Microtubule binding of the *Drosophila* DMAP-85 protein is regulated by phosphorylation in vitro

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Abstract The phosphorylation of microtubule-associated proteins (MAPs) is thought to be a key factor in the regulation of microtubule (MT) stability. Previously we isolated DMAP-85, a *Drosophila* MAP shown to be associated with stable MTs. In this work we show that DMAP-85 phosphorylated in cell-free early embryo extracts is released from MTs. MPM-2 antibodies recognize the phosphorylated protein. In vitro, DMAP-85 can be phosphorylated by the mitotic kinase Polo affecting its binding to MTs and creating MPM-2 epitopes on the protein. The results suggest that phosphorylation of DMAP-85 might affect its MT stabilizing activity during early mitotic cycles.

Key words: Microtubule-associated protein; DMAP-85; Polo kinase; MPM-2; Microtubule; *Drosophila*

1. Introduction

In living cells, microtubule (MT) dynamic is thought to be modulated by a number of post-translational modifications of both tubulin and microtubule-associated proteins (MAPs). MAPs bind to MTs in a nucleotide-independent way, promoting MT assembly and stabilization [1–3]. Several lines of evidence indicate that phosphorylation plays a major role in the regulation of MAP activity. Most identified MAPs are regulated by phosphorylation, and this modification interferes with their MT stabilizing capabilities [4–8]. In neurons, phosphorylation of MAPs seems to modulate MT organization during morphogenesis and process outgrowth [9,10]. In proliferating cells, phosphorylation of MAPs is tightly coupled to mitotic cycles and appears to regulate changes in MT dynamic during the cell cycle [11].

Drosophila has emerged as an important experimental system for the analysis of cytoskeletal functions [12,13]. The studies on *Drosophila* MAPs have revealed the existence of a MAP of 205 kDa, which colocalizes with interphase and mitotic MTs [14]. Based on their ability to bind MT affinity columns, Kellogg et al. [15] identified several MAPs, some of them have been cloned and their association to centrosomes has been determined [16–19]. Recently, the gene products of *abnormal spindle* (*Asp*) [20] and *mini-spindle* (*Msp*) [21] genes, have been identify as MAPs, based on both their capacity to bind MTs in vitro and molecular similarities with other MAPs. Asp associates with centrosomes and, together with the γ-tubulin ring complex, can restore MT nucleation activity to salt-stripped centrosome preparations in vitro [22,23]. Msp localizes to mitotic spindles and centrosomal regions during mitosis and is required to maintain the integrity of the bipolar spindle [21].

The effects of phosphorylation upon the MT binding capacity of *Drosophila* MAPs have only been shown in vitro for CP-60 [19]. Nevertheless, a strong genetic interaction has been demonstrated between the mitotic Polo kinase and Asp suggesting that the activity of Asp might be modified by this kinase [24]. Polo is an essential protein kinase required for multiple stages of mitosis. It has been shown to phosphorylate the activator of $p34^{cdc2}$ promoting entry into mitosis [25]. It has also been shown to phosphorylate tubulin and at least two MAPs in cell-free extracts [26]. More recently it has been reported that Polo phosphorylates components of the anaphase promoting complex [27] and PAV-KLP, a kinesin-like protein required for cytokinesis [28]. When phosphorylated by Polo, a number of these proteins display reactivity to the monoclonal antibody MPM-2 [29–31].

To explore further the regulatory activities of MAPs upon MT behavior, we have isolated DMAP-85, a *Drosophila* 85 kDa MAP present in embryos, larvae and adults, as a protein that can associate to taxol-stabilized MTs [32,33]. The biochemical analyses of DMAP-85 indicated that this protein cosediments with tubulin and promotes tubulin polymerization in vitro through its interaction with exposed domains of MTs, specifically with MAPs binding sequences present at the C-terminal end of β-tubulin [1,32,33]. These results suggest that one of the functions of DMAP-85 could be to regulate the dynamics of MT assembly in the cell.

In the work reported here, we have extended the biochemical characterization of DMAP-85 and demonstrate that the interaction of the protein with MTs in vitro is regulated by phosphorylation. The MPM-2 antibody recognizes the DMAP-85 phosphorylated in cell-free extracts. In vitro phosphorylation of DMAP-85 by Polo kinase causes its release from MTs and creates MPM-2 epitopes on this protein. Our data indicate that phosphorylation of DMAP-85 may be an important regulatory mechanism for controlling the interaction of this protein with MTs in vivo.

