PGGG motif. The boundaries between the repeats can be chosen in different ways; we prefer the alignment shown in Table I because in this case the second repeat of tau coincides exactly with one alternatively spliced exon (number 10, Val^{275}-Ser^{305}).

In addition it was realized that other stretches in the repeat domain were repeat-like, albeit with even lower homology (e.g. the 38-residue repeat following repeat "1" in MAP4 and the 32-residue repeat following repeat "4" in tau, Chapin and Bulinski (1992)). In order to unify the nomenclature we will denote the "classical" repeats (containing the higher degree of homology) as 1, 2, 3, and 4. One of these, 2, maybe absent due to alternative mRNA splicing. The low homology repeat of MAP4 will be called 1a. The repeat following 4 will be "4a." Thus Table I shows 6 repeats, 5 of which are common to all three MAPs (1–4 and 4a), and 1a is specific for MAP4 (note that 4a of MAP4 shows less homology than the corresponding tau and MAP2 sequences). These repeats correspond to repeats 1–6 in Chapin and Bulinski (1992), and 4a is found in MAP2, MAP3, and MAP4.

| Repeat | Sequence | Description |
|---|---|---|
| 1 | MAFPKQG | Acidic domain Thr^{243}-Lys^{566} |
| 2 | EFPQTTEGSSGFGSADKTPAE | Basic proline-rich domain Asn^{659}-Arg^{895} |
| 3 | DYTAPLVO...ROAP..QGEAAQRTPEPFT | Basic repeats Ala^{897}-Gly^{1090} |
| 4 | ATVYRNPEFPPSPAPGCTAALER* | Acidic C-terminal tail A{^{1091}-I^{1125}} |
| 5 | IATFPEGAP..QEGQANATRALKTAOPPSFPPSPGFGDROGTT | Tau-specific repeat |
| 6 | GSYSSPS... | Basic repeats |
| 7 | QTAIVFPMDFQLEHVSQGK | Repeat 1 |
| 8 | VQIZEFEDLQESVCHEDKEVPGGG | Repeat 2 |
| 9 | VQWVKEVSYHISQKVCVSDKVPCCN | Repeat 3 |
| 10 | VQFQKFVQLEKTSQCVHSNGIHEPPGGQ | Repeat 4 |
| 11 | VQFQKFVQLEKTSQCVHSNGIHEPPGGQ | Repeat 4a |

The repeat following 4 will be "4a." Thus Table I shows 6 repeats, 5 of which are common to all three MAPs (1–4 and 4a), and 1a is specific for MAP4 (note that 4a of MAP4 shows less homology than the corresponding tau and MAP2 sequences). These repeats correspond to repeats 1–6 in Chapin and Bulinski (1992), and 4a is found in MAP2, MAP3, and MAP4.
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RESULTS

Phosphorylation of MAP2 and MAP4 by the Protein Kinase p110^mark_. Tau protein is phosphorylated efficiently on the KXGS motifs in its microtubule repeat region by a novel protein kinase, particularly at Ser^262^ in repeat 1 (Drewes et al., 1995). This type of phosphorylation leads to the loss of tau-microtubule interaction, and therefore we termed the kinase p110^mark_ (microtubule affinity regulating kinase). This posed the question if p110^mark_ could play a more general role, by phosphorylating other MAPs in an analogous way. As shown in Fig. 2A, brain MAP2 and its juvenile isoform, MAP2c (lanes 2-5) and heart/tung MAP4 (lanes 8 and 9), are readily phosphorylated by p110^mark_ with an efficiency comparable to the six tau isoforms (lanes 10 and 11). An E. coli expressed MAP4 fragment termed MAP4-BDC, comprising the C-terminal half which binds to microtubules (residues 640-1125), is also readily phosphorylated. As described previously for tau protein, phosphorylation of the other MAPs also leads to a small but significant shift toward higher M, the time course of phosphorylation of tau, MAP2c, and MAP4-BDC is also similar, leading to a saturation of the incorporation of ^32^P after 2-3 h (Fig. 2B). The final stoichiometry of phosphorylation was around 3 to 4 mol of phosphate/mol of MAP. The phosphorylation of recombinant MAP2c and tau by p110^mark_ is characterized by a similar K_m value of around 30-50 μM, whereas the MAP4-BDC fragment is a somewhat better substrate with a K_m of approximately 10 μM (Fig. 2C).

