Phosphorylation of MAP4 affects microtubule properties and cell cycle progression

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ABSTRACT

In human cells MAP4, a microtubule-associated protein ubiquitously expressed in proliferating cells, has been shown to undergo \textit{in vivo} phosphorylation. Two phosphorylation sites, serines 696 and 787, lie within the proline-rich region of its microtubule-binding domain. To test the hypothesis that phosphorylation at these sites influences microtubule properties or cell cycle progression, we prepared cells that inducibly express versions of MAP4 in which phosphorylation of these two serines was prevented by their replacement with alanine, lysine, or glutamate residues (AA-, KK-, or EE-MAP4). All non-phosphorylatable mutant forms of MAP4 expressed in mouse Ltk" cells were localized to MT arrays that were unremarkable in appearance. Expression of non-phosphorylatable mutants of MAP4 did not affect cell doubling time; however, expression of some mutants altered progression into or through cell division. Interactions of mutant MAP4 with MTs were examined \textit{in vitro}. KK mutant MAP4 bound MTs more avidly than its wild-type counterpart, WT-MAP4. \textit{In vivo} MT polymer also differed among the mutants: MTs in cells expressing the KK- and AA-MAP4 forms were more resistant to nocodazole depolymerization than those in cells expressing EE- or WT-MAP4 forms. Our results demonstrate that phosphorylation alters MAP4 properties and suggest a raison d'être for phosphorylation of the MAP4 microtubule-binding domain during cell cycle progression.
INTRODUCTION

Microtubules (MTs) are dynamic polymers of tubulin that are involved in many diverse functions, including spindle formation, vesicle and organelle transport, and cell motility. The fact that MTs polymerized in vivo are more dynamic than those polymerized in vitro (Desai and Mitchison, 1997; Verdé et al., 1990) has been attributed to the actions of cellular factors that destabilize MTs, such as Op18 (Marklund et al., 1996; Larsson et al., 1999), and the opposing action of factors that stabilize MTs, such as microtubule-associated proteins (MAPs) (Mandelkow and Mandelkow, 1995; Andersen, 2000). MTs undergo a dramatic increase in dynamics during mitosis, relative to interphase (Saxton et al., 1984; Hamaguchi et al., 1985), possibly due to activation of MT-destabilizing factors, deactivation of MT-stabilizing MAPs, or a combination of both. MAP4, a 210 KDa MAP present in all proliferating cells, binds to and stabilizes MTs both in vitro (e.g., Bulinski et al., 1980) and in vivo (e.g., Nguyen et al., 1997). Thus, altered MAP4 activity is a mechanism that might contribute to increased MT turnover at the mitotic stage (Ookata et al., 1995).

Consistent with this hypothesis, MAP4’s level of phosphorylation has been shown to increase at the G2/M transition (Vandré et al., 1991). In vitro, MAP4 interacts directly with cyclin B-cdc2, the major kinase responsible for mitosis-specific phosphorylation of structural proteins (Ookata et al., 1995). In addition, in vivo phosphorylation of MAP4 by cyclin B-cdc2 has been shown to occur at numerous sites throughout the molecule (Ookata et al., 1997). Phosphorylation of MAP4 by cyclin B-cdc2 kinase renders MTs more dynamic in vitro, apparently by decreasing the frequency of rescue (Ookata et al., 1995). This mechanism is consistent with the observed in vivo behavior of MTs during mitosis in mammalian cells.
Mitotic behavior of MAP4 homologs has been studied both in mammalian cells and in *Xenopus*. However, the *Xenopus* MAP4 homolog (XMAP4, a.k.a. XMAP230) (Cha et al., 1999; Andersen et al., 1994; Shiina et al., 1992) shows only limited sequence identity with mammalian MAP4 (~30% sequence identity between *Xenopus* and human MAP4) (Shiina et al., 1999; Andersen, 2000). Other data suggest that there may also be mechanistic differences between the cellular actions of these homologs. For example, XMAP4 was reported to dissociate from MTs during mitotic prophase (Andersen et al., 1994), in contrast to mammalian MAP4, which remains associated with MTs throughout the cell cycle (Bulinski and Borisy, 1980; Olson et al., 1995). It should be noted, though, that other investigators, Cha et al. (1999) and Shiina et al. (1999), demonstrated continuous association of XMAP4 with MTs during mitosis. Because human MAP4 is a MT-stabilizing protein that is continuously localized on MTs throughout the cell cycle, we and others have hypothesized that mitosis-specific phosphorylation of MAP4 toggles its MT stabilizing function during portions of the cell cycle.

Accordingly, we focused on phosphorylation events within MAP4’s MT-binding domain. We previously showed that cyclin B-cdc2 kinase phosphorylates serine-787 *in vivo* during mitosis (Ookata et al., 1997), and that *in vitro* phosphorylation by cyclin B-cdc2 is sufficient to reduce MAP4’s capacity to stimulate *in vitro* polymerization of MTs (Kitazawa et al., 2000). In contrast, we showed that serine-696 is continually phosphorylated throughout the cell cycle in proliferating cells (Ookata et al., 1997), while phosphorylation at this site is not detectable in quiescent, serum-starved cells (Srsen et al., 1999). Because we predicted that phosphorylation at sites within the MT-binding domain would manifest effects on MT dynamics, we focused on the function of MAP4 phosphorylation at the serine-696 and -787 sites.
Materials and Methods

Materials

Except as noted, all chemicals were purchased from Sigma (St Louis, MO) or from Fisher Scientific (Tustin, CA). All tissue culture materials were from Gibco Life Sciences (Gaithersburg, MD). Immunochemicals were obtained from Organon Teknika (Durham, NC). All restriction enzymes were purchased from Promega Biotech (Madison, WI).

Preparation of stable cell lines overexpressing wild-type and mutant MAP4

MAP4 cDNA was mutated at amino acids serine-696 and serine-787 and inserted into pEGFP-C1 vector, modified to be dexamethasone-inducible. Full-length MAP4 cDNA encoding the five-repeat isoform of human MAP4 (isoform IV; Chapin et al., 1995) was mutagenized using the Muta-Gene in vitro Mutagenesis Kit (Biorad, CA) and the following primers for the S696A/S787A (AA-MAP4) construct: AACAAGGAGCTCCCACCAGCC

|  CCAGAG and |
| TGGCTTGGATGGTGC |
| GGCCCGCTT. | Corresponding primers for S696K/S787K and S696E/S787E were substituted, respectively, with AAA/TTT and GAG/CTC at the positions underlined in the AA-MAP4 primers. All mutant MAP4 cDNAs were then cut with Sma1 and partially digested with Bgl II, which deleted the N-terminal 669 amino acids, corresponding to the projection domain. Each fragment was then cloned into a modified pEGFP-C1 (Clontech, CA), generated by replacing its promoter with MMTV-LTR, a dexamethasone (dex)-inducible promoter from the pMAMneo vector (Clontech, CA). Transfection of wild-type and mutant MAP4 into mouse fibroblast Ltk<sup>-</sup> cells (ATCC, MD) and selection of stably transfected cell lines were performed according to protocols described in Nguyen et al. (1997). Cell lines were induced to express WT or mutant forms of MAP4 by adding 1µM dex for 36 hours prior to analysis.
Quantification of MT polymer
Following induction with dex, confluent cell monolayers in 60 mm dishes were washed once with Earle’s buffered salt solution (EBSS) at 37°C and extracted with and without saponin (200 µg/mL) in PEM buffer (100mM PIPES, pH 6.9, 5mM MgCl₂, 1mM EGTA) for 2 min at 37°C. Identical volumes of SDS-gel sample buffer were added to pellets containing total cell protein (extracted in PEM alone) and MT polymer (extracted in PEM with saponin). Western blots of 20 µg extract protein (Chapin and Bulinski, 1991), stained with anti-β-tubulin antibody (3F3, courtesy of Dr. James Lessard, University of Cincinnati, OH), were scanned with an HP ScanJet 6200C scanner and intensities of each immunostained band determined with MetaMorph software (Universal Imaging Corp., West Chester, PA). The percentage of tubulin in polymer was defined as the ratio (tubulin in MT polymer sample) : (tubulin in total protein extract).

Analysis of cell growth and phenotype
Cells in 100 mm tissue culture plates, induced with dex, were released from the substratum with Viokase solution, and counted in a haemocytometer before and after an additional 36 hr of growth. Cell cycle stages of each cell line were determined following 36 hr dex-induction on coverslips. The coverslips were fixed in ice-cold methanol for 15 min, mounted in the presence of 1µg/ml DAPI, and cell cycle stages were determined by scoring GFP-MAP4-labeled spindles and DAPI-labeled condensed chromosomes. Fluorescence-activated cell sorting (FACS) was performed as previously described (Nguyen et al., 1999).

Assay of MT stability
Following dex induction, cells on coverslips were incubated in medium containing the MT-depolymerizing drug nocodazole (10µM) for 0-30 min, washed once with Hanks’ Balanced Salt
Solution (HBSS) containing 10 μM nocodazole, and immediately fixed in ice-cold methanol for 15 min. GFP-expressing cells were scored for the presence of a MT array at each time of nocodazole treatment. Since all expressing cells showed an extensive MT array initially (T=0) and no MTs at T=30, the proportion of expressing lines with a MT array at the ten-minute time point (t=10), when a disparity was noted, was quantified.

**Affinity of mutant and wild-type GFP-MAP4 forms for MTs**

Suspension cultures of each cell line, induced with 1μM dex for 20-28 hr, were used to prepare Taxol-stabilized MTs, as described in Chapin et al. (1991). After washing MT pellets four times in buffer containing 0.1 M PIPES, pH 6.9, 1mM dithiothreitol, 1mM EGTA, and 1mM MgCl₂ (PDEM), the MTs were resuspended in fresh PDEM plus the applicable concentration of NaCl (0-0.6 M), incubated for 10 min at 37°C, and centrifuged (40,000xG, 15min). Supernatant fractions containing unbound MAP were assayed by Western blotting with guinea pig antibodies prepared against the MT-binding domain of human MAP4 (MTB antibody, Nguyen et al., 1997) or to the bacterially expressed proline-rich domain of murine MAP4 (MuPro antibody; used at 1:1000 dilution). MAP4 released at each salt concentration was quantified and normalized to that eluted with 0.6 M NaCl, which was set at 100%.
RESULTS

Mutation of MAP4 at positions 696 and 797 abolishes major cyclin B-cdc2 phosphorylation sites

In previous work, we identified sites within MAP4 that were phosphorylated in vitro and in vivo by cyclin B-cdc2 kinase (Ookata et al., 1997). Two of the major spots detectable in 2-D phosphopeptide maps were of special interest, since they corresponded to sites within the MT-binding domain of the MAP, and their sequences were conserved amongst species (Fig. 1). To determine in vivo sequelae of phosphorylation at these sites, we performed in vitro mutagenesis of the two relevant serines (ser-696 and ser-787). Figure 2 shows that cyclin B-cdc2 phosphorylation at these sites was abolished in constructs containing mutations of either or both serines to alanine residues, thus confirming that these constructs are appropriate for the proposed studies.

Phosphorylation mutants of MAP4 colocalize with apparently normal MT arrays in vivo

Human MAP4 with serines at positions 696 and 787 (WT) or with residues 696 and 787 mutated to alanines (AA, imparting a neutral charge), to lysines (KK, introducing two constitutive positive charges) or to glutamates (EE, adding two constitutive negative charges, mimicking phosphorylation) were cloned into pEGFP-C1. Since MAP4 is believed to bind to MTs through ionic interactions (Vallee, 1982; Ookata et al., 1995), we hypothesized that substitution of amino acids of different charges within the MAP4 MT-binding domain might change MAP4’s binding to and stabilization of MTs. To simplify interpretation of phenotypes caused by altered phosphorylation, in each construct we also deleted the N-terminal 669 amino acids, i.e., most of the projection domain of MAP4, which is known to contain additional phosphorylation sites (Ookata et al., 1997).
We were unable to isolate Ltk- transfectants that constitutively expressed any of the GFP-MAP4 forms. Transfectants appeared to exhibit a growth disadvantage, and were consequently overgrown quickly by non-expressing cells (data not shown). For this reason, we cloned a dexamethasone (dex)-inducible promoter sequence from pMAMneo into pEGFP-C1 along with the MAP4 mutant constructs, yielding transfectants whose expression of MAP4 mutant forms was inducible. Interestingly, cells expressing any of the dex-inducible mutant MAP4 forms decreased expression by at least two-fold after three days of induction in dex. In contrast, there was no detectable decrease in GFP-MAP4 expression observed after dex-induction of WT-MAP4 for an identical period (data not shown). This was the first suggestive evidence that expression of the MAP4 mutants was more deleterious to cell growth than expression of WT-MAP4. Because of these observations, we induced cells expressing each construct with dex for 24-36 hr in all experiments.

We localized both WT and mutant MAP4 forms in vivo by imaging the fluorescence of the GFP attached to each. All transfected MAP4 species localized to MTs, and the pattern of those MTs was unremarkable when compared to MTs in untransfected cells, visualized via tubulin immunofluorescence (Figure 3A, and data not shown). The morphology of the transfectants was also indistinguishable from that of untransfected cells. In mitotic cells, both wild-type and mutant MAP4 forms were localized to spindle MTs throughout cell division; spindles appeared to be normal in morphology.

We isolated two clones each of cells expressing WT and mutant versions of MAP4. Expanded clones showed significant cell-to-cell heterogeneity, even though FACS sorting or selection of clones was performed as closely as possible to the time of the experiment. In each clone we measured the average level of transfected MAP4, detected by Western blot, and the
percentage of expressing cells, detected by GFP fluorescence (this varied from 27-96%). Figure 3B shows blots of the cell lines; when corrected for the proportion of expressing cells, expression levels varied from 0.28- to 2.2-fold the MAP4 : tubulin ratio (mol : mol) found in HeLa, the human cell line used for comparison (e.g., Nguyen et al., 1997). As shown in Table 1, we used these data to categorize each line as low (<0.4-fold expression relative to HeLa), medium (0.5-1.5-fold), or high (>1.6-fold) expressers.

We determined that the tubulin content of each transfectant line was indistinguishable from the other transfectants or from naïve L^tk^- cells (determined as a proportion of total soluble protein, shown in Figure 3B). These data are consistent with results of a previous study of four cell lines induced to express varying levels of wild-type MAP4 (full- or partial-length); only the line whose MAP4 expression level was 8-fold higher than the level endogenous to HeLa cells showed any increase in tubulin level (Nguyen et al., 1997). In cells expressing MAP4 mutant forms, the proportion of tubulin that was polymeric was similar in all of the transfected cell lines (Table 1), again consistent with previous results on MAP4 transfected cells. Thus, expression of modest levels of mutant MAP4 forms in L^tk^- cells did not significantly alter cell morphology, content of tubulin, or appearance of the MT array, nor did it affect the distribution of the MAP4, itself, during the cell cycle.

**Expression of MAP4 mutants alters cell cycle progression**

Next, we measured the growth rate of each transfectant line to determine if expression of mutant MAP4 species changed cell growth properties. Cell doubling times of 28-30 hours were measured for all lines, regardless of the mutant or its expression level (Table 2). Because growth rate data could be skewed by the growth of non-expressing cells within the population, we analyzed the cell cycle further, using FACS. As shown in Table 2, cells expressing a
medium-level of EE-MAP4 exhibited a higher proportion of cells in G1 phase and a lower proportion in G2/M phase, suggesting that EE-MAP4 mutant cells either transit G1 phase more slowly or pass through G2/M phase more quickly, or both, as compared with cells expressing WT-MAP4 or other mutant MAP4 species.

FACS analysis is only amenable to the quantification of progression through three broadly defined categories of cell cycle events, that is, G1, G2 + M, and S phases. To delineate cell cycle progression of individual cells more precisely, we used the localization pattern of their GFP fluorescence to assay each MAP4-expressing transfectant morphologically. We scored the proportion of log phase MAP4-expressing cells that contained mitotic spindles, separated centrosomes with an intact interphase MT array and condensed chromatin, and midbodies connecting daughter cells. These three morphologically-defined categories corresponded to cells in M phase, in late G2 or the G2-M transition, and in early G1 phase, respectively. As shown in Figure 4, KK-MAP4 cells possessed more than twice the proportion of cells in the late G2 stage as compared with any other cell line, suggesting that KK-MAP4 cells transit the G2-M transition half as quickly as cells expressing the other MAP4 mutants. In contrast, neither the duration of transit through M-phase, itself, nor through early G1 differed significantly among the cell lines. Note that the morphological scoring of the low-expressing EE-MAP4 clone showed a higher proportion of cells in all three stages. Because these are very faintly expressing cells, we believe that this measurement is an artifact caused by the difficulty of scoring dimly fluorescent cells. In these cells, EE-GFP-MAP4 fluorescence may have been more readily detectable when it was concentrated into discrete structures such as separated centrosomes, spindles, and midbodies.
Phosphorylation-site mutations alter MAP4’s binding to MTs

A possible explanation for the increased abundance of late G₂ stage cells in KK-MAP4 cell lines is that the KK-MAP4 mutant is a more potent stabilizer of MTs than other MAP4 forms. KK-MAP4 would be expected to stabilize MTs more effectively than other forms if it bound to MTs more tightly. If this were the case, the interphase MT array containing these stable MTs might break down more slowly, and this could result in a delayed progression through late G₂ and into M phase, as we observed for KK-MAP4-expressing cells. Accordingly, we tested the MT-binding properties of the MAP4 mutant forms, exploiting the capacity of MAP4, which binds ionically to MTs, to be eluted from Taxol-stabilized MTs with moderate salt concentrations (Vallee, 1982). By quantifying the efficacy of various concentrations of salt in eluting MAP4 from crude MTs, we assessed how tightly each MAP4 mutant form bound to MTs. Unlike a conventional affinity measurement, this assay, which relied upon MTs assembled in cell extracts, did not require the availability of pure proteins.

Results of the salt-elution assay (Figure 5C) showed that KK-MAP4 bound to MTs more tightly than any other MAP4 form, mutant or WT (p< 0.05 at salt concentrations <0.3 M). AA-MAP4 bound less tightly than KK-MAP4, but significantly more tightly than WT-MAP4 or EE-MAP4 (e.g., p<0.05 at salt concentrations <0.25 M). MT binding of the latter two forms was indistinguishable from one another, or from the endogenous (murine) MAP4 in Ltk⁻ cells (Figure 5B).

Stability of MT polymer is altered by expression of MAP4 mutants

Inducing expression of transfected MAP4, either mutant or wild-type forms, did not significantly alter either the total amount of tubulin in the cells (Figure 3) or the proportion of tubulin that existed in the polymeric state (Table 1). However, from MT-binding data described
above, in which KK-MAP4 and AA-MAP4 exhibited tighter \textit{in vitro} binding to MTs than either WT- or EE-MAP4, one might predict that these mutants would show corresponding differences in MT stabilization \textit{in vivo}, that is, in the stability or longevity of their MTs. To test for differences in MT stabilization, we measured the capacity of MAP4 mutant forms to protect MTs from \textit{in vivo} depolymerization by the MT-depolymerizing drug, nocodazole.

We assayed the nocodazole sensitivity of MTs, to determine whether any of the cell lines expressing MAP4 mutants possessed MTs that differed in their sensitivity to nocodazole. This assay entailed exposing cells, all of which initially contained the same quantity of MT polymer, to nocodazole in cell culture medium for a limited time (10\textmu M, 10 min), and then scoring individual cells for the presence or absence of a nocodazole-resistant array of MTs. Table 3 shows that, of the four MAP4 forms, cells expressing AA-MAP4 were least sensitive to nocodazole, followed by those expressing KK-MAP4. MTs in either WT or EE-MAP4 cells were equivalent to each other in their nocodazole-sensitivity, and they were significantly more nocodazole-sensitive than MTs in cells expressing AA- or KK-MAP4 (p<0.05 for each line). These results are quite consistent with MT binding data for the two mutants and suggest that MT stability can be varied by altering the charge of residues within the MAP's MT-binding domain.
DISCUSSION

Previous studies demonstrated in vivo phosphorylation of two residues (ser-696 and ser-787) within the MT-binding domain of MAP4 by cyclin B-cdc2 kinase (Ookata et al., 1997). Here, we examined effects of mutating these sites on cell cycle progression and MT stabilization. We found that transfecting mouse cells with MAP4 in which ser-696 and ser-787 had been replaced with lysines increased the proportion of cells in late G2 or at the G2/M transition, suggesting that expression of KK-MAP4 retards progression through this portion of the cell cycle.

In contrast to single-cell assays, neither measurements of population doubling times nor FACS analysis detected any differences in cell cycle progression among cells expressing the MAP4 mutants. This is not surprising since these methods are not sufficiently sensitive to detect differences of only 15-30 minutes, within a 28-30 hour cell cycle time. All transfectants showed heterogeneous expression levels of MAP4 mutant forms, no matter whether the cells had been cloned, drug-selected, or pre-sorted by FACS. Diversity in expression would tend to obscure differences in average growth rate of the population. Similarly, bulk analyses by FACS, which demarcates only three cell cycle stages, would not be expected to discriminate as finely among defined cell cycle stages as would morphological analysis of individual cells.

Expression of MAP4 mutants with differently-charged residues also allowed us to test the hypothesis that the phosphorylation state of MAP4’s MT-binding domain modulates its strength of MT binding and ultimately, its ability to stimulate MT polymerization. Specifically, we predicted that mutation of ser-696 and ser-787 to positively-charged lysines (KK-MAP4) would increase the affinity of positively charged MAP4 for negatively-charged tubulin within the MT. Conversely, we proposed that adding negatively-charged glutamic acid residues to mimic the phosphorylated state of wild-type MAP4 (EE-MAP4) might decrease the affinity of MAP4 for
MTs. Our results showed that, among the MAP4 mutants, KK-MAP4 indeed displayed the tightest binding to MTs, followed by neutrally charged AA-MAP4. EE-MAP4 bound the least tightly; which was also as expected. However, EE-MAP4’s binding profile was indistinguishable from that of WT-MAP4. The similarity in behavior of EE- and WT-MAP4 was consistent with previous findings that ser-696 in wild-type MAP4 is continuously phosphorylated throughout interphase (Ookata et al., 1997). This result also implies that the phosphorylation state of ser-696 impacts more on binding strength of MAP4 than does the phosphorylation state at ser-787.

Both KK-MAP4 and AA-MAP4 bound more tightly to MTs than did WT-MAP4 in the salt elution assay, and both KK- and AA-MAP4 showed greater in vivo MT stabilizing activity than WT-MAP4 in the nocodazole depolymerization assay. However, KK-MAP4 bound to MTs more tightly than AA-MAP4, while AA-MAP4 showed greater in vivo MT stabilization than KK-MAP4. We are uncertain why the binding and stabilization activities of KK-MAP4 and AA-MAP4 were not better correlated. One possibility is that the extra positively charged residues in KK-MAP4 resulted in higher MT affinity but also brought about conformational changes that lead to less optimal MT stabilization.

MAP4 homologs exist in many species, including human, cow, chicken, mouse, and more recently discovered, *Xenopus*. The *Xenopus* MAP4 homolog, XMAP230 (XMAP4), shows only 30% sequence identity with mammalian MAP4, possibly explaining behavioral differences between it and mammalian MAP4. For example, injecting XMAP4 function-blocking antibodies into *Xenopus* blastomeres disrupted assembly of mitotic spindles (Cha et al., 1999), whereas removal of human MAP4 from MTs by function-blocking antibodies, its depletion by antisense, or its overexpression in mammalian cells did not detectably affect spindle formation (Wang et al., 1996; Nguyen et al., 1997; Yoshida et al., 1996; Olsen et al., 1995; Nguyen et al., 1999).
Finally, XMAP4 and mammalian MAP4 also differ in the effects each manifests on the parameters of MT dynamics: XMAP4 was found to increase MT growth rate, decrease the rate of MT shrinkage, and suppress catastrophes \textit{in vitro} (Andersen et al., 1994), whereas human MAP4 was shown to increase the frequency of rescue events \textit{in vitro} under steady-state conditions (Ookata et al., 1995).

Shiina et al. (1999) used \textit{Xenopus} cultured cells to test the effects of mutating ten XMAP4 consensus sites for cyclin B-cdc2 or MAP kinase, including sites homologous in position and sequence to human ser-696 and ser-787. Mutating a minimum of six cyclin B-cdc2 consensus sites within the projection domain, i.e., not including the sites homologous to human ser-696 and ser-787, affected chromosome separation during mitotic anaphase A. In particular, Shiina et al. (1999) observed that chromosome segregation towards spindle poles was preempted by spindle elongation in about 20% of mitotic cells. Shiina et al. (1999) also observed a phenomenon they termed “back-and-forth peristaltic movement”, whereby multiple cleavage furrows prematurely formed prior to spindle elongation and appeared to push the spindle from one side to another. These experiments show that the projection domain of \textit{Xenopus} MAP4 and/or its appropriate phosphorylation is required for some aspect of anaphase chromosome separation.

It is germane to compare our results to those Shiina et al. (1999) obtained when they mutated the two MT-binding region sites in XMAP that are homologous to human ser-696 and ser-787, as well as six of the projection domain phosphorylation sites of XMAP discussed above. These mutants phenotypically resembled mutants of the six projection domain sites only, suggesting that the phenotype(s) of MT-binding site mutations were obscured by the striking phenotypic defects that arose from mutations in the projection domain sites. Therefore, we
tested the role(s) of phosphorylation of MT-binding domain sites directly, focusing specifically on
ser-696 and ser-787. As expected, mutating these two residues in each of three possible ways
yielded more subtle, yet mechanistically more understandable effects than those Shiina et al.
(1999) observed for their XMAP projection domain mutants. We can only speculate on whether
expression of XMAP4 MT-binding domain mutants would yield the same phenotypes we
observed for human MAP4 MT-binding domain mutants, since no one has yet dissected the
role(s) of the MT-binding domain sites of XMAP4. Since the MT-binding domain functions not
only in binding to, but also in stabilizing MTs, cells may use subtle modulation of MT-binding
activity in concert with other changes in MAPs or MTs to fully effect the MT transitions needed
for error-free mitosis.

Others have studied phosphorylation of MAP4 by other kinases, such as protein kinase C
(Mori et al., 1991), MAP kinase (Shiina et al., 1992; Hoshi et al., 1992), and MARK (Ebneth et
al, 1999; Illenberger et al., 1996). These data, as well as the large number of spots detected in
2D phosphopeptide mapping of MAP4 (Shiina et al., 1992; Ookata et al., 1997; Andersen et al.,
1994; Mori et al., 1991; Illenberger et al., 1996) show that MAP4 undergoes multiple
phosphorylation events involving several kinases, in addition to phosphorylation of ser-696 and
ser-787 sites. Indeed, Ookata et al. (1997) showed that, in human MAP4, at least six additional
sites, some within the MT-binding domain and some not, are phosphorylated in vivo during M-
phase. These phosphorylation events may be carried out by cyclin B-cdc2, by other kinases
studied in vitro, or by kinases not yet identified. Determining the effects of each phosphorylation
event on mitosis or MT function is a complicated and daunting task.

Because expression of mutants showed that phosphorylation at each site affected MT
stability and MAP binding, it may be informative to perform time-lapse studies to observe how
each affects MT dynamics. MAP4 was previously shown in vitro to affect MT dynamics by increasing the frequency of rescue, that is, the transition between MT depolymerization and pausing or polymerization (Ookata et al., 1995). In contrast, XMAP4 was shown to decrease the frequency of catastrophe, the transition between MT polymerization or pausing and depolymerization (Andersen et al., 1994). Studies of in vivo MT dynamics may provide a clearer picture of how MAP4 controls the dynamics of individual MTs and whether mutant MAP4 forms with different MT-binding capabilities exert different effects on MT dynamics.

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REFERENCES


FIGURE LEGENDS

Figure 1: Conservation of phosphorylation site sequences within the MAP4 MT-binding domain.

Comparison of amino acid sequences from human (Chapin et al., 1995), mouse (West et al., 1991), bovine (Aizawa et al., 1990), and *Xenopus* (Shiina et al., 1999) shows conservation of serines among species (696 and 787 positions in human MAP4 sequence), residing within proline-directed consensus sites for cyclin B-cdc2 kinase phosphorylation, highlighted in boxes. Identical amino acids among species are represented by bars and deletions by asterisks. Note that the region surrounding ser-696 is more highly conserved than that around ser-787.

Figure 2: Mutation of ser-696 or ser-787 to alanine blocks their phosphorylation by cyclin B-cdc2 kinase.

2D phosphopeptide mapping of recombinant MAP4 bacterial proteins, showing wild-type (Panel A) and wild-type mutated singly (Panels B and C) or doubly (Panel D) at residues 696 and 787 from serines to alanines and subjected to cyclin B-cdc2 kinase phosphorylation as described in Ookata et al. (1997).

Figure 3: Wild-type and mutant MAP4 colocalize with MTs in mouse L<sup>tk</sup>- cells.

(A) GFP fluorescence images of living L<sup>tk</sup>- cells expressing WT-, KK-, AA-, or EE-MAP4 mutants. Cells were induced with dex for 36 hours prior to capturing these images. (B) Immunoblots of L<sup>tk</sup>- cell extracts containing transfected MAP4 constructs. Two clones each of transfectants expressing wild-type (WT) and each mutant MAP4 form were isolated, and 50 µg of each cell extract was immunoblotted with an antibody against human MAP4 (top lanes) and a monoclonal tubulin antibody (bottom lanes). GFP fluorescence of EE-MAP4 from the last clone was visible under microscopy, albeit at low levels, but EE-MAP4 was not readily detected by Western
 blotting. This clone of cells showed a very low level of expression (see Table 1 for details).

**Figure 4: Cell cycle progression in Ltk- cells expressing WT and mutant MAP4 constructs.**

Cells grown on coverslips were fixed and expressing cells were quantified by scoring GFP-MAP4 fluorescence. Cells were scored for presence of 1) two centrosomes with an intact nucleus (late G2 or G2-M transition), 2) mitotic spindle (M), or 3) midbody separating two daughter cells (early G1) as illustrated in the schematic diagrams and fluorescence images. Cells expressing WT- and mutant MAP4 species (2 clones for each) were scored for the proportion of the total GFP-MAP4-expressing cells that were in late G2, M, or early G1 phase.

**Figure 5: Binding of mutant MAP4 forms to MTs.**

(A) WT-MAP4-expressing Ltk- cells before (ext) and after (H1P) a single step of Taxol-dependent MT polymerization (Chapin et al., 1991) The Coomassie-stained electropherogram shows an enrichment of the tubulin band at ~50 kDa in the H1P fraction. (B) H1P fractions from WT- and KK-MAP4 cells (AA- and EE-MAP4 are not shown) were eluted with 0-0.6 M NaCl and the eluted material was assayed by Western blotting with an antibody against human MAP4 (top two panels) and an antibody against endogenous mouse MAP4 (bottom panel). Essentially all MAP4 was released with 0.6 M NaCl.

(C) Quantification of MAP4 eluted from crude MTs (H1P) prepared from cells expressing transfected WT- and mutant MAP4 forms. Western blots such as those shown in Figure 6B, above, were scanned and the percentage of MAP4 released from H1P fraction at each salt concentration is shown. The MAP4 in each eluted sample was quantified and normalized to the total MAP4 that was released by elution with 0.6M NaCl. ND indicates that no MAP4 was detected in this sample; error bars show ± S.D.
<table>
<thead>
<tr>
<th>Species</th>
<th>Start Position</th>
<th>Amino Acid Sequence</th>
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</thead>
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<tr>
<td>Human</td>
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<td>NDITPPNKEELPPSPEKKTKPLATTQPAKTS</td>
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<tr>
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<td>----A-----------------</td>
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<tr>
<td>Xenopus</td>
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<tr>
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<td>TSKAKTQPTSLPKQPAPT*TIGGLNKKPMSL</td>
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<tr>
<td>Mouse</td>
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<tr>
<td>Bovine</td>
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<td>------------------*L--S--------</td>
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<tr>
<td>Mouse</td>
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<tr>
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<tr>
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<tr>
<td>Xenopus</td>
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<td>--*TL-L--S--V--**--D--K--ALKQTPTSAT</td>
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Figure 2
<table>
<thead>
<tr>
<th>Cell line</th>
<th>% MAP4 * (w/w)</th>
<th>% MAP4/Tub (mol/mol)</th>
<th>% expressors</th>
<th>% MAP4/Tub ** (mol/mol)</th>
<th>expression level relative to Hela ***</th>
<th>% Tubulin in polymer</th>
<th>Expression category</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.0855 ± 0.0247</td>
<td>4.23 ± 1.20</td>
<td>96.40</td>
<td>4.39 ± 1.24</td>
<td>2.19 ± 0.62</td>
<td>50.00 ± 22.88</td>
<td>high</td>
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<tr>
<td>WT</td>
<td>0.0305 ± 0.0049</td>
<td>1.49 ± 0.27</td>
<td>69.80</td>
<td>2.15 ± 0.36</td>
<td>1.08 ± 0.18</td>
<td>45.80 ± 14.89</td>
<td>medium</td>
</tr>
<tr>
<td>KK</td>
<td>0.0205 ± 0.0007</td>
<td>1.02 ± 0.05</td>
<td>75.80</td>
<td>1.34 ± 0.06</td>
<td>0.67 ± 0.03</td>
<td>44.40 ± 19.35</td>
<td>medium</td>
</tr>
<tr>
<td>KK</td>
<td>0.0420 ± 0.0028</td>
<td>2.08 ± 0.015</td>
<td>75.10</td>
<td>2.76 ± 0.10</td>
<td>1.38 ± 0.05</td>
<td>63.33 ± 8.96</td>
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<tr>
<td>AA</td>
<td>0.0600 ± 0.0042</td>
<td>2.99 ± 0.23</td>
<td>88.99</td>
<td>3.36 ± 0.26</td>
<td>1.68 ± 0.13</td>
<td>47.00 ± 7.81</td>
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<tr>
<td>AA</td>
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<td>89.45</td>
<td>2.38 ± 0.11</td>
<td>1.19 ± 0.67</td>
<td>48.60 ± 19.31</td>
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<tr>
<td>EE</td>
<td>0.0165 ± 0.0007</td>
<td>0.84 ± 0.06</td>
<td>88.12</td>
<td>0.98 ± 0.10</td>
<td>0.49 ± 0.03</td>
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<tr>
<td>EE</td>
<td>0.0040 ± 0.0028</td>
<td>0.20 ± 0.14</td>
<td>27.15</td>
<td>0.57 ± 0.27</td>
<td>0.28 ± 0.07</td>
<td>61.50 ± 29.60</td>
<td>low</td>
</tr>
</tbody>
</table>

* MAP4 content, expressed as percent of total extract protein (w : w)
** Adjusted for percent expressing cells
*** Expression relative to HeLa MAP4. Values for HeLa are from Bulinski and Borisy (1979).
Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expression category</th>
<th>Growth rate* n=5</th>
<th>FACS**</th>
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<td></td>
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<td>G1</td>
<td>S</td>
<td>G2/M</td>
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<tr>
<td>WT</td>
<td>high</td>
<td>28.5 ± 1.5</td>
<td>69</td>
<td>14</td>
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<tr>
<td>WT</td>
<td>medium</td>
<td>29.8 ± 1.6</td>
<td>62</td>
<td>20</td>
</tr>
<tr>
<td>KK</td>
<td>medium</td>
<td>28.7 ± 2.5</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>KK</td>
<td>medium</td>
<td>29.2 ± 2.2</td>
<td>65</td>
<td>21</td>
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<td>AA</td>
<td>high</td>
<td>29.1 ± 1.6</td>
<td>58</td>
<td>23</td>
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<tr>
<td>AA</td>
<td>medium</td>
<td>28.8 ± 2.0</td>
<td>65</td>
<td>17</td>
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<tr>
<td>EE</td>
<td>medium</td>
<td>30.1 ± 3.5</td>
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<td>EE</td>
<td>low</td>
<td>28.9 ± 3.0</td>
<td>67</td>
<td>20</td>
</tr>
</tbody>
</table>

* Doubling time in hours. Values are shown as mean ± SD.
** Percentage of cells in each indicated cell cycle stage, as determined by FACS analysis.
Figure 5

A. 

B. 

Salt elutes from H1P

WT   K-K   A-A   E-E

MAP4

C.

MAP4 released, % of total

NaCl concentration, M
Table 3: Expression of MAP4 mutant forms reduces MT sensitivity to nocodazole.
Values listed are the percentage of MAP4-expressing cells that showed nocodazole-sensitive MT arrays; that is they lost their MT array during nocodazole treatment. Cells induced to express WT and mutant MAP4 were incubated with 10µM nocodazole for 10 minutes and fixed in cold methanol. Coverslips were scored for the presence of a GFP-fluorescent MT array following treatment, and calculations were adjusted to include expressing cells only. KK- (p=0.029) and AA-MAP4 (p=0.017) are significantly less nocodazole-sensitive than WT. Mean ± S.D. is given; N=3 for each cell line.