2. Materials and methods

2.1. Phosphorylation/dephosphorylation assays

High speed supernatant extracts were prepared from 200 μl of 0–2 h

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embryos. Embryos were dechlorionated in 50% sodium hypochlorite for 2–3 min, washed in HEM buffer (20 mM HEPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA), containing 0.1% Triton X-100, resuspended in 500 μl of this solution plus protease inhibitors (1 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 μg/ml aprotinin, 100 μg/ml PMSF), and homogenized at 4°C by several passes of a dounce tissue grinder. The homogenates were clarified by centrifugation at 12 000×*g* and then centrifuged at 50 000×*g* for 20 min at 4°C. Phosphorylation of native DMAP-85 was assessed by adding 10× phosphatase inhibitor stock (10 μM microcystin LR, 0.2 M NaF and 0.2 M β-glycerophosphate) to 200 μl of high speed supernatants, followed by 30 min incubation at 22°C, in the presence or in the absence of 1.0 μl [^{32}P]ATP. The mixtures were diluted to 500 μl with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) containing phosphatase inhibitors, and DMAP-85 was immunoprecipitated as described below. Immunocomplexes were resuspended in gel sample buffer and analyzed by SDS–PAGE followed by Western blot (non-radioactive samples) or by Coomassie blue staining and autoradiography (radioactive samples). Phosphorylated proteins were cut out from stained dried gels and radioactivity was measured by Cerenkov counting. For dephosphorylation assays, protein A beads with immunoprecipitated DMAP-85 were washed twice with phosphatase buffer (50 mM Tris–HCl, pH 7.8, 5 mM DTT, 2 mM $MnCl_2$, 100 μg/ml BSA), resuspended in 25 μl of the buffer and incubated at 30°C for 30 min with 270 U of *Lambda* phosphatase (Calbiochem, San Diego, CA, USA). In order to phosphorylate recombinant DMAP-85, samples of high speed supernatants were diluted 0-, 5-, 15-, 25- and 35-fold in a preparation of purified recombinant protein (0.4 mg/ml) and the phosphorylation assay was carried out by adding to the mixture 10 μl of 3× HEM buffer, 10× phosphatase inhibitor stock and 1.5 μl [^{32}P]ATP (10 mCi/ml). The mixture was incubated 30 min at 20°C and the reaction quenched by addition of gel sample buffer and boiling. Proteins were analyzed by SDS–PAGE and autoradiography.

2.2. Purification of recombinant protein

Escherichia coli cells JM109 containing pTrcHis-DMAP-85 vector (Cambiazo et al., unpublished results) were cultured in LB medium with 50 μg/ml ampicillin at 37°C. Expression of recombinant DMAP-85 was induced by addition of IPTG to a final concentration of 1 mM and the culture was further incubated at 37°C for 3 h. The induced cells were then harvested by centrifugation and soluble cell extracts were prepared in lysis buffer from the Xpress System Protein Purification (Invitrogen Co., San Diego, CA, USA) with addition of protease inhibitors. DMAP-85 was purified using Probond columns (Invitrogen Co.). After elution, DMAP-85 enriched fractions were concentrated by centrifugation in Ultrafree microcentrifuge filters (molecular mass cut-off: 30 000). Fractions were pooled, dialyzed against HEM buffer overnight at 4°C, and then clarified by centrifugation at 12 000×*g*.

