Orbit, a Novel Microtubule-associated Protein Essential for Mitosis in Drosophila melanogaster

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Abstract. We describe a Drosophila gene, orbit, that encodes a conserved 165-kD microtubule-associated protein (MAP) with GTP binding motifs. Hypomorphic mutations in orbit lead to a maternal effect resulting in branched and bent mitotic spindles in the syncytial embryo. In the larval central nervous system, such mutants have an elevated mitotic index with some mitotic cells showing an increase in ploidy. A morphic alleles show late lethality and greater frequencies of hyperploid mitotic cells. The presence of cells in the hypomorphic mutant in which the chromosomes can be arranged, either in a circular metaphase or an anaphase-like configuration on monopolar spindles, suggests that polyploidy arises through spindle and chromosome segregation defects rather than defects in cytokinesis. A role for the Orbit protein in regulating microtubule behavior in mitosis is suggested by its association with microtubules throughout the spindle at all mitotic stages, by its copurification with microtubules from embryonic extracts, and by the finding that the Orbit protein directly binds to MAP-free microtubules in a GTP-dependent manner.

Key words: mitosis • microtubule-associated protein • Drosophila melanogaster • mitotic spindle • centrosome

Introduction

The requirement for microtubules in the mitotic spindle is self-evident, and yet the role of nonneuronal microtubule-associated proteins (MAPs) in its function is poorly understood (for review, see Hyman and Karsenti, 1996). Formation of the spindle requires that the interphase microtubule network be reorganized as a result of an increase in microtubule turnover at mitotic entry. This is possibly due to the dynamic instability of microtubules that oscillate between periods of growth and shrinkage and is due to an increase in the frequency of transitions from the polymerization to depolymerization phases, the frequency of catastrophe (for review, see Desai and Mitchison, 1997). How the catastrophe rate increases at the onset of mitosis has been puzzling, as most known MAPs have the property of stabilizing microtubules. However, recent studies have identified proteins that promote microtubule catastrophe, such as op18 or stathmin, the microtubule-severing ATPase katanin, and the kin I family of kinesins (Belmont and Mitchison, 1996; Hartman et al., 1998; Desai et al., 1999).

Studies on the mitotic roles of MAPs have concentrated upon the use of Xenopus as a model system as it offers advantages for experimentation in vitro. This work has identified several MAPs that localize to the mitotic spindle, including XMAP230, MAP4, XMAP215, and XMAP310 (Gard and Kirschner, 1987; Andersen et al., 1994; Vasquez et al., 1994; Ookata et al., 1995; Andersen and Karsenti, 1997; Charrasse et al., 1998) and offers the possibilities for direct studies of their effects upon microtubule dynamics.

We have chosen to search for mitotic regulators in Drosophila melanogaster, which offers the possibility of studying the effects of mitotic mutations within the intact cell. The characterization of maternal-effect mutants of Drosophila is a powerful route towards the identification of such genes. In many cases, maternal-effect mitotic defects reflect a specific requirement for the product of the affected gene for cell division throughout development. The proteins encoded by such genes may have either regu-
latory roles or be part of the structural components of the mitotic apparatus. The protein kinases encoded by the polo and aurora (aur) genes, for example, were first identified through hypomorphic mutations, which, when homozygous in the mother, result in gross mitotic defects within the embryo (Sunkel and Glover, 1988; Glover et al., 1995). However, the functions of these kinases in cell division throughout development has been revealed through the study of series of mutant alleles that show developmental arrest at different stages. Sullivan et al. (1993) identified a number of maternal-effect mutations affecting the cytoskeletal organization of syncytial embryos. One of these, nuclear fallout (nuf), encodes a protein that concentrates at the centrosomes during prophase and is cytoplasmic during the rest of the nuclear cycle (Rothwell et al., 1998). However, none of the genes originally identified by maternal-effect mutations have yet been shown to encode a MAP, although the potential for identifying such mutants is evident. In fact, some alleles of abnormal spindle (asp), which encodes a protein associated with the polar regions of the mitotic spindle, exhibit a maternal effect on syncytial mitoses (Gonzalez et al., 1990; Saunders et al., 1997).

A number of biochemical approaches to identify Drosophila MAPs also take advantage of the maternal dosage of proteins essential for the syncytial mitoses. One strategy has been to use libraries of mAbs to search for proteins that display dynamic patterns of localization during the mitotic cycle (Frasch et al., 1986). This set of antibodies was successful in identifying genes encoding a Drosophila homologue of the vertebrate regulator of chromatin condensation (RCC1; Frasch, 1991), and a centrosomal antigen known now as CP190 (Whitfield et al., 1989), and then raise antibodies against individual proteins. Some 50 proteins were identified that would bind to microtubules and mAbs raised to 24 of them. One of the proteins, CP190, which in turn was used as an affinity reagent to identify a second centrosomal associated antigen, CP60 (Kellogg and Alberts, 1992). The function of the majority of these proteins still remains uncertain because of the lack of mutations or assays of their molecular function.

We have continued the direct genetic approach in a search for mutants that identify genes encoding MAPs, expecting that these would give rise to spindle defects in the syncytial mitoses, and also show defective cell divisions at other developmental stages. In this paper, we report the characterization of one such novel gene, orbit. We show that orbit encodes a novel 165-kD MAP and discuss possible functions for this protein suggested by the phenotypes of an allelic series of orbit mutants.

Materials and Methods

Immunofluorescent Staining of Embryos

Immunostaining of embryos from wild-type or mutant flies was carried out either as described by Gonzalez and Glover (1993) or by fixing dechorionated embryos with freshly prepared 4% paraformaldehyde in buffer B (45 mM KCl, 15 mM NaCl, 10 mM phosphate buffer, pH 6.8) at room temperature for 5 min and a further 25 min at 4°C. Microtubules were detected with the rat anti-α-tubulin antibody, YL1/2 (Sera-Lab) and either an FITC or Texas red-conjugated anti-rat IgG antibody (Jackson Laboratories). Centrosomes were revealed with the polyclonal rabbit serum, P238 (Whitfield et al., 1989) and Cys-conjugated secondary anti-rabbit IgG antibody (Jackson Laboratories). Orbit was localized using an affinity-purified rabbit antibody against residues 1-632 (see below) diluted 1:100 in PBS containing 0.1% Triton X-100, and an FITC-conjugated anti-rabbit IgG antibody. DNA was stained with 1 μg/ml of propidium iodide. The preparations were observed using either an MRC 600 or MRC 1024 laser confocal microscope (Bio-Rad). Images were processed and merged in pseudocolor using Photoshop version 5 (Adobe Systems).

Cytological Analysis of Larval Central Nervous System and Chromosome In Situ Hybridization

Wild-type and mutant larval CNSs were fixed and squashed in acetocarmine, and whole-mount preparations of the larval CNSs were carried out as previously described (Inoue and Glover, 1998). For in situ hybridization, polytene chromosomes were prepared from salivary glands of late third instar larvae heterozygous for orbit and wild-type. Biotin-labeled probe was prepared from the P-lacW plasmid. Hybridization and signal detection were performed as described earlier (Deak et al., 1997). The specimens were examined using phase-contrast optics and hybridization signals were assigned to chromosome bands referring the revised salivary chromosome maps of Heino et al. (1994).

Reversion Analysis of orbit

Reversion analysis to test whether the P-lacW integrated at 78C is responsible for sterility and mitotic phenotypes of orbit were carried out by mating y w; orbit/ TM3, 5B 2; orbit w; y w females to w; TM6C, 2; orbit w; y w males. A total of 270 F1 progenies were scored for proper spacing, growing, and motility. None of the first to be cloned also proved to be CP190, which in turn was used as an affinity reagent to identify a second centrosomal associated antigen, CP60 (Kellogg and Abers, 1992). The function of the majority of these proteins still remains uncertain because of the lack of mutations or assays of their molecular function.

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P Element Mediated Rescue

Genomic DNA fragments of 1.3-kb and 1.4-kb flanking the P-lacW element as probes. It was confirmed that all three revertants are imprecise and have internal deletions in the third chromosome. Both reduced viability and sterility in neither nor in females and males were scored for proper spacing, growing, and motility. None of the first to be cloned also proved to be CP190, which in turn was used as an affinity reagent to identify a second centrosomal associated antigen, CP60 (Kellogg and Abers, 1992). The function of the majority of these proteins still remains uncertain because of the lack of mutations or assays of their molecular function.

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bined chromosome carrying orbit\(^2\) and P[\(w^+\) orbit\(^4\)]. Females of genotype w; orbit\(^2\) P[\(w^+\) orbit\(^4\)] / sr e f/TM 6C were mated to w; Df(3L)orbit\(^2\)/TM 6C males. Among the F1 progeny, expected numbers of Sb; P\(r\) flies were scored and those flies showed normal fertility in both sexes.

**Orbit Antibody and Western Blot Analysis**

A 2.0-kb EcoRI fragment of the cDNA clone pOrb1 was inserted in frame into the EcoRI site of the expression vector pGEX-2T (Pharmacia). The resulting plasmid expresses a fusion protein of a polypeptide corresponding to amino acids 1-632 and a stretch of 10 amino acids from the \(\delta\) untranslated region of the cDNA with the COOH terminus of glutathione S-transferase (GST) protein. The recombinant Orbit protein was purified on a glutathione-Sepharose (Pharmacia) column. A nitrocellulose was prepared by injecting rabbits as described (Harlow and Lane, 1988). We affinity-purified antibodies specific to the Orbit protein from antisera with GST-Orbit conjugated Sepharose after preabsorption with GST-conjugated Sepharose. For Western blot analysis, Canton-S females or orbit\(^2\) females were dissected and approximately equal volumes of ovaries were collected. Generally, ten larval wild-type brains or an approximately equal volume of brains from orbit\(^2\)/Df(3L) orbit\(^2\) third instar larvae were collected separately. Samples containing \(\sim 20\ \mu\)g of protein were electrophoresed and transferred to a PVDF membrane (Bio-Rad). To detect Orbit, the blots were incubated with the affinity-purified antibody diluted 1:1,500, followed by incubation with HRP-conjugated anti-rabbit IgG.

**Microtubule Preparation and Overlay Assays**

Microtubules were purified from 0-3-h-old Drosophila embryos essentially as described previously (Saunders et al., 1997). About 3 ml of embryos were homogenized with a Dounce homogenizer in 2 vol of ice-cold lysis buffer (0.1 M Pipes/NaOH, pH 6.6, 5 mM EGTA, 1 mM MgSO\(_4\), 0.9 M glycerol, 1 mM DTT, 1 mM PM SF, 1 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupeptin, and 1 \(\mu\)g/ml pepstatin). The microtubules were depolymerized by incubation on ice for 15 min, and the extract was then centrifuged at 16,000 \(g\) for 30 min at 4°C. The supernatant was recentrifuged at 135,000 \(g\) for 90 min at 4°C. Microtubules in this later supernatant were polymerized by addition of GTP to 1 \(M\) and incubated at room temperature for 30 min. A 3-ml aliquot of the extract was layered on top of 3-ml gradients of Sepharose. For Western blot analysis, Canton-S females or orbit\(^2\) females were dissected and approximately equal volumes of ovaries were collected. Generally, ten larval wild-type brains or an approximately equal volume of brains from orbit\(^2\)/Df(3L) orbit\(^2\) third instar larvae were collected separately. Samples containing \(\sim 20\ \mu\)g of protein were electrophoresed and transferred to a PVDF membrane (Bio-Rad). To detect Orbit, the blots were incubated with the affinity-purified antibody diluted 1:1,500, followed by incubation with HRP-conjugated anti-rabbit IgG.

**Microtubule Binding Assays**

M. microtubules were polymerized with taxol in the absence of GTP as described in the previous section. Different concentrations of tubulin were used. Bacterially expressed Orbit was diluted to 200 ng/\(\mu\)l in microtubule lysis buffer (see previous section) and centrifuged for 30 min in a refrigerated Eppendorf centrifuge at top speed in order to remove any insoluble protein. This was mixed with the microtubule preparation (final volume of 20 \(\mu\)l) and incubated for 30 min at 37°C in the presence of GTP, GDP, or GTP\(-\gamma\)-S (1 mM each). Microtubules were sedimented by centrifugation for 30 min in an Eppendorf centrifuge and both the pellet and the supernatant were saved. The pellet was washed twice with 200 \(\mu\)l of lysis buffer and loaded, together with the supernatant on 10% polyacrylamide gels. The presence of microtubules after immunoblotting was assessed by staining the membranes with Pronase S (Sigma Chemical Co.).

**Results**

**orbit\(^1\) Identifies a Novel Locus Required for the Nuclear Division Cycles in Syncytial Embryos**

We identified the original orbit\(^1\) mutation within a subset of a collection of P element-induced mutants (Deak et al., 1997) that showed maternal-effect lethality. Homozygous orbit\(^1\) mutants were fully viable, except that their development was delayed by a number of days in crowded cultures. However, homozygous orbit\(^2\) females laid fewer than 10% of the eggs of wild-type females, the numbers decreasing markedly as mutant females aged. Consistently, ovaries of orbit\(^1\) females showed degeneration and contained fewer egg chambers than wild-type. A approximately one third of the orbit\(^2\)-derived eggs possessed no nuclei, suggesting that there might have been defective premeiotic and/or meioetic divisions, and of the remainder, >90% showed perturbation of the uniform distribution of the nuclei. 10–20% of embryos derived from orbit\(^2\) females cellularized, at least partially, but <1% hatched. Homozygous males for orbit\(^2\) mutation were also sterile.

To better understand how the abnormal nuclear density might arise in orbit\(^2\)-derived embryos, we examined the distribution and organization of centrosomes and spindle microtubules during the syncytial nuclear division cycle. Whereas in wild-type embryos there is a regular distribution of mitotic spindles at metaphase (Fig. 1 A), in orbit\(^2\)-derived embryos at a similar stage there are regions devoid of nuclei that contain free centrosomes that nucleate aster-like microtubules (Fig. 1 C). Moreover, additional centrosomes appeared to become incorporated into spindles to form multipolar structures (Fig. 1 C, arrowheads). Free centrosomes could also be observed in fields of anaphase figures from the mutant (Fig. 1 D) in which the spindles were frequently excessively curved, bent, and sometimes wavy (Fig. 1 D, arrowheads). This defect appeared accentuated at telophase (Fig. 1 E) where the midbodies could be disoriented so that they are aligned at 90° rather than 180° to each other (Fig. 1 E, arrowheads). When syncytial orbit\(^2\)-derived embryos were stained with Hoechst to reveal DNA, we frequently observed nuclei that were more brightly stained than their neighbors. These appear to contain more than a diploid amount of DNA, and could either be the outcome of failed nuclear separation or the fusion of nuclei (Fig. 1 F, large arrowheads). In addition, nuclei connected by thin chromatid bridges were occasionally seen suggesting failure of chromatid disjunction (Fig. 1 F, arrows).

**Molecular Mapping of orbit**

Sterility of the orbit\(^1\) homoygotes of both sexes could be reverted under dysgenic conditions suggesting that the P element is responsible for the mutation. In situ hybridization to polytene chromosomes showed a single P element at 78C. Consistently, the locus maps by recombination to 46.6 between st and cu. Deficiency mapping (Fig. 2 A) placed the orbit locus in the cytological interval 78B3-78C1 to 78C2, defined by the proximal breakpoints of the chromosome deficiencies Df(3L)Pc-12h and Df(3L)Pc-14d. One male sterile, sa (White-Cooper et al., 1998), and four lethal mutations, l(3)neo29, l(3)07615, l(3)78Cb, and...
suggests a requirement for orