The EMBO Journal vol.15 no.18 pp.4873–4883, 1996

The conserved mitotic kinase polo is regulated by phosphorylation and has preferred microtubule-associated substrates in *Drosophila* embryo extracts

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The *Drosophila* gene *polo* encodes a protein kinase required for progression through mitosis. Wild-type polo protein migrates as a tight doublet of 67 kDa which is converted to a single band by phosphatase treatment, which also inactivates the kinase. We have determined putative polo substrates in a cell-free system derived from mutant embryos. Exogenous polo protein kinase phosphorylates proteins of sizes 220 kDa, 85 kDa and 54 kDa, to a greater extent when added to extracts of *polo[1]*-derived embryos compared with extracts of wild-type embryos, which in both cases have been subject to mild heat treatment to inactivate endogenous kinases. Proteins of the same size are predominantly phosphorylated by the endogenous kinases present in wild-type extracts, and are either not phosphorylated or are poorly phosphorylated in extracts of *polo[1]*-derived embryos. We show that a specific monoclonal antibody to β-tubulin precipitates the phosphorylated 54 kDa protein together with an associated 85 kDa protein also phosphorylated by polo protein kinase. Moreover polo binds to an 85 kDa protein which is enriched in microtubule preparations. We discuss the extent to which these *in vitro* phosphorylation results reflect the effects of mutations in *polo* on microtubule behaviour during the mitotic cycle.

Keywords: *Drosophila*/kinase/mitosis/polo/tubulin

Introduction

Cycles of protein phosphorylation and dephosphorylation play a central role in regulating the progression through mitosis; of the protein kinases required for these processes, considerable attention has focused on the mitotic kinase $p34^{cdc2}$ and its cyclin sub-units required for the G_2–M transition (for reviews see Nurse, 1990; Nigg, 1993; Morgan, 1995). This enzyme has been implicated in the phosphorylation of a variety of cellular substrates including chromosomally associated proteins, intermediate filaments and spindle-associated proteins which mediate the changes in cytoarchitecture upon entry into mitosis. The enzyme has been remarkably conserved throughout evolution, such that homologous genes from human, fly and other organisms are able to complement a temperature-sensitive fission yeast mutant (for example, Lee and Nurse, 1987; Jimenez *et al.*, 1990). Moreover, the mitotic activity of $p34^{cdc2}$ is regulated by its association with evolutionarily conserved cyclins and by its state of phosphorylation which, in turn, is also regulated by a highly conserved set of enzymes such as the cdc25 phosphatase (Draetta *et al.*, 1988; Gautier *et al.*, 1991; Millar *et al.*, 1991; Lee *et al.*, 1992).

Another highly conserved protein kinase required for progression through mitosis is the product of the *Drosophila* gene *polo* (Sunkel and Glover, 1988; Llamazare *et al.*, 1991). Mutations in this gene result in a high mitotic index and abnormal chromosome segregation in larval neuroblasts. Many of these cells do not proceed into anaphase and chromosomes become hyper-condensed. A proportion of those cells that proceed to anaphase are characterized by having broad or disorganized poles and some are clearly monopolar. Abnormal networks of microtubules associated with condensed chromosomes are often observed and centrosomes do not seem to be correctly organized. Multipolar spindles and non-disjunction are also seen in male meiosis indicating that the polo protein kinase is required for normal function of the mitotic apparatus not only in mitosis but also in meiosis.

Recent reports have shown that the *Drosophila polo* gene has been conserved in a number of other species. Protein kinases which display extensive sequence similarity to polo include the kinase Cdc5 of *Saccharomyces cerevisiae* (Kitada *et al.*, 1993), the kinase plo1 of *Schizosaccharomyces pombe* (Ohkura *et al.*, 1995), the murine kinases Snk, Fmk and Plk (Simmons *et al.* 1992; Clay *et al.*, 1993; Lake and Jelinek, 1993; Hamanaka *et al.*, 1994; Donohue *et al.*, 1995) and the human kinase Plk1 (Lake and Jelinek, 1993; Golsteyn *et al.*, 1994; Hamanaka *et al.*, 1994; Holtrich *et al.*, 1994). Computer analysis of the protein sequences of these enzymes indicates that they define a new subfamily of protein kinases (Hanks and Hunter, 1995). All these kinases share the characteristic of being highly expressed in tissues with a high mitotic index, consistent with a possible function in cell proliferation. Moreover, the levels of mRNA of CDC5 and Plk, accumulate periodically in the cell cycle, reaching a peak preceding or during mitosis (Kitada *et al.*, 1993; Lake and Jelinek, 1993; Golsteyn *et al.*, 1994), and protein levels may fluctuate during the cell cycle in concert with the fluctuations of their mRNA, as described for the human Plk1 kinase (Golsteyn *et al.*, 1994).

The phenotypes of the *Drosophila polo[1]* mutation suggest a role in the normal function of the mitotic spindle. This is reinforced by the analysis of the human kinase Plk1, which localizes to the area of the mitotic spindle during metaphase and to the midbody during telophase (Golsteyn *et al.*, 1994, 1995). Furthermore, the Plk1 protein kinase is maximally active during mitosis and

displays a cyclical activity similar to that of $p34^{cdc2}$ kinase (Golsteyn *et al.*, 1995). The *Drosophila* polo protein kinase, on the other hand, although also showing a cyclical activity with a peak at mitosis appears to be maximally active slightly later than $p34^{cdc2}$ kinase during anaphase and telophase of the mitotic cycles in syncytial embryos (Fenton and Glover, 1993). It is not clear whether this discrepancy in the timing of maximal activity in these two organisms simply reflects the greater accuracy with which the mitotic stage can be assessed in the synchronous mitosis of the *Drosophila* embryo, or whether it is a fundamental difference in the pattern of mitotic regulation in these two systems.

Loss of $plo1^+$ function in *S.pombe* also leads to the formation of monopolar spindles, as in the *Drosophila* mutant (Ohkura *et al.*, 1995). Such disruptants also fail to septate. Overexpression of $plo1^+$ can drive the formation of septa at any stage of the fission yeast cell cycle and, in the presence of active $p34^{cdc2}$, can also lead to the formation of monopolar spindles. These observations define two major roles for the $plo1^+$ in fission yeast; as an enzyme which co-operates with $p34^{cdc2}$ to regulate spindle behaviour and as the primary kinase that can independently drive the late cell division events of septation. Mutations in the *CDC5* gene in *S.cerevisiae* block nuclear division late in mitosis with chromosomes having segregated on an elongated spindle (Hartwell *et al.*, 1973; Byers and Goetsch, 1974; Kitada *et al.*, 1993). The kinase Cdc5 may also regulate microtubule behaviour, as temperature-sensitive *cdc5* mutants lose susceptibility to the microtubule destabilizing drug methyl-benzimidazole-2-ylcarbamate (MBC), following release from a temperature-induced block (Wood and Hartwell, 1982). Based on these parallels it is tempting to conclude that the polo-like kinases may not only be structurally related but may also share similar function in the progression through mitosis.

Here we report a series of biochemical experiments that show that polo is a Ser/Thr protein kinase which has to be phosphorylated to show kinase activity. We also search for putative substrates of the polo protein kinase using extracts of total embryos derived from homozygous $polo^1$ mothers ($polo^1$ extracts). We predicted that in these extracts the endogenous polo substrates would be found in a predominantly dephosphorylated state (or phosphorylated to a lesser extent than in wild-type embryo extracts). We have been able to detect three putative substrates phosphorylated by exogenous polo to a greater extent in the mutant extracts than in wild-type embryo extracts. One of the proteins specifically phosphorylated by polo under these conditions was identified by immunoprecipitation as β-tubulin and another polypeptide was shown to be an 85 kDa microtubule-associated protein (MAP) to which polo can bind specifically. Our results are consistent with the hypothesis that polo might be involved in orchestrating changes in microtubule organisation that have to occur for progression through mitosis.