2.3. Immunoprecipitations

Affinity-purified anti-DMAP-85 antibody (10 μg) was incubated 2 h at 4°C with 100 μl of protein A beads (Sigma Chemical Co., St Louis, MO, USA) diluted 1:10 in RIPA buffer, and the beads were washed three times with 500 μl of RIPA buffer. For Polo phosphorylation assays, immunoprecipitated DMAP-85 was obtained as follows: cell-free extracts were made from 0–2 h embryos (25°C) in RIPA buffer, using ∼0.1 g of embryos in 0.3 ml of buffer. After centrifugation of the embryo homogenate at 12 000×*g*, the supernatant, diluted 1:2 in RIPA buffer and pre-cleared with 20 μl of protein A beads 2 h at 4°C, was incubated overnight at 4°C with anti-DMAP-85 antibody pre-bound to protein A beads. The immunocomplexes were thoroughly washed with RIPA buffer by pelleting at 100×*g* and resuspending in 500 μl of RIPA buffer five times. MPM-2 antibody reactive phosphoproteins were immunoprecipitated from 0–2 h embryos as described above; using 0.4 μg/μl anti-MPM-2 antibody (Upstate Biotechnology Inc., Lake Placid, NY, USA) pre-bound to 50 μl of protein G beads. Depending on the experiment, phosphatase inhibitors were or were not added to the RIPA buffer during immunoprecipitation assays. Control immunoprecipitations were performed in parallel, in which either protein A/G beads or antibodies were not included. Rabbit preimmune serum was also used as control in DMAP-85 immunoprecipitations. Proteins were resuspended in 50 μl of gel sample buffer and analyzed by SDS–PAGE and Western blot. Polo kinase was immunoprecipitated from 0–2 h embryos according to Tavares et al. [26] except that 0.2 μg/μl anti-Polo antibody (MA294) pre-bound to 80 μl of protein G was added to 500 μl of embryo extracts diluted 1:4 in TM buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgSO_4$, 1 mM Na_3VO_4, 0.5 M NaF) that contained 0.5% NP-40 and 1 μM microcystin LR [31]. The immunocomplexes were washed five times in TM buffer, and immediately used in phosphorylation assays.

2.4. Assays for MT binding activity

Tubulin was purified from bovine brains by three cycles of temperature-dependent assembly–disassembly followed by P-11 phosphocellulose chromatography [34]. High speed supernatants from 0–2 h (300 μl) embryos were incubated for 15 min at 37°C with or without 1 μM microcystin LR, before the addition of 20 μM taxol (Sigma Chemical Co.), 1 mM GTP and 0.3 mg/ml brain tubulin. In other experiments, 100 μl of tubulin (1.0 mg/ml) was admixed with phosphorylated or unphosphorylated recombinant protein diluted to 90 μl with HEM buffer, and assembly was initiated by the addition of 1 mM GTP and 20 μM taxol to the assay mixture (0.25 ml final volume). In both cases, after 30 min incubation at 37°C, the mixtures were layered over 100 μl of 15% sucrose in HEM buffer plus 20 μM taxol and centrifuged at 125 000×*g* for 30 min at 20°C. The pellets were washed twice with HEM buffer plus 20 μM taxol and resuspended in gel sample buffer. Equivalent fractions of supernatants and pellets were analyzed by SDS–PAGE and Western blot.

2.5. Polo kinase assays

Protein A beads with immunoprecipitated DMAP-85 and protein G beads with immunoprecipitated Polo kinase were rinsed twice in kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 1 μM microcystin LR, 20 mM NaF, 1 mM Na_3VO_4 and 20 mM β-glycerophosphate). Polo and DMAP-85 beads were mixed, pelleted and resuspended in 30 μl of kinase buffer containing 1 mM ATP and 1.0 μl [^{32}P]ATP (10 mCi/ml). In control experiments, dephosphorylated casein (2.0 μl of a 1 mg/ml solution) was used as a substrate of Polo kinase. In some experiments, Polo beads were mixed with 7.5 μl of recombinant DMAP-85 (0.9 mg/ml), in the presence or absence of [^{32}P]ATP for 20 min at 20°C. The final volume of the mixtures was 30 μl. Phosphorylated proteins were or were not tested for binding to MTs, and then analyzed by SDS–PAGE followed by Western blot or by autoradiography. For Polo immunodepletion, cell-free extracts were made from 0–2 h embryos in HEM buffer containing 0.5% NP-40 and protease inhibitors and incubated for 2 h at 4°C in the absence or presence of 0.2 μg/μl anti-Polo antibody. Extracts were incubated with protein G beads for 1 h at 4°C. Supernatants were saved and the incubation repeated overnight with or without the anti-Polo antibody, followed by 1 h incubation with protein G beads. Supernatants were diluted to 5 μg/μl in HEM buffer (0.5 ml final volume) containing 0.5 mM ATP, 1 mM DTT phosphatase and protease inhibitors. They were incubated for 20 min at 20°C and DMAP-85 was immunoprecipitated from the extracts using an anti-DMAP-85 antibody pre-bound to protein A beads as described above. Immunoprecipitated proteins were analyzed by SDS–PAGE and Western blot.

