Metaphase Arrest with Centromere Separation in polo Mutants of Drosophila

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Abstract. The Drosophila gene polo encodes a conserved protein kinase known to be required to organize spindle poles and for cytokinesis. Here we report two strongly hypomorphic mutations of polo that arrest cells of the larval brain at a point in metaphase when the majority of sister kinetochores have separated by between 20–50% of the total spindle length in intact cells. In contrast, analysis of sister chromatid separation in squashed preparations of cells indicates that some 83% of sisters remain attached. This suggests the separation seen in intact cells requires the tension produced by a functional spindle. The point of arrest corresponds to the spindle integrity checkpoint; Bub1 protein and the 3F3/2 epitope are present on the separated kinetochores and the arrest is suppressed by a bub1 mutation. The mutant mitotic spindles are anastral and have assembled upon centrosomes that are associated with Centrosomin and the abnormal spindle protein (Asp), but neither with γ-tubulin nor CP190. We discuss roles for Polo kinase in recruiting centrosomal proteins and in regulating progression through the metaphase–anaphase checkpoint.

Key words: mitosis • spindle checkpoint • Drosophila • Polo kinase • cell cycle

Introduction

The Polo-like protein kinases (Plks)† are a conserved family of enzymes that play a variety of roles in the passage of cells through M phase (for reviews, see Glover et al., 1998; Nigg, 1998). They are named after the Drosophila polo gene originally identified through a recessive maternal effect lethal mutation. Flies homozygous for the original polo allele can develop to adulthood due to the weakly hypomorphic nature of this mutation and the provision of wild-type protein from their heterozygous mothers. However, they do exhibit spindle pole defects at several developmental stages including multiply branched spindles in syncytial polo-derived embryos, and spindles with broad poles and a low frequency of circular mitotic figures in larval neuroblasts (Sunkel and Glover, 1988; Llamazares et al., 1991). Requirements for polo during male meiosis are also evident from the chromosome nondisjunction and failures in cytokinesis seen during meiosis of polo testes (Sunkel and Glover, 1988; Carmena et al., 1998; Herrmann et al., 1998). A more recent examination of meiosis in polo eggs and at the onset of their zygotic development indicates abnormalities in microtubule organizing centres in the meiotic spindle, the sperm aster, and the astral arrays of microtubules associated with the polar bodies (Riparbelli et al., 2000).

These roles of Polo kinase are echoed in other organisms, suggesting conserved function. Disruption of the fission yeast counterpart, plo1, leads to formation of monopolar spindles (Ohkura et al., 1995). Interfering with enzyme activity using antibodies can also lead to the formation of monopolar spindles in Xenopus, or human cells (Lane and Nigg, 1996; Qian et al., 1998). In human cells, this appeared to be linked to a failure of the centrosome to increase its microtubule nucleating activity upon mitotic entry. Indeed, a direct role for polo in mitotic entry is suggested by the finding that Xenopus Polo-like kinase, Plx1, copurifies with and can activate cdc25, and may thus play a role in the positive feedback loop that operates during p34cdc2 activation at the G2-M transition (Kumagai and Dunphy, 1996; Abrieu et al., 1998; Qian et al., 1998).

A requirement for the Plks to promote the onset of cytokinesis also appears to have been conserved from the yeasts to the metazoans. Disruption of the fission yeast plo1 leads also to the formation of multinucleate cells in which neither an actin ring nor a septum has been formed. Overexpression of plo1 in fission yeast, on the other
hand, leads to the formation of multiple septa at any stage of the cell cycle, indicating the potential of the enzyme to overcome the dependence of this process upon the completion of mitosis (Ohkura et al., 1995). Expression of an activated form of mammalian Plk in budding yeast has also been found to drive the formation of multiple septa, suggesting this is a conserved property of the enzyme (Lee and Erikson, 1997). In Drosophila, the requirement of polo for cytokinesis appears to be linked to the need to reorganize the central region of the spindle in late M phase (Herrmann et al., 1998). In budding yeast, Cdc5p is also shown to play a role in regulating cytokinesis, and the polo-box, a conserved motif in the noncatalytic domain of Plks, is required for this (Song and Lee, 2001).