Results

polo^1 *mutant embryos contain inactive polo protein kinase*

In order to determine possible substrates of the polo protein kinase we sought to devise an *in vitro* assay in which *Drosophila* cell extracts devoid of polo activity could be incubated with polo protein kinase and the subsequent phosphorylation events could be monitored. We wished to know whether extracts of embryos derived from $polo^1$ homozygous females could be used as a source of potential kinase polo substrates. $polo^1$ is an EMS-induced semi-lethal mutation with ~7% of homozygotes surviving as adults. The homozygote mutant adult females produce embryos with severe mitotic abnormalities. Multiple rounds of DNA replication appear to take place with limited nuclear division. As a result the nuclei become highly polyploid and the embryos do not cellularize and never hatch (Sunkel and Glover, 1988). We set out to determine whether such embryos contained polo protein and if the enzyme was active.

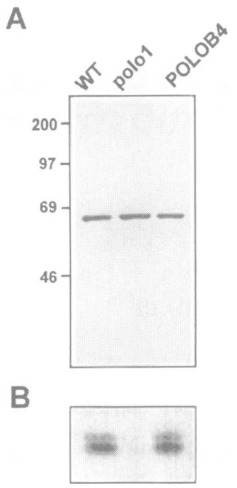

Fig. 1. Comparison of the activity of polo protein kinase from wild-type and $polo^1$ mutant embryo extracts with POLOB4 kinase from Sf9 cells infected with recombinant baculovirus. (**A**) Extracts prepared from 10 wild-type or $polo^1$ embryos were subjected to SDS–PAGE together with an extract of Sf9 cells producing recombinant POLOB4. Following transfer to nitrocellulose, polo was detected with the monoclonal antibody MA294. (**B**) Polo protein kinase was immunoprecipitated from the same extracts using MA294 and assayed for its ability to phosphorylate casein, as described in Materials and methods.

As a source of enzyme, we have been able to immunoprecipitate active polo protein kinase from wild-type *Drosophila* embryos (Fenton and Glover, 1993), and we also cloned a complete *polo* cDNA into a baculovirus expression vector for the purpose of expressing functional protein (poloB4, see Materials and methods). Total embryo extracts from either wild-type or $polo^1$-derived embryos were separated by SDS–PAGE, blotted to nitrocellulose and probed with an anti-polo monoclonal antibody (MA294). On these gels there is no apparent difference in the size or abundance of the mutant protein as compared with the wild-type protein (Figure 1A). This indicates that $polo^1$ is either a point mutation or a small deletion in the coding region. The poloB4 protein expressed in the baculovirus expression vector has the same apparent molecular weight in SDS–PAGE as the enzyme from both wild-type and $polo^1$-derived embryo extracts (Figure 1A). All three proteins were then tested for kinase activity using casein as substrate (Figure 1B). The results indicate that polo protein kinase immunoprecipitated from early wild-type embryos and poloB4 is able to phosphorylate

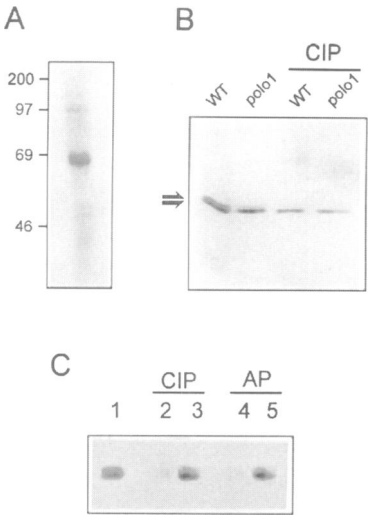

Fig. 2. Active polo protein kinase is phosphorylated. (**A**) polo was immunoprecipitated from extracts of Kc cells cultured for 16 h in medium containing [^{32}P]orthophosphate. Cells were lysed and polo protein kinase immunoprecipitated. The precipitate was then subjected to SDS–PAGE and autoradiography. (**B**) Extracts of 10 wild-type or *polo^{1}* embryos were fractionated on 8% SDS–PAGE before (first two lanes) or after incubation with calf intestinal phosphatase (CIP) (last two lanes). After transfer to nitrocellulose polo was detected with the MA294 antibody. (**C**) Immunoprecipitated polo was used in the casein kinase assay without preincubation with phosphatases (lane 1), or after treatment with calf intestinal phosphatase (lane 2) or potato acid phosphatase (AP) (lane 4). In order to test whether any phosphatase remained active after the treatment, the same amount of the immunocomplex used in lane 1 was mixed with immunocomplexes used in lane 2 and lane 4, before the kinase reaction. Results are shown in lanes 3 and 5, respectively.

casein efficiently. However, the kinase activity of polo1 immunoprecipitated from early mutant embryos is only at the limits of detection.

Subsequent experiments that require the addition of exogenous polo protein kinase have been performed with recombinant poloB4 protein and with polo protein kinase immunoprecipitated from wild-type embryos, and these gave equivalent results.

polo has to be phosphorylated to be an active kinase

Many kinases involved in the control of the cell cycle are themselves substrates of other kinases. We therefore tested whether polo is itself a phosphoprotein and also whether phosphorylation alters its kinase activity. We were able to immunoprecipitate a 67 kDa phosphoprotein, using the specific anti-polo antibody MA294, from extracts of *Drosophila* Kc cells cultured in medium containing [^{32}P]orthophosphate (Figure 2A). This suggests that polo is phosphorylated *in vivo*.

In order to assess the role of polo phosphorylation upon its kinase activity, we immunoprecipitated the enzyme from wild-type embryos and treated it with alkaline phosphatase or acid phosphatase before assaying its activity (Figure 2C). After treatment with either phosphatase the kinase activity is completely abolished (lanes 2 and 4). To control for the removal of all the phosphatase in the samples, untreated (lane 1) and phosphatase-treated enzyme (lane 2 or lane 4) were mixed in equal amounts before the kinase assay. As shown in lanes 3 and 5 these mixtures are still able to phosphorylate casein. Thus we conclude that polo needs to be phosphorylated in order to be an active protein kinase.

These conclusions were reaffirmed by analysing the polo protein kinase protein in whole extracts made from wild-type and *polo^{1}*-derived embryos (Figure 2B). Embryonic extracts were prepared and run in SDS gels under conditions that increase the resolution of bands in the 60 kDa range. The gel was then blotted and the polo protein kinase identified with the antibody MA294. Under these conditions it is possible to separate the 67 kDa band of polo into a closely spaced doublet (lane WT). If the extract is treated with alkaline phosphatase prior to SDS–PAGE then the higher molecular weight band is absent, suggesting that this band corresponds to the phosphorylated form and the lower molecular weight band to a dephosphorylated form. In *polo^{1}*-derived extracts, we observed mainly the band corresponding to the low molecular weight form which showed no change in the migration of the mutant protein following phosphatase treatment. Because the polo1 protein is not a fully active kinase, these results are consistent with the interpretation that the *polo^{1}* mutation prevents efficient phosphorylation thus reducing the activity of the kinase. However, as some residual molecules of 'shifted mobility' remain in polo1-derived embryos, we cannot rule out the possibility of another activating phosphorylation site (see Discussion).

Detection of putative substrates of polo in embryo extracts

To further elucidate the role of *polo* in regulating the passage through mitosis, it is essential to identify potential substrates. In order to identify possible endogenous substrates we developed a new approach using total protein extracts devoid of an active polo kinase. We followed the reasoning that as *polo^{1}*-derived embryos do not have polo protein kinase activity, its endogenous substrates should be in a dephosphorylated state. On the other hand, in wild-type embryo extracts, where the polo protein kinase is functioning normally, the substrates should exist both in a phosphorylated and unphosphorylated state depending upon the cell cycle stage of any given embryo. If polo protein kinase is then added to such extracts together with radioactive ATP, it might be expected that its substrates should be strongly labelled in extracts of *polo^{1}*-derived embryos and weakly labelled in wild-type extracts. Any non-specific phosphorylation would be expected to result in bands of equal intensity in both wild-type and *polo^{1}*-derived embryo extracts.

