Determinants of Drosophila zw10 protein localization and function

Byron C. Williams and Michael L. Goldberg*

Section of Genetics and Development, Biotechnology Building, Cornell University, Ithaca, NY 14853-2703, USA

*Author for correspondence

SUMMARY

We have examined several issues concerning how the Drosophila l(1)zw10 gene product functions to ensure proper chromosome segregation. (a) We have found that in zw10 mutant embryos and larval neuroblasts, absence of the zw10 protein has no obvious effect on either the congression of chromosomes to the metaphase plate or the morphology of the metaphase spindle, although many aberrations are observed subsequently in anaphase. This suggests that activity of the zw10 protein becomes essential at anaphase onset, a time at which the zw10 protein is redistributed to the kinetochore region of the chromosomes. (b) The zw10 protein appears to bind to kinetochores in mitotically arrested cells, eventually accumulating to high levels within the chromosome mass. Our results imply that zw10 may act as part of a novel feedback pathway that normally renders sister chromatid separation dependent upon spindle integrity. (c) The localization of zw10 protein is altered by two mitotic mutations, rough deal and abnormal anaphase resolution, that specifically disrupt anaphase. These findings indicate that the zw10 protein functions as part of a multicomponent mechanism ensuring proper chromosome segregation at the beginning of anaphase.

Key words: kinetochore, centromere, mitosis, chromosome segregation, microtubule poison, anaphase onset

INTRODUCTION

The end result of mitosis is the equal apportionment of sister chromatids to two daughter cells. To avoid the deleterious consequences of aneuploidy, the movements of chromosomes and their interactions with the mitotic apparatus must be coordinated precisely in space and in time. One strategy to identify molecules required for proper mitotic chromosome segregation and to help define their associated biochemical activities is the study of mutations that result in altered karyotypes. A variety of methods for the isolation of such mitotic mutations in Drosophila have been developed (Gatti and Goldberg, 1991).

We have previously shown that mutations abolishing the function of one such gene, l(1)zw10 (hereafter abbreviated zw10), result in high levels of aneuploidy in larval neuroblast cells (Williams et al., 1992). Aberrations occurring during anaphase appear to be the immediate cause of chromosomal mis-segregation: many mutant anaphase figures display lagging chromatids that remain near the metaphase plate at a time when the majority of chromatids have arrived at the poles. In many cases, it appears that the separation of sister chromatids is delayed, resulting in the migration of both sister chromatids to the same pole. At times we observe anaphase figures with more severe defects, such as the appearance of grossly unequal chromosome complements at the two poles. Another facet of the zw10 mutant phenotype is ‘precocious sister chromatid separation’ (PSCS) triggered by mitotic arrest: the connection between sister chromatids in severed in mutant, but not wild-type larval neuroblast cells treated with the mitotic poison colchicine (Smith et al., 1985; Williams et al., 1992).

The relationship, if any, of this PSCS phenomenon to the generation of aneuploidy is unclear.

The zw10 protein is found at different locations at various times during the Drosophila embryonic cell cycle. It is excluded from the nuclei during interphase. The zw10 product migrates into the nuclear volume at prometaphase, eventually becoming part of a filamentous structure associated with a portion of the spindle and extending through the metaphase plate. Because the zw10 protein colocalizes with only a subset of the tubulin found in the spindle, we have suggested that zw10 is associated either with one class of microtubules (most likely the kinetochore microtubules) or with an unknown microtubule-independent structure within the spindle. Within a few seconds of anaphase onset, zw10 protein relocates to an area at or near the kinetochores of the separating sister chromatids and remains there until telophase (Williams et al., 1992).

These observations together suggest that the movement of zw10 protein to the vicinity of the kinetochores at the metaphase-anaphase transition is important for subsequent proper chromatid separation. However, these initial findings do not exclude the possibility that zw10 function is important at earlier times in mitosis. The localization of zw10 protein to different structures through mitosis could indicate that it has multiple sites of function. For example, zw10 protein could be required for the bipolar attachment of chromosomes to the spindle, for spindle assembly or integrity, for prometaphase chromosome movements, or for the formation of the metaphase plate. In addition, we have been unable to incorporate the phenomenon of PSCS into a consistent model of zw10
action, because we did not know the fate of zw10 protein in mitotically arrested cells.

In this paper, we describe more detailed analyses, both of the mutant phenotype and of the cell cycle-dependent intracellular distribution of the zw10 protein, which provide clues to the role played by zw10 in mitotic chromosome distribution. We show that in zw10 mutants the first obvious mitotic defect is seen in anaphase chromatid movement. Prometaphase centromere movement, metaphase spindle structure, and metaphase chromosome alignment all appear to be unaffected, suggesting that zw10 function is not required prior to anaphase onset.

We have furthermore made two observations of potential relevance to the PSCS phenomenon described above. First, we have found that the zw10 protein localizes to the region of the kinetochores in cells mitotically arrested by either colchicine or taxol treatment. Second, we demonstrate that PSCS in mitotically arrested zw10 mutant cells occurs in the absence of cyclin B degradation. This supports the recent finding by Holloway et al. (1993) that sister chromatid separation can occur without MPF destruction. We believe that this PSCS phenomenon indicates that the zw10 protein participates in a signalling pathway that coordinates sister chromatid separation or movement at the metaphase-anaphase transition.

MATERIALS AND METHODS

Examination of zw10 germline clone embryos

Homozygous zw10 germline clones were generated by the FLP recombinase system (Chou and Perrimon, 1992). Three null alleles of zw10 were used (zw10b1, zw10b2 and zw10b5), all of which gave the same germline clone phenotype. Briefly, the FRT site (the site of FLP-catalyzed recombination) was crossed onto the zw10 chromosome by mating y,w,FRT[w+]Y males with zw10/FM7a virgin females. The male progeny of genotype y,w,FRT[w+]/yw,FRT[w+]Y males. Female progeny from this second cross included desired recombinants with the genotype zw10,FRT[w+]/yw,FRT[w+]. These could be identified by individually mating female progeny with wild-type eye color to FM7a/Y males. If crossing-over occurs so that zw10 is on the same chromosome as the FRT site, this last cross will yield no white-eyed males. Furthermore, a stock can be established with the zw10,FRT[w+] males by a subsequent cross with FM7a/Y males.