As reported previously, p110^mark_ phosphorylated tau exclusively on serine residues. While phosphoamino acid analysis showed that the same is true for MAP2c, we found that MAP4 is also phosphorylated on threonine (Tables II and III).

Identification of the p110^mark_ Phosphorylation Sites on MAP2c and MAP2—For the determination of phosphorylation sites we used a strategy applied previously to tau protein in materials and Methods”. Locations of phosphorylation sites are indicated (see Table I).

Relative amount of phosphorylation at different sites, and thereby allows a distinction between major and minor sites. The specificity of p110^mark_ for sites on MAP2 was examined by tryptic digestion of ^32^P-phosphorylated MAP2 and MAP2c, the juvenile isoform, whose sequence is fully contained within the adult MAP2 isoforms. A comparison of the phosphopeptide maps obtained from recombinant MAP2c (Fig. 3A) and full-length brain MAP2 (Fig. 3B) indicates that the majority of spots, including the most prominent ones, stem from sites located within the MAP2c sequence. This was confirmed by mapping a mixture of equivalent amounts of both samples (Fig. 3D). HPLC fractionation of the digest (Fig. 3E) allowed the isolation and subsequent sequence determination of four major labeled peptides. Each purified peptide was localized within the pattern of the MAP2c digest by TLE/TLC of the peptide in combination with a small aliquot of the digest (not shown). The results are compiled in Table I. The major phosphorylation site is Ser^1713 within the KCGS motif of repeat 3 (spot 2). A second major site is Ser^1682 within the KIGS motif of repeat 1. Minor sites are located outside the repeat region. Comparison of these data to the results obtained previously with tau shows that p110^mark_ displays a general specificity for the KXGS motifs in the microtubule binding domain. It is, however, inter-
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Peptides were obtained from tryptic digests of E. coli-expressed MAP2c and MAP4 isolated from porcine brain. Peptides from MAP2c purified by HPLC were matched with peptides from brain MAP2 using two-dimensional TLE/TLC. The sequences are derived from the main radioactive peaks. Listed are the amount of material which was used for sequencing after a second purification run, the sequence with the phosphorylated residue (identified as S-ethylcysteine) starred, the phosphorylation site, the molecular mass of the peptide obtained by MALDI-MS, and the identification of the peptide in the two-dimensional HV-TLE/TLC maps (see Fig. 3).

| cpm | Picomoles | Sequence found and domain | Mass calculated/ observed | Phosphorylated residue(s) | TLC spot No. |
|-----|-----------|---------------------------|---------------------------|--------------------------|-------------|
| 740,000 | 1000 | CGS*LK* (KXGS in "3") | 555 | 635* | S1713 | 2 |
| 470,000 | 500 | IG*TDN* (KXGS in "1") | 847 | 931* | S1682 | 3 |
| 320,000 | 300 | LS*NVSS*GS*INLLESPQALTDVAX ("C") | 3100 | 3186* | S178/1802/1805 | 1 |
| 220,000 | 800 | DQGGG*SEGS*R (Acidic) | 1062 | 1064* | S37/42 | 4 |
| 800 | 800 | SY*LP* (Basic) | 863 | 863* |

- a Cysteine was found as cysteic acid.
- b Corresponds to the molecular weight of the peptide phosphorylated on a single site.
- c These residues appear to be only partially phosphorylated.
- d The phosphorylated peptide was probably not separated from the unphosphorylated in both purification runs.
- e Peptide eluted together with the Ser37/Ser42 peptide in both purification runs.

