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Protein phosphatase 4 is an essential enzyme required for organisation of microtubules at centrosomes in *Drosophila* embryos

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SUMMARY

The protein serine/threonine phosphatase 4 (PP4), which localises to centrosomes/spindle pole bodies in human cells, is shown to exhibit a similar localisation in *Drosophila* cells and embryos and possess a highly conserved (91% identical) amino acid sequence from humans to invertebrates. A homozygous *Drosophila melanogaster* strain mutant in the PP4 gene at 19C1-2 has been produced using *P* element mutagenesis. This strain, termed *centrosomes minus microtubules* (*cmm*), has reduced amounts of PP4 mRNA, ~25% of normal PP4 protein in early embryos and exhibits a semi-lethal phenotype with only 10% viability in certain conditions. Reversion mutagenesis shows that the phenotype is due to the presence of the *P* element in the PP4 mRNA. In early *cmm* embryos, nuclear divisions become asynchronous and large regions containing centrosomes with no well defined radiating microtubules are visible. In such areas, most nuclei arrest during mitosis with condensed DNA, and mitotic spindle microtubules are either absent, or aberrant and unconnected to the centrosome. A reduction in the staining of γ-tubulin at centrosomes in *cmm* embryos suggests a conformational change or relocation of this protein, which is known to be essential for initiation of microtubule growth. These findings indicate that PP4 is required for nucleation, growth and/or stabilisation of microtubules at centrosomes/spindle pole bodies.

Key words: Protein phosphatase, Phosphorylation, Centrosome, Microtubule, *Drosophila*, *P* element

INTRODUCTION

Centrosomes are the main microtubule organising centres of eukaryotic cells and play a key role as spindle pole bodies during cell division (reviewed by Kalnins, 1992; Kellogg et al., 1994). The centrosome duplicates during S-phase to form a centrosome pair which nucleate highly dynamic astral microtubules. As the two centrosomes move apart, polar microtubules elongate from the centrosomes forming the mitotic spindle which joins to each chromosome at the kinetochore. During chromosome segregation, the mitotic spindle microtubules shorten before disappearing and the centrosome in each daughter cell renucleates a distinct array of more stable cytoplasmic interphase microtubules, which influence cell shape and mediate intracellular transport. However, the processes by which microtubule growth is initiated and regulated either during mitosis or interphase are unclear.

Protein phosphorylation plays a central role in controlling many aspects of cell division and has been implicated in the regulation of microtubule dynamics. $p34^{cdc2}$ kinase can shorten microtubules by increasing the frequency of transitions from growth to shrinkage (Belmont et al., 1990; Verde et al., 1990), while PP2A (or a type-2A like phosphatase) is required to maintain a short steady state length of microtubules by dephosphorylation of Op18/stathmin, a protein which interacts with microtubules (Tournebize et al., 1997; Belmont and Mitchison, 1996). The phosphorylation of other microtubule associated proteins (MAPs), such as MAP4 and XMAP230 are also implicated in regulation of microtubule stability (Vandré et al., 1991; Ookata et al., 1995; Shiina et al., 1992; Andersen et al., 1994).

A number of lines of evidence suggest protein phosphorylation may also play a role in initiation of microtubule growth at centrosomes/spindle pole bodies. A phosphorylated epitope, recognised by the monoclonal antibody MPM2 is required for microtubule nucleation (Centonze and Borisy, 1990) and other centrosomal proteins are known to be phosphorylated (reviewed by Kellogg et al., 1994). The protein kinase, $p34^{cdc2}$ and cyclin A localise to

centrosomes in prophase (Bailly et al., 1989; Pagano et al., 1992) and the addition of cyclin A to certain isolated centrosome preparations can increase the microtubule nucleating activity of centrosomes (Buendia et al., 1992). More recently, a protein serine/threonine kinase, termed LK6, has been identified in *Drosophila* and found to associate with microtubules and centrosomes (Kidd and Raff, 1997). Several studies suggest a role for an okadaic sensitive serine/threonine phosphatase in microtubule nucleation, although the phosphatase involved was believed to be PP2A (Picard et al., 1989; Rime and Ozon, 1990; Alexandre et al., 1991). However, our recent analyses have shown *Drosophila* embryos deficient in PP2A are able to nucleate microtubules from centrosomes and elongate microtubules, while being unable to connect them to the kinetochore (Snaith et al., 1996). In addition, PP4 and PP2A have been found to exhibit similar sensitivities to okadaic acid and PP4, unlike PP2A, is localised at centrosomes (Brewis et al., 1993), making PP4 a more likely candidate for regulation of microtubule nucleation.

The amino acid sequence of PP4 is highly conserved in mammals (Brewis and Cohen, 1992) and the function of PP4 might be expected to be conserved in *Drosophila*, an organism more amenable to genetic manipulations. We therefore sought to analyse the physiological role of PP4 by producing a *Drosophila* strain defective in PP4 by *P* element mutagenesis. We report here that *Drosophila* embryos with reduced levels of PP4 show a substantial loss of viability. Centrosomes appear reduced in their ability to initiate and/or maintain microtubule growth, indicating that PP4 is an essential enzyme and required for organisation of microtubules at centrosomes.

MATERIALS AND METHODS

Drosophila strains and maintenance of *Drosophila* stocks

Maintenance of the *Birm-2* strain and crosses involving this strain were performed at 16°C. Other *Drosophila* strains were maintained at 18°C and experiments were performed at 18-25°C unless otherwise stated. Genetic markers and chromosomes are detailed by Lindsley and Zimm (1992).

Oligonucleotides and nucleic acid sequencing

Oligonucleotides used for the polymerase chain reaction (PCR) and DNA sequencing were synthesized by Audrey Gough (University of Dundee) on an Applied Biosystems 394 DNA synthesizer. The oligonucleotide primers identified in Fig. 1B had the following sequences:

Primer O1, 5′TTACGCTGACGATCCTTCTGCTCG3′;
O2, 5′GCTAGCGTTGCCAGATGGAG3′;
O3, 5′AGTTGCTCGATCTGTCGGTCCAG3′;
O4, 5′GAAGCGCTGCGAGATCATCAAGG3′;
O5, 5′ACTCCGTTCCACCGGATTGTCAG3′.

Primers within the *P* element sequences were:
P1, 5′CGACGGGACCACCTTATGTTATTTCATCATG3′;
P2, 5′GTGTATACTTCGGTAAGCTTCGG3′.

DNA sequences were determined by dideoxy-terminator sequencing followed by manual electrophoresis (Sequi-Gen, Bio-Rad) and/or automated DNA sequence analysis (Applied Biosystems 373A).