There is also evidence that the polo-like kinases can activate certain functions of the anaphase-promoting complex (APC), an E3 ubiquitin-protein ligase that directs the degradation of anaphase inhibitors, Pds1p in budding yeast (Cohen-Fix et al., 1996) and Cut2p in fission yeast (Funabiki et al., 1996), and the mitotic cyclins (for reviews, see Townsley and Ruderman, 1998; Yanagida, 1998). Descombes and Nigg (1998) showed that the addition of catalytically inactive polo-like kinase to colony-stimulating factor (CSF)-arrested extracts of Xenopus eggs blocks the Cdk2/Cdc21-triggered destruction of cyclin B and inactivation of p34(cdc2) and also prevented the destruction of exogenous APC-dependent substrates. Furthermore, M phase exit would not take place in this system after immunodepletion of Plxl, but could be restored by the addition of catalytically active enzyme. The mouse polo-like kinase will also phosphorylate and activate the bacterially expressed APC components Cdc16 and Cdc27 in vitro (Kotani et al., 1998). The mitotic cyclin Cbl2p is not degraded in mutants for the budding yeast plk, Cdc5p (Shirayama et al., 1998), whereas overexpression of Cdc5p increased APC activity and decreased the levels of Cbl2p, an outcome not obtained with a “kinase-dead” mutant (Charles et al., 1998).

Until now, a requirement for polo in regulating APC in Drosophila has not been apparent from the phenotypes of the alleles that have been studied. polo1 mutants, for example, are able to progress through development, as a function of the weakly hypomorphic protein is sufficiently supplemented by maternally provided wild-type protein. Consequently, it has been difficult to assess fully the functions of Polo kinase in polo1 somatic cells which are capable of progression through multiple cell cycles. In this paper, we describe the mitotic phenotype of two strongly hypomorphic alleles, polo9 and polo10, which block the proliferation of diploid tissues. Cells of these mutants appear poised to initiate anaphase and sister kinetochores are pulled apart. Chromatids undergoing partial separation can remain attached through their telomeres and do not migrate to the spindle poles. We discuss potential roles for Polo kinase in regulating specific functions of checkpoint proteins and the APC at the metaphase–anaphase transition.

Materials and Methods

Immunoblotting

Extracts of larval brains were prepared for immunoblotting according to Scaerou et al. (1999). 6 brains were used per lane for wild-type, 7 for polo1, and 12 per lane for polo9/10. This ensured equal protein loading, which was tested by probing with the C4 mouse monoclonal antibody to actin. The Polo and γ-tubulin proteins were identified by probing with MA81 rabbit anti-Polo and GTU88 mouse anti-γ-tubulin antibodies.

Giems-stained Preparations of the Larval Central Nervous System

Third-instar larvae were washed and dissected in PBS. The associated imaginal discs were removed and the brains were incubated in 45% acetic acid for 30 s, then in 60% acetic acid for 3 min. The brains were then squashed hard between a poly-lysine–coated slide (Sigma-Aldrich) and a siliconized coverslip. The slides were frozen in liquid nitrogen and the coverslip flipped off with a scalpel blade. The preparations were then dehydrated in 75 and 100% ethanol for 5 min each before being left to air dry. The preparations were then stained in Giemsa and mounted in DPX. Preparations were observed at 60 and 100× objective on a compound microscope. In experiments where colchicine was used, the dissected brains were incubated with 10−4 M colchicine, in Schneider's tissue culture medium (Trueman and Bate, 1988) containing FCS, for 30 min, 1 h, 2 h, or 4 h, before being incubated in 45% acetic acid.

Immunostaining

Immunostaining of whole mount preparations of third-instar larval brains was carried out by first dissecting the larvae in PBS and then fixing in 3.7% formaldehyde for 20 min. Brains were washed in PBS, then permeabilized in 0.3% Tween 20 in PBS for 10 min. The tissue was “blocked” by a 40-min incubation in PBS containing 10% FCS before being incubated overnight with the primary antibodies in PBS containing 0.1% Tween 20 (PBST) containing 2.5 μg/ml RNase, at 4°C. Brains were then washed in PBST and incubated with a 10−2 dilution of secondary antibodies in PBST containing 10% FCS for between 2 and 4 h. Brains were finally washed in PBST before being incubated in propidium iodide to stain DNA. The preparation was mounted in Vectashield.

Immunostaining with the 3F3/2 antibody was performed according to Bousbaa et al. (1997), except that no taxol was used, and the staining of α-tubulin carried out simultaneously by adding YL1/2 into the primary antibody incubation, and the appropriate suitable secondary antibody into the subsequent incubation.

Microscopy was performed using a Nikon Optiphot compound microscope with a Bio-Rad Laboratories MRC 1024 Scanning Confocal attachment. Images were captured using Lasersharp software and manipulated in Adobe Photoshop.