2.6. Gel electrophoresis, immunoblotting and autoradiography

SDS–PAGE was performed according to the method of Laemmli [35], with 10% or 15% acrylamide gels. For immunoblotting, gels were transferred to nitrocellulose membranes according to standard procedure [36]. Blots were processed as described in Cambiazo et al. [37]. Bound antibodies were revealed using DAB Liquid substrate System (Sigma Chemical Co.) or ECL reagents (Amersham Corp, Arlington Heights, IL, USA). Anti-DMAP-85 antibody [33] was used at 1:500, anti-MPM-2 antibody was diluted 1:10 000. For autoradiography, gels were dried and exposed to X-OMAT AR films (Kodak) at −70°C.

3. Results

A direct interaction of DMAP-85 with MTs has been previously characterized [32,33]. In this work we test whether DMAP-85 is phosphorylated and whether binding to MTs is affected by the phosphorylation state of the protein. To determine if DMAP-85 is phosphorylated, the protein was immunoprecipitated from 0–2 h embryo extracts, in the presence

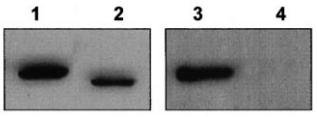

Fig. 1. Phosphorylation of DMAP-85. DMAP-85 was immunoprecipitated from 0–2 h embryo extracts in the absence (lanes 1 and 2) or in the presence of [γ-^{32}P]ATP (lanes 3 and 4), and either left untreated (lanes 1 and 3), or treated with *Lambda* phosphatase (lanes 2 and 4). Samples were then analyzed by SDS–PAGE and the gels were transferred to nitrocellulose membranes and probed with an anti-DMAP-85 antibody (lanes 1 and 2), or dried and exposed for autoradiography (lanes 3 and 4).

of phosphatase inhibitors, and its electrophoretic mobility analyzed after treatment with *Lambda* phosphatase. The results indicate that phosphatase treatment causes a shift in the electrophoretic mobility of DMAP-85 (Fig. 1, lanes 1 and 2). To confirm that the change in mobility is due to phosphorylation, the extract was incubated with [γ-^{32}P]ATP, followed by immunoprecipitation with anti-DMAP-85 specific antibodies (Fig. 1, lane 3). However, if the pellet is treated with *Lambda* phosphatase the radioactive label is removed (Fig. 1, lane 4). The results show that under these conditions DMAP-85 incorporates ^{32}P from [γ-^{32}P]ATP, with a stoichiometry of 6.2 ± 2.3 mol/mol protein ($n = 3$).

The ability of MAPs to bind MTs has been shown to be affected by its phosphorylation state. Therefore, we investigated the effect of the phosphatase inhibitor microcystin LR on the capacity of DMAP-85 to bind MTs in crude extracts from 0–2 h embryos. A high speed supernatant from embryo extracts was incubated with taxol and bovine brain tubulin in the absence or in the presence of microcystin LR. The binding of DMAP-85 to taxol MTs was assayed by centrifugation and analysis of supernatants and pellets by SDS–PAGE and Coomassie blue staining (Fig. 2A) or Western blot (Fig. 2B). Fig. 2A shows that the pattern of protein sedimentation with MTs is not broadly affected by the addition of microcystin LR. In

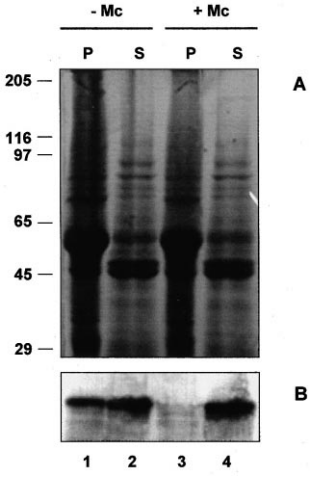

Fig. 2. Effects of microcystin LR on MT binding of DMAP-85 in cell-free extracts. Cell-free extracts from 0–2 h embryos were incubated without (−Mc) or with (+Mc) 1 μM microcystin LR for 15 min at 37°C, before the addition of 20 μM taxol and 0.3 mg/ml tubulin. After 30 min incubation at 37°C and centrifugation, pellet (lanes 1 and 3) and supernatant (lanes 2 and 4) samples were analyzed by SDS–PAGE followed by Coomassie blue staining (A) or by immunoblotting with a polyclonal antibody anti-DMAP-85 (B).

the absence of microcystin LR, a significant fraction of DMAP-85 cosediments with MTs (Fig. 2B, lanes 1 and 2). In the presence of 1 μM microcystin LR, a concentration sufficient to inhibit both type 1 and 2A phosphatases, a negligible amount of DMAP-85 binds to MTs and appears in the pellet (Figs. 2B, 3 and 4). These results indicate that DMAP-85 is a substrate for phosphorylation by a kinase activity present in the embryo high speed supernatant. Also, a phosphatase activity present in the extracts appears to modulate the MT binding of native DMAP-85, so that the protein only binds MTs when dephosphorylated.