Isolation of *Drosophila PP4* cDNA and gene

A cDNA probe encoding amino acids 108-295 of rabbit PP4 (Brewis et al., 1993) was used to screen 1.8×10^5 pfu of a *Drosophila melanogaster* (Oregon R) eye imaginal disc cDNA λgt10 library (kindly provided by D. Kalderon and G. M. Rubin) under low stringency conditions (Orgad et al., 1990). Four positive cDNAs were subcloned into the *Eco*RI sites of the plasmid Bluescript M13$^+$ KS. Two were sequenced and contained the complete coding region of PP4. Several genomic clones were subsequently isolated by screening 2.0×10^5 pfu of a *D. melanogaster* (Canton S) genomic library in λEMBL3 (Clontech, Palo Alto, CA) at high stringency with the *Drosophila* PP4 cDNA. 1.1 kb and 2 kb *Pst*I fragments and a 1.7 kb *Acc*I fragment (Fig. 1B) were subcloned into Bluescript and sequenced. Only one difference between the Oregon R cDNA and Canton S gene sequences was found, nt −858 being G in the cDNA and C in the gene sequence.

In situ chromosomal hybridisation and RNA analysis

Polytene chromosomes from salivary glands of female *D. melanogaster* larvae were hybridised to biotin labelled *Drosophila* PP4 cDNA or *P* element DNA as described by Dombrádi et al. (1993). Total RNA was isolated from 0-4 hour *D. melanogaster* embryos and analysed by northern blotting as previously described (Armstrong et al., 1995).

P element mutagenesis and isolation of a *PP4*$^-$ mutant *Drosophila* strain

Transposase defective *P* elements in the *Birm-2* strain of *D. melanogaster* were mobilised by crossing *Birm-2* males to *w/w*; *Cyo/Sp; Dr, P[Δ2-3, ry+]99B/TM6* virgin females, which possess a *Δ2-3* transposase on the third chromosome (Kaiser and Goodwin, 1990). Male offspring were collected and the transposase was removed by mating these to Oregon R virgin females. DNA was prepared from embryos collected from this cross and examined by the polymerase chain reaction (PCR) using the oligonucleotide P1 in combination with either O3, O4 or O5 (Fig. 1B). The PCR products were analysed by electrophoresis, transferred to nylon membranes (Amersham) and hybridised with *PP4* cDNA. Any batch of females laying embryos where a PCR product gave a positive signal were subdivided further to identify the female fly carrying the mutagenised *PP4* gene. The positive female carrying a *P* element in the *PP4* gene was mated with a *FM7c*/*Y* male to obtain a *PP4*$^-$/*FM7c* female, which could be used to maintain a balanced *PP4*$^-$ stock from which a *PP4*$^-$ homozygous strain was derived. The position of the *P* element was determined by PCR with primers P2 and O3, insertion of the PCR product into the Bluescript plasmid using a *Hin*dIII site in the *P* element DNA and a *Pst*I site in the 5′ untranslated region of the *PP4* gene, followed by DNA sequencing.

In order to remove the *P* elements at 1D and 3A in the *PP4*$^-$ strain, while retaining the *P* element at 19C, *PP4*$^-$ homozygous female flies were crossed with males carrying an X-chromosome possessing the markers *y w ct m f*. Male flies were collected from the second generation which carried *y w* but not *f*. Balanced lines were established from these males and the presence of the *P[0.68kb]19C* element was confirmed by PCR in several of these lines. A homozygous line was then derived which carried y w and the *P[0.68kb]19C* element. DNA from this stock was examined by restriction analysis and Southern blotting with a *P* element probe, as well as by in situ hybridisation, to confirm that the *P[0.68kb]* element was still present at 19C and that the *P* elements at 1D and 3A had been removed. This line still contained an additional *P* element at 10F.

Excision of the *P[0.68kb]* element at 19C

The homozygous *PP4*$^-$ mutant was crossed with a strain carrying the *Δ2-3* transposase essentially as described by Snaith et al. (1996). After removal of the transposase, lines were screened for excision of the *P* element by PCR of DNA with primers P1 and O1 or O3. Excision of the *P* element in lines where PCR products were not generated was verified by PCR with primers O1 and O3, followed by DNA sequencing.

Immunological techniques

For immunoblotting, protein extracts were prepared from various *D. melanogaster* developmental stages by sonication in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride and centrifuged at 14,000 ***g*** for 10 minutes at 4°C. Supernatants containing 10 μg of protein were fractionated by 12.5% acrylamide sodium dodecyl sulphate gel electrophoresis, transferred to nitrocellulose and probed with 0.1 μg/ml affinity purified anti-PP4 antibodies. Staining was detected using anti-sheep IgG antibodies conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (Amersham, UK). Blots were reprobed with affinity purified anti-PPV (1-55 amino acids) antibodies (H. Snaith, unpublished data) after removal of the anti-PP4 antibodies by washing in 2% sodium dodecyl sulphate, 50 mM Tris-HCl, pH 7.5, 0.1% (v/v) β-mercaptoethanol at 60°C for 30 minutes. Densitometric analysis of signal intensity was performed using a Bio Image whole band analyser (MilliGen/Biosearch).

For most in situ immunological analyses, 0-2 hour *Drosophila* syncytial embryos were collected on apple juice agar, dechorionated in 100% chloros and fixed in 38% formaldehyde as described by Snaith et al. (1996). Microtubules were detected with rat anti-tyrosinated-—tubulin antibody (YL1/2, Harlan Sera-Lab) and donkey anti-rat CY5-conjugated antibody or goat anti-rat FITC-conjugated antibody. γ-Tubulin at centrosomes was detected with mouse anti-—tubulin antibody (Sigma) and donkey anti-mouse FITC-conjugated antibody. Centrosomes were identified with rabbit anti-CP190 antibody and goat anti-rabbit Texas red-conjugated antibody or goat anti-rabbit CY5-conjugated antibody. Antibodies labelled with fluorescein isothiocyanate (FITC), indodicarbocyanine (CY-5) and Texas red were from Jackson ImmunoResearch Laboratories. Centrosomal protein CP190 antibody was kindly provided by Will Whitfield and David Glover (Whitfield et al., 1995). For immunocytological analysis of PP4, embryos were fixed in 97% methanol as described by Kellogg et al. (1988). Two separate anti-PP4 antibodies were raised in sheep (at the Scottish Antibody Production Unit, Carluke, Lanarkshire, UK) against the N-terminal 57 amino acids of human and *Drosophila* PP4 expressed in *E. coli* as fusions with glutathione-S-transferase. The antibodies were affinity purified against full length rabbit or *Drosophila* PP4 expressed in *E. coli*. *Drosophila* cells from the embryonic cell line Kc were fixed as described by Brewis et al. (1993) before immuonstaining for PP4.

RESULTS

Protein phosphatase 4 catalytic subunit is highly conserved from mammals to *Drosophila* and localises to centrosomes in *Drosophila* cells and embryos

Drosophila PP4 cDNA and genomic DNA from nt −2,273 to nt 1,412 (deposited in the GenEMBL databases under accession no. Y14213) encode a protein which possesses all the conserved motifs of the PPP family of protein serine/threonine phosphatases (Barton et al., 1994; Cohen, 1997). The amino acid sequence of *Drosophila* PP4 is most closely related to mammalian PP4 showing 91% identity and

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Dros PP4     1 MSDYSDLDRQIEQLKRCEIIKENEVKALCAKAREILVEEGNVQRVDSPVT  50
               | : ||||||||||:|||:||| |||||||||||||||| ||||||||||
Human PP4    1 MAEISDLDRQIEQLRRCELIKESEVKALCAKAREILVEESNVQRVDSPVT  50

Dros PP4    51 VCGDIHGQFYDLKELFKVGGDVPEKNYLFMGDFVDRGYYSVETFLLLLAL 100
               |||||||||||||||:||||||| |||||||||||:||||||||||||
Human PP4   51 VCGDIHGQFYDLKELFRVGGDVPETNYLFMGDFVDRGFYSVETFLLLLAL 100

Dros PP4   101 KVRYPDRITLIRGNHESRQITGVYGFYDECLRKYGSTAVWRYCTEIFDYL 150
               |||||||||||||||||||||| ||||||||||||  ||||||||||||
Human PP4  101 KVRYPDRITLIRGNHESRQITQVYGFYDECLRKYGSVTVWRYCTEIFDYL 150

Dros PP4   151 SLSAIIDGKIFCVHGGLSPSIQYLDQIRSIDRKQEVPHDGPMCDLLWSDP 200
               |||||||||||||||||||||| ||||||:||||||||||||||||||||
Human PP4  151 SLSAIIDGKIFCVHGGLSPSIQTLDQIRTIDRKQEVPHDGPMCDLLWSDP 200

Dros PP4   201 EDQTGWGVSPRGAGYLFGSDVVSQFNRTNDIDMICRAHQLVMEGFKWHFN 250
               || |||||||||||||||||||| |||  ||||||||||||||||:|||||
Human PP4  201 EDTTGWGVSPRGAGYLFGSDVVAQFNAANDIDMICRAHQLVMEGYKWHFN 250

Dros PP4   251 ETVLTVWSAPNYCYRCGNVAAILELNEYLHRDFVIFEAAPQESRGIPSKK 300
               |||||||||||||||||||||||||| | | :||:||||||||:|||||||
Human PP4  251 ETVLTVWSAPNYCYRCGNVAAILELDEHLQKDFIIFEAAPQETRGIPSKK 300

Dros PP4   301 PQADYFL                                          307
               | |||||
Human PP4  301 PVADYFL                                          307
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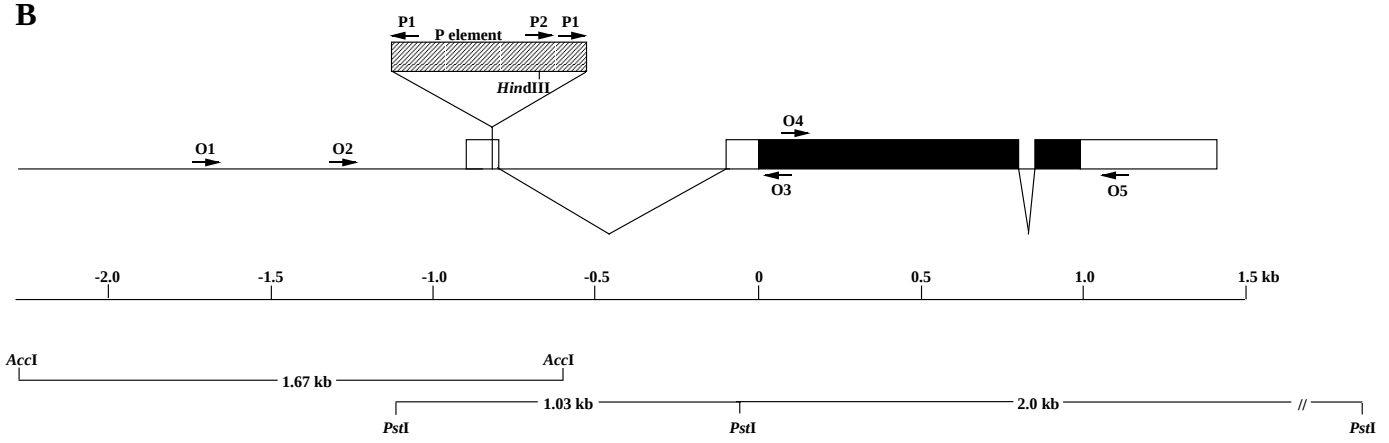

Fig. 1. (A) Comparison of the amino acid sequences of *Drosophila* melanogaster PP4 with human PP4 (Brewis and Cohen, 1992). (B) Genomic organisation of the *PP4* gene locus at 19C. The 5′ and 3′ untranslated regions are represented as open boxes, the coding exons as black boxes, the non-transcribed sequences are shown as lines and the intron sequences as lines which depict their removal by splicing. Intron I in the 5′ untranslated region of the mRNA is at −0.792 to −0.101 and intron II in the coding region is at 0.795 to 0.858. Four putative TATA boxes between −0.932 and −1.258, a CAAT box at −918 to −914 and two DRE sequences (TATCGATA), putative promoter activating elements for cell-proliferation related genes (Matsukage et al., 1995), precede the start of the cDNA (−0.893). The site of insertion of the *P[0.68kb]* element is between −0.818 and −0.819. A potential open reading frame encoding 278 amino acids on the complementary strand exists from nucleotide −1.175 to −2.008. The scale is in kilobases. Relevant restriction sites are shown below.

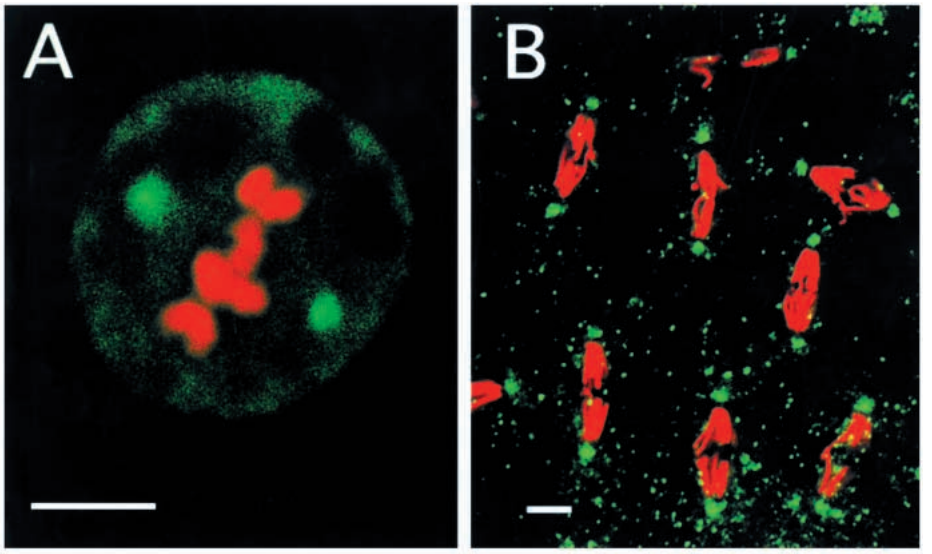

Fig. 2. Indirect immunofluorescent staining of (A) *Drosophila* embryonic cell line (Kc), and (B) Oregon R 0-2 hour embryo. PP4 (green) was detected with sheep anti-PP4 antibody and donkey anti-sheep FITC-conjugated antibody. DNA (red) was stained with propidium iodide. Bars, 5 μm.

94% similarity to human PP4 (Fig. 1A). It shows less than 63% identity to other members of the PP2A subfamily including *Drosophila* PP2A (62% identity) and PPV (59% identity) and less than 46% identity to members of other subfamilies in the PPP family including *Drosophila* PP1 isoforms (44-45% identity), PPY (39% identity), PP5 (41% identity) and PP2B isoforms (39-40% identity). Based on sequence similarities, *S. cerevisiae* does not possess a clear PP4 homologue. *The S. cerevisiae* phosphatases Pph3 (Ronne et al., 1991) and Sit4 (Arndt et al., 1989) show only 62% and 61% identity to *Drosophila* PP4, respectively. The level of PP4 conservation from mammals to *Drosophila*, although slightly less than that observed for PP1 and PP2A, is higher than that of many conserved proteins, such as histone 2A, and suggests that all or most of the functions of PP4 will be conserved between mammals and *Drosophila*.

Immunocytological analysis of *Drosophila* Kc cells shows that, as observed with human cells, anti-PP4 antibodies intensely stain the centrosome, while being present at lower levels in other regions of the cell (Fig. 2A). In interphase cells PP4 is visible in the nucleus and the cytoplasm as well as being located at the centrosomes (data not shown). Centrosomal PP4 staining is also seen in *Drosophila* embryos (Fig. 2B).

Generation of a *Drosophila* strain carrying a *P* element in the PP4 catalytic subunit gene at 19C1-2

The *PP4* gene was mapped to a single site at cytological location 19C1-2 on the X-chromosome. Unfortunately no strains were available possessing mutations that were likely to be in the *PP4* gene. We therefore employed *P* element mutagenesis in conjunction with a polymerase chain reaction (PCR) strategy in order to isolate a mutant carrying a *P* element in the *PP4* gene (Kaiser and Goodwin, 1990). This method has the advantage that mutants of a gene can be isolated without having to predict the ensuing phenotype. A PCR product, which hybridised to *PP4* cDNA, was obtained with a *P* element specific primer (P2) and a *PP4* specific primer (O3) in one batch of embryos and this allowed eventual isolation of a single fly carrying a *P* element in the *PP4* gene. No further mutants were identified out of 10,000 flies screened. Sequencing of a PCR product generated with primers P2 and O3 (Fig. 1B) showed that the *P* element had inserted in the 5′ untranslated region of the *PP4* gene 818 nucleotides 5′ of the initiating ATG (Fig. 1B). Since there is a 692 nucleotide intron in the 5′ untranslated region, the *P* element is located only 126 nucleotides 5′ of the initiating AUG in the mature mRNA. The sequence of the *PP4* gene 3′ of the *P* element (GTCCAGAT) shows high identity with the octomeric consensus sequence (GGCCAGAC) which is duplicated on *P* element insertion (O'Hare and Rubin, 1983). The size of the *P* element was determined to be 0.68 kb by PCR with oligonucleotides O2 and O3 and sequence analysis of this fragment.

The *P[0.68kb]19C* element insertion increases the size of the *PP4* mRNA and reduces the level of PP4 protein

Total RNA from *Drosophila* embryos homozygous for the *P[0.68kb]19C* element was examined by hybridisation with *PP4* cDNA (Fig. 3A). The size of the *PP4* mRNA was increased by approximately 0.7 kb in *P[0.68kb]19C* homozygous embryos compared with wild-type embryos. This is consistent with the position of the *P[0.68kb]19C* element in the 5′ untranslated region of the gene and retention of the *P* element in the mRNA. The quantity of PP4 mRNA in P[0.68kb]19C homozygous embryos was considerably lower than the PP4 mRNA levels present in wild-type embryos.

Examination of the level of PP4 protein by immunoblotting during the *Drosophila* life cycle showed that in early embryonic stages of development *P[0.68kb]19C* homozygotes had only ~25% of the levels of PP4 found in early wild-type embryos (Fig. 3B). The allele carrying the *P[0.68kb]19C* element was therefore termed *PP4*$^{-}$. In later stages of *Drosophila* embryonic development, the level of PP4 protein in *PP4*$^{-}$ homozygotes rose to 45% of wild-type levels. In adult *Drosophila*, the level of PP4 protein was not significantly different from wild type (data not shown). Reduced levels of PP4 in early embryos probably occur prior to initiation of zygotic mRNA transcription which replaces the falling levels of maternally deposited mRNA. The presence of the *P* element may lower the rate of transcription of the *PP4* gene and/or