Peptides were obtained from tryptic fragment MAP4-BDC by HPLC and matched with peptides from MAP4 (isolated from mouse heart and lung tissue) using two-dimensional TLE/TLC. The sequences are derived from the main radioactive peaks. Listed are the number of counts in radioactive labeled fractions of the peaks from the first HPLC run, the amount of material which was used for sequencing after a second purification run, the sequence with the phosphorylated residue (identified as S-ethylcysteine) starred, the phosphorylation site, the molecular mass of the peptide obtained by MALDI-MS, and the identification of the peptide in the two-dimensional HV-TLE/TLC maps (shown in Fig. 4).

| cpm | Picomoles | Sequence and domain | Mass calculated/ observed | Phosphorylated residue(s) | TLC spot No. |
|-----|-----------|-------------------|---------------------------|--------------------------|-------------|
| 240,000 | 500 | SPATT*LPK (Pro-rich) | 814 | 897* | Thr* | 4 |
| 240,000 | 600 | VGS*TENIK (KXGS in "3") | 847 | Ser* | 3 |
| 170,000 | 300 | LATTTS*APDLK ("1") | 1115 | 1200* | Ser* | 5 |
| 80,000 | 400 | NTTPTGAAAPGMT*ST*R (Pro-rich) | 1663 | 1745* | Thr* | 2 |
| 190,000 | 500 | VGS*LDNWHG*LPAGGAVK (KXGS in "4") | 1591 | 1675* | Ser* | 7 |
| 80,000 | 150 | LATTTS*APDLK ("1") | 863 | 863* | Thr* | 1 |
| 100,000 | 100 | SS*GALS*VDK (Pro-rich) | 863 | 863* | Ser* | 6 |

- a Corresponds to the molecular weight of the peptide phosphorylated on a single site.
- b Methionine is oxidized to methionine sulfone.
- c From sequencing, Ser* appears to be only partially phosphorylated.
- d This sample was not modified because of its small amount.
- e Corresponds to the molecular weight of the nonphosphorylated peptide.

It is also thought to be involved in microtubule binding (Aizawa et al., 1991; Olson et al., 1995), but there is no pronounced homology to the proline-rich regions in MAP2 or tau (Fig. 1). The extent of phosphorylation at other N-terminal sites was minor.

Effects of MAP Phosphorylation on Dynamic Instability of Microtubules—Under certain conditions, microtubules show abrupt transitions between phases of rapid shortening (“catastrophe”) and elongation (“rescue”), and are termed “dynamically unstable” (Mitchison and Kirschner, 1984). MAPs are able to suppress microtubule dynamic instability by decreasing the frequency of catastrophe or increasing rescue (Pruyer et al., 1992; Dredesel et al., 1992; Panda et al., 1995; Trinczek et al., 1995)). Phosphorylation of MAPs affects this stabilizing capacity by lowering microtubule affinity, and as a result the mean length of microtubules decreases. This effect can be observed in a time resolved manner by video dark field microscopy of individual microtubules.

In the experiment shown in Fig. 5 the concentration of tubulin was 10 μM to ensure that microtubules did not self-assemble. However, microtubules nucleated and grew upon addition of native MAP4 prepared from brain (Fig. 5A, open circles), native MAP2 (Fig. 5B, open circles), or recombinant MAP2c (Fig. 5C, open circles). In these control experiments, p110* was added together with the MAPs but without ATP so that phosphorylation could not proceed. In a parallel experiment under otherwise identical conditions, 1 mM MgATP was added together with the kinase. The effect of the phosphorylation by p110* appeared rapidly (Fig. 5, A-C, closed circles). After about 5 min, growth is largely inhibited. Some microtu-
bule nucleation initially took place while the MAPs were not yet phosphorylated, but polymerization after this time was suppressed due to progressive phosphorylation of the MAPs. In another type of experiment, the MAPs were phosphorylated by p110\(^{mark}\) for 30 min prior to their addition to tubulin (Fig. 5, A-C, triangles). In this case, nucleation was also abolished. However, tubulin could still form polymers, as short microtubules of about 2\(\mu\)m length could be observed when axonemes were added to promote nucleation. Analysis of mutant forms of MAP2c shows that the loss of binding capacity depends on the phosphorylation of both Ser\(^{1682}\) in repeat 1 and Ser\(^{1713}\) in repeat 3 (Fig. 5D). If only one of these sites is mutated, microtubule growth is still induced by the phosphorylated mutants.

The length histograms show the distribution of microtubule lengths at 5 min (Fig. 6, A-C) and 30 min (Fig. 6, D-F) after the addition of MAP and kinase. At 5 min, where the microtubules are still in the growing phase, the length distributions, peaking around 10\(\mu\)m, are comparable in the presence or absence of ATP (kinase active or inactive, Fig. 6, A-C, open and closed circles). After 30 min the distribution of the control microtubules (no ATP) has become broader (Fig. 6, D-F, closed circles). However, incubation with ATP strongly suppresses long microtubule and shifts the distribution to short lengths (open circles in Fig. 6, D-F).