To correlate the ability of DMAP-85 to bind MTs with its phosphorylation state, we used recombinant DMAP-85 as a source of unmodified protein. Incubation of this protein with [γ-^{32}P]ATP in the presence of a low protein concentration of high speed supernatant (dilution 1:35) results in its phosphorylation (Fig. 3A, lane 1), while the phosphorylation of endogenous DMAP-85 is hardly detectable (Fig. 3A, lane 2). In the control reaction, absence of diluted supernatant, there is no

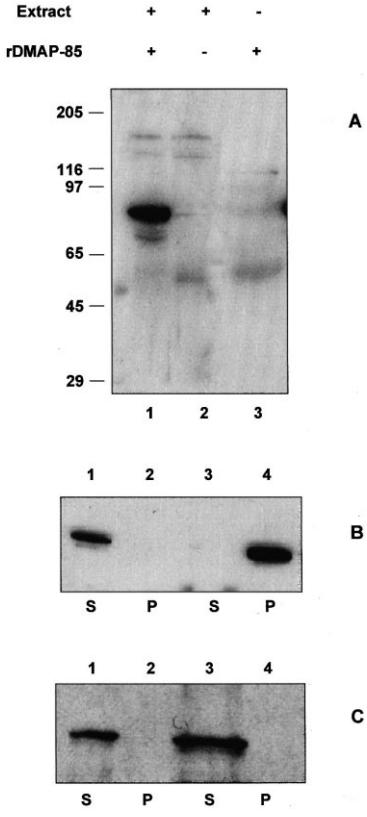

Fig. 3. In vitro phosphorylation of recombinant DMAP-85 in the presence of endogenous kinases and interaction with MTs. A: Cell-free extract from 0–2 h embryos diluted 1:35 in a preparation of recombinant DMAP-85 (lane 1), cell-free extract diluted 1:35 in HEM buffer (lane 2) or recombinant DMAP-85 (lane 3) were incubated with [γ-^{32}P]ATP at 20°C for 30 min. Samples were analyzed by means of SDS–PAGE and autoradiography. B: Recombinant DMAP-85 (7.5 μg) was phosphorylated with endogenous kinases from embryo extracts and tested for binding to MTs. Supernatants (S) and pellets (P) were analyzed by SDS–PAGE, transferred to nitrocellulose and probed with an antibody against DMAP-85. Phosphorylated recombinant protein fails to cosediment with MTs (lanes 1 and 2), while unphosphorylated protein is found in the pellet fraction (lanes 3 and 4). C: In the absence of MTs, both phosphorylated (lanes 1 and 2) and unphosphorylated (lanes 3 and 4) proteins remain in the supernatant fractions. (rDMAP-85 = recombinant DMAP-85.)

incorporation of ^{32}P into DMAP-85 (Fig. 3A, lane 3). Once the conditions for phosphorylation of the recombinant DMAP-85 were established we tested whether the phosphorylation of the protein affects its ability to bind MTs in a cosedimentation assay. The results show that when recombinant DMAP-85 is incubated with the extract under conditions that favor its phosphorylation, ~100% of protein remains in the supernatant (Fig. 3B, lanes 1 and 2). However, the unphosphorylated recombinant DMAP-85 cosediments with tubulin and is recovered in the pellet fraction after centrifugation (Fig. 3B, lanes 3 and 4). In the absence of MTs both phosphorylated (Fig. 3C, lanes 1 and 2) and unphosphorylated proteins (Fig. 3C, lanes 3 and 4) are found in the supernatant fractions. These results suggest that the state of phosphorylation of both recombinant and native DMAP-85 is directly related to their ability to bind MTs.