Once such lines were established, zw10,FRT/FM7a virgin females were mated with ovoD1,FRT;FLP/FLP males in vials and their progeny heat-shocked at 37°C in a water bath for two hours at the third instar larval and pupal stages. The heat shock at 37°C activates expression of the FLP recombinase under the control of the hsp70 promoter, which acts on the paired FRT sites to catalyze mitotic

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**Fig. 1.** Non-specific spindle abnormalities in zw10 mutant germline clone embryos. Embryos were stained with Hoechst 33258 (A,C) and anti-β-tubulin antibodies (B,D; see Materials and Methods). (A,B) Wild-type syncytial blastoderm embryo, which contains a field of evenly spaced metaphase nuclei (A). In each mitotic figure, the spindle (outline arrow, B) is organized around a metaphase plate (outline arrow, A) and has been nucleated from centrosomes at opposite poles as revealed by the positions of the asters (arrowheads, B). (C,D) In zw10 germline clone syncytial blastoderm embryos, nuclei commonly are unevenly spaced. Defects in nuclear division in mutant embryos can lead to free asters (arrowheads in D; see text) unassociated with nuclei (arrowheads in C). These free asters may in turn result in the formation of tripolar spindles when they become attached to nearby nuclei (outline arrow, C and D). The uneven spacing of nuclei can lead to spindle fusions, since a single pole is shared by two spindles (long arrow, C and D). Bars, 10 μm.
crossing-over (Chou and Perrimon, 1992). Any mitotic cross-over products that can produce functional ovaries due to absence of oo D1 will be simultaneously homozygous for zw10. Females (zw10,FRT1ovo,D1,FRT;FLP) were collected and mated to sibling males (FM7a,Y;FLP), and allowed to lay eggs on yeasted grape agar plates.

Embryos were collected, fixed with formaldehyde, and stained for immunofluorescence according to previously described procedures (Karr and Alberts, 1986; Theurkauf, 1992; Williams et al., 1992). Fixed embryos were rehydrated in Tris-buffered saline + Triton (TBST; 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.02% sodium azide, 0.1% Triton X-100) and incubated with a 1:10 dilution of anti-β-tubulin monoclonal antibody (Calbiochem, La Jolla, CA) in the presence of 1 μg/ml DNase-free RNase (Boehringer Mannheim) overnight at 4°C. After washing with TBST, embryos were incubated in a 1:100 dilution (12.5 μg/ml final concentration) of FITC-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN). DNA was stained with both 1 μg/ml propidium iodide and 0.05 μg/ml Hoechst 33258 for 5 minutes, and mounted in 80% glycerol containing 2% n-propyl gallate. For examination of...
zw10 protein localization in embryonic polar body nuclei, wild-type embryos were fixed with formaldehyde and incubated with affinity-purified anti-zw10 antibodies as previously described (Williams et al., 1992).

Laser-scanning confocal microscopy was primarily carried out using a Zeiss Axiosvert 10 attached to a Bio-Rad MRC600 confocal imaging system equipped with a Krypton/Argon laser (Bio-Rad Laboratories, Cambridge, MA) at Cornell (Biotechnology Flow Cytometry and Imaging Facility), with additional images collected using a similar system in the laboratory of Professor David Glover (University of Dundee, Scotland). Image collection was performed utilizing a no. 2 neutral density filter to attenuate photobleaching, and assisted by Kalman averaging to improve the signal/noise ratio. Double fluorescence images were collected simultaneously either as two full screen images or on a split screen, and could be merged in pseudocolor using COMOS software. Images were photographed with a Screenstar camera (Presentation Technologies, Sunnyvale, CA).

**Immunostaining of brain mitotic figures**

Larval brains were dissected and fixed following the protocol of González et al. (1990), except that, after fixation in formaldehyde, brains were exposed to methanol for 2 minutes, which appeared to improve the antibody staining. This methanol treatment was not used for brains fixed for subsequent staining with anti-cyclin B antibodies. For drug effect studies, brains were treated with 0.5 μg/ml colchicine (Sigma Chemical Co., St. Louis, MO) in 0.7% NaCl for 1.5 hours, or 9 μM taxol (Molecular Probes, Eugene, OR) in 0.7% NaCl, for 1 hour at 24°C before fixation. In certain cases (described in Results), hypotonic treatment (8 minute incubation of the brains in 0.5% sodium citrate) preceded the fixation (Gatti and Goldberg, 1991). Control brains were incubated in 0.7% NaCl alone.