In summary, the results show that phosphorylation by p110\(^{mark}\) has similar dramatic effects on the function of MAP2c, MAP2, and MAP4. Microtubule stabilization is progressively impaired when the kinase and ATP are added together with the MAP to the tubulin sample. Moreover, pre-phosphorylated MAPs are not able to support microtubule growth or even nucleation. These effects are comparable to the previously reported effects of p110\(^{mark}\) phosphorylation on tau (Drewes et al., 1995).

Effects of MAP Phosphorylation on MAP-microtubule Binding—To determine whether the effects of MAP phosphorylation on dynamic instability was due to a reduced interaction with microtubules, we performed binding studies with taxol-stabilized microtubules. As we had found previously for tau protein...
FIG. 5. Effects of unphosphorylated and p110<sub>mark</sub>-phosphorylated MAP4 (A), MAP2 (B), MAP2c (C), and MAP2c point mutants (D) on the length of self-nucleated microtubules measured by dark field microscopy. For each condition, 500–800 microtubules were analyzed, and the mean length were plotted against time. Tubulin concentration was 10 μM in all cases, the concentration of MAP4 and MAP2 was 1 μM, and that of MAP2c, 2 μM. In control experiments, AT was omitted (−ATP). Open circles in A–C: the MAPs were preincubated for 30 min with 2.5 milliunits/ml p110<sub>mark</sub> (final concentration), but without AT. By adding 10 μM tubulin, microtubules were nucleated and the mean microtubule length increased up to about 20 μM within 30 min. If AT was preincubated without self-nucleation occurred, showing that the phosphorylation of the MAPs prevented microtubule formation. Short microtubules of about 2 μM length could only be observed by adding axonemes (10–100 fmol) to promote seeded nucleation (open triangles in A–C). Closed circles in A–C: tubulin and MAP were mixed at 4°C with 2.5 milliunits/ml of p110<sub>mark</sub> (final concentration) and 1 μM AT, and the temperature was shifted immediately to 37°C (so that initially the MAPs were unphosphorylated). Microtubule growth was promoted in all three cases, but the final mean microtubule length was only about half of that observed for the unphosphorylated MAPs (compare to open circles). D, the effect of phosphorylation site point mutations of MAP2c. All proteins were preincubated with kinase and ATP as described above. Triangles, wild type MAP2c; closed circles, MAP2cA319/X350 (KKXS in repeat 1 mutated to KXS); squares, MAP2cA350 (KKXS in repeat 3 mutated to KXS); closed squares, MAP2cA319/A350 (KKXS in both repeats mutated to KXS).

FIG. 6. Microtubule length histograms obtained at 5 min (A–C) and 30 min (D–F) derived from the experiments shown in Fig. 5 (open and closed circles). Each sample shows a pronounced peak at around 10 μM after 5 min (closed circles in A–C). If Mg-ATP was absent (closed circles in D–F), the distribution became broader and shifted to greater length at 30 min. By contrast, phosphorylation of the MAPs with p110<sub>mark</sub> successfully decreased the mean microtubule length within 30 min of incubation (open circles in D–F), n, number of microtubules analyzed.

FIG. 7. Effect of the phosphorylation by p110<sub>mark</sub> on the binding of recombinant wild type MAP2c and MAP2c point mutants to taxol-stabilized microtubules (30 μM tubulin dimers). The concentrations are Ser to Ala at positions 319 and/or 350 of MAP2c, corresponding to 1682 and 1713 in the full MAP2 sequence (Table I). Open circles, non-phosphorylated wild-type MAP2c. The binding is tight (K<sub>d</sub> of 0.25 μM) and saturates around 17 μM ligand (1 MAP2c molecule per 2 tubulin dimers). Closed circles, wild-type MAP2c, phosphorylated previously with p110<sub>mark</sub> (2.5 milliunits/ml) for 2 h. Note that there is essentially no binding. Closed and open squares, MAP2cA319 and MAP2cA350 phosphorylated previously with p110<sub>mark</sub> (2.5 milliunits/ml) for 2 h. The affinity to microtubules has decreased markedly (K<sub>d</sub> of 7 μM), although the stoichiometry remains similar to the wild type MAP2c. Triangles, MAP2cA319/A350, phosphorylated previously with p110<sub>mark</sub> (2.5 milliunits/ml) for 2 h. The binding is similar to the unphosphorylated protein, showing that the sensitivity to phosphorylation has disappeared.

DISCUSSION

Phosphorylation Sites on MAPs—Microtubules are dynamic polymers, and their dynamic behavior is regulated by cells according to their needs. MAPs and their phosphorylation state have a pronounced effect on microtubule dynamics, and indeed changes in MAP phosphorylation patterns accompany major rearrangements of microtubules during the cell cycle or during differentiation. The previous studies have focused mainly on the kinases and phosphatases regulating the phosphorylation of the neuronal tau protein, with emphasis on the hyper-
phosphorylation that plays a role in the neurofibrillary pathology of Alzheimer’s disease. It was intriguing to note that many “abnormal” phosphorylation sites were in Ser-Pro or Thr-Pro motifs and can be phosphorylated by proline-directed kinases (such as MAPK, cdk5, or GSK-3) which are important in cellular signal transduction (for review, see Mandelkow and Mandelkow (1993)). Indeed, using phosphorylation-sensitive antibodies we observed that kinases of this class phosphorylate both MAP2 and tau in cells in a cell-cycle or differentiation dependent fashion (Berling et al., 1994; Preuss et al., 1995). Similar observations were made in other laboratories (e.g. Dinsmore and Solomon (1991) and Ookata et al. (1995)). There was, however, the puzzle that proline-directed phosphorylation had only a comparatively mild effect on microtubule stability; it was dwarfed by the much larger effect of another kinase activity which phosphorylated mainly KXGS motifs in tau (Biernat et al., 1993; Trinczek et al., 1995). The search for the kinase lead to a 110-kDa protein which was termed MARK because of its regulation of the affinity of tau to microtubules (Drewes et al., 1995). Given the specificity of the enzyme it was natural to ask whether the kinase would also phosphorylate related MAPs such as MAP2 and MAP4, and whether this would have similar consequences on microtubule stability. We show here that this is indeed the case.

Our results imply that the role of MARK in regulating MAP interactions with microtubules may be more general than expected. Because tau, localized primarily to axons, and MAP2, distributed in dendrites, are both substrates, MARK or related kinases could be active in different neuronal cell compartments. An even more general role is implied by the results with MAP4, since this ubiquitous MAP has been inferred to affect microtubule stability in dividing cells (Bulinski and Borisy, 1980; Parysek et al., 1984; Chapin and Bulinski, 1994; Olson et al., 1995). Thus far it has been difficult to determine what combination of MAPs, phosphorylation sites, kinases, and other factors are responsible for the pronounced increase in microtubule dynamics during mitosis. MAP4 was considered a likely candidate, as well as other related ones (e.g. XMAP from Xenopus eggs, Faruki and Karsenti (1994)). Regarding kinases, cdc2 and MAP kinases were suggested as potential triggers of microtubule reorganization (Gotth et al., 1991; Verde et al., 1992; Lieuvin et al., 1994; Ookata et al., 1995). However, it remains to be seen whether these kinases act directly or via other intermediate steps. The weak effect of proline-directed phosphorylation on microtubule dynamics makes us believe that other kinases, such as MARK, may be involved. In this regard it is interesting that MARK is itself activated by phosphorylation, pointing to other kinases upstream in the signaling pathway.

The phosphorylation of MAPs has been studied by a number of authors, and it is pertinent to ask how the results compare with ours. In most cases it was concluded that phosphorylated MAPs bound less tightly to microtubules and supported their assembly less efficiently (although exceptions were also noted, see Brugg and Matus, 1991). However, in the majority of studies, the phosphorylation sites involved in the regulation were not known, and indirect information, such as kinase consensus motifs, are not reliable (as illustrated for tau and CaM kinase by Steiner et al. (1990), or for MAP4 and cdc2 by Ookata et al., 1995). There are, however, a few cases where phosphorylation sites have been determined directly. Examples include the sites in MAP2 altered by PKC (Ainsztein and Purich, 1994), or the sites on tau phosphorylated by several kinases (PKA, PKC, Ca/calmodulin dependent kinase II, and the proline-directed kinases MAPK, GSK-3, cdc2, and related kinases, see below).

While additional parameters need to be measured for the influence of site-specific modifications on microtubule dynamics to be rigorously assessed, the observations with tau (the MAP studied most comprehensively) allows a distinction to be made between the sites within and outside the repeat domain. The sites outside the repeats examined so far have either no effect on microtubule binding and dynamics, or only a moderate effect, reducing the stabilizing power of tau from “high” to “medium” (in the classification of Trinczek et al. (1995)). This includes the many Ser-Pro or Thr-Pro sites (phosphorylated by proline-directed kinases), as well as PKA or Ca/calmodulin dependent kinase II sites (Steiner et al., 1990; Scott et al., 1993; Brandt et al., 1994). Inside the repeats there are the KXGS motifs affected by MARK. One of these (Ser262 in repeat 1) eliminates the stabilizing power of tau, the others have only a modulatory influence. The KXGS motifs of tau can also be phosphorylated to some extent by PKC (Ser324 in repeat 3, Correas et al., 1992), PKA (mostly Ser324 and Ser256 in repeats 3 and 4, Scott et al. (1993) and Drewes et al. (1995)), and GSK-3 when activated by heparin (Ser262 in repeat 1, Song and Yang (1995)). In vivo the phosphorylation at KXGS motifs is normally low (Seubert et al., 1995), consistent with its tight association with microtubules. This implies that the kinases affecting KXGS motifs are normally down-regulated.

The results on the other MAPs echo those of tau. Phosphorylation sites outside the repeats may reduce the interaction with microtubules, but they do not eliminate it. For MAP4, this includes the sites Ser667 and Ser760 in the proline-rich domain (our numbering, see Table I) which are potential targets of cdc2 (Ookata et al., 1995). Sites inside the repeats include the KPXS motifs which cooperate to eliminate the interaction with microtubules (see Fig. 7). They also include reported PKC sites in MAP2 at serines 1705, 1713, and 1730 (our numbering, Fig. 1; see Ainsztein and Purich, 1994). The second of these is in the KXGS motif of repeat 3. The example illustrates how PKC could exert a modulatory effect by phosphorylating one KXGS motif, while MARK would eliminate microtubule interactions by phosphorylating two motifs (in 1 and 3). In the case of MAP4, point mutants of KXGS motifs are not available, but in analogy with MAP2 and tau we expect that the full inhibition of microtubule binding by MARK resides in repeats 1, 4, or both. Other phosphorylation sites of MAP4 have not been determined thus far.

Most studies on MAPs emphasize their role as microtubule stabilizers, but it is worth noting that they have at least two additional functions. One is their role as “spacers” between microtubules and other cellular components (Chen et al., 1992). This is achieved mainly by the acidic N-terminal domain which may be short (as in MAP2c or tau) or long (as in MAP2 or MAP4). A third function is that of a docking site for cellular enzymes, including kinases and phosphatases or their cofactors (PKA, cdc2, PKC-ζ, MAP kinase, PP-1, see Obar et al. (1989), Mandelkow et al. (1992), Baumann et al. (1993), Ookata et al. (1995), Lehrich and Forrest (1994), Reszka et al. (1995), and Santog et al. (1995)). It is intriguing to speculate that the docked kinases may be activated by some signaling cascade and then phosphorylate their host protein or others nearby, thus modulating their association with the microtubule cytoskeleton.

Structural Implications—We conclude by commenting on possible structural implications of the phosphorylation by

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2 G. Drewes, H. E. Meyer, E.-M. Mandelkow, and E. Mandelkow, unpublished data.

3 R. Godemann, G. Schmitt-Ulms, S. Illenberger, J. Biernat, E.-M. Mandelkow, and E. Mandelkow, unpublished observations.
MARK. The main sites affecting microtubule binding and dynamics are in the repeat domain although this domain, taken by itself, interacts only rather weakly with microtubules (En

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Phosphorylation of Microtubule-associated Proteins MAP2 and MAP4 by the Protein Kinase p110: PHOSPHORYLATION SITES AND REGULATION OF MICROTUBULE DYNAMICS
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