In proliferating cells or tissues a similar effect has been described for several other MAPs. In the case of MAP-4, it has been reported that phosphorylation by $p34^{cdc2}$ creates an MPM-2 reactive epitope that correlates with its effect on MT stability [38,39]. The MPM-2 monoclonal antibody has been shown to recognize a subset of proteins that become phosphorylated during the transition from interphase to mitosis [40–43]. Accordingly, we proceed to test whether the phosphorylation of DMAP-85 is associated with MPM-2 reactivity. For this we immunoprecipitated DMAP-85 from embryo extracts in the presence or absence of microcystin LR (Fig. 4). The immunoprecipitates were separated by SDS–PAGE, blotted to nitrocellulose and then probed with an MPM-2 antibody. The results (Fig. 4A) show that a specific anti-DMAP-85 antibody can immunoprecipitate an MPM-2 reactive protein in the presence of the phosphatase inhibitor, but not in its absence (Fig. 4A, lanes 2 and 3). In order to confirm these results, we have carried out the converse experiment by immunoprecipitating with the MPM-2 specific antibody and probing with the anti-DMAP-85 antibody. As shown in Fig. 4B, a fraction of DMAP-85 specifically immunoprecipitates with MPM-2 antibody, in the presence but not in the absence of microcystin LR. These results indicate that when phosphorylated, DMAP-85 is an MPM-2 reactive phosphoprotein.

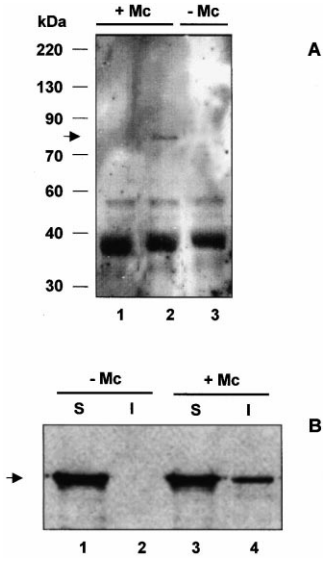

Fig. 4. MPM-2 reactivity of phosphorylated DMAP-85. A: Cell-free extracts from 0–2 h embryos were prepared in the presence (+Mc) or in the absence (−Mc) of 1 μM microcystin LR and incubated with pre-immune serum (lane 1) or anti-DMAP-85 antibody (lanes 2 and 3). Immunoprecipitated proteins were analyzed by Western blots, using a monoclonal anti-MPM-2 antibody. B: Cell-free extracts prepared in the presence (+Mc) or in the absence (−Mc) of microcystin LR were incubated with an anti-MPM-2 antibody. Both fractions, immunoprecipitates (I) and supernatants (S) were analyzed by SDS–PAGE, transferred to nitrocellulose and probed with the anti-DMAP-85 antibody. A fraction of DMAP-85 immunoprecipitates with the anti-MPM-2 antibody in the presence (lanes 3 and 4) but not in the absence (lanes 1 and 2) of microcystin LR. Arrows indicate DMAP-85 position.

A number of protein kinases have been shown to be responsible for the phosphorylation of MPM-2 reactive epitopes [42,44,45]. Among them, the Polo mitotic kinase is particularly important since its phosphorylation of CDC25 leads to activation of $p34^{cdc2}$ and entry into mitosis [25]. Furthermore, for the purpose of our study, previous work showed that the *Drosophila* Polo kinase specifically phosphorylated an 85 kDa protein in embryo extracts [26]. Thus, we tested whether Polo is able to phosphorylate DMAP-85 in vitro (Fig. 5A). For this, we first immunoprecipitated Polo from embryo extracts in the presence of phosphatase inhibitors and tested its kinase activity against casein. The data (Fig. 5A, lane 1) show that under these conditions Polo is immunoprecipitated as an active kinase. We then immunoprecipitated DMAP-85 from embryo extracts in the absence of phosphatase inhibitors and use it as a substrate for a phosphorylation reaction with immunoprecipitated Polo. The result shows that DMAP-85 is phosphorylated by the active Polo kinase (Fig. 5A, lane 2). This reaction is specific since it is not observed in the absence of immunoprecipitated DMAP-85 (lane 3), when pre-immune serum was used (lane 4) or in the absence of Polo (lane 5). These results indicate that Polo phosphorylates DMAP-85 in vitro. In order to investigate whether Polo is involved in the creation of MPM-2 epitopes on DMAP-85, this protein was immunoprecipitated from Polo-immunodepleted extracts, in the presence of phosphatase inhibitors and probed with the MPM-2 antibody. Whereas incubation of extracts with protein G beads has no effect on the formation of MPM-2 epitopes (Fig. 5B, lane 2), immunodepletion of Polo kinase from the extract results in a loss of the MPM-2 reactivity of immunoprecipitated DMAP-85 (Fig. 5B, lane 3). These results strongly suggest that DMAP-85 phosphorylation by Polo kinase generates MPM-2 epitopes on this protein.

Our results show that phosphorylation of DMAP-85 affects its ability to bind MTs and also that Polo can specifically phosphorylate the protein in vitro. Therefore we assessed whether phosphorylation by Polo can modify the binding of DMAP-85 to MTs. Unphosphorylated or Polo phosphorylated recombinant DMAP-85 was incubated with purified MTs and its binding ascertained by a sedimentation assay (Fig. 5C). Western blots of supernatant and pellet fractions indicate that unphosphorylated recombinant protein cosediments with MTs (Fig. 5C, lanes 1 and 2). However, when the recombinant protein is phosphorylated by Polo, approximately 40% of the protein does not associate to MTs and remains in the supernatant (Fig. 5C, lanes 3 and 4). In order to know whether the protein fraction bound to MTs was phosphorylated, we added [γ-^{32}P]ATP to the Polo phosphorylation assay and used the ^{32}P-labeled protein in the sedimentation procedure. Our data from Fig. 5D show that unbound protein has incorporated ^{32}P from [γ-^{32}P]ATP (Fig. 5D, lane 1), while the fraction of DMAP-85 that cosediments with MTs (Fig. 5D,

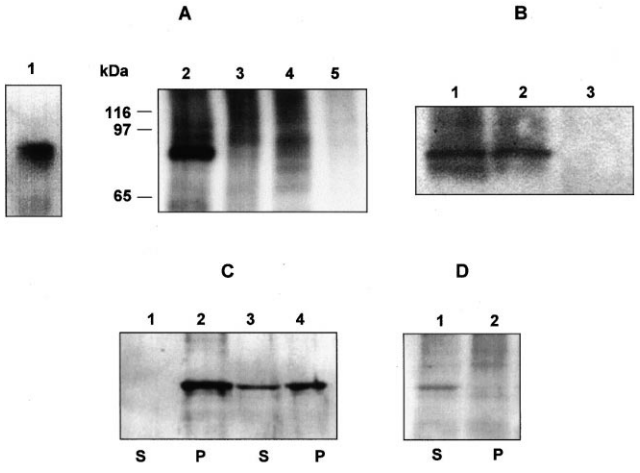

Fig. 5. In vitro phosphorylation of DMAP-85 by Polo kinase. A: The autoradiographs show the phosphorylation of casein (lane 1) and immunoprecipitated DMAP-85 (lane 2) by immunoprecipitated Polo kinase. In control experiments, anti-DMAP-85 or anti-Polo antibodies were omitted (lanes 3 and 5) and immunoprecipitation assays were carried out in the presence of pre-immune serum (lane 4). B: DMAP-85 was immunoprecipitated from 0–2 h embryo extract in the presence of phosphatase inhibitors followed by Western blot analysis with an anti-MPM-2 monoclonal antibody (lane 1). Pre-incubation of the extracts with protein G beads had little effect on the formation of MPM-2 epitopes (lane 2). In contrast, immunodepletion of Polo kinase results in a loss of the MPM-2 reactivity of DMAP-85 (lane 3). C: Recombinant DMAP-85 (6.7 μg) was phosphorylated with immunoprecipitated Polo kinase and assayed for binding to MTs. Supernatants (S) and pellets (P) were analyzed by SDS–PAGE, transferred to nitrocellulose and probed with an anti-DMAP-85 antibody. The immunoblot indicates that unphosphorylated recombinant protein cosediments with MTs (lanes 1 and 2) while approximately 40% of Polo phosphorylated protein is found in the supernatant fraction (lanes 3 and 4). D: Recombinant DMAP-85 was incubated with immunoprecipitated Polo in the presence of 1 mM [γ-^{32}P]ATP at 20°C for 30 min, and tested for binding to MTs. The autoradiograph indicates that phosphorylated protein fails to cosediment with MTs. Supernatant (lane 1) and pellet (lane 2).

lane 2), remains unphosphorylated. These results indicate that Polo phosphorylation affect the MT binding ability of DMAP-85.