All antibody incubations were done at 4°C overnight in phosphate-buffered saline (PBS; formula of Karr and Alberts, 1986) containing 0.3% Triton-X (PBST), 10% fetal calf serum (FCS, Sigma Chemical Co.), DNase-free RNase (1 μg/ml), and 0.02% sodium azide (González et al., 1990). Washes were done for 30 minutes with several changes of PBST lacking FCS and RNase. Prior to staining, brains were first equilibrated in PBST, then blocked in PBST + FCS for 1 hour. For spindle staining, brains were incubated in a 1:5 dilution of anti-β-tubulin monoclonal antibody (Calbiochem), washed, and incubated in a 1:50 dilution (final concentration 25 μg/ml) of FITC-conjugated goat anti-mouse IgG (Boehringer Mannheim). For zw10 staining, brains were incubated with either crude rabbit anti-zw10 serum (1:300 dilution), mouse anti-zw10 serum (1:100) or affinity-purified rabbit anti-zw10 antibody (1:50), all of which gave identical results. After washing, the appropriate biotinylated goat anti-mouse IgG (Boehringer Mannheim). For zw10 staining, brains were incubated with either crude rabbit anti-zw10 serum (1:300 dilution), mouse anti-zw10 serum (1:100) or affinity-purified rabbit anti-zw10 antibody (1:50), all of which gave identical results. After washing, the appropriate biotinylated goat anti-rabbit or anti-mouse IgG (Vector Laboratories, Burlingame, CA) was added at a dilution of 1:400 (to a final concentration of 4 μg/ml). After washing again, brains were incubated in a 1:600 dilution of avidin-FITC (4 μg/ml final concentration; Vector Laboratories). After a final wash, DNA was stained with propidium iodide, mounted, and examined by confocal microscopy as for embryos (see above). Alternatively, rabbit anti-zw10 antibodies were detected by TRITC-conjugated anti-rabbit IgG antibodies (7.5 μg/ml; Jackson Immunoresearch) and DNA stained with YOYO-1 (benzoxazolium-4-quinolinium dimer) iodide (Molecular Probes, Eugene, OR) at 0.2 μM for 5 minutes, with identical results. BX63, a mouse monoclonal antibody that stains centrosomes (Frasch et al., 1986), and Rhb271, a rabbit polyclonal antibody against cyclin B (Whitfield et al., 1990), were gifts from D. Glover (University of Dundee, Scotland). For double labelling with anti-mouse and anti-rabbit antibodies (e.g. anti-tubulin and anti-zw10) the positions of the chromosomes were obtained by Hoechst 33258 fluorescence.

Brain squashes for immunostaining were prepared according to González and Glover (1993). Brains were fixed as above, except that incubation in the fixative was reduced to 30 minutes. Up to four brains at a time were placed in 4% acetic acid for 30 seconds, then in drops of 60% acetic acid for 3 minutes on a non-siliconized coverslip. A glass slide was placed on the coverslip and the sandwich inverted; excess liquid was removed and the brains were then vigorously squashed. After freezing the slide in liquid nitrogen, the coverslip was removed with a razor blade, the squash was dehydrated in ethanol for 30 minutes, immediately rehydrated in PBST without letting the slide dry out, and stained with antibodies as above except that incubation with the secondary antibodies was reduced to 2 hours at room temperature. When examining brains of other mitotic mutants, one wild-type brain was placed on each slide as a control for antibody staining.

**RESULTS**

The zw10 mutant phenotype and zw10 protein distribution in syncytial and cellular mitoses

Previously, we examined the phenotypic effects of zw10 mutations in larval neuroblast (brain) cells, but determined the cell cycle-dependent distribution of zw10 protein in syncytial blastoderm embryos (Williams et al., 1992). Mitosis exhibits significant differences in these two tissue types, including variations in cell cycle controls, source of mitotic components (maternal vs zygotic), and syncytial vs cellularized modes of division. To correlate these sets of observations preliminary to the studies reported here, it was thus important to analyze the phenotype and the distribution of zw10 gene product in both tissues.

**Phenotype of zw10 mutant embryos**

The first 13 nuclear divisions of *Drosophila* embryogenesis rely almost entirely on maternally supplied gene products stored in the oocyte. Although it was difficult to obtain zw10 homozygotes given their almost complete lethality, in rare instances such females can be obtained. Embryos collected from these females showed a variety of mitotic defects at the syncytial blastoderm stage, including uneven nuclear density and aberrant anaphases often containing lagging chromatids or chromatin bridging. Larger numbers of zw10-deficient embryos, necessary for more detailed examination of the mutant phenotype, can be generated in homozygous germline clones produced from zw10/zw10 heterozygous females using the FLP recombinase system (Chou and Perrimon, 1992; see Materials and Methods).

To determine the lethal phase, embryos derived from homozygous zw10 germline clones were allowed to develop, then fixed and stained with Hoechst 33258 to visualize nuclei. zw10 embryos terminated development primarily in the late syncytial blastoderm stages, though degenerating embryos apparently representing earlier developmental stages are also commonly found (data not shown). A subpopulation (15-25%) develop through later stages of embryogenesis and die, though a small fraction (1%) of them eventually hatch into larvae, most likely due to paternal rescue of zw10 function by wild-type sperm.

To examine associated nuclear division abnormalities, zw10 mutant embryos were collected at timed intervals, fixed, and stained to visualize DNA and microtubules (see Materials and Methods). In wild type, the syncytial blastoderm stage is typified by a spatially even layer of metasynchronously dividing nuclei at the surface of the egg (Foe and Alberts, 1983). Spindles are bipolar and prominent asters at the poles are seen
Drosophila zw10 protein

(Fig. 1A,B). In zw10 embryos, however, mitotic synchrony is lost. Nuclei often have a highly uneven distribution in the cortex, and free centrosomes and multipolar spindles are common (Fig. 1C,D). However, these defects are unlikely to be the direct consequence of lesions in the zw10 gene. Many mutations or drug treatments that affect virtually any aspect of nuclear division produce similar effects (Freeman et al., 1986; Raff and Glover, 1988; Sunkel and Glover, 1988; González et al., 1990; Lin and Wolfner, 1991; Girdham and Glover, 1991; Gomes et al., 1993). This is thought to be due to loss of coordination between the nuclear and centrosome cycles, which is particularly sensitive to disruption during the rapid syncytial divisions. More importantly, anaphase and post-anaphase aberrations similar to those seen in zw10 mutant brains (Williams et al., 1992) are immediately visible in zw10-deficient embryos. These include chromatin bridges between nuclei (Fig. 2B; compare to wild-type in Fig. 2A), unequal segregation of chromatids at anaphase, and lagging chromatids at anaphase (Fig. 2B). We believe these phenomena to be the primary defects associated with the absence of zw10 function in embryos. Additional aspects of this embryonic phenotype are discussed below.

