Serum-Dependent Phosphorylation of Human MAP4 at Ser696 in Cultured Mammalian Cells

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ABSTRACT. In the previous paper [Ookata *et al.*, (1997) *Biochemistry*, 36: 249–259], we identified two mitotic cdc2 kinase phosphorylation sites (Ser696 and Ser787) in the proline-rich region of human MAP4. One (Ser696) of them was also phosphorylated during interphase. A protein kinase responsible for interphase phosphorylation of Ser696 could necessarily be distinct from cdc2/cyclin B kinase. To get insights into a physiological role for Ser696 phosphorylation, we searched for a Ser696 kinase and for cellular conditions under which Ser696 is dephosphorylated. Because Ser696 conforms to the MAP kinase phosphorylation consensus motif (PXSP), MAP kinase was tested as a possible kinase phosphorylating Ser696. MAP kinase, in fact, did phosphorylate Ser696 in MTB3, the carboxy-terminal half of human MAP4 *in vitro*. Phosphorylation of Ser696 in HeLa cell extract was suppressed by a MAP kinase inhibitor, DBTM-0004. Also consistent with the notion that Ser696 is a MAP kinase site were the fact that serum-starvation induced dephosphorylation of Ser696 in HeLa cells, TIG-3 and MRC-5-30 human fibroblasts, while readdition of serum recovered Ser696 phosphorylation, albeit after a surprisingly long interval. Thus, phosphorylation of Ser696 of MAP4, most likely carried out by MAP kinase, may play a role in modulation of MAP4 activity in proliferating versus quiescent cells.

Key words: microtubule/MAP4/phosphorylation/G0/cell cycle/serum starvation

Introduction

Microtubules are major cytoskeletal fibers which are involved in various cellular activities including organ-

elle transport, chromosome movement and cell shape determination. Individual microtubules undergo rapid and repeated polymerization/depolymerization, known as dynamic instability, both *in vivo* and *in vitro* (13, 21). Dynamic instability, which is an inherent property of microtubules composed of tubulin, is modified by microtubule-associated proteins (MAPs), group of which are proteins capable of polymerizing and stabilizing microtubules (12, 15, 29). Further, the microtubule-stabilizing and -polymerizing abilities of MAPs are known to be regulated by their phosphorylation (8, 17). Because microtubules are associated with MAPs *in vivo*, it is important to understand how MAPs modify the dynamic property of microtubules and how phosphorylation regulates microtubule-stabilizing ability of MAPs.

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Abbreviations: MAPs, microtubule-associated proteins; MAP4, microtubule-associated protein 4; MAP kinase, mitogen activated kinase; MTB3, microtubule binding domain of human MAP4.

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The dynamic property of microtubules changes dramatically at mitosis (19, 20, 31). Stable interphase microtubules become labile upon the onset of mitosis; that is, there is an increased turnover rate of microtubules during mitosis (11, 33). This is at least partially due to decreased microtubule-stabilizing activity of MAPs by phosphorylation. Mitosis is induced by activation of cdc2/cyclin B kinase, called M-phase promoting factor (MPF) (25). In HeLa cells, cdc2 kinase has been shown to phosphorylate MAP4, a major MAP in all proliferating non-neuronal cells (4), at mitosis and this phosphorylation reduced the microtubule-stabilizing activity of MAP4 (3, 27). We previously identified two cdc2 kinase phosphorylation sites at Ser696 and Ser787 within the proline-rich region of MAP4 (28). When their phosphorylation states were examined with phosphorylation-dependent antibodies during the cell cycle, however, one (Ser696) of them was shown to be phosphorylated in interphase as well as during mitosis (28). Because cdc2 kinase is not active in interphase, some other kinase would have to phosphorylate Ser696 in interphase. For several reasons, it was of interest to identify this kinase and examine this phosphorylation event. First, the amino acid sequence surrounding Ser696 is highly conserved evolutionarily, for 15 residues, from human to *Xenopus* (2, 6, 37, 38, 41). Further, *in vitro* experiments have demonstrated that mutation of Ser696 to Glu slightly enhanced, the microtubule-polymerization ability of MAP4. This was in contrast to Glu mutation at Ser787, which remarkably reduced polymerization stimulatory ability (our unpublished observation). For several reasons, we carried out to identify a Ser696 kinase in interphase. Although we could not unequivocally determine the kinase, we show here that Ser696 can be phosphorylated by MAP kinase *in vitro* and that its phosphorylation in cultured mammalian cells is serum-dependent, also suggestive of a phosphorylation event carried out by MAP kinase. The role of phosphorylation at Ser696 as well as the putative Ser696 kinase are discussed.

Materials and Methods

Cell culture and preparation of cell extract

HeLa cells, TIG-3 human fibroblasts and MRC-5-30 human fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5 or 10% fetal calf serum (FCS) and antibiotics (28). For serum starvation, medium was replaced by serum-free DMEM medium. Heat stable cell extracts used for immunoblotting with anti-MAP4 antibodies were prepared by boiling cell homogenates in 10 mM HEPES, pH 7.4, 1 mM EGTA, 0.75 M NaCl, 0.5% Nonidet P-40 and 10% sucrose, followed by centrifugation at $100,000 \times g$ for 30 min at 4°C.

Preparation of MTB3 and protein kinases

Preparation of a pET-MTB3 expression plasmid was described in Nguyen *et al.* (24). MTB3 (amino acid residues 668–1152 of human MAP4) was purified from a heat-stable fraction of the extract prepared from *Escherichia coli* carrying pET-MTB3 plasmid, using a Mono-S column (Pharmacia Biotech, Uppsala, Sweden) (28).

Cdc2 kinase that was purified from starfish oocytes at the first meiotic M-phase by p13^{suc1} affinity column chromatography was provided by Dr. E. Okumura (Tokyo Institute of Technology) (26). *E. coli* carrying *Xenopus* MAP kinase cDNA was provided by Dr. E. Nishida (Kyoto University, Kyoto, Japan). Bacterially expressed MAP kinase, which was purified and activated by thiophosphorylation with *Xenopus* MAP kinase kinase as described previously (9), was provided by K. Nonoyama (Tokyo Institute of Technology).

In vitro phosphorylation of MTB3

HeLa cell extracts for phosphorylation of MTB3 were prepared by freeze-thawing in 20 mM MOPS, pH 6.8, 0.5 M NaCl, 5 mM EGTA, 1 mM MgCl₂, 0.1 mM DTT, 20 mM β -glycerophosphate, 10% sucrose, and 0.5% Nonidet P-40, followed by centrifugation at $100,000 \times g$ for 30 min at 4°C. MTB3 (0.17 mg/ml) was phosphorylated in 0.1 mM [γ -³²P]ATP by cdc2 kinase or MAP kinase or by incubating with extracts prepared from HeLa cells, arrested at G1/S phase by thymidine block, in the presence or absence of DBTM-0004, a MAP kinase inhibitor, provided by Dr. A. Okuyama (Banyu Tsukuba Institute in Collaboration with Merk) at 30°C for 30 min. The phosphorylation reaction was stopped by the addition of the SDS-PAGE sample buffer or by boiling, and was monitored by autoradiography or western blotting following SDS-PAGE.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to the method of Laemmli (16). Separated proteins were transferred to Immobilon membranes (Millipore Corp., Bedford, MA, USA). The blots probed with anti-MAP4 or anti-phospho-Ser696 antibody were labeled with alkaline phosphatase-conjugated secondary antibody and developed with a BCIP/NBT phosphate substrate system. Anti-phospho-Ser696 antibody was prepared as described previously (28).

Results

Phosphorylation of Ser696 by MAP kinase in vitro

Ser696 of MAP4 is present within the amino acid sequence, PPSPEK (6, 41), which conforms the phosphorylation consensus motif not only for cdc2 kinase

{(S/T)PX(R/K)}, but also for MAP kinase (PXSP) (25). Although MAP kinase is generally believed to be activated just after mitogenic stimulation, a small fraction of MAP kinase, which is associated with microtubules (30), has been shown to be constitutively active (23). Therefore, we first examined whether Ser696 could be phosphorylated *in vitro* by MAP kinase, as well as by cdc2 kinase. Autoradiography documents phosphorylation of MTB3 (C-terminal fragment of MAP4 containing Ser696) by cdc2 kinase or MAP kinase (Fig. 1, lane 1 and 2). That the Ser696 site was indeed phosphorylated was examined by western blotting with an antibody specifically reactive with phosphorylated Ser696 (anti-phospho-Ser696 antibody). As expected, MTB3 expressed in *E. coli* was not originally phosphorylated (Fig. 1, lane 3). MTB3 phosphorylation at Ser696 caused by MAP kinase, cdc2 kinase, and by interphase HeLa cell extracts, could be detected by anti-phospho-Ser696 antibody (Fig. 1, lanes 4–6). Furthermore, phosphorylation of Ser696 in the presence of HeLa interphase extracts was suppressed by MAP kinase inhibitor DBTM-0004 at a concentration specifically inhibitory for MAP kinase (Fig. 1, lanes 7 and 8). These results allowed us to conclude that Ser696 of MTB3 can be phosphorylated *in vitro* by MAP kinase.

Serum-dependent phosphorylation/dephosphorylation at Ser696 of MAP4 in HeLa cells

If Ser696 is indeed phosphorylated by MAP kinase *in vivo*, the removal of a mitogenic stimulus, in our case serum, from the cell culture medium should be sufficient to bring about its dephosphorylation. We followed the phosphorylation state of Ser696 in HeLa cells cultured in the absence of serum. HeLa cell extracts were prepared from cells cultured for 0, 1, 2, 3, and 4 days in the absence of serum and immunoblotted with anti-bovine MAP4 (phosphorylation-independent) and with anti-phospho-Ser696 antibodies. Since the proportional content of MAP4 in cell extracts tended to de-

Fig. 1. Phosphorylation at Ser696 by cdc2 kinase, MAP kinase and HeLa cell extract *in vitro*. Autoradiograph of MTB3 phosphorylated by cdc2 kinase (lane 1) or MAP kinase (lane 2). Western blots of *E. coli* expressed MTB3 (lane 3) and that phosphorylated by cdc2 kinase (lane 4), MAP kinase (lane 5), HeLa cell extract (lane 6) and HeLa cells extract in the presence of 10 μ M (lane 7) and 50 μ M DBTM-0004 (lane 8) were detected with anti-phospho-Ser696 antibody.

crease to some extent during starvation, the amount of MAP4 was roughly adjusted by blotting, as shown with phosphorylation-independent bovine MAP4 antibody (Fig. 2A, the upper panel). MAP4 in randomly growing HeLa cells was reactive with anti-phospho Ser696 as previously shown (Fig. 2A, lane 1 in the lower panel), this reactivity gradually decreased during culture in the absence of serum (lanes 2–5). Only a faint reaction was detected after 4 day culture in the absence of serum; thus phosphorylation of Ser696 was decreased during growth in the absence of serum.

We also examined rephosphorylation after serum addition to serum-starved cells. The amount of MAP4 loaded can be seen in the upper panel of Figure 2B, by western blotting with anti-MAP4 antibody. MAP4 prepared from HeLa cells starved for 5 days did not react with anti-phospho-Ser696 (Fig. 2B, lane 1). Reaction appeared faintly at 12 hr (Fig. 2B, lane 6) and increased to an intensity similar to that before serum depletion, by 24 hr after serum was readded to the cultures (Fig. 2B, lane 7). This result also documents that HeLa cells were viable during 5 days of serum starvation and that phosphorylation/dephosphorylation reaction was reversible.

Typical HeLa cell morphologies are shown in Figure 3. HeLa cells which were relatively flat and adhered to substrate in the presence of serum (Fig. 3A), detached and became spherical several days after serum-depletion (Fig. 3B). Return to serum-containing medium brought the cells to the same morphology as before starvation (Fig. 3C).

Fig. 2. Serum-dependent phosphorylation of Ser696 in HeLa cells. Effects of serum starvation (A) or return to serum supplemented medium (B) on phosphorylation at Ser696, probed by western blotting with anti-phospho-Ser696. (A) Cell extracts prepared from HeLa cells cultured in the presence of serum (lane 1), and in the absence of serum for 1 (lane 2), 2 (lane 3), 3 (lane 4) and 4 days (lane 5). (B) Cell extracts prepared from HeLa cells cultured in the absence of serum for 5 days (lane 1), and 0.5 (lane 2), 1 (lane 3), 6 (lane 5), 12 (lane 6) and 24 hr (lane 7) after serum addition.

Fig. 3. Morphology of HeLa cells cultured in the presence or absence of serum. Phase contrast micrographs of HeLa cells cultured in the presence of 10% serum (A), in the absence of serum for 5 days (B), and cells 24 hr after return to serum supplemented medium (C).

Phosphorylation of Ser696 in fibroblast cells

HeLa cells are cancer cells in which the mitogenic signaling pathway might operate constitutively. To confirm that phosphorylation at Ser696 is similarly serum-dependent in non-cancer cells, the phosphorylation state of Ser696 was examined using two types of human fibroblasts, TIG-3 and MRC-3-20. Ser696 was phosphorylated in both cell types when the cells were cultured in the presence of serum, and was dephosphorylated when cells were grown in serum-free medium (Fig. 4), just as observed in HeLa cells.

Discussion

Several pieces of evidence suggest that phosphorylation at Ser696 or Ser787 in the proline-rich region of MAP4 appears to play different roles in microtubule-organization. First, Ser696 and Ser787 displayed different phosphorylation patterns during the cell cycle: while Ser787 was phosphorylated specifically at mitosis, Ser696 was phosphorylated in both interphase and mitosis (28). Although phosphorylation at Ser787 was

indicated to contribute to mitotic destabilization of microtubules, the role of phosphorylation at Ser696 is unclear. However, because the amino acid sequence around Ser696 is highly conserved in MAP4 in different species (2, 6, 37, 38, 41), it might play an important role in microtubule-mediated functions in interphase cells.

We have shown here that site specific phosphorylation at Ser696 is serum-dependent. This correlates this phosphorylation with mitogenic stimuli. While the interphase phosphorylation, originally detected in HeLa cell (28), could be a particular paradigm in tumor cells whose signaling pathway might be constitutively active. This was not the case, here; the same pattern of interphase phosphorylation of Ser696 obtained in two types of human fibroblasts TIG-3 and MRC-5-30. Serum starvation usually withdraws proliferating cells from the cell cycle, and results in G0 arrest. Considering that Ser787 was shown to be phosphorylated specifically at mitosis (28), there would exist three combinations of different phosphorylation states of MAP4 with respect to Ser696 and Ser787, depending on growth conditions (Fig. 5).

Serum stimulation of quiescent cultured cells has been reported to induce phosphorylation of MAPs or MAP-like proteins. Sato *et al.* showed rapid phosphorylation of MAP2-related 300 kDa protein after growth stimulation of quiescent 3Y1-B cells (32). Shaw *et al.* documented transient phosphorylation of 70–80 kDa MAPs in Swiss 3T3 cells as early as 15 min after serum stimulation and suggested the existence of two pathways for MAP phosphorylation, one that was protein kinase C-dependent and one that was independent (35). They also noted the presence of 200 kDa MAP4-like protein in Swiss 3T3 cells, but its phosphorylation was not described. Although MAP4 has been shown to be phosphorylated by protein kinase C, the phosphoryla-

Fig. 4. Serum-dependent phosphorylation of MAP4 at Ser696 in fibroblasts. Cell extracts of TIG-3 fibroblasts (lanes 1 and 2) and MRC-5-20 fibroblasts (lanes 3 and 4) cultured in the presence (lanes 1 and 3) or absence of 10% serum for 5 days (lanes 2 and 4) were blotted with anti-phospho-Ser696 antibody.

Fig. 5. Three different phosphorylation states of Ser696 and Ser787 of MAP4. MAP4 is a filamentous molecule whose N-terminal half construct the projection domain (NR) and C-terminal half the microtubule-binding domain consisting of the proline-rich region (Pro), the assembly promoting repeat region (AP) and a C-terminal region (C). Ser696 and Ser787 phosphorylation sites are located in the Pro-rich region. With respect to these two sites, MAP4 displays three different possible combinations of phosphorylation states depending on growth conditions of cells. Both sites are phosphorylated at mitosis (M-phase). Only Ser696 is phosphorylated during interphase. Neither is phosphorylated in G0 phase, which can be induced by serum starvation.

tion site, Ser815 in bovine MAP4, corresponding to Ser868 in human MAP4 (22), is distinct from Ser696. MAP4 may not be a target for these protein kinases, which are activated immediately after serum addition.

We have examined whether MAP kinase can phosphorylate Ser696 *in vitro*, because the amino acid sequence around Ser696 (PPSPEK) conforms to the MAP kinase consensus sequence, PXSP (25). MTB3, the C-terminal fragment of MAP4, was in fact phosphorylated at Ser696 by MAP kinase *in vitro*. Phosphorylation by HeLa cell extract also occurred, this was suppressed by a MAP kinase inhibitor. Serum deprivation induced dephosphorylation of Ser696. These results are consistent with the hypothesis that MAP kinase is the Ser696 kinase. Rephosphorylation at Ser696, however, was not induced immediately after serum addition but instead required about 24 hr, which is the time of one round of the cell cycle. This timing is in contrast to the well known fact that MAP kinase activation follows immediately upon mitogenic stimulation (1, 40). This discrepancy could occur because of distinct regulation of microtubule-associated MAP kinase (23). It was shown recently that a portion of MAP kinase bound to kinetochores is activated at mitosis (34, 42). Thus MAP kinase associated with microtubules might be independently activated after one round of the cell cycle.

Phosphorylation by MAP kinase was shown to decrease the microtubule-polymerization activity of MAP4 (14, 36). This apparently disagrees with the effect of Glu mutation of Ser696 and with its phosphorylation by MAP kinase. However, phosphorylation *in*

vitro could occur at sites which are not phosphorylated *in vivo*, as has been noted with phosphorylation of MAP4 by cdc2 kinase (28). MAP kinase has been shown to incorporate 1.7 mol phosphate into 1 mol of bovine adrenal MAP4, though Hoshi *et al.* described that 1 mol phosphate incorporated was sufficient to abolish tubulin-polymerization activity (14). *In vitro* phosphorylation at SP sites other than Ser696 could therefore reduce the microtubule polymerization activity of MAP4, some limiting mechanism(s) therefore must exist *in vivo*.

What cellular activity does serum-dependent phosphorylation at Ser696 relate to? Integrity of cytoplasmic microtubules is known to influence the biochemical events leading to initiation of DNA synthesis (39). Compounds that depolymerize microtubules potentiate the mitogenic effects of growth factors and other growth-promoting agents (18). On the other hand, Taxol, a compound specifically promoting microtubule-assembly, prevents induction of DNA synthesis mediated by growth factors and other mitogens (7). However, the timing of rephosphorylation after serum addition is inconsistent with these effects. While Ser696 required about 24 hr for to return to phosphorylated state, DNA commences starts about 12–16 hr after serum stimulation.

Serum starvation induces destabilization of microtubules in cultured cells (5). This was documented by disappearance of long-lived, nocodazole resistant "Glu"-microtubules in 3T3 cells cultured for 48 hr in serum-free medium (10). If phosphorylation at Ser696 is involved in serum-dependent stabilization of microtubules, our findings can explain the above observation very well. Phosphorylation at Ser696 was shown to be required for maintaining the microtubule-polymerization activity of MAP4 (our unpublished observation). Since serum starvation induced dephosphorylation at Ser696 (Fig. 2 and 4), that would result in decreasing the microtubule-polymerization activity of MAP4, leading to the destabilization of microtubules (10). Although the physiological significance of the serum-dependent change of microtubule stability is not yet known, this change may in part explain the morphological change of cells, as they switch from an adhering form to non adhering state.

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