4. Discussion

In previous reports we have shown that DMAP-85 stabilizes MTs, promotes MT assembly [32] and associates to stable MTs in late *Drosophila* embryos [1]. In this report we examine the post-translational modifications of this protein during developmental stages known to be characterized by rapid mitotic divisions. Our in vitro studies suggest that DMAP-85 is phosphorylated by the Polo mitotic kinase, and that this modification alters its MT binding capacity.

We first show that when DMAP-85 is purified from early embryo extracts (0–2 h), it displays an altered pattern of electrophoretic mobility that is correlated with the protein being phosphorylated. Furthermore, in this cell-free system we find that the binding of DMAP-85 to MTs can be regulated by the presence or the absence of phosphatase inhibitors, which are known to alter the equilibrium between phosphorylated and dephosphorylated proteins. Reversible phosphorylation of MAPs is well established as a mechanism for the rapid regulation of MT rearrangements through the activity of many kinases modulated by extracellular factors. In fact, activation of cellular phosphorylation mechanisms, after treatments with phosphatase inhibitors, has been shown to modify MT dynamics in vitro [46] and in vivo [47,48].

In order to study the effects of phosphorylation of DMAP-85 upon its ability to bind MTs, we have produced a recombinant protein and phosphorylate it by endogenous kinases in a cell-free assay. We showed that the phosphorylated protein is unable to bind to MTs, suggesting that the activity of this protein is regulated in vivo by phosphorylation. Accordingly, we propose that the MT stabilizing activity of DMAP-85 is abolished during early stages of embryonic development when most of the MTs are highly dynamic polymers, characterized by the absence of any tubulin modifications associated with stable MTs [49,50]. Similar results have been obtained for other MAPs, including XMAP-4 [8], p220 [4,51] MAP-4 [5], EMAP-115 [52], CP-60 [19] and EMAP [53]. The phosphorylation of these MAPs correlates with the transition from interphase to mitosis, and it has been associated with the activation of the general mitotic kinases, such as p34^{cdc2} [11]. It has been proposed that this modification alters the MT stabilizing capacity of these MAPs [54].

In proliferating cells the transition from interphase to mitosis has been shown to be associated with the phosphorylation of a large group of proteins. A subset of these proteins is recognized due to their reactivity with the MPM-2 monoclonal antibody [40–43]. At least one MAP has been reported to be an MPM-2 reactive phosphoprotein in vitro [38,39]. Our results show that the mitotic phosphorylation of DMAP-85 creates MPM-2 reactive epitopes in this protein. Thus, these results suggest that the presence of MPM-2 reactive epitopes is not only correlated with the activation of mitotic proteins but can also result in the inactivation of proteins that promote MT stabilization.

A number of protein kinases are known to phosphorylate mitotic substrates thereby creating MPM-2 reactive phosphoepitopes [29,30,44,45,55]. One of these kinases corresponds to Polo kinase, which has been shown to bind a number of compartments of the mitotic apparatus [31], and it is required throughout mitotic progression [56,57]. Our data indicate that DMAP-85 is phosphorylated in vitro by Polo kinase and that this phosphorylation generates MPM-2 epitopes on the protein. In our hands, the maximum extent of DMAP-85 Polo-dependent phosphorylation was 40% with respect to the total DMAP-85 present in the assays. This result is in good agreement with the fraction of recombinant DMAP-85 that becomes unable to bind to MT after Polo phosphorylation, and indicates that phosphorylation mediated by Polo kinase regulates the binding of DMAP-85 to MTs. Considering that Polo phosphorylates DMAP-85 in vitro, and that MPM-2 reactivity of DMAP-85 is dependent on endogenous Polo kinase activity, we propose that Polo might be responsible for the phosphorylation of DMAP-85 during early stages of embryogenesis, affecting its MT binding activity, and thus modulating the MT stabilizing capacity of DMAP-85.

Polo activity is essential for the G2/M transition [56,57], and it has been associated with direct phosphorylation of tubulin [26]. Furthermore, it has been shown that in the absence of Polo, spindle MTs are more resistant to depolymerization by colchicine and display a morphology resembling taxol treated MTs [58]. Therefore, it has been proposed that Polo might be required to promote an increase in MT dynam-

ics associated with entry into mitosis [58]. Our results show that the phosphorylation of DMAP-85 by Polo affects its MT binding properties. Therefore, we suggest that Polo might alter MT dynamics through the phosphorylation of MT stabilizing proteins like DMAP-85.

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