Localization of zw10 protein in neuroblast cells

We have characterized the distribution of zw10 antigen during mitosis in wild-type neuroblast cells by examining fixed, whole-mounted brains stained with anti-zw10 antibody using confocal microscopy. The localization of zw10 protein is essentially identical to that previously described for syncytial blastoderm embryos (Williams et al., 1992). The zw10 protein remains primarily cytoplasmic at interphase and prophase (not shown). By metaphase, it becomes associated with, or forms, filaments that pass through the central region of the metaphase plate (Fig. 3A-C). At anaphase, zw10 is found almost exclusively at the leading edges of the chromosomes in the region of the kinetochores (Fig. 3D,E) until telophase when it is dispersed into the cytoplasm (not shown). Importantly, none of these structures was recognized by anti-zw10 antibodies in brains of zw10<sup>S1</sup>/Y larva (not shown), which do not contain zw10 protein on Western blots (Williams et al., 1992). This last observation confirms that these structures do indeed contain zw10 protein.

In summary, these results combined with previously published observations (Williams et al., 1992) show that the zw10 mutant phenotype and the cell cycle-dependent distribution of zw10 protein are similar in two very diverse types of tissue. The biochemical function of zw10 is therefore likely to be the same in many kinds of cell division.

Organization of mutant mitoses prior to anaphase

One of the central goals of this study was to determine whether...
absence of zw10 function disrupts processes that occur prior to the onset of anaphase. Are there any obvious abnormalities in the migration of chromosomes to the metaphase plate or in metaphase spindle structure in either zw10-deficient embryos or in mutant neuroblast cells?

Metaphase structures in zw10-deficient embryos
As noted above, embryos devoid of zw10 activity contain many free centrosomes, which appears to be a non-specific consequence of disrupted nuclear division. These free centrosomes are often shared between closely adjacent nuclei, leading to ‘frayed’ or fused tri- and multi-polar spindles. Thus, in order to assess the direct effect of zw10 mutations on metaphase plate formation and the spindle in embryos, we restricted our observations to nuclei that were in regions of normal nuclear density and in which the spindles were clearly

Fig. 5. Metaphases in zw10 mutant brains. Metaphase plate (blue; arrows) and spindle formation (green) were assessed in whole mounted brains examined by confocal microscopy. Single optical sections from typical wild-type (A) and zw10S1/Y (B,C) neuroblasts are shown; no differences between the two are apparent. Head-on views of the metaphase plate in zw10 brains (C) show no alteration in the orientation of chromosomes (compare with wild-type in Fig. 3B,C above). Anti-tubulin staining of the mitotic

Fig. 6. Cyclin B levels in wild-type and zw10 brains treated with colchicine. Cyclin B abundance in mitotically arrested colchicine-treated neuroblasts appears to be the same in (A) wild-type and (B) zw10/Y mutant brains. Brains were treated with colchicine as described (see Materials and Methods) and immunofluorescence performed to show chromosomes (blue) and cyclin B (purple). Wild-type and zw10/Y brains were incubated with colchicine for the same duration, and all conditions for staining were identical. Images were collected using the same settings on the confocal microscope and merged without adjustment. Individual metaphase-arrested cells (arrows) are recognized by their highly condensed chromosomes. Bars, 10 μm.

Fig. 7. Polar body localization of zw10 protein. Early embryos were collected and fixed (see Materials and Methods), and stained with propidium iodide to visualize chromosomes (blue), and with affinity-purified anti-zw10 antibody to locate zw10 antigen (red). (A,B,C) are superimposed views of zw10 staining and chromosomes in single optical sections of polar bodies visualized by confocal microscopy. Arrows point to relatively clear instances of zw10 antigen localization to regions at or near kinetochores. Bars, 5 μm.
nucleated from only two centrosomes. In these cases, the spindles are comparable to wild-type in appearance (Fig. 4A-D). All chromosomes congress to a tight metaphase plate; we see no evidence of DNA that is unassociated with the metaphase plate.

Metaphase structures in zw10 larval neuroblasts
Because of the complications introduced by free centrosomes in syncytial mutant embryos, we wished to confirm our impressions about spindle and metaphase plate organization by examining these structures in third instar larval brain tissue. In this cellularized material, centrosomes cannot be shared by adjacent cells, thus clarifying the analysis.

To this end, confocal microscopy of fixed, whole-mounted zw10 brains were used to examine metaphase structures in three dimensions. We looked in particular for chromosomal abnormalities (defects in metaphase plate formation or the presence of maloriented chromosomes) and mitotic spindle defects (monopolar or multipolar spindles or aberrations in gross morphology). Examination of a large population of cells in 40 larval brains revealed that the overall appearances of the metaphase plate and spindle in zw10 brains are comparable to those in wild-type brains (Fig. 5A,B). Head-on views of the metaphase plate in zw10 brains are particularly revealing, showing the normal chromosome configuration with centromeres pointed inward, which are typically seen in wild-type (Fig. 5C). We have never observed chromosomes that are clearly unattached to the metaphase plate. Operationally, this could be distinguished only if individual chromosomes were separated from the metaphase plate by more than approximately one-fifth of the distance from the main mass of chromosomes to the pole. Such events were rarely, if ever, observed in zw10 mutant brain metaphases. However, it should be noted that subtle defects in zw10 brains, such as chromosomes at the metaphase plate that are unconnected to kinetochore microtubules, or effects on small subpopulations of microtubules,
would not have been detectable in these immunofluorescence experiments.

Interphase and prophase microtubule distribution in zw10 mutants appears to be normal as well; centrosome localization is not obviously altered in any stage of the cell cycle (not shown). In contrast, many anaphases in whole-mounted brains contain lagging chromatids, as previously seen in squashed preparations (Williams et al., 1992). The shape of these lagging chromatids, indicative of kinetochore microtubule attachment, suggests strongly that they are being translocated towards the pole, albeit in a delayed fashion. Otherwise, anaphase spindle morphology appears to be normal (not shown).