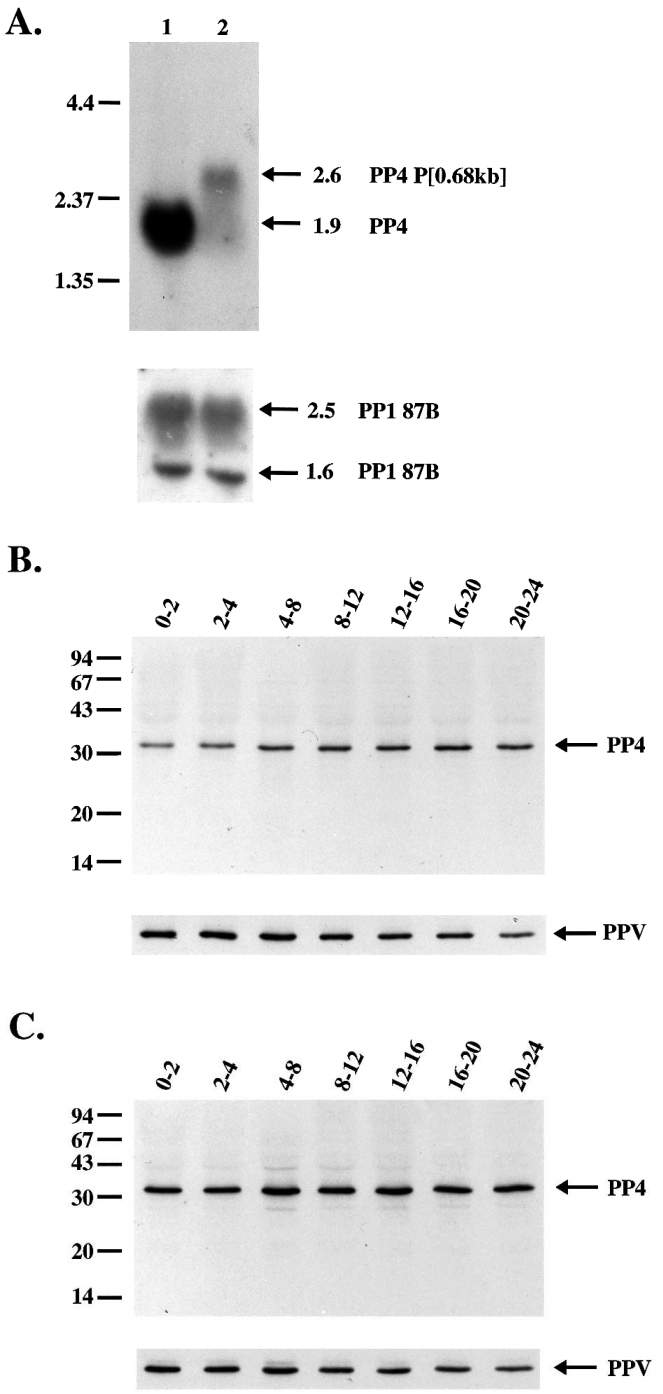

Fig. 3. Analysis of PP4 expression in *Drosophila* embryos homozygous for a P element at chromosomal location 19C. (A) *PP4* mRNA in 0-4 hour wild-type (Oregon R) and mutant embryos. 30 μg of total RNA from embryos was analysed by northern blotting using a ^{32}P-labelled *PP4* cDNA probe (upper panel), followed by a ^{32}P-labelled *PP1 87B* cDNA probe as a control (lower panel). Lane 1, Oregon R; lane 2, homozygous mutant carrying the *P[0.68kb]* element in the *PP4* gene at 19C. (B and C) Levels of PP4 protein detected by immunoblotting in *PP4*$^{-}$ mutant and wild-type (Oregon R) embryo extracts. The age of the embryos in hours is given above each lane. (B) PP4^{-} mutant homozygous for the *P[0.68kb]19C* element. (C) Wild-type (Oregon R). The upper panels are probed with sheep anti-PP4 antibody and the lower panels probed with rabbit anti-PPV antibody to control for variations in loading of the samples.

reduce the stability of the *PP4* mRNA. Consequently, in the early embryo, there is insufficient *PP4* mRNA to synthesise normal levels of PP4 protein, while in the adult the levels of *PP4* mRNA may be sufficient to replenish PP4 protein turnover.

Sequencing of 2.273 kb 5′ of the initiating ATG of the *PP4* coding region (Fig. 1B) identified a putative open reading frame on the complementary strand encoding a 278 amino acid protein and therefore we analysed RNA for transcripts synthesized from this region. A 2.6 kb mRNA transcribed from the strand encoding the 278 amino acid protein, which is present in early embryos and decreases in abundance during later stages of development, was identified using an *Acc*I/*Pst*I fragment (–2.273 to –1.086). The level and size of this transcript is unaffected by the presence of the *P* element in *PP4*$^{-}$ embryos (data not shown).

Drosophila carrying the *P[0.68kb]19C* element insertion exhibit a semi-lethal phenotype

In situ hybridisation of chromosomes from *PP4*$^{-}$ larvae revealed the presence of other *P* elements on the X-chromosome at locations 1D, 3A and 10F. *PP4*$^{-}$ homozygous female flies were crossed with males carrying an X-chromosome possessing the markers *y w ct m f*. Balanced lines were then derived which carried *y w* and the *P[0.68kb]19C* element. Although these lines were free of the *P* elements at 1D and 3A, they still carried the *P* element at 10F. *Drosophila* homozygous for *P[0.68kb]19C* with the genotype *y w PP4*$^{-}$/*y w PP4*$^{-}$ were produced and three homozygous lines were examined phenotypically. These strains had decreased viability with respect to wild type. The proportion of embryos hatching under standard growth conditions at 25°C in the *PP4*$^{-}$ strains was approximately 30% of that observed in wild type (Fig. 4), indicating that this is a semi-lethal mutation. Analysis at different temperatures in the range 16-30°C did not enhance the difference in viability between the *PP4*$^{-}$ and wild-type strains. However, examination by plating out early embryos on agar dishes and counting the number which hatched into larvae, revealed that the *PP4*$^{-}$ strain unexpectedly possessed only 10% of normal viability under these conditions (Fig. 4). This increased mortality may be due to different culture conditions and loss of moisture by the embryos during the plating out procedure or due to development being retarded over the critical early embryonic period when the levels of PP4 protein are at their lowest.

To determine whether the *PP4*$^{-}$ allele is a maternal effect mutation, embryos from crosses of homozygous *PP4*$^{-}$ females with wild-type males were examined. The viability of embryos was very similar (approximately 25%) to that obtained from crosses of homozygous *PP4*$^{-}$ females with hemizygous *PP4*$^{-}$ males (approximately 30%), indicating that the *PP4*$^{-}$ allele is a maternal effect mutation.

Decreased viability of embryos homozygous for *PP4*$^{-}$ is caused by the *P[0.68kb]* element insertion at 19C

In order to ascertain whether the semi-lethal phenotype of *PP4*$^{-}$ *Drosophila* was due to the presence of the *P[0.68kb]* element at 19C and not to the *P* element at 10F or another mutation, reversion mutagenesis was performed. The *P[0.68kb]* element in the *PP4*$^{-}$ strain was mobilised by

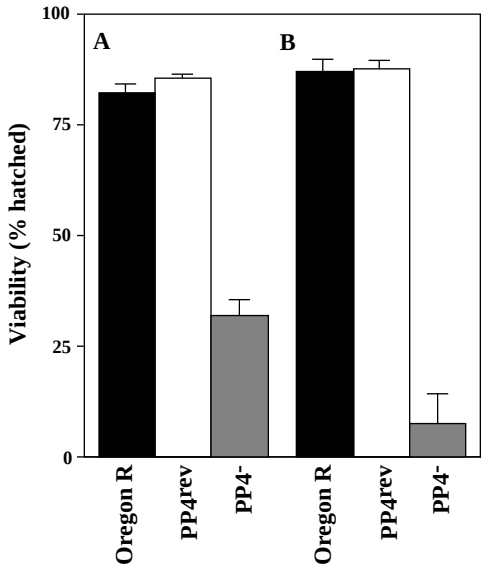

Fig. 4. Comparison of viability of wild-type (Oregon R), PP4^{rev} (revertant) and *PP4*$^{-}$ (*cmm*) embryos. (A) Embryos allowed to develop under standard laboratory conditions. (B) Embryos collected and plated out on agar dishes. Two additional lines for both *PP4*$^{-}$ and *PP4*^{rev} also showed the same viabilities.

introduction of the *Δ2-3* transposase. Flies in the second generation were examined for excision of the *P* element by PCR of DNA from their embryos. In six out of 254 flies examined the *P* element had been excised and sequencing of the PCR fragment showed that in three of these the *P* element had been precisely excised. Analysis of these three *Drosophila* lines homozygous for the revertant allele showed that, as expected, the viability of embryos plated out on agar was not significantly different from wild type (Fig. 4). In addition the level of the PP4 protein in early embryos returned to that seen in wild-type embryos. The phenotype of the revertant embryos was normal, cytological abnormalities described below for the *PP4*$^{-}$ embryos being absent. Although three lines were also recovered with imprecise excision of the *P* element, none were obtained in which the *PP4* gene had been fully or partially deleted. In situ chromosomal analysis with a *P* element probe showed that the *P* element at 10F was still present and confirmed that there was no *P* element at 19C (data not shown).

Examination of the phenotype of *Drosophila centrosomes minus microtubules* (*cmm*) embryos homozygous for the *P[0.68kb]19C* element in the *PP4* gene

Since the level of PP4 in *PP4*$^{-}$ embryos was lowest in the initial stages of development, 0-2 hour embryos were chosen for phenotypic examination. The embryos were dechorionated, fixed and examined with anti-tyrosinated α-tubulin antibodies to reveal the mitotic spindle and anti-—tubulin or centrosomal protein-190 (CP-190) antibodies to locate the centrosomes.

During early embryonic development, the first 14 mitotic nuclear division cycles are synchronous in the wild-type embryo (Fig. 5A). In contrast, nuclei in different regions of homozygous *PP4*$^{-}$ embryos were often observed to be in different stages of mitosis, presumably as a result of asynchronous nuclear divisions. Although asynchrony was occasionally observed in wild-type embryos, the frequency and extent of asynchrony was much greater in *PP4*$^{-}$ embryos and was the first cytological abnormality to be observed during the development of *PP4*$^{-}$ embryos. Subsequently, regions were evident in *PP4*$^{-}$ embryos where centrosomes were present but not associated with any polar microtubules or DNA, suggesting that centrosomes are unable to nucleate mitotic spindle microtubules (Fig. 5B). Regions containing free centrosomes occurred randomly throughout the embryo, sometimes in more than one place, and were of variable size. Hence this strain was termed *centrosomes minus microtubules* (*cmm*). Although occasional mitotic division errors lead to free centrosomes being seen in wild-type embryos (Debec et al., 1996), large areas of free centrosomes never occur. In *cmm* embryos, extensive regions of unattached centrosomes were repeatedly seen (Fig. 5B-D), often coupled with an arrest of nuclei in mitosis and aberrant polar microtubule structures (Fig. 5C). Arrested mitotic structures usually had condensed DNA and often possessed associated polar microtubules, but these were mostly unconnected to the centrosomes. These types of cytological abnormalities (excluding asynchronous mitoses) were seen in 20% of *cmm* embryos in standard conditions and this figure rose to approximately 50% after plating out of the embryos. Although these values are lower than the mortality rates (Fig. 4), presumably some embryos showing milder phenotypes at this stage of development (4-6 hour) eventually become inviable. The percentage of eggs that were fertilised as judged by their ability to initiate some nuclear divisions was similar in wild-type and *cmm* mutant strains (approximately 80%). Often where large patches of free centrosomes were seen in *cmm* embryos, recently duplicated centrosomes were visible (Fig. 5D) indicating that centrosome duplication continues in the absence of correct microtubule formation at the centrosome.

Since γ-tubulin has been shown to be essential for the nucleation of microtubules at centrosomes, we examined embryos for the localisation and distribution of this protein. Fig. 5E and F shows γ-tubulin staining of centrosomes was consistently and markedly weaker in *cmm* embryos showing abnormalities than in wild-type embryos, suggesting a loss or change in structure of γ-tubulin at centrosomes. In the batches of mutant embryos examined, those not yet exhibiting an abnormal phenotype showed wild-type levels of γ-tubulin, providing a good internal control for the level of staining. The total level of γ-tubulin was shown to be identical in *cmm* and wild-type embryo extracts by immunoblotting with anti-—tubulin (data not shown). It therefore appears that there must be a relocation or conformational change of γ-tubulin in *cmm* embryos. To confirm that centrosomes were still present in severely abnormal embryos, the localisation of CP190 was examined and found to be similar in the wild-type (Fig. 5E) and *cmm* embryo, even when γ-tubulin staining was very weak in the latter (Fig. 5F).

Since viability of *cmm* embryos was decreased from 30% to 10% by plating out the embryos on agar dishes, this procedure was used to determine whether embryos would be arrested at a specific stage of the cell cycle. A large proportion of 4-6 hour *cmm* embryos (approximately 50%) arrested with condensed or partially condensed DNA while only 2% of PP4 revertant embryos arrested under these conditions. The lower viability

presumably results from additional embryos arresting in later stages of development. Well defined chromosome arms were often not observed. These results indicate that the nuclei are arrested in mitosis, although it is not possible to discern a specific stage of mitosis at which the block occurs.

PP4 is present throughout the wild-type *Drosophila* early embryo and occurs at higher concentrations at the centrosomes (Fig. 2B). However, centrosomal staining is not as clear as in *Drosophila* tissue culture cells (Fig. 2A) and it was necessary to fix embryos in methanol rather than formaldehyde in order to visualise the staining. The structure of centrosomes in the early *Drosophila* embryo has been reported to differ from that in adult tissues, possibly because the rapid mitoses in the embryo may mean there is insufficient time to build more than a minimal centrosome (Moritz et al., 1995a). This could lead to reduced PP4 levels at the centrosome in embryos. There also appears to be a greater overall abundance of PP4 in the syncytial embryo relative to adult cells, which may partially occlude the centrosomal staining. The variability of the PP4 staining in wild-type embryos precluded a meaningful analysis of lower levels in *cmm* embryos.

DISCUSSION

PP4 is essential for viability in *Drosophila* and is required for the progression through mitosis

We have determined the sequence of the *PP4* cDNA and gene at 19C1-2 in *Drosophila* and produced a mutation by *P* element mutagenesis in the 5′ untranslated region of this gene. The *P* element sequences present in the *PP4* mRNA decrease both the mRNA and PP4 protein levels in early embryos. *cmm* embryos homozygous for this mutation exhibit a semi-lethal phenotype, which was shown by reversion mutagenesis in three independent lines to be due to the presence of the *P* element at 19C1-2. Since the expression of PP4 in *cmm* embryos was not eliminated by this mutation, but reduced to approximately 25% of wild-type level, it is very likely that abrogation of PP4 production would lead to a fully lethal phenotype. The extremely high identity of *Drosophila* PP4 amino acid sequence to rabbit (Brewis et al., 1993) and human (Brewis and Cohen, 1992) PP4 suggests that this phosphatase will serve at least one critical function. PP4 was also shown to be present in all tissues examined in rabbits and therefore it might also be expected to participate in a function common to all tissues.