In order to use these extracts as a source of substrates for the polo protein kinase it is first necessary to inactivate the endogenous kinases present in the embryo extracts, the activity of which can be detected by the simple addition of [γ-^{32}P]ATP to the extracts (Figure 3A, lane 1). These kinases would mask phosphorylation due to polo protein kinase. We found that this inactivation could be achieved by a relatively mild heat treatment (15 min at 55°C; see Figure 3A, lane 2). Under these conditions, all endogenous kinase activity was abolished and the extracts showed no sign of protein degradation (data not shown). Addition of immunoprecipitated polo protein kinase together with [γ-^{32}P]ATP to the heat-inactivated extract results in the appearance of three strongly labelled bands

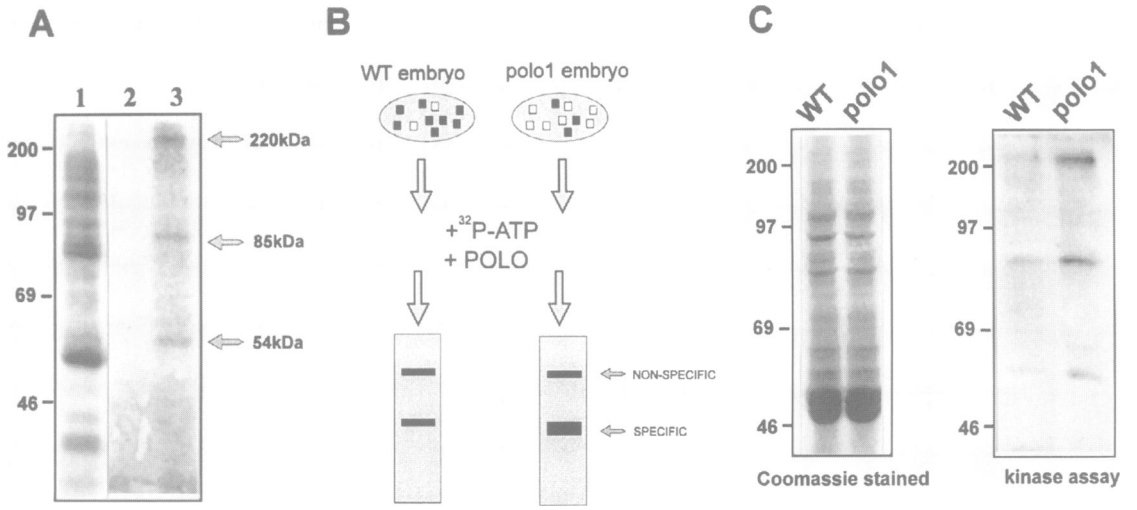

Fig. 3. Polo protein kinase phosphorylates primarily three proteins in embryo extracts. (**A**) Extracts from 35 wild-type embryos were assayed for total endogenous kinase activity before (lane 1) and after (lane 2) heat treatment for 15 min at 55°C. Another aliquot of heat-treated embryo extracts was used as substrate for polo protein kinase (lane 3). (**B**) In polo1 embryos the endogenous substrates of polo protein kinase should be found mainly dephosphorylated (white squares) while in wild-type embryos a larger proportion should be found phosphorylated (black squares). The addition of exogenous polo protein kinase together with [γ-^{32}P]ATP should then permit a stronger labelling of the substrates in extracts made from polo1 embryos. (**C**) Extracts from 35 wild-type embryos or 35 *polo^{1}* embryos were used as substrates for polo protein kinase after heat treatment. The Coomassie-stained SDS–PAGE gel of wild-type and *polo^{1}* extracts before the kinase assay is shown in the left hand panels, together with the autoradiograph of a similar gel to detect proteins phosphorylated in the kinase assay (right hand panels).

with apparent molecular weights of 220 kDa, 85 kDa and 54 kDa (Figure 3A, lane 3).

A comparison of the proteins phosphorylated by exogenously added polo protein kinase to wild-type or *polo^{1}* heat-inactivated embryo extracts is shown in Figure 3C (second panel). The same three bands were more strongly labelled in *polo^{1}*-derived than in wild-type embryo extracts, when comparable amounts of total protein are used in the kinase assay. This suggests that the three polypeptides could be endogenous substrates of the kinase. We have controlled the amount of protein used from both type of extracts by preparing them from an equal number of embryos (see Material and methods). The extracts of wild-type embryos and *polo^{1}* embryos show no difference in protein composition or concentration, and the three proteins which appear to be specifically phosphorylated by polo do not correspond to unusually high abundance proteins in the extracts (Figure 3C, first panel). When these assays were repeated with protein kinase A and protein kinase C none of the phosphorylated proteins were labelled to different levels in mutant and wild-type extracts. Furthermore none of the three proteins phosphorylated by polo was a preferential substrate for either of the two kinases (data not shown).

We also performed kinase assays in embryo extracts to which no exogenous polo protein kinase was added and without inactivating the endogenous kinases. As polo is active in wild-type embryos and inactive in *polo^{1}*-derived embryos, we expected to observe the opposite result of the previous experiment, that is upon addition of [γ-^{32}P]ATP to the extracts physiological substrates of the kinase polo should appear labelled in wild-type embryo extracts and remain unlabelled (or appear less labelled) in *polo^{1}* embryo extracts. Consistent with our previous result, we observed in this type of assay the labelling of 220 kDa, 85 kDa and 54 kDa proteins to be stronger in the wild-type extract than in the polo1-type extract (Figure 4A). This gel also

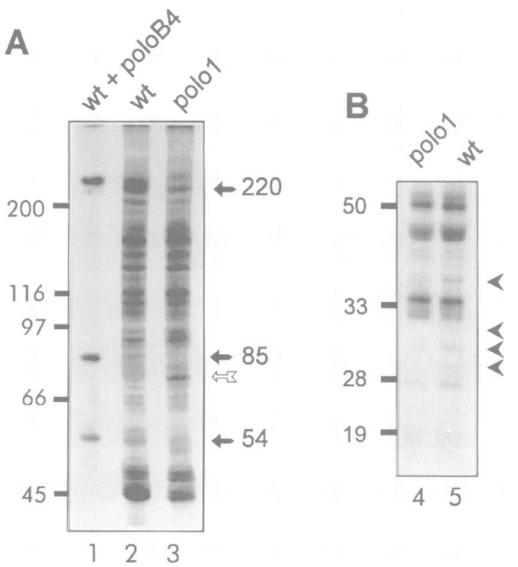

Fig. 4. Endogenous kinase activity in cell-free extracts of wild-type and *polo^{1}* embryos. (**A**) Lane 1: an extract of 35 wild-type embryos was used as substrate for POLOB4 after heat-inactivation, as in Figure 3. Lanes 2 and 3: 15 embryos (wild-type or *polo^{1}*) were homogenized in a minimum volume of IP-buffer and incubated in the presence of [γ-^{32}P]ATP. After the incubation period the phosphorylated proteins were analysed on 7.0% SDS–PAGE. The dried gel was exposed for autoradiography. (**B**) 20 wild-type or *polo^{1}* embryos were homogenized directly in 4 µl of kinase buffer, incubated with [γ-^{32}P]ATP and the products separated on 12% SDS–PAGE. The dried gel was exposed for autoradiography. Numbers at the left represent the molecular mass of protein markers (kDa). Black arrows indicate the 220 kDa, 85 kDa and 54 kDa proteins. Solid arrowheads indicate additional proteins phosphorylated in the wild-type but not in the *polo^{1}* mutant extract.

shows proteins of a heat-treated wild-type embryo extract phosphorylated by poloB4 for comparison (Figure 4A, lane 1). These results show that polypeptides corresponding in

size to the putative substrates, defined in the heat-inactivated extracts, are natural phosphoproteins that are preferentially phosphorylated in extracts which contain an active form of polo protein kinase. In addition, a number of other lower molecular weight proteins are also more strongly phosphorylated in wild-type than in *polo*^{1} extracts (arrowheads, Figure 4B). These could either represent direct substrates of polo protein kinase not detected in the previous assay due to heat denaturation, or they could be phosphorylated by other downstream cell cycle kinases in events that are prevented as a secondary consequence of the *polo* mutation. It is also interesting to note a preferential labelling of some proteins in the *polo*^{1}-derived extracts (e.g. white arrow, Figure 4A).

The 54 kDa polo protein kinase substrate is β-tubulin

Aspects of the phenotypes of mutations in *D.melanogaster polo*, *S.cerevisiae CDC5* and *S.pombe plo1* and the immunolocalization of Plk1 in human cells raise the possibility that one of the roles of polo-like kinases may be to regulate the function of the mitotic spindle. In view of this, we decided to use microtubule extracts partially purified from early embryos according to a modification of the procedure of Goldstein *et al.* (1986) as substrates for the polo protein kinase assay. The extent of the tubulin purification was determined by staining the preparation with Coomassie Blue after SDS–PAGE (Figure 5A, first panel). The results show that after the final steps, the preparation contains mostly tubulin. Western blotting of these extracts using a highly specific β-tubulin specific antibody (Bx69) shows that the lower molecular weight band corresponds to β-tubulin (Figure 5A, second panel). The addition of radioactive ATP to the extract followed by a short incubation at 20°C, showed that the microtubule preparation also contained an endogenous kinase activity (Figure 5B, lane 1) that was readily inactivated by the same heat treatment as described previously (Figure 5B, lane 2). When this extract is used as a substrate for the poloB4 kinase we observed that polo is able to phosphorylate β-tubulin very efficiently, and that α-tubulin is only weakly phosphorylated (Figure 5B, lane 3).

Knowing that polo is able to phosphorylate β-tubulin in microtubule extracts we wished to determine whether the 54 kDa protein phosphorylated in the kinase assays with total embryo extracts (Figures 3 and 4) corresponded to β-tubulin. We, therefore, repeated the polo protein kinase assays on extracts of *polo*^{1} embryos and on partially purified microtubule extracts as before, then at the end of the incubation period we divided the resulting reaction mixture into two equal parts. To the first half we added SDS-sample buffer and loaded it onto an SDS–polyacrylamide gel, to observe the total pattern of protein phosphorylation. The other half of the reaction mixture was first subjected to immunoprecipitation with Bx69 antibody, and the precipitate loaded onto an SDS–polyacrylamide gel (Figure 5C). A 54 kDa-labelled phosphoprotein is immunoprecipitated by the antibody Bx69 from the *polo*^{1}-embryo extract and therefore corresponds to one of the β-tubulin isotypes. The same result is obtained with the microtubule extract (Figure 5C). From these results we conclude that the 54 kDa protein phosphorylated by polo protein kinase in embryo extracts is β-tubulin.

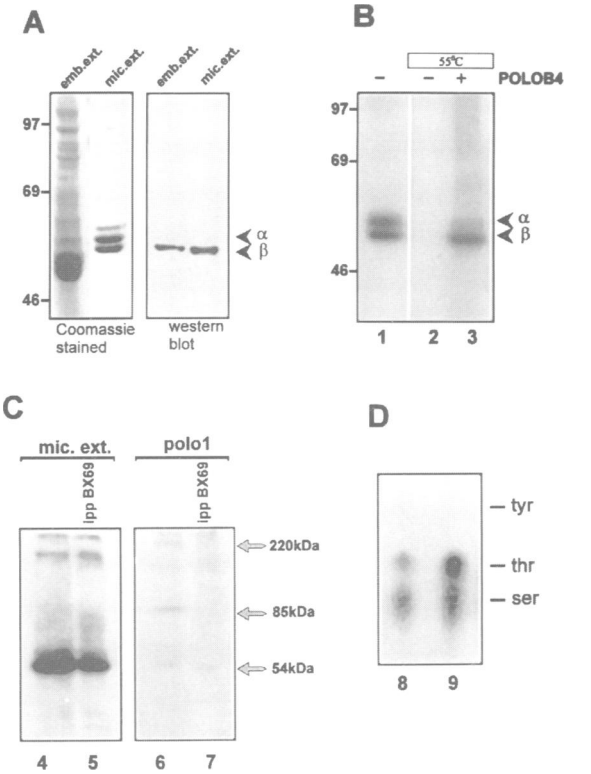

Fig. 5. The 54 kDa protein is β-tubulin. (**A**) Total embryo extracts (emb.ext.) and microtubule preparations (mic.ext.) were fractionated by SDS–PAGE and either stained with Coomassie Blue to reveal total protein, or blotted with Bx69 antibody to detect β-tubulin. (**B**) The microtubule extract was incubated with [γ-^{32}P]ATP, before (lane 1) or after heating to 55°C for 15 min (lane 2). The heat treated preparation was also used as a substrate for polo protein kinase (lane 3). (**C**) Heat-inactivated *polo*^{1} embryo extracts and microtubule preparations were used as substrate for polo protein kinase. After the incubation with polo protein kinase (lanes 4 and 5), half of the reaction mixture was immunoprecipitated with Bx69, and the immunoprecipitate subsequently analysed on SDS–PAGE followed by autoradiography (lanes 5 and 7). (**D**) Acid hydrolysis of casein (lane 8) and tubulin (lane 9) phosphorylated by polo protein kinase was carried out as described in Materials and methods. Phosphoaminoacid identification was carried out on TLC using phosphorylated amino acid standards as markers. The position of the markers is indicated.

Conserved residues in the primary structure of the polo protein indicate that this kinase belongs to the Ser/Thr protein kinase family, and should therefore phosphorylate target proteins on serine and/or threonine residues (Llamazares *et al.*, 1991). To test this hypothesis we performed a phosphoaminoacid analysis on casein and β-tubulin phosphorylated *in vitro* by the polo protein kinase. The results revealed that although polo labels serine and threonine residues to the same extent in casein, a greater proportion of phosphothreonine is detected in tubulin (Figure 5D). In neither case is there any phosphotyrosine labelling, confirming polo as a Ser/Thr protein kinase.

A proportion of polo molecules are associated with microtubules

To analyse the association of polo with microtubules we blotted the protein fractions obtained during each step of a microtubule preparation and incubated the membrane with anti-β-tubulin antibody Bx69 and, after a wash, reprobed the membrane with anti-polo MA294 antibody.

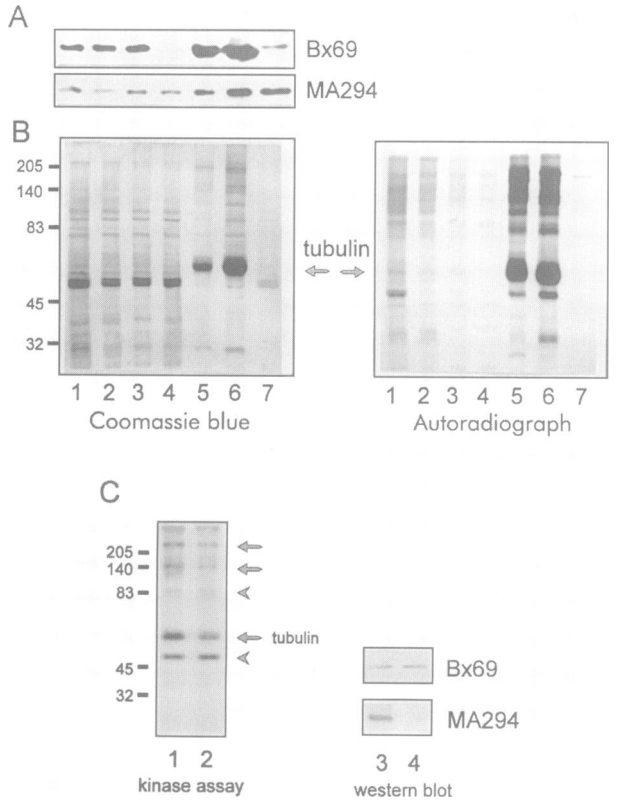

Fig. 6. Association of polo protein kinase with microtubules. (**A**) Fractions obtained during microtubule purification were analysed by Western blotting with anti-tubulin monoclonal antibody (Bx69) or anti-polo monoclonal antibody (MA294). Lane 1, total embryo extract; lane 2, supernatant after centrifugation at 16 000 *g*; lane 3, supernatant after centrifugation at 135 000 *g*; lanes 4 and 5, supernatant and pellet after incubation with taxol and centrifugation at 54 000 *g*; lanes 6 and 7, pellet and supernatant after incubation with 0.4 M NaCl and centrifugation. (**B**) The same microtubule fractions analysed in (A) were incubated for 15 min at 20°C in the presence of [γ-^{32}P]ATP and analysed in 10% SDS–PAGE followed by autoradiography. As in (A) 5 μg of protein was loaded on each lane. (**C**) The activity of polo protein kinase was analysed in fraction 6 of the microtubule preparation. Aliquots with 1 μg protein were incubated with protein G beads (lane 1) or with protein G beads and anti-polo antibody MA294 (lane 2). After removal of beads the kinase activity that remains associated with the microtubules was analysed by incubation with [γ-^{32}P]ATP, followed by SDS–PAGE and autoradiography. The right panels (lanes 3 and 4) show the corresponding Western blot with Bx69 antibody and reprobed with MA294 antibody.

The results (Figure 6A) reveal that in addition to free enzyme, a substantial proportion of polo kinase is associated with polymerized microtubules. This is consistent with immunolocalization studies that have shown polo kinase to be associated with the mitotic spindle as well as being generally abundant in the cytoplasm of mitotic cells (Golsteyn *et al.*, 1995; our unpublished observations).

The presence of kinase activity associated with microtubule proteins and its ability to phosphorylate β-tubulin and some associated MAPs has been described previously (Rappaport *et al.*, 1976; Sandoval and Cuatrecasas, 1976; Serrano *et al.*, 1987; see also Mandelkow and Mandelkow, 1995; Ookata *et al.*, 1995 and references therein). It was also reported that taxol, a drug which stabilizes polymerized microtubules, stimulates an increase in tubulin phosphorylation (Gard and Kirschner, 1985). Our finding that polo protein kinase can be found associated with polymerized microtubules raises the possibility that part of the kinase activity we find associated with microtubules (Figure 5B) could be due to polo. To determine if this is the case we performed two different *in vitro* kinase assays. In the first assay we determine the total endogenous kinase activity in the protein fractions obtained from the steps of the microtubule preparation. The addition of [γ-^{32}P]ATP to these fractions induces the labelling of numerous proteins. The weak labelling of tubulin in the total embryo extract (Figure 6B, lane 1) increases dramatically when taxol is present in the buffer (Figure 6B, lanes 5 and 6). It is interesting to notice that in lane 7, the high salt wash of microtubules, no tubulin phosphorylation is observed even though both polo protein kinase and β-tubulin are present (panel A, Lane 7).

In the second assay we measured the kinase activity associated with microtubules when depleted of polo protein kinase. In this experiment an aliquot of microtubule preparation was incubated with antibody MA294 at 4°C for 1 h, after which time protein G beads were used to pull out the immunocomplex. After the immunoprecipitation, [γ-^{32}P]ATP was added to the aliquots and the remaining kinase activity measured. As shown in Figure 6C depletion of polo results in a drop of β-tubulin phosphorylation (2- to 3-fold) as well as in the phosphorylation of some other proteins (arrows) but not all (arrowheads) relative to the control. We conclude that polo is responsible for at least part of the observed microtubule-associated kinase activity capable of phosphorylating β-tubulin, and that residual phosphorylation is due to other kinases in the microtubule preparation.

polo binds to an 85 kDa MAP in ligand blotting assays

If, during the immunoprecipitation of β-tubulin following the polo protein kinase mediated phosphorylation of wild-type extracts, we use a large number of embryos as a substrate (50 embryos) we were able to observe a co-precipitating 85 kDa phosphoprotein (Figure 7A, lane 2), suggesting that it might be a MAP. We therefore prepared an enriched microtubule fraction that should retain most MAPs by eliminating the last incubation with a high concentration of salt. We then tested whether polo could bind to any of the proteins in this microtubule preparation or in an extract of wild-type embryos blotted onto a nitrocellulose membrane. The filter was incubated with poloB4 (in an extract of cells infected with recombinant virus) and the bound poloB4 on the membrane was detected using the antibody MA294 (Figure 7B, lanes 5 and 6). As a negative control, a similar membrane was incubated with an extract of cells infected with wild-type baculovirus (Figure 7B, lanes 3 and 4). Interestingly we found that recombinant polo can only bind to two small molecular weight proteins (40 kDa and 36 kDa) in the embryo extract (lane 5). On the other hand poloB4 binds to a 85 kDa protein in the purified microtubule extracts, but binding to the smaller molecular weight proteins is not observed (lane 6). This result suggests that the concentration of the 85 kDa protein is highly enriched in the microtubule extract reinforcing the idea that this 85 kDa protein is a MAP. In the conditions of our assays we never observed a poloB4 binding to the β-tubulin monomer.

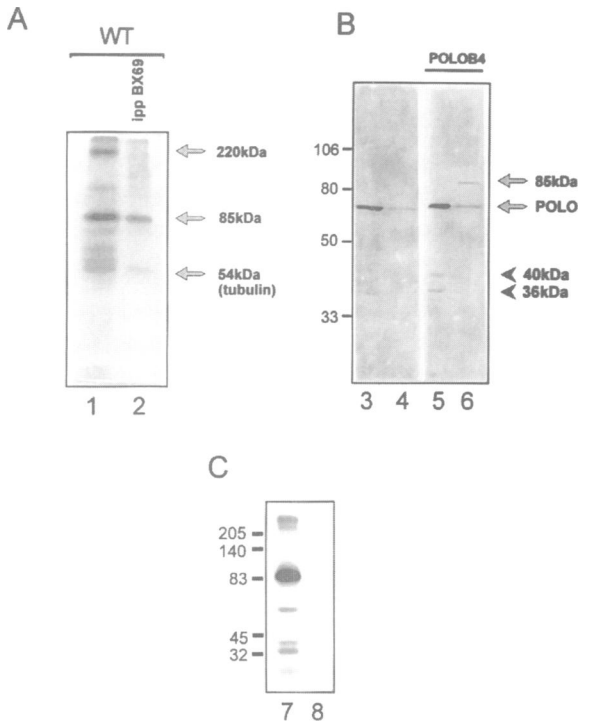

Fig. 7. Polo protein kinase binds to an 85 kDa MAP. (**A**) Heat-inactivated wild-type embryo extract (from 50 embryos) was used as substrate for polo protein kinase. Half of the reaction mixture was subjected to immunoprecipitation using Bx69 antibody as in Figure 5. The result of the kinase assay and of the immunoprecipitation (lanes 1 and 2 respectively) were analysed in SDS–PAGE and autoradiography. (**B**) A total embryo extract (lanes 3 and 5) and a preparation of microtubules (not washed with salt solution) (lanes 4 and 6) were separated on SDS–PAGE and transferred to nitrocellulose. The blots were incubated with extracts of Sf9 cells producing wild-type viral proteins (lanes 3 and 4) or producing recombinant poloB4 kinase (lanes 5 and 6). Bound poloB4 was then detected with the antibody MA294. (**C**) polo immunocomplexes, washed with low salt concentration, were incubated at 20°C in the presence of [γ-^{32}P]ATP, and analysed by SDS–PAGE and autoradiography. Lane 7, immunocomplexes from wild-type embryo extracts; lane 8, immunocomplexes from *polo*^{1} extracts. Numbers at the left represent the molecular mass of protein markers (kDa).

To further analyse the association between polo and the 85 kDa protein we immunoprecipitated polo from wild-type embryo extracts with MA294 antibody under physiological conditions (see Material and methods). In order to detect whether polo is able to phosphorylate any co-precipitating proteins the immunoprecipitate was then used in an *in vitro* kinase assay by mixing it with kinase buffer and [γ-^{32}P]ATP (Figure 7C, lane 7). We observed the strongest labelling of a protein of 85 kDa, together with significant labelling of proteins of 220 kDa, 52 kDa, 40 kDa and 36 kDa. The 52 kDa band is not β-tubulin as determined by Western blot (data not shown). To exclude the possibility that non-specific kinases in the polo immunocomplex were responsible for these phosphorylations we performed the same type of assay with extracts from *polo*^{1} embryos. As shown, in Figure 7C lane 8, no phophorylation is detected in *polo*^{1} immunocomplexes. These results show that several proteins corresponding in size to those detected in ligand blotting experiments co-precipitate with polo and are substrates of this kinase.

Discussion

The *Drosophila* polo protein kinase is the founding member of a family of kinases which are expressed at high levels in proliferating cells, and which, from the phenotypes of mutants in *Drosophila*, budding yeast and fission yeast would seem to have a mitotic role. This is also supported by observations on the timing of the maximal activity of the polo and Plk1 kinases in syncytial *Drosophila* embryos and human cells respectively. In both systems the enzyme reaches a peak of activity during mitosis, and in *Drosophila*, where this activity has been examined during the rapid nuclear division cycles of syncytial embryos, the interval of activity could be narrowed down to anaphase/telophase following the decline of activity of p34^{cdc2} kinase (Fenton and Glover, 1993). In fission yeast, the timing of enzyme activation has not yet been measured, but observations on the loss-of-function phenotype in either germinating *plo1* disruptant spores or in exponentially growing cells, following the switching off of transcription of an episomally maintained *plo1*$^{+}$ gene, suggests that different thresholds of enzyme activity are required for two distinct roles of the enzyme: in the generation of a bipolar spindle, and in septation (Ohkura *et al.*, 1995). It is, therefore, likely that the level of activity of the polo-like kinases is regulated during the mitotic cycle. Moreover, in cultured human cells and in the *Drosophila* syncytial embryo, the cyclical increase in kinase activity is far greater than can be accounted for by *de novo* synthesis of the enzyme suggesting that regulation occurs by post-translational modification.

We present several lines of evidence that indicate polo protein kinase can be regulated by its state of phosphorylation. If polo protein kinase from either wild-type embryos or the product of recombinant baculovirus expression is treated with phosphatase, its kinase activity is lost. It is clearly a possibility that phosphorylation of the enzyme in wild-type embryos could reflect cyclical phosphorylation during the cleavage divisions. The phosphorylated form of the enzyme can be seen in Western blots of wild-type embryos as a phosphatase-sensitive higher molecular weight band in a doublet. In *polo*^{1}-derived embryos, however, almost all the enzyme is of the lower molecular weight form suggesting that the mutant protein is not phosphorylated at this site. This suggests that the *polo*^{1} mutation renders the protein inactive because it cannot be phosphorylated efficiently. This provides a biochemical basis for the earlier suggestion that *polo*^{1} was a hypomorph (Sunkel and Glover, 1988). It remains possible, however, that there is another activating phosphorylation site that is not revealed as a mobility shift on SDS–PAGE. It remains an important future objective to identify the precise phosphorylation sites in wild-type and mutant polo kinases. Several other protein kinases involved in cell cycle regulation are known to be controlled by phosphorylation/dephosphophosphorylation reactions. Examples include p34^{cdc2}, which is activated by phosphorylation on the residue Thr161. This residue is conserved in many other kinases, and in most cases its phosphorylation is dynamic and correlates with activation (for examples see Marshall, 1994; Clark, 1995). In common with these other kinases, polo protein kinase has a potential site of phosphorylation at the residue T182 in

the sub-domain VIII of the catalytic region (corresponding to T161 of $p34^{cdc2}$). Although this observation points towards phosphorylation as a primary means of regulating polo protein kinase activity, it has yet to be determined whether or not the phosphorylation state of polo fluctuates during the cell cycle. It is interesting to notice that in human cells arrested in M-phase, it as also been found that Plk1 is specifically phosphorylated (K.Mundt, R.Golsteyn and E.Nigg, unpublished results).

The identification of the physiological substrates of a specific kinase is generally very difficult. We are encouraged that two different assays identify putative substrates of comparable size. In one assay, three phosphorylated polypeptides of 220 kDa, 85 kDa and 54 kDa were more strongly labelled following the addition of exogenous polo protein kinase to extracts of *polo*^{1}-derived embryos compared with wild-type embryos. It is important that although the results of this experiment were obtained in an *in vitro* assay they reflect the situation *in vivo*: the fact that the polo kinase phosphorylates these proteins to a higher extent in *polo1* embryo extracts mean that these proteins have sites free for phosphorylation in the mutant embryos. It is possible that the same result could be obtained if the proteins were less abundant (as a result of degradation for example) in wild-type embryos, yet we observed no specific protein degradation in any type of embryo extract. In this type of experiment it is crucial to use exactly the same amount of extract from both types of embryos. This was carefully controlled by preparing samples from the same number of embryos that are homogenized individually. When analysed on SDS–PAGE early wild-type embryos are indistinguishable from *polo*^{1} embryos. Moreover a second assay of the endogenous kinase activities, in wild-type and *polo*^{1}-derived extracts, identifies proteins of identical size phosphorylated in the wild-type extract to a higher degree than in the mutant extract. The relative intensities of the labelling of the three bands was maintained in the two types of assay. In addition, the latter assay identified a number of other low molecular weight proteins strongly phosphorylated in wild-type but not mutant extracts. These could either be heat-labile substrates of the polo protein kinase, or the substrates of downstream protein kinases that fail to become activated in the *polo*^{1} embryos. Another possibility is that, as we are working with asynchronous cell-free extracts, reactions downstream of polo will also occur normally in wild-type extracts, so we may be observing the products of phosphorylation reactions occurring at a different stage of the cycle. We cannot completely rule out the possibility that *in vivo* the putative substrates we identified are not phosphorylated directly by polo, but by another kinase that is activated by polo or that is active after the polo execution point. However, as proteins of the same size appear to be specifically phosphorylated in both types of experiment this would mean that polo is able to phosphorylate the proteins on the same sites as the other hypothetical kinase. Taking these results together we favour the interpretation that polo is directly responsible for their phosphorylation and that the three proteins are candidates for being *in vivo* substrates of the polo kinase.

We were able to identify one of the three polypeptides phosphorylated by polo protein kinase in embryo extracts as β-tubulin, and we also show that the kinase is able to phosphorylate β-tubulin from polymerized microtubules prepared by a taxol-dependent polymerisation method. It is possible that the heat treatment used to inactivate endogenous kinases may expose sites for phosphorylation not normally available. However, we show that tubulin can be phosphorylated to a greater extent when polo kinase is added to *polo*^{1} extracts compared with wild-type extracts. Thus one would have to argue for differential denaturation of tubulin in the types of extracts. We also show that the kinase activity associated with microtubules and able to phosphorylate β-tubulin is in part due to polo protein kinase. Our findings of the phosphorylation of β-tubulin in these extracts are therefore consistent with the possibility that β-tubulin may be a substrate for polo protein kinase *in vivo*.

Several kinases have been shown to phosphorylate tubulin *in vitro*, including calmodulin-dependent protein kinase, casein kinase II, and the kinase c-mos (Wandosell *et al.*, 1986; Serrano *et al.*, 1987; Zhou *et al.*, 1991). However there is yet no conclusive evidence about the identity of the kinase(s) responsible for the phosphorylation *in vivo* (Eipper, 1972, 1974; Pfeffer *et al.*, 1983; Gard and Kirschner, 1985). Upon phosphorylation by calmodulin-dependent kinase, tubulin undergoes conformational changes that inhibit its capacity to self-assemble into microtubules, and prevents it binding to some MAPs (Wandosell *et al.*, 1986). Phosphorylation by casein kinase II, on the other hand, seems to promote of the assembly and stabilisation of the resulting microtubules (Serrano *et al.*, 1989). In this work we have not examined the effect of polo protein kinase on tubulin polymerisation/depolymerization, but from our results it would appear that polo is able to phosphorylate tubulin under conditions that favour tubulin oligomerization (Figures 5 and 6). Conversely when conditions favour depolymerization little phosphorylation occurs. Our results are in agreement with the observations of Gard and Kirschner (1985) who found that when cultured cells were treated with taxol, the tubulin phosphorylation increased dramatically, and when depolymerizing drugs (nocodazole and colcemid) were used phosphorylation was greatly reduced. In our *in vitro* assays the same correlation is observed and there is a striking increase in tubulin phosphorylation when taxol is used to stabilize microtubules.

Another substrate that may be of physiological importance is an 85 kDa protein that appears to be associated with microtubules. We have shown that a monoclonal antibody that specifically recognizes β-tubulin is able to precipitate not only phosphorylated β-tubulin, but also a phosphorylated 85 kDa protein from heat-treated extracts incubated with polo protein kinase. Furthermore the kinase is able to bind an 85 kDa protein highly enriched in the microtubule extract, and an 85 kDa protein is the major substrate that co-immunoprecipitates with polo protein kinase. Recently, Cambiazo and colleagues (1995) described the isolation of an 85 kDa MAP expressed in *Drosophila* larvae that shares common features with mammalian MAPs, including binding to a short region on the C-terminal of β-tubulin and induction of microtubule assembly. This protein was isolated from purified microtubule extracts prepared according to protocol similar to the one we have used, and is recovered from taxol-stabilized microtubules by exposure to high salt concentra-

tion. We have been able to detect the phosphorylation or binding to an 85 kDa protein only when low salt concentrations were used to prepare microtubules (Figure 7B) or when the immunocomplexes were washed with low salt concentrations (Figure 7C). When high salt concentration was used no phosphorylation of an 85 kDa is observed (Figure 5B and C). Experiments are in progress to determine whether this corresponds to the same protein as that described by Cambiazo *et al.* (1995).

The ligand blotting assay also shows that polo binds to two other low molecular weight proteins. It is possible that these could correspond to the proteins of lower molecular weight phosphorylated in wild-type extracts but not in extracts from *polo*^{1}-derived embryo extracts, or to the ones that co-precipitate with polo. The nature of these proteins, as well as the 220 kDa protein substrate, remains elusive.

Genetic evidence strongly suggests that the activity of the polo protein kinase is required for proper assembly and function of a bipolar mitotic spindle. Therefore the finding in the present study that polo protein kinase can phosphorylate MAPs may well have physiological relevance in light of the effects of mutations in this gene family upon microtubule behaviour. It is extremely difficult to prove that the substrate of an *in vitro* assay for any kinase is the one used in the cell. It will be of future interest to determine the precise site at which polo phosphorylates tubulin, and to study whether or not this site is differentially phosphorylated during the cell cycle. If such events occur during mitosis they may take place at specific locations on the mitotic apparatus to modify microtubule behaviour, for example in the vicinity of centrosomes early in the mitotic cycle. The demonstration by immunofluorescence of an association of the human polo-like kinase with the poles of the spindle until metaphase, and with the equatorial region of the spindle at subsequent stages suggests an association with MAPs (Goldsteyn *et al.*, 1995). This could be mediated through proteins such as the 85 kDa polypeptide identified by the ligand blotting assay. We cannot rule out the possibility that polo protein kinase might modify tubulin for return to interphase. In syncytial *Drosophila* embryos the kinase is active late in the mitotic cycle (Fenton and Glover, 1993). The phenotypes of the fission yeast *plo1* mutant indicates that in addition to a role in regulating spindle microtubules, the enzyme encoded by this gene plays a primary role in driving the late events of septation (Ohkura *et al.*, 1995). The distribution of the human enzyme in the midbody of the telophase spindle (Goldsteyn *et al.*, 1994) suggests that this enzyme might be targeted towards a specific role in cytokinesis late in the mitotic cycle. It will therefore be of interest to search for polo substrates amongst the molecules known to be required for this process. Some proportion of the enzyme appears to be found in other cellular compartments and it may have other substrates in addition to those associated with microtubules. Indeed polo also shows binding to and phosphorylation of 40 kDa and 36 kDa proteins that do not appear to be associated with microtubules. Whether or not these are bona fide substrates remains to be determined.

Materials and methods

Immunoprecipitation of polo kinase

The kinase was immunoprecipitated either from *Drosophila* embryo extracts or from extracts of Sf9 cells producing recombinant poloB4. Embryos were homogenized in 5 volumes of IP-buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.5% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin) supplemented with 1 mM PMSF. After a short centrifugation at 4°C to pellet cellular debris, the homogenates were pre-cleared with protein G in IP-buffer (usually 20 μl protein G/100 μl homogenate). During the pre-clearing, monoclonal anti-polo antibody MA294 was coupled to protein G beads, by incubation at 4°C for 1 h after which the excess of antibody was washed away with IP-buffer. The coupled antibody was then incubated with the extracts for 1 h at 4°C to immunoprecipitate polo. The immunoprecipitates were then washed once in IP-buffer containing 500 mM NaCl, twice in IP-buffer and used in kinase assays or kept at −20°C until use. To obtain an Sf9 cell extract cells were collected by centrifugation, washed twice in PBS, resuspended in 10 volumes of IP-buffer supplemented with 1 mM PMSF, 1 μg/ml aprotinin, leupeptin and pepstatin, and disrupted by passing several times through a 27G needle. The extract was then cleared by centrifugation and recombinant polo immunoprecipitated as for embryos.

To analyse proteins co-precipitating with polo the *Drosophila* embryos were homogenized in 10 mM HEPES pH 8.0, 1 mM EDTA, 0.5% Triton X-100, 1 μg/ml aprotinin, pepstatin, leupeptin and 1 mM PMSF. After incubation with MA294 coupled to protein G beads the immunocomplexes were washed three times in the same solution supplemented with 200 mM NaCl and once in kinase buffer.