Effect of mitotic arrest on zw10 localization

Colchicine treatment

Colchicine, an inhibitor of microtubule assembly, causes cell cycle arrest in wild-type brain cells; this block is generally thought to be at prometaphase (Rieder and Palazzo, 1992). Colchicine treatment, followed by a short incubation in hypotonic solution, has long been used for examining karyotypes in many organisms (Hsu, 1952; Gatti and Goldberg, 1991). Chromosomes in cells treated in this manner are highly condensed and do not assemble into a compact metaphase plate. The sister chromatids of these chromosomes appear in squashed preparations to remain attached at the centromeres, suggesting that centromere separation is normally dependent upon the action of the mitotic spindle (González et al., 1991). In contrast, zw10 mutant brains treated in the same way exhibit precocious sister chromatid separation (PSCS); sister chromatids are separated from each other in a high proportion (30-40%) of mutant cells (Williams et al., 1992).

In order to understand the PSCS phenomenon in terms of possible models for zw10 action, we used confocal microscopy to determine where the zw10 product is found in colchicine-treated wild-type cells. Unexpectedly, this localization primarily consists of very intensely staining concentrations of zw10 antigen around, on, and between chromosomes (Fig. 6A-C). Localization in the vicinity of kinetochores is often clearly visible in whole mounts, especially with shorter times of incubation of brains in colchicine (Fig. 6D). This effect is particularly noticeable in squashed preparations, where the locations of the centromeres are more easily distinguished (Fig. 6E).

Further characterization of these colchicine-treated cells provides additional evidence that they are indeed in classical prometaphase arrest. No organized spindles are observed; in addition, centrosomes do not separate to opposite poles of the cell (not shown).

Taxol treatment

Taxol, a drug that stabilizes microtubules and therefore promotes microtubule assembly, is known to cause mitotic arrest in mononuclear blood cells (Sondell et al., 1988). We wished to determine whether taxol treatment of zw10 mutant Drosophila brains would induce the PSCS phenomenon. When zw10/Y brains are treated with 9 μM taxol for one hour, followed by a short hypotonic shock, a far higher proportion of nuclei display PSCS (60% of cells) than is the case in cells in similarly treated wild-type brains (7%). We therefore suggest that PSCS is a generalized response to mitotic arrest in the absence of the zw10 gene product.

The state of cells arrested by taxol is different in many respects from those arrested with colchicine. In wild type and in zw10 mutant brains treated with taxol, mitotically arrested cells contain large asters averaging 5-7 μm in diameter. Each cell usually contains one aster; the condensed chromosomes encircle or lie off to one side of the monoaster (Fig. 6F). In 69% of mitotically arrested cells, these large asters are associated with unseparated or unduplicated centrosomes (not shown). In the remainder (31%), an abnormally short separation (0.5-2.0 μm) of centrosomes was observed, compared with the approximately 10 μm separation of centrosomes at metaphase in untreated brains (not shown). These observations are in agreement with studies in other organisms, where taxol has been reported to produce large monoasters and to prevent the movement of centrioles to opposite poles (Daub and Hauser, 1988; Rao et al., 1989; Buendia et al., 1990).

In spite of the differences in the appearance of the taxol- and colchicine-arrested cells, the zw10 antigen is localized to essentially the same positions in both types of drug-treated brains. High concentrations of zw10 protein are again observed within the chromosomal mass of taxol-arrested cells (Fig. 6G). Occasionally, localization to the kinetochore regions of individual chromosomes can be seen (Fig. 6H). It is important to note that, with taxol treatment, zw10 protein preferentially localizes to the chromosomal region rather than to the extensive array of microtubules in the monoaster (Fig. 6I).

Cyclin B levels in mitotically arrested zw10 brains

One possible explanation of the PSCS phenomenon is that drug-treated zw10 mutant (but not wild-type) cells actually transit through anaphase onset, as signified by their separated sister chromatids. The cyclin B-cdc2 kinase complex (MPF or maturation-promoting factor) would thus be expected to be inactivated in these cells, an event that accompanies anaphase onset (Whitfield et al., 1990). However, the subsequent events of anaphase, such as chromatid movement, could not occur because of the absence of spindle function in the presence of microtubule poisons.

In this scenario, the zw10 protein’s normal function would be to provide feedback control, ensuring that cells stay in metaphase until assembly of the spindle and metaphase plate is complete; the existence of such controls has previously been inferred (Murray and Kirschner, 1989; Rieder and Alexander, 1989). The bub and mad gene products in Saccharomyces cerevisiae provide a precedent for this model of zw10 action. In wild-type yeast, treatment with the microtubule poison benomyl arrests the cell cycle in a metaphase-like state characterized by high MPF levels. When yeast carrying mutations in their bub or mad genes are incubated in benomyl, the cells exit from mitosis and MPF levels are not sustained. Because the spindle is compromised by the drug, massive nondisjunction and cell death result (Li and Murray, 1991; Hoyt et al., 1991).

To test if zw10 mutants were defective in this type of feedback control of anaphase onset, levels of cyclin B protein (the labile component of MPF that is degraded at the metaphase-anaphase transition; Whitfield et al., 1990; Hunt et al., 1992) were assayed by indirect immunofluorescence. Fig. 7A verifies previous reports (Whitfield et al., 1990) showing that cyclin B levels remain high in colchicine-treated wild-type brain cells that contain condensed chromosomes characteristic
of mitotic arrest. In zw10 brains incubated in colchicine, cyclin B levels are approximately equivalent to those in treated wild-type cells (Fig. 7A,B). Furthermore, elevated cyclin B levels are observed in both wild-type and zw10 neuroblasts arrested with taxol (not shown). Thus, PSCS in mutant brains is unlikely to result from a feedback control defect in which events dependent upon MPF degradation occur in the absence of a functional spindle.

zw10 localization in mitotically arrested polar bodies

As described above, one means of studying the influence of mitotic arrest on zw10 localization is treatment with microtubule poisons. Another method is to examine zw10 in naturally occurring mitotically arrested nuclei, such as those of the early embryonic polar bodies. Polar bodies are the three haploid products of meiosis that remain in the egg after formation of the female pronucleus (Doane, 1960; Campos-Ortega and Hartenstein, 1985; Matthews et al., 1993). They move to the egg cortex and become arrested at metaphase, often coalescing with one another so that sometimes only one or two discrete polar bodies are seen (Doane, 1960). The chromosomes in a polar body are arranged with their centromeres pointing inwards and telomeres directed towards the periphery, suggesting a monopolar spindle (Hatsumi and Endow, 1992). In our hands, anti-tubulin staining shows MTs originating at or near the chromosomes and extending a short distance into the surrounding cytoplasm. At the surface of the embryo, the polar body is “capped” by a proliferation of MTs (not shown).

The zw10 protein is localized at or near kinetochores on polar body chromosomes (Fig. 8A-C). When compared with the mitotically dividing nuclei at the syncytial blastoderm stage, the immunofluorescent signal is much greater on polar body chromosomes, suggesting a higher concentration of zw10 protein. The polar body chromosomes are condensed and presumably in a metaphase-arrested state, so it is possible that this staining pattern reflects the concentrated localization of zw10 in larval neuroblast cells arrested in mitosis by spindle poisons (see above).

Effect of other mitotic mutations on zw10 localization

To determine the role of the zw10 gene product with respect to the action of other proteins important in chromosome segregation, the localization of the zw10 protein was examined in brains of other Drosophila mitotic mutants. In particular, we closely examined the effects of mutations in three genes required for proper chromatid behavior during anaphase: namely, rough deal (rod; Karess and Glover, 1989), lodestar (Girdham and Glover, 1991), and abnormal anaphase resolution (aar; Gomes et al., 1993). At least 50 larval brains homozygous for each mutation were examined for the localization of zw10 protein during the cell cycle. These observations are described for each mutant in the following sections.

rough deal (rod)

rod mutations result in levels of aneuploidy comparable to those of zw10, and analogous anaphase defects such as lagging chromatids are commonly observed (Fig. 9B; Karess and Glover, 1989). Unlike zw10, however, nuclear bridging (where a strand of chromatin stretches continuously from one nucleus to the other during anaphase/telophase) is often seen in rod brains; also, PSCS does not result when rod brains are exposed to colchicine (Karess and Glover, 1989).

rod mutations severely affect zw10 protein localization. In brains of larvae homozygous for either weak (X-6) or strong (H4.8, X-5) alleles of rod, proper metaphase and anaphase zw10 staining was greatly diminished or abolished (Fig. 9A,B; compare with wild-type, Fig. 3A-F). In addition, the intense staining of zw10 antigen near kinetochores seen in colchicine-treated wild-type brains (Fig. 6A-E) was clearly not evident in colchicine-treated rod brains (Fig. 9C). Mutant rod alleles do not affect the overall accumulation of the zw10 protein in the larva, as detected on Western blots (Fig. 10). The zw10 protein in rod neuroblasts is distributed uniformly throughout the extranuclear volume (Fig. 9A). Thus, rod mutations most likely result in defective zw10 protein aggregation or localization.

lodestar

Like rod, mutations at the lodestar locus also affect anaphase. The primary defect of lodestar mitoses is the failure to separate chromatin at anaphase, resulting in chromatid bridging and fragmentation. The lodestar gene encodes a member of the helicase superfamily (Girdham and Glover, 1991). Our examination of lodestar mutant brains revealed that zw10 localization was similar to that in wild-type throughout mitosis; even anaphases with tangled chromatids contained the zw10 protein correctly localized to the kinetochore regions (Fig. 9D). However, zw10 protein was not localized on chromatin that remained behind at the former position of the metaphase plate during anaphase (Fig. 9E). The chromatin most likely represents chromosome fragments lacking centromeres previously described in lodestar anaphases (Girdham and Glover, 1991). In accordance with this idea, such chromatin exhibits no consistent poleward orientation, as would be expected if it contained centromeres capable of attachment to the spindle (Fig. 9E). These findings suggest that the lodestar helicase is probably not required for proper zw10 localization during metaphase and anaphase.

abnormal anaphase resolution (aar)

The aar1 mutant allele causes high levels of chromatin bridging and lagging chromatids at anaphase. The latter class of aar1 anaphase figures are identical in appearance to those seen in zw10 mutants and have been demonstrated to contain complete centromeres and telomeres (Gomes et al., 1993; Mayer-Jaekel et al., 1993). The aar gene encodes the 55 kDa regulatory subunit of protein phosphatase 2A involved in the metaphase-anaphase transition (Mayer-Jaekel et al., 1993).

The localization of zw10 protein was altered in aberrant aar1 anaphase figures. Typically, chromatids that have moved properly to the poles show zw10 occupying its normal position at the kinetochore regions, but chromatids or chromosomes lagging at the metaphase plate completely lack zw10 staining (Fig. 9F-H). In aar1 anaphases, chromosome bridges, which have been shown to contain intact centromeres (Mayer-Jaekel et al., 1993), also lack zw10 antigen at the expected locations (Fig. 9D). In aar1 brains, localizations of zw10 at other stages in mitosis and during colchicine-induced mitotic arrest, were comparable to those in wild-type (not shown). Thus, the mis-localization of zw10 antigen in aar1 cells appears to be specific to chromosomes that mis-segregate during anaphase.
DISCUSSION

The role of the zw10 protein in syncytial and cellularized mitoses

The experiments reported previously (Williams et al., 1992) and those in this paper demonstrate that both the distribution of the zw10 protein and the phenotype resulting from the absence of zw10 gene product are similar in syncytial embryos and in cellularized larval neuroblasts. Thus, the zw10 protein plays a consistent and important role in governing accurate mitotic chromosome segregation. This role is similar in both syncytial and cellularized divisions, which differ in several aspects of cell cycle control. Maternally and zygotically supplied zw10 proteins thus appear to have identical properties.

Time and location of zw10 action

Our evidence strongly suggests that zw10 function is first required at anaphase onset. Null mutations in the zw10 gene appear to have no obvious effect on either the morphology of metaphase spindles or the congression of chromosomes to the metaphase plate in the two tissue types examined. It is nonetheless conceivable that the absence of zw10 activity could cause subtle though important disruptions in structures or processes prior to the start of anaphase. Moreover, it is possible that zw10 function might be required prior to anaphase onset, but the effects of this activity would be undetectable until anaphase onset. Despite these caveats, we believe it most likely that the role of the zw10 protein first becomes critical at the metaphase-anaphase transition. This protein undergoes a rapid relocalization within a few seconds of anaphase onset (Williams et al., 1992), suggesting a mechanism by which mutant anaphase figures can display aberrations while mutant metaphase figures appear normal.

According to this hypothesis, the localization of zw10 antigen to spindle-associated filaments during normal metaphase would essentially be for the purpose of sequestering this protein from its normal site of action near or at kinetochores, thereby preventing zw10 function until anaphase onset. In support of this notion, our findings suggest that the intrinsic affinity of zw10 protein is for the centromere/kinetochore. Localization of zw10 product to the spindle-associated metaphase filaments is clearly not an obligatory precondition for subsequent movement to the chromosomes, as zw10 protein is concentrated in the chromosomal mass even when spindles are depolymerized by colchicine treatment. Moreover, when faced with a decision between chromosomes and a large population of monoaster microtubules in taxol-poisoned cells, zw10 protein chooses the chromosomes.

The idea that zw10 protein plays no essential role prior to anaphase will remain speculative until we understand at the ultrastructural level where it is found in the region of the metaphase spindle. We have previously suggested that during metaphase, zw10 is a component of kinetochore microtubules, consistent with its location in the central portion of the spindle and with its rapid relocalization to kinetochore regions at anaphase onset (Williams et al., 1992). However, it is by no means clear that this supposition is correct. There is some evidence for the existence of non-microtubule-associated structures in the spindle (Paddy and Chelsky, 1991; Steffen and Linck, 1992). In addition, preliminary results indicate that zw10 is not purified on microtubule affinity columns (B. Williams, J. Raff and T. Karr, unpublished). It should be noted in particular that our indirect immunofluorescence studies to date have insufficient resolution to dismiss the possibility that some fraction of zw10 protein is located at the centromere/kinetochores during prometaphase or metaphase, even if the majority of the antigen at metaphase is found extra-chromosomally on the spindle-associated filaments.

The zw10 protein and PSCS

PSCS occurs in zw10 mutant brain cells treated with colchicine or taxol, both of which cause mitotic arrest. We propose that the occurrence of PSCS in the absence of zw10 protein function reflects the unusual localization of zw10 protein in arrested cells. Our results suggest that zw10 first localizes to the kinetochores and accumulates there over time, forming large aggregations of zw10 antigen around the chromosomes. This accumulation does not result from an increase in zw10 protein synthesis, since the zw10 protein levels in untreated and colchicine-treated brains are indistinguishable on Western blots (data not shown). It should be noted that other molecules, including calmodulin, the ‘32-9’ antigen, and the INCENP proteins, also redistribute to kinetochores after drug-induced mitotic arrest (Welsh and Sweet, 1989; Balczon et al., 1990; Earnshaw and Cooke, 1991).

At least in a formal sense, the chromosomal aggregation of zw10 protein in mitotically arrested cells acts as part of a checkpoint control that holds sister chromatids together when the spindle is disrupted by microtubule poisons. Because cyclin B levels remain high in these cells, it is unlikely that the zw10

Fig. 9. Effects of rod, lodestar and aar mutations on zw10 protein localization. Larval brains from rodH4.8 (A-C), lodestarH79.2 (D-E), and aar (F-I) mutant homozygotes were fixed and stained (see Materials and Methods) to detect zw10 (red) and DNA (blue). Regions of overlap between zw10 and DNA appear white. (A) Metaphase cell from rodH4.8 showing chromosomes situated on the metaphase plate (arrow) accompanied by absence of zw10 staining in filaments that pass through the metaphase plate (see Fig. 3A). Instead, zw10 is diffuse in the cytoplasm. (B) Anaphase figure from rodH4.8 (Kares and Glover, 1989) showing a lagging chromatid (open arrow). zw10 protein is absent from kinetochore regions of all chromatids. (C) Colchicine-treated rodH4.8 brain showing two cells in mitotic arrest as indicated by their highly condensed chromosomes (open arrows). zw10 protein does not accumulate in high levels around kinetochores as wild-type (Fig. 6A-E) but instead is scattered in the cytoplasm. (D-E) Anaphase figures from lodestar brains (Girdham and Glover, 1991) displaying chromatin tangling (D, open arrow) and chromatin filaments that remained at the metaphase plate (E, open arrow). In both instances, zw10 protein is present at the kinetochore regions of properly situated chromatids (filled arrows in D,E). (F-H) Anaphase figures from aar1 brains with lagging chromatids (described by Gomes et al., 1993). Chromatids that have moved properly to the poles contain zw10 on the kinetochore regions (filled arrows) while lagging chromatids completely lack zw10 staining (open arrows). (I) aar2 anaphase figure with a stretched chromosome (chromosome bridge) between the two poles (arrow). Whereas zw10 is properly localized on separated chromatids at the poles, it is absent from the entire length of the chromosome bridge. Similar chromosome bridges in aar4 anaphases have been shown to contain two centromeric regions (Mayer-Jaekel et al., 1993). Bars, 5 μm.
proteins make cell cycle progression dependent upon spindle integrity, a checkpoint that is abolished by the yeast mad and bub mutations (Li and Murray, 1991; Hoyt et al., 1991). However, Holloway et al. (1993) have recently shown that sister chromatids can separate even if MPF remains active. Our finding that PSCS occurs while cyclin B levels remain high verify their conclusions, at least in zw10 mutant cells. Thus, it may be possible that the zw10 product participates in a previously unknown type of feedback control that renders sister chromatid separation, but not other events that occur at or subsequent to anaphase onset, sensitive to the state of the spindle.

The rod+ product is required for zw10 localization

Mutations in the Drosophila mitotic gene rough deal (Karess and Glover, 1989) eliminate proper zw10 protein localization in untreated and in colchicine-treated cells. This effect appears to be quite specific. Global accumulation of zw10 protein is not reduced, nor is this due to general disruptions in the mitotic apparatus: we have confirmed previous findings (Karess and Glover, 1989) that rod metaphase and anaphase spindles have normal morphology (data not shown). The complete inability of zw10 to localize in rod mutants is unique among a large number of Drosophila mitotic mutants examined (B. Williams and M. L. Goldberg, unpublished results).

The phenotypic effects of rod and zw10 mutations have strong similarities: both cause disruptions in anaphase leading to high levels of aneuploidy. Preliminary examination of zw10;rod double mutants shows that the levels of aneuploidy are no worse than in either mutant alone, suggesting that both genes may be part of the same pathway (R. Karess, personal communication). We speculate that the rod protein may be directly responsible for the attachment of zw10 protein to specific sites within the spindle or on the kinetochore. It should be noted, however, that the rod phenotype cannot be completely explained by mislocalization of zw10 protein because rod mutations do not appear to cause PSCS (Karess and Glover, 1989).

zw10 protein is missing from mis-segregating chromosomes in aar anaphase figures

The aar gene encodes the 55 kDa regulatory subunit of the serine/threonine-specific protein phosphatase 2A (Mayer-Jaekel et al., 1993). Mutations in the aar gene result in the appearance of lagging chromosomes and chromosome bridges at anaphase (Gomes et al., 1993), which have been shown by in situ hybridization procedures to contain intact centromeres and telomeres (Mayer-Jaekel et al., 1993). It is remarkable that zw10 protein is absent from the kinetochore region of these missegregating chromosomes, while it is present on chromosomes that have properly migrated to the poles of the anaphase spindle. This observation underscores the importance of the zw10 protein for coordinating chromosome segregation.

Dephosphorylation of many mitotic phosphoproteins occurs at anaphase onset (Vandere and Borisy, 1989). It thus seems likely that the phosphorylation state of the zw10 protein, or of some other component with which it interacts, is necessary for the movement of zw10 protein to the kinetochore region and its function there. Our results imply that protein phosphatase 2A participates in such a dephosphorylation event. It is surprising that the consequences of defects in dephosphorylation are highly compartmentalized: zw10 protein is apparently completely absent from particular chromosomes, yet it is present on others. This suggests that highly cooperative interactions are required for zw10 localization to the kinetochore region.

Possible functions of the zw10 protein

The cytological behavior of zw10 mutants (Williams et al., 1992; this paper) clearly indicates that the zw10 gene product plays an essential role in ensuring the proper segregation of chromosomes during mitosis. The precise biochemical function of this protein must be consistent with its site and timing of action. In this paper, we present evidence that the location of zw10 activity is at the centromere/kinetochore. Normally, the function of this protein is not required until anaphase onset, correlating with its movement to the centromere/kinetochore at this stage of mitosis. However, it appears that the zw10 protein can have an effect prior to the beginning of anaphase when the cell cycle is arrested by treatment with microtubule poisons. Presumably, these cells are blocked in a state similar to metaphase, as evidenced by their condensed chromosomes and elevated levels of cyclin B. However, in this abnormal circumstance, zw10 protein apparently again finds its way to the centromere/kinetochore, where we suggest that its activity is manifested.

These observations are most simply explained by a role for zw10 either in sister chromatid separation or in anaphase chromatid movements. The latter possibility appears unlikely because we have been unable to observe the presence of tubulin at kinetochores in colchicine-treated Drosophila neuroblasts (not shown). Chromosome movement is dependent on kinetochore microtubules, yet the absence of zw10 protein has phenotypic consequences (i.e. PSCS) in cells without obvious microtubules. However, it remains possible that we were
unable to detect a small population of short microtubules attached to the kinetochores in colchicine-treated cells; such microtubules have been observed in colchicine-treated mammalian and plant cells (Mitchison and Kirschner, 1985; Utrilla et al., 1989; Meininger et al., 1990).

Two considerations must be kept in mind while evaluating any potential explanation of zw10 action, including the hypothesis that we favor: that zw10 participates in the process of sister chromatid separation. First, mutants lacking zw10 gene product show no evidence of a mitotic block in any stage of the cell cycle (Williams et al., 1992). This suggests that zw10 is required for the timing and/or accuracy of chromosome segregation, but is not essential for transit through the cell cycle or for chromosome distribution to occur at all.

A second conceptual difficulty in understanding the role of the zw10 protein is that zw10 mutations appear to have opposite effects in the absence or presence of microtubule poisons. For example, untreated zw10 mutant cells contain anaphase figures suggesting a delay in sister chromatid separation, while mitotically arrested zw10 mutant cells show PSCS. In another view of the same phenomenon, if the arrival of zw10 protein at the centromere/kinetochore of wild-type untreated cells could be imagined to trigger sister chromatid separation, then the presence of zw10 at these structures must inhibit sister chromatid separation in drug-arrested wild-type cells. PSCS in mutants is thus paradoxically associated with the absence of zw10 product from the centromere/kinetochore. One way to reconcile these observations is to suggest that zw10 activity can vary with the cell cycle. The zw10 product, or some other molecule it associates with, might be phosphorylated during normal or drug-blocked metaphase, but then become dephosphorylated at anaphase. This could lead to very different effects when the protein reaches the centromere/kinetochore normally at anaphase onset, or artefactually during drug-induced metaphase arrest. The zw10 protein contains at least one potential phosphorylation target; we are currently investigating whether this site is indeed employed to regulate zw10 activity.

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