Results

Strong polo Hypomorphs Show Mitotic Arrest in Larval Brain Cells

We wished to isolate strong hypomorphic alleles of polo in order to examine their effect upon mitosis in somatic cells. As P-elements generally insert either into the 5′ regulatory regions or coding sequences to downregulate or inactivate their target genes, we sought to identify such alleles in a collection of mutants generated by P-element–mediated mutagenesis (Deak et al., 1997). We discovered two new polo alleles, polo9 and polo10, that have their lethal phase during late third larval instar development and have respective mitotic indices that are elevated about 11- and 8-fold above cells of wild-type larval brains (Fig. 1 and Table I). This is considerably higher than polo7 that has a mitotic index about fourfold greater than wild-type in this tissue. polo7 mutants produce comparable levels of protein to wild-type, but the kinase is thought to be defective due to a failure of an activating phosphorylation (Tavares et al., 1996). In contrast, the amounts of Polo kinase in the polo9 and polo10 mutants are barely detectable (Fig. 2 B). This is a consequence of the insertion of P-elements in the two lines into the noncoding regions of the first exon of
Figure 1. Mitotic figures from wild-type, polo<sup>9</sup>, and polo<sup>10</sup> brains. (A) A wild-type metaphase figure. (B) A wild-type anaphase figure. (C) Hypercondensed mitotic chromosomes from polo<sup>9</sup>. The arrow points to a pair of separated sister chromatids. (D) polo<sup>10</sup> cell showing separated sisters (arrow) that could be at early anaphase. (E) An anaphase (arrow) and metaphase (arrowhead) figure from polo<sup>9</sup>. (F) A tetraploid polo<sup>10</sup> cell in which several sister chromatids appear separated throughout their length, and yet joined at their telomeres (arrow). (G) A polyploid polo<sup>9</sup> cell in which chromosomes form long chains attached at their telomeres (arrows). (H) Bar graph showing the proportion of cells at prophase and metaphase (Pro. & Met.) or anaphase and telophase (Ana. & Tel.) in wild-type (wt), polo<sup>1</sup>, polo<sup>9</sup>, and polo<sup>10</sup> brains. (I) Wild-type cell from a larval brain treated for 4 h with colchicine. (J) polo<sup>10</sup> cell after 30 min colchicine treatment. The arrow marks separated sisters. (K) polo<sup>10</sup> cell after 2 h colchicine treatment.
the polo gene at a position that affects either one (polo10) or both (polo9) of the two polo transcription units (Fig. 2A). The levels of enzyme in polo9 are less than in polo10, consistent with the sites of insertion, and they account for the relative severity of the mutant phenotype indicated by the mitotic indices.

Examination of mitotic chromosomes in squashed preparations of the central nervous systems from polo9 or polo10 larvae indicated several characteristic features. First, by comparison with wild-type mitotic cells (Fig. 1, A and B), chromosomes are much more highly condensed, indicating that the cells have been delayed in mitosis for some time (Fig. 1, C, E, and F). The majority of cells appear arrested in a metaphase-like state in that most sister chromatids (83%) are still joined at their centromeres, and some time (Fig. 1, C, E, and F). The majority of cells appear arrested in a metaphase-like state in that most sister chromatids (83%) are still joined at their centromeres, and the proportion of figures in which chromosomes are well separated at anaphase is relatively low, although in absolute numbers it is comparable to that in wild-type cells (Fig. 1 H and Table I). A proportion of cells are polyploid (Fig. 1, F and G, and Table I), suggesting that they have been able to escape the metaphase block and undergo a further cell cycle in the absence of chromosome segregation or cytokinesis. A low proportion of circular mitotic figures could be seen, as reported previously for polo13. In ~40% of cells, at least one set of the sister chromatids appear to have prematurely separated (arrows in Fig. 1, C and D; see also the legend to Table I). In addition, chromosomes frequently appeared to be connected through their telomeric regions. Fig. 1, F and G, show polyploid cells in which many chromosomes are linked in this way (arrows). This phenotype strongly resembles that described previously in the mutant UbcD1 gene that encodes a class I ubiquitin-conjugating (E2) enzyme (Cenci et al., 1997). The mitotic index of the polo9 and polo10 brains did not change significantly after colchicine treatment, suggesting that the population of cells in this tissue could not respond further to disruption of spindle integrity (Fig. 1, J and K). A similar frequency of sister chromatid separation was observed independently of whether the preparation had been made in the presence or absence of colchicine.

**polo Differentially Affects Centrosomal Components**

Defects in spindle poles and other microtubule organizing centres have been reported previously for polo1. However, the polo10 mutants have allowed us to examine for the first time the effects of greatly diminished levels of the Polo kinase on the spindles and centrosomes of somatic cells. Examination of whole mount preparations of polo10 larval brains stained with antibodies to α-tubulin to reveal the mitotic spindle confirms the results seen with Giemsa-stained preparations. It reveals a very high mitotic index with the majority of cells arrested in a metaphase-like state. The arrested cells all have bipolar spindles with robust arrays of spindle microtubules, but lacking asters at their poles (Figs. 3–6). Consistent with the previously observed failure of the CP190 antigen to assemble onto centrosomes in syncytial polo1-derived embryos, CP190 does not concentrate in the centrosomes of polo10 neuroblasts, but is scattered throughout the spindle with some tendency to accumulate around the condensed chromosomes (Fig. 3, K and L). γ-Tubulin is also missing from the spindle poles (Fig. 3, E and F). The protein cannot be seen in these particular micrographs, although other preparations show weak staining, suggesting it is dispersed throughout the mutant cells. This interpretation is supported by the detection of undiminished levels of γ-tubulin by Western blotting of extracts of these mutant brains (Fig. 2). In contrast, both centrosomin (Fig. 3, B and C) and abnormal spindle protein (Asp) (Fig. 3, H and I) are both present at the spindle poles in the mutant cells, indicating that Polo kinase function is not required for their localization.

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**Table I. Analysis of the Mitotic Phenotype of polo Alleles in the Larval Central Nervous System**

|                  | Cells scored* | Prophase and metaphase§ | Circular mitotic figures | Anaphase and telophase | Mitotic index\(^{\text{\%}}\) | Mitotic index\(^{\text{\%}}\) | M/A* |
|------------------|---------------|-------------------------|--------------------------|------------------------|-------------------------------|-------------------------------|-----|
| Wild-type        | 2,521         | 2.8                     | 0                        | 0.5                    | 1.2                           | 3.3                           | 5.8 |
| polo1            | 1,793         | 10.4                    | 0.7                      | 1.0                    | 2.3                           | 11.9                          | 11.6|
| polo9            | 1,616         | 32.9                    | 1.4                      | 0.7                    | 16.1                          | 35.6                          | 51.4|
| polo10           | 1,723         | 22.9                    | 1.2                      | 1.0                    | 11.3                          | 25.0                          | 26.1|
| polo10 bab1      | 1,766         | 3.1                     | 0.5                      | 0.4                    | 49.2                          | 3.7                           | 8.3 |

In addition to the above quantitation, numbers of pairs of sister chromatids showing separation were also counted in polo9 cells in the presence and absence of colchicine. Of 272 chromatid pairs scored in untreated polo9 cells, 17.2% were separated, compared with 17.0% of 176 chromatid pairs scored in colchicine-treated preparations. 40% of untreated polo10 cells had at least one separated pair of sister chromatids. This figure was similar (36.6%) after colchicine treatment.

*Total number of cells scored in 10 fields (60× objective) from 5 brains for each genotype.

§Cells were scored as being metaphase if chromosomes were closely associated and if they contain some sister chromatids that appeared to be still attached.

\(^{\text{\%}}\)Note, this is percentage of mitotic cells and not total cells.

\(^{\text{\%}}\)Metiotic index is scored as the number of mitotic cells as a percentage of total cell number.

\(^{\text{\%}}\)Metaphase/anaphase ratio.
Figure 3. Distribution of centrosomal antigens in wild-type, polo9, and polo10 cells. In all cases, wild-type cells are shown in the left panels, polo9 cells the middle panels, and polo10 cells the right panels. Spindle microtubules revealed by immunostaining with the YL1/2 antibody are shown in green, DNA stained with propidium iodide in red, and the centrosomal antigen in blue. (A–C) Centrosomin (Cnn) is revealed using a rabbit antibody from Heuer et al. (1995). (D–F) γ-Tubulin was detected using the mouse monoclonal antibody GTU88 (Sigma-Aldrich). (G–I) The Asp was detected using the rabbit antibody Rb3133 (Saunders et al., 1997). (J–L) CP190 was detected using the rabbit antibody RB188 (Whitfield et al., 1988). Bar, 5 μm.
Sister Chromatids Have Undergone Partial Separation in Strong polo Hypomorphs

It appeared from the above immunostaining experiments that the condensed chromatid had a broader distribution about the central part of the spindle in the polo or polo10 mutant cells than in wild-type cells at metaphase. We were curious to know whether this reflected any separation of the centromeric regions of the chromatids, so we localized the product of the gene proliferation disrupter (prod) in polo and polo10 cells. This protein localizes principally to the centromeric regions of chromosomes 2 and 3 (Torok et al., 1997). In wild-type cells at metaphase in which chromosomes are fully aligned on the metaphase plate, four punctate regions of Prod staining can be seen corresponding to the second and third chromosome pairs in which the sister centromeric regions are still tightly adjoined (Fig. 4 A). In polo (Fig. 4 B) or polo10 cells (Fig. 4 C), we frequently observed eight dots in two lines of four separated by 2–5 μm in the central region of the 10-μm long spindle. Approximately 80% of pairs of centromeric regions showed separation in these whole mount preparations of brains (see the legend to Fig. 4). This appearance suggests that movement of the centromeric regions of the chromatids towards the spindle poles has been initiated and that the sister kinetochores are being pulled apart. The broad distribution of the chromosomes in these preparations contrasts with their highly condensed appearance in the squashed preparations. This may indicate that the chromatim is highly stretched when associated with the intact mitotic spindle. This broad distribution of chromatim in whole mount preparations is in contrast to compact condensed chromosomes in the metaphase arrest in fizzy (Fig. 4, D–F), a gene required to activate the anaphase promoting complex for degradation of both cyclin A and cyclin B (for a review, see Hershko, 1999). The distribution of Prod in fizzy cells indicates that the sister centromeres have not separated; only four dots of staining are seen and these are not aligned but randomly positioned within the area occupied by the condensed chromosomes.

polo and polo10 Cells Bear the Signature of Checkpoint Arrest

The segregation of sister chromatids into daughter cells at anaphase is normally prevented until a stable bipolar attachment is made between the kinetochores and the spindle microtubules. This process is monitored by the spindle integrity checkpoint which requires the function of a conserved complex of proteins originally identified in budding yeast as the products of the genes BUB1, BUB3 (Hoyt et al., 1991), MAD1, MAD2, and MAD3 (Li and Murray, 1991). In metazoan cells this complex localizes to unattached kinetochores to inhibit the activation of the APC (for a review, see Amon, 1999). Mutations in the Drosophila counterpart of one of these proteins, Bub1, indicate that it is required for the fidelity of chromosome segregation (Basu et al., 1999). The Bub1 protein associates with kinetochores as wild-type cells from the larval central nervous system progress to metaphase (Fig. 5 A), and is abruptly lost at anaphase (Fig. 5 B). We found metaphase-like arrested polo or polo10 cells in which 16 dots of Bub1 staining could be seen corresponding to the separated sister kinetochores (Fig. 5, C and D). Thus, the kinetochores appear to have undergone alignment and the first stages of separation and yet Bub1 remained associated with them.

A phosphoepitope recognized by the 3F3/2 monoclonal antibody is also found at kinetochores that are reportedly not under tension (Gorbsky and Ricketts, 1993). It has been thought that when bipolar attachments are made, and tension at the kinetochore is established, the epitope becomes dephosphorylated and so it is no longer recognized by the 3F3/2 antibody. In Drosophila wild-type neuroblasts, the 3F3/2 epitope can be seen weakly at the kinetochores and at the spindle poles at metaphase (Fig. 5 E) and is lost from the chromosomes at anaphase (Fig. 5 F). Interestingly, spindle pole staining of 3F3/2 is not seen in the polo and polo10 mutants, probably reflecting the disruptions of the centrosome discussed above (Fig. 5 G and H). However, staining remains often as two separate lines of eight dots corresponding to the sister kinetochores of the four pairs of chromosomes (Fig. 5, G and H). Thus, this tension-sensing checkpoint epitope is present at kinetochores in the mutant cells even though one might expect the kinetochores to be under tension as they are pulled toward the spindle poles.

In the unperturbed mitotic cycle, cyclin A is abundant in prophase (Fig. 6 A) but is degraded ahead of cyclin B which is still abundant at metaphase (Fig. 6 D). This is accentuated during spindle integrity checkpoint arrest. After colchicine treatment, for example, cells arrest at a point at which cyclin A has undergone degradation allowing cyclin B to accumulate to higher than usual levels (Whitfield et al., 1990). In metaphase-arrested polo and polo10 neuroblasts, cyclin A has been degraded (Fig. 6, B and C), whereas cyclin B is present at elevated levels, particularly concentrated in the regions between chromosomes on the metaphase spindle (Fig. 6, E and F).

Thus, by three criteria both polo and polo10 cells appear to be delayed at the spindle integrity checkpoint, and yet they have initiated anaphase chromosome movements that have pulled apart the centromeric regions of the sister chromatids.

bub1 Releases polo10 Cells from Checkpoint Arrest

To determine whether indeed polo10 cells were in a state of checkpoint arrest, we made a double mutant construct with bub1 and monitored mitotic progression in the larval central nervous system. The mitotic index in the double mutant was comparable to wild-type (Table I), and cells could be observed at all mitotic stages (Fig. 7, A–D). The spindles still showed the characteristic polo defects at their poles, which were anastral and showed no staining for CP190 (Fig. 7, E and F). CP190 antigen was present in the vicinity of the mitotic chromosomes (Fig. 7 E) and remained associated with telophase nuclei (Fig. 7 F). Stained squashed preparations were similar to those reported for the bub1 mutant alone (Basu et al., 1999) and showed sister chromatids already separated at metaphase (Fig. 7 G), and lagging chromatids at anaphase (Fig. 7 H, arrow). This would account for the increased number of aneuploid cells that were observed (Table I). The double mutant also showed an elevated number of polyploid figures that could
Figure 4. Localization of Prod in wild-type (A), polo (B and C), and fizzy (D–F) cells. Spindle microtubules stained with the rat monoclonal antibody YL1/2 are stained green. DNA is stained red. Prod (blue) was detected using a rabbit antibody (Torok et al., 1997). Merged images are shown in the top panels with the separated channel for Prod staining below. Bar, 5 μm. 79.3% of 213 clear pairs of sister centromeric regions were scored as having separated in immunostained polo⁹ cells. A similar frequency of separation (78.6% of 112 pairs) was observed in polo¹⁰ cells. Centromere separation was not observed by anti-Prod staining in the fizzy⁴³ mutant.
Figure 5. Association of Bub1 and the 3F3/2 epitope with the mitotic apparatus in wild-type and polo mutant cells. In all panels, spindle microtubules are stained green and DNA stained red. Bub1 (A–F) or 3F3/2 (G–L) staining are shown in blue. (A and B) Wild-type cells at metaphase and anaphase, respectively. (C and D) polo9 cells showing Bub1 staining with the rabbit antibody Rb666 (gift of C. Sunkel, University of Porto, Porto, Portugal) on the separated kinetochores. (E and F) Wild-type cells at metaphase and anaphase, respectively, stained with the 3F3/2 mouse monoclonal antibody (gift of G. Gorbsky, University of Oklahoma, Oklahoma City, Oklahoma). Note the presence of the 3F3/2 epitope at centrosomes. (G and H) polo9 cells showing 3F3/2 staining on the separated kinetochores, and absent from the spindle poles. Bar, 5 μm.
be explained either by a complete failure of chromosome segregation or cytokinesis.

Thus, it appears that although sister kinetochores are under sufficient tension in \textit{polo} or \textit{polo}^{10} cells to pull them apart by up to 5 \(\mu\)m, the cells are delayed in a metaphase-like checkpoint arrest that can be suppressed by \textit{bub1}.

**Discussion**

The Recruitment of Centrosomal Antigens

Previous studies on the \textit{Drosophila polo} allele have revealed requirements for its protein kinase for the function of microtubule organizing centres at several stages of development, and in both chromosome segregation and cytokinesis in male meiosis (Sunkel and Glover, 1988; Herrmann et al., 1998; Riparbelli et al., 2000). The strongly hypomorphic mutant alleles we now describe, \textit{polo}^{9} or \textit{polo}^{10}, suggest a new requirement for Polo kinase in the metaphase–anaphase transition, but also give additional insight into the requirements for Polo kinase to organize microtubule nucleating centers. It is known that both the \(\gamma\)-tubulin ring complex (\(\gamma\)-TuRC) and Asp are required for the integrity of mitotic microtubule organizing centers (Moritz et al., 1998; Schnackenberg et al., 1998; Avides and Glover, 1999), and yet neither appears to be required for localization of the other at the spindle pole. \(\gamma\)-Tubulin is found at the spindle poles in \textit{asp} mutants (Avides and Glover, 1999), and Asp, which only associates with spindle poles during mitosis (Saunders et al., 1997; Avides and Glover, 1999), still localizes to either the poles or the unfocused minus ends of microtubules in mutants of the \textit{dd4} gene that encodes the 91-kD component of the \(\gamma\)-TuRC (Barbosa et al., 2000). The effect of the \textit{polo}^{9/10} mutations on the constitution of centrosomes for the limited number of antigens that we have studied is in fact remarkably similar to that seen when the \(\gamma\)-TuRC is disrupted in \textit{dd4} mutants. In \textit{dd4} cells, \(\gamma\)-tubulin is dispersed throughout the cytoplasm and a large proportion of the CP190 antigen shows a punctate distribution around the condensed chromosomes in much the same way as in \textit{polo}^{9/10} mutants. More-
over, in addition to Asp, Centrosomin is present at the focused spindle poles both in dd4 and polo9/10 mutants. We suggest that these common aspects of phenotype result from a common primary failure to correctly assemble and/or localize the γ-TuRC at the centrosome. This would be consistent with the observation that γ-tubulin is recruited to the centrosome at mitosis (Khodjakov and Rieder, 1999) and the finding that centrosomes fail to grow and effectively nucleate microtubules in human cells injected with antibodies to Polo kinase (Lane and Nigg, 1996). However, it is of interest that although centrosomes appear to lack γ-tubulin and do not nucleate astral microtubules, they are nevertheless capable of assembling a spindle and of establishing a metaphase-like array of chromosomes.

Figure 7. Suppression of the polo10 mitotic phenotype by bub1. In A–F, microtubules are stained green and DNA stained red. (A–D) polo10 bub1^{k6109} cells at metaphase, early anaphase, late anaphase, and telophase, respectively, showing the localization of Prod (blue). (E and F) polo10 bub1^{k6109} cells at early anaphase and telophase showing the localization of CP190 (blue). Bar, 5 μm. (G and H) Giemsa-stained squashed preparations of polo10 bub1^{k6109} cells showing, respectively, sister chromatid separation at metaphase and a lagging chromatid (arrow) at anaphase.
Metaphase or Anaphase Arrest in polo Cells?

If plks play an essential role in the activation of cdk1, an arrest at the G2/M transition in polo9 and polo10 might have been expected. As this is not observed, it suggests either that this function is not required at this stage of Drosophila development, or it is redundant, or it can be fulfilled by the little amount of detectable Polo protein. The phenotype of polo910 is more typical of cells unable to pass beyond the metaphase–anaphase checkpoint, as they have low levels of cyclin A and high levels of cyclin B. It is generally believed that the inhibitory signal of this checkpoint arises from unaligned chromosomes and requires a complex containing the Bub1 and 3 and Mad1, 2, and 3 proteins that associates with unattached kinetochores. Mad3 binds to and inactivates Cdc20p-fizzy, a protein that directs the APC to a specific set of substrates. In yeast, one of the Cdc20-APC substrates is Pds1, whose proteolysis releases the separin Esp1 that in turn catalyses the degradation of Sec1p resulting in sister chromatid separation (for a review, see Nasmyth et al., 2000). In the presence of the Bub1 complex of checkpoint proteins, the APC cannot be activated and cells become arrested in metaphase (for a review, see Gardner and Burke, 2000), polo910 cells have Bub1 and the 3F3 epitope at their kinetochores, suggesting they are held in a state of checkpoint arrest by the Bub1 complex. This is confirmed by our observation that bub1 mutation suppresses the mitotic phenotype of polo10 and so releases the metaphase-like arrest.

However, polo910 cells exhibit a paradox that sets them apart from other metaphase arrest mutants of Drosophila. Their chromosomes appear to have undergone alignment and anaphase movements activated in that sister kinetochores appear to be not only attached through microtubules to the poles but also pulled apart by about 2–5 μm. This itself might have been expected to relieve the checkpoint arrest, and usually the 3F3 epitope is lost from chromosomes under tension. In contrast, Giemsa-stained squashed preparations reveal that only ~17% of sister chromatids remain separated in squashed preparations of polo10 brain cells made in the presence or absence of colchicine. This suggests that the majority of sister chromatids are still held together, explaining their failure to move to the spindle poles. In addition, some chromosomes appear connected in chains through telomeric linkages. Chaining of chromosomes in this way has been described previously in the mutants for the UbcD1 gene that encodes a class I ubiquitin-conjugating (E2) enzyme (Cenci et al., 1997). This observation suggests that Polo kinase may in part play a role in regulating the proteolytic system responsible for breaking such linkages.

Centromere separation seen in intact polo10 cells must be accounted for by pole-directed forces of motor proteins on the chromatids. Although several explanations are possible to resolve the paradox of the polo phenotype, the simplest is that Polo kinase is actively required to relieve the function of the Bub1 checkpoint complex in the progression through mitosis. This would have some parallels with the finding that in budding yeast, CDC5 is essential to escape the metaphase arrest imposed by DNA damage surveillance mechanisms (Toczyński et al., 1997). This hypothesis implies that the polo arrest point lies on the cusp of the metaphase–anaphase transition. This is at a later stage than the requirement for the fizzy gene product function. In fizzy mutants, neither A- nor B-type cyclins are degraded and as we show here, there is no separation of centromeric regions as seen in polo mitotic arrest. This incidentally contrasts with mutants for CDC20, the fizzy homologue of budding yeast where centromeric regions are observed to be separated (Tanaka et al., 2000). However, the transient separation of sister centromeres while chromosome arms still show cohesion does appear to reflect a general aspect of normal progression through metaphase in wild-type budding yeast (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). If similar events occur in metazoan cells, this aspect of normal progression through metaphase might be accentuated in polo cells that appear to have initiated anaphase by the criterion of the apparent poleward tension imposed at the centromeres, and yet are not released from “checkpoint” arrest.

Mitotic Exit

Polo kinase may also be required to promote complete separation of sister chromatids and for those specific aspects of APC activity that mediate cyclin B degradation. The latter would have some similarities to budding yeast where mutants in the plk gene CDC5 are reported to show no effect on Cdc20-APC function and so permit Pds1p to be degraded, in the absence of degradation of the mitotic cyclin Clb2p (Shirayama et al., 1998). Moreover, overexpression of Cdc5p results in proteolysis of Clb2p but not Pds1p, suggesting that the CDC5 plk promotes Hct1 (Schwab et al., 1997; Visintin et al., 1997; Charles et al., 1998) (fizzy related)/APC functions to regulate cyclin B levels. In fact, the phenotype of polo910 mutants resembles that of mutants expressing nondegradable cyclin B in several respects. Expression of stable cyclin B (lacking its destruction box) from a GAL4 responsive promoter in Drosophila larval neuroblasts results in a mitotic arrest in which the greater proportion of cells arrest in anaphase (Rimmington et al., 1994). In embryonic cells, the phenotype resulting from expression of stable forms of cyclins A, B1, and B3 support a sequential requirement for the three proteins: stable cyclin A leads to a metaphase delay, stable cyclin B an early anaphase arrest, and stable cyclin B3 a late anaphase arrest (Sigrist et al., 1995). Sister chromatids clearly separate in those cells expressing stable cyclin B as indicated by in situ hybridization with a dodoca-satellite probe to identify the centromeric region of the third chromatids. A role for polo in promoting those aspects of APC activity that mediate cyclin B degradation would be consistent with the observations that mammalian Plk can phosphorylate and activate the APC in vitro (Kotani et al., 1998). However, the ability of the polo10 bub1 double mutant to progress through mitosis would suggest that this APC function can also be activated at some level in the absence of polo function.

In budding yeast, there is now considerable evidence that Hct1p can be activated by the Cdc14p protein phosphatase (Alexandru et al., 1999; Fraschini et al., 1999; Gardner and Burke, 2000). Cdc14p appears itself to be activated downstream of the GTP-bound active form of Tem1p. This pathway is held in check in response to spin-
dle damage by a two component GTPase activating protein (GAP) formed between Bub2p and the Bfa1p/Byr4p protein that promotes formation of the GDP-bound state of Tem1p which does not favor Cdc14 activation. The CDC5 polo-like kinase appears to regulate the phosphorylation of Bfa1p/Byr4p in this process (Lee et al., 2001). In fusion yeast, the equivalent pathway regulates the onset of the septation process and appears to be under the control of Plo1 protein (Tanaka et al., 2001). It is of considerable interest to know the extent to which the pathway might be conserved and whether an analogous process regulates the onset of cytokinesis in metazoans. The requirement for polo for cytokinesis in Drosophila is obscured by earlier mitotic defects seen in somatic cells with all alleles. The exception is male meiosis, where it seems that spindle checkpoint pathways do not lead to metaphase arrest. However, cells from the polo_bub1 double mutant do attain high levels of polyploidy, suggestive of a failure in cytokinesis once the metaphase arrest is bypassed. It will be a future challenge to determine the extent to which this mitotic exit network has been conserved in metazoans and its relationship to the regulation of the onset of anaphase.

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