The frequent occurrence of asynchronous nuclear divisions in *Drosophila cmm* embryos with only 25% of wild-type PP4 levels implicates PP4 in the regulation of cell cycle events. In more severely abnormal *cmm* embryos developing under a variety of conditions, nuclei arrested at different stages of mitosis with condensed or partially condensed DNA, presumably because the PP4 level dropped below that required for the completion of mitosis. The presence of aberrant polar microtubule structures and free centrosomes suggests that a dysfunction of microtubule nucleation, growth or stabilisation, resulting from reduced levels of PP4, is likely to be the cause of the cell cycle arrest. Although we cannot exclude the possibility that lower amounts of a phosphatase catalytic subunit are affecting more than one cellular process, it is likely that the one most sensitive to reduction of PP4 will cause the major phenotype observed. The presence of recently duplicated centrosomes in *cmm* embryos indicates that the centrosome cycle is not blocked by mutation of PP4.

PP4 is required for the organisation of microtubules at centrosomes

Assembly of microtubules can take place in vitro in the absence of centrosomes, but the addition of centrosomes decreases the tubulin concentration at which nucleation of microtubules occurs (Fuller et al., 1992) and in vivo, microtubule growth is thought to initiate only from centrosomes or other microtubule organising centres (MOTCs) (Kalnins, 1992). In *cmm* embryos that have reduced levels of PP4, the presence of centrosomes with no attached polar microtubules in some areas of *cmm* embryos suggests that PP4 may play a role in the initiation of microtubule growth at centrosomes and/or in the maintenance of their attachment to centrosomes. Reduced or absent microtubule growth from centrosomes contrasts with the situation in PP2A deficiency, where although nuclei in syncytial embryos arrest in mitosis, microtubules emanate from centrosomes in all directions and are elongated (Snaith et al., 1996). PP4 localises to centrosomes in *Drosophila* Kc cells (Fig. 2A), *Drosophila* embryos (Fig. 2B) and mammalian cells, where high magnification reveals that PP4 is located in the pericentriolar material that initiates microtubule growth (Brewis et al., 1993). This location coupled with the phenotype of *cmm* embryos indicates that PP4 is involved in the initiation of growth or maintenance of microtubule attachment at centrosomes. Recent data shows that mitotic spindle structures can form in the presence of DNA but absence of centrosomes, indicating that centrosomes are not necessarily required for spindle assembly but can regulate the organisation of microtubules into a bipolar array (Heald et al., 1996, 1997). The presence of mitotic spindle structures associated with DNA but not centrosomes in Fig. 5C is also consistent with this hypothesis. Alternatively, spindle/DNA structures without centrosomes could arise after microtubule nucleation and growth, by loss of attachment of polar microtubules to the centrosomes as the level of PP4 drops. The absence of visible astral microtubules emanating from centrosomes in large patches of free centrosomes in *cmm* embryos suggests that microtubule nucleation is blocked by reduced levels of PP4. However, although astral microtubules can be visualised in nuclei that appear to be dividing normally in *cmm* and wild-type embryos with more intense microtubule staining (data not shown), it is difficult to detect them on the occasional free centrosomes that occur in wild-type embryos (Debec et al., 1996), using either formaldehyde or methanol fixation of embryos. Therefore although our results suggest that astral and polar microtubules are not being nucleated from centrosomes, it is difficult to exclude the possibility that growth or stability of the microtubules near the centrosome are being decreased by reduced levels of PP4.

Microtubules consist of polymers of α- and β-tubulin and a variety of minor components termed microtubule associated proteins (MAPS). The polarity of microtubules is organised with the minus (slow growing) ends in the pericentriolar material of the centrosome and the plus (fast growing) ends orientated away from the centrosome. γ-Tubulin is the only component of the pericentriolar material that has so far been found by deletion analyses and immunodepletion experiments to be essential for the initiation of microtubule growth (Oakley

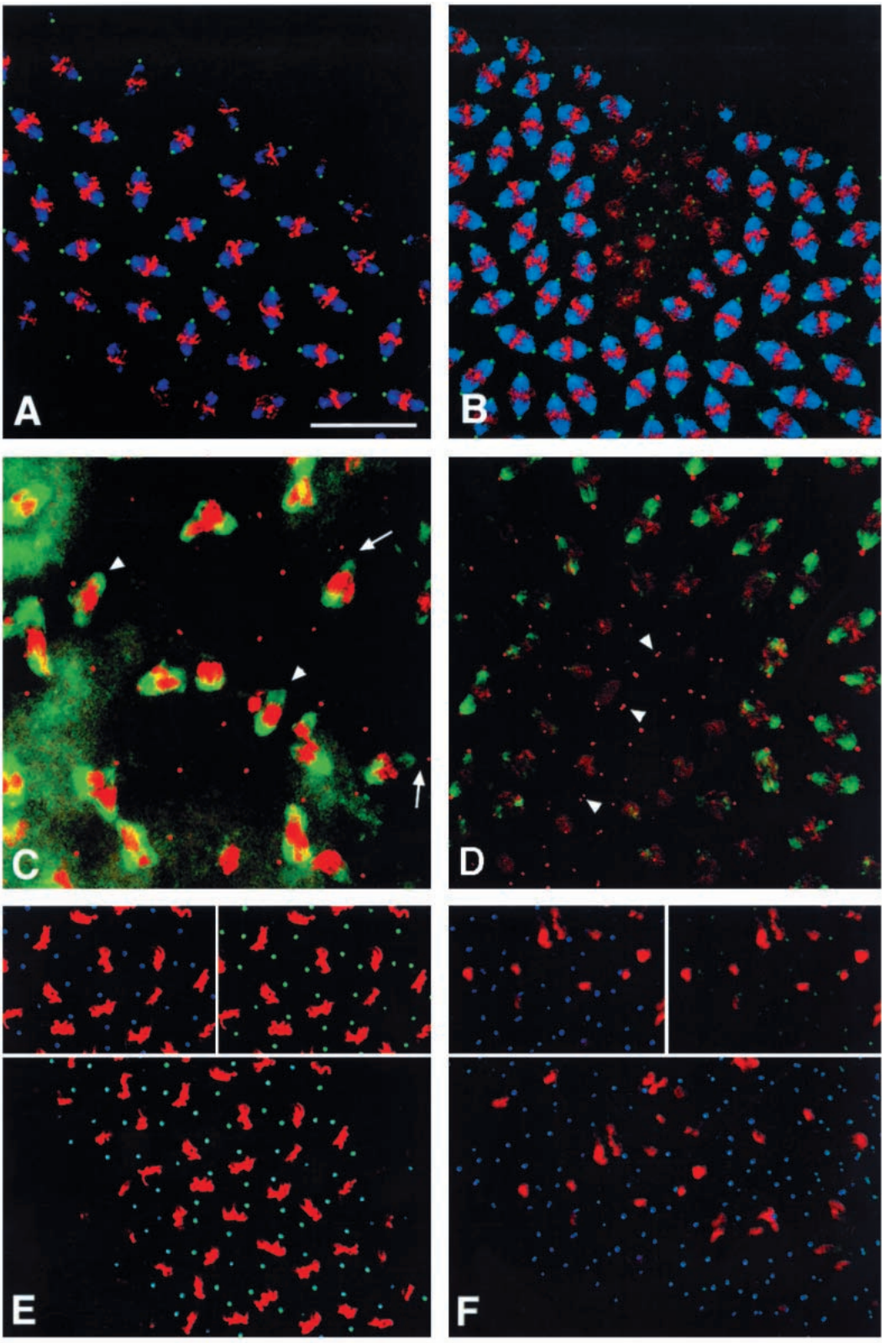

Fig. 5. Indirect immunofluorescent staining of *Drosophila cmm* embryos homozygous for the *P* element at 19C and wild-type (Oregon R) embryos. (A and B) Microtubules (blue) were detected with rat anti-tyrosinated-α-tubulin antibody; centrosomes (green) were detected with mouse anti-γ-tubulin antibody. (C and D) Centrosomally associated CP190 (red) was detected with rabbit anti-CP190 antibody and microtubules (green) were detected with rat anti-tyrosinated-α-tubulin antibody. (E and F) Centrosomally associated CP190 (blue) was detected with rabbit anti-CP190 antibody. γ-tubulin (green) was detected as in A and B. DNA (red) was stained with propidium iodide in all panels. (A) Wild-type embryo undergoing synchronous nuclear divisions and showing the nuclei in metaphase (microtubules blue, centrosomes green and DNA red). (B) *cmm* embryo showing a region where centrosomes are present, but not associated with any microtubules or DNA (colours as in A). (C) *cmm* embryo showing nuclei arrested in mitosis with condensed DNA and abnormal polar microtubules, which are mostly unconnected to centrosomes. Arrowheads point to mitotic structures lacking centrosomes. Arrows indicate mitotic structures where centrosomes are not properly attached to polar microtubules. (D) *cmm* embryo in metaphase showing an area of free centrosomes. Arrowheads point to recently duplicated centrosomes. Centrosomally associated CP190 and DNA are stained red and microtubules are stained green. (E) 0-2 hour Oregon R embryo with nuclei in metaphase showing co-localisation of CP190 (blue) and γ-tubulin (green), resulting in a turquoise colouration. (F) *cmm* embryo showing nuclei arrested in mitosis with CP190 (blue) staining at centrosomes but virtual absence of γ-tubulin (green) staining. Insets in E and F show the DNA (red) and separate channels for CP190 (blue) and γ-tubulin (green) used to produce part of the merged image. Bars: 25 μm (A,B,E,F); 16 μm (C and D).

et al., 1990; Horio et al., 1991; Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994; Sunkel et al., 1995). γ-Tubulin binds to the minus ends of the microtubules and it is present in ring shaped complexes which form the nucleating material for the polymerisation of α- and β-tubulin dimers into microtubules (Zheng et al., 1995; Moritz et al., 1995b). Reconstitution studies in *Xenopus* indicate that γ-tubulin is recruited from the egg cytoplasm to sperm centrioles during centrosome assembly. Furthermore relocation of γ-tubulin to the centrosome is independent of microtubule polymerisation (Stearns and Kirschner, 1994). Therefore our immunolocalisation and immunoblotting studies, which show that in *cmm* embryos there is a relocation or change in conformation of γ-tubulin at the centrosome, indicate that organisation of γ-tubulin in the percentriolar material is being affected by decreased levels of PP4 and not merely by an absence of microtubules. This may be the result of PP4 altering the structure of γ-tubulin by dephosphorylation either directly or indirectly. In this respect it is interesting that γ-tubulin structure is highly divergent in *Saccharomyces cerevisiae* (Keeling and Logsdon, 1996) and that there is no clear homologue of PP4 in *S. cerevisiae* (Cohen, 1997).

The factors that regulate the ability of γ-tubulin to initiate microtubule growth and coordinate it with the cell cycle are not known, but reversible phosphorylation is an attractive mechanism. $p34^{cdc2}$ cyclin A localises to centrosomes in prophase and can increase the microtubule nucleation of centrosomes in vitro (Buendia et al., 1992). However, although $p34^{cdc2}$ plays a well documented role in mitosis, in vivo experiments that implicate $p34^{cdc2}$ cyclin A in microtubule nucleation are lacking. LK6 kinase can associate with centrosomes and microtubules, and overexpression of LK6 causes defects in microtubule organisation in early embryos (Kidd and Raff, 1997). The phenotype of embryos overexpressing LK6 shows some similarities to *cmm* embryos including the presence of isolated centrosomes, suggesting the attractive possibility that PP4 could reverse phosphorylations catalysed by LK6. However, no mutant in LK6 kinase was available for analysis and overexpression of kinases can lead to phosphorylation of non-physiological substrates. The generation of *cmm* embryos expressing reduced levels of PP4 provides in vivo evidence demonstrating that reversible phosphorylation regulates the initiation of microtubule growth.

Mammalian PP4 expressed from baculovirus has been shown to dephosphorylate serine and threonine residues (Brewis et al., 1993). However, the specific activity of PP4 against all substrates tested was lower than that of PP2A and the in vivo substrate(s) of PP4 have not been identified. It is of interest to consider possible protein(s) that PP4 may dephosphorylate at centrosomes. Proteins or epitopes that are required in phosphorylated forms for microtubule initiation and growth, like the epitope MPM-2 (Centonze and Borisy, 1990), are unlikely to be substrates, since reduction of PP4 levels would be expected to increase protein phosphorylation. Proteins that are dephosphorylated during microtubule nucleation, undergo multiple phosphorylations or where the phosphorylation state is not known are more likely candidates for substrates of PP4. For example, CP60 (Kellogg et al., 1995) binds to microtubules in a dephosphorylated form and association is disrupted by phosphorylation with $p34^{cdc2}$ kinase. In the case of γ-tubulin, the phosphorylation state does not appear to have been investigated. It will be interesting to examine *cmm* embryos for hyperphosphorylation of γ-tubulin and other proteins. The present data show that PP4, a protein serine/threonine phosphatase, is an essential enzyme required for microtubule nucleation, growth or stabilisation at centrosomes/spindle pole bodies.

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