Embryo extracts and kinase assays

Embryo extracts to be used as the substrate for the polo protein kinase were prepared from 1–2 h old *Drosophila* embryos collected on feeding plates, washed with 0.7% NaCl containing 0.1% Triton X-100, dechorionated for 60 s in 50% sodium hypochloride, washed again, and finally the dechorionated embryos were rinsed three times with IP-buffer. Individual embryos that had yet to form pole cells were selected and placed on a microtitre plate in a volume of 5 μl IP-buffer. They were disrupted and homogenized with two sterile needles, centrifuged immediately for 20 s in a microfuge and the supernatant was either used immediately or kept at −70°C until use. Before being used as substrate for the polo protein kinase these extracts were first heat treated to inactivate the endogenous kinases. Heat-inactivated extracts were obtained by incubating the extracts for 15 min at 55°C, after which the extracts were centrifuged for 1 min on a microcentrifuge, and the kinase assay was carried out by adding to the supernatant an equal volume of 2× kinase buffer (20 mM HEPES pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 2 mM DTT and 1 mM EGTA), typically 5 μl. To this mixture was added 5 μl of protein G beads with immunoprecipitated polo (recombinant or immunoisolated from embryo extracts), 1.0 μl 2 mM ATP and 0.5 μl [γ-^{32}P]ATP (10 mCi/ml, Amersham). The mixture was incubated for 20 min at 20°C, and the phosphorylated substrates were identified by SDS–PAGE followed by autoradiography. Dephosphorylated casein (5 μl of a 1 mg/ml) was alternatively used as a substrate (as in Fenton and Glover, 1993).

When the total endogenous kinase activity in *Drosophila* embryos was to be determined, extracts of 15 embryos were prepared as above but in 1.0 μl of IP-buffer, to keep the total volume to a minimum, and stored on ice until used (never more than 15 min later). To start the reactions we added 1.2 μl of 2× kinase buffer and 0.4 μl of [γ-^{32}P]ATP (10 mCi/ml) to the extract and the mixture was incubated for 15 min at 20°C. The reactions were stopped with the addition of SDS-sample buffer, heated for 2 min to 95°C and run in SDS–PAGE.

Microtubule extracts

Microtubules were prepared from *Drosophila* embryos following a modification to the method described by Goldstein *et al.* (1986). Embryos were collected over a period of 2 h, washed with PBS containing 0.1% Triton X-100, dechorionated by incubating for 60 s in 50% sodium hypochloride and washed in lysis buffer (0.1 M PIPES pH 6.6, 5.0 mM EGTA, 1 mM $MgSO_4$, 0.1 M glycerol, 2 mM PMSF, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The embryos were them homogenized in 2 volumes of lysis buffer previously chilled on ice. The extract was incubated on ice for 15 min to depolymerize microtubules, and then centrifuged at 16 000 *g* for 30 min at 2°C. The supernatant was further centrifuged at 135 000 *g* for 90 min at 2°C. Microtubules in the clarified supernatant were polymerized by addition of taxol to 20 μM and GTP to 1.0 mM and incubated at 37°C for 10 min. This mixture was layered on a 15% sucrose cushion prepared in lysis buffer containing 20 μM taxol and 1.0 mM GTP, and centrifuged at 54 000 *g* for 30 min at 20°C. The resulting pellet is a standard microtubule pellet containing MAPs. Extraction of MAPs was accomp-

lished by resuspending the microtubule pellet in lysis buffer containing 20 μM taxol, 1.0 mM GTP and 0.4 M NaCl, incubating the mixture for 30 min at room temperature and re-centrifuging at 54 000 *g* for 30 min at 20°C. The final pellet was resuspended in lysis buffer and stored at −70°C before use.

Western blotting

Extracts were subjected to SDS–PAGE and proteins transferred to nitrocellulose by electroblotting for 2 h at 300 mA. The nitrocellulose membrane was preincubated in 7% milk powder in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The filter was washed for 10 min with TBST + 1% milk powder, and then incubated with primary monoclonal antibodies [MA294 anti-polo (Llamazares *et al.*, 1991); BX69 anti-β-tubulin] used at a dilution of 1/3 in TBST+1% milk powder for 1 h. Blots were washed for 30 min in TBST+1% milk powder, and incubated with peroxidase-labelled donkey anti-mouse antibodies (Jackson ImmunoResearch) for 1 h. After extensive washing with TBST (usually 30 min), the immune complex was detected using ECL reagents (Amersham) according to the manufacturer's instructions.

Ligand blotting assay

Samples were subjected to SDS–PAGE, transferred to nitrocellulose membranes as described for Western blotting, and were preincubated in 5% milk powder in PBS for 30 min. The membranes were then washed in PEMF buffer (0.1 mM PIPES–NaOH, pH 6.6, 1 mM EGTA, 1 mM $MgSO_4$, 25 mM NaF, 1 mM DTT) for 5 min. They were then incubated for 2 h in PEMF buffer with an extract of SF9 cells producing recombinant poloB4 kinase or, as a control, with an extract of Sf9 cells infected with wild-type virus. After washing the filter 3× with TBST buffer, the bound poloB4 kinase was detected using the monoclonal antibody MA294 in the Western blotting procedure described above.

Phosphoaminoacid analysis

Phosphoaminoacid analysis was performed on proteins recovered from dried SDS–PAGE gels according to Van der Geer *et al.* (1993). Briefly, the protein bands of interest were cut out of the dried gel and cut into small pieces. The pieces were rehydrated in 400 μl 50 mM ammonium bicarbonate pH 7.3 and ground into smaller bits. An additional 400 μl of 50 mM ammonium bicarbonate were then added, with 40 μl 2-mercaptoethanol and 8 μl 10% SDS. This mixture was boiled for 2 min, incubated 90 min at room temperature and centrifuged 3 min at 12 000 *g* in a microcentrifuge. The supernatant was saved and the pellet was incubated again with 200 μl 50 mM ammonium bicarbonate, 4 μl 10% SDS and 20 μl 2-mercaptoethanol. After centrifugation the two supernatants were combined, mixed with 20 μg of albumin (BSA) and precipitated on ice 1 h after the addition of 250 μl 100% TCA. The precipitated protein was collected by centrifugation (15 min at 12 000 *g*), washed with ethanol, air dried and subjected to acid hydrolysis in 50 μl 6 M HCl at 110°C for 1 h. The phosphorylated residues were then separated on ascending TLC (cellulose plates, Sigma) using isobutiric acid : 0.5 M NH_4OH (5:3) as solvent. Unlabelled standards (Sigma) were run on the same plate as standards.

Expression of poloB4 in baculovirus

cDNA encoding the entire sequence of polo was subcloned into the *Sma*I and *Not*I restriction sites in the transfer vector pVL1393, using *Pvu*I and *Not*I restriction sites present outside of the coding region. Recombinant baculovirus encoding full-length polo were then generated using the MAX-bac kit (Invitrogen) according to the manufacturer's instructions. The recombinant baculoviruses were used to infect Sf9 cells grown in Ex-Cell 401 medium (Sera-Lab) in the presence of 5% FCS. Cells were harvested 2 days after infection, and poloB4 was immunoprecipitated as described above.

Phosphatase treatment

Calf intestinal alkaline phosphatase (CIP) and phosphatase buffer (Boehringer Mannheim) were added to extracts (0.4 U/ml) and the mixture was incubated for 30 min at 37°C. After the incubation the reaction was stopped with SDS-sample buffer and the mixture was analysed on SDS–PAGE. Immunoprecipitated polo was treated the same way with either CIP or with potato acid phosphatase (AP) (Boehringer Mannheim), and after the incubation with phosphatase the immuno-complexes were washed four times with kinase buffer for remotion of the phosphatases. The phosphatase-treated polo was immediately assayed for kinase activity using dephosphorylated casein as substrate.

Acknowledgements

We wish to thank Carol Midgley for advice on preparing recombinant baculovirus. We also like to thank Ines Chaves and Servaas Morré for their collaboration during different stages of this project. This work was supported by the JNICT in Portugal, and by grants from the Cancer Research Campaign, the European Union Human Capital and Mobility Programme, and the Biotechnology and Biological Sciences Research Council. A.T. was supported by a scholarship from JNICT.

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Received on January 15, 1996; revised on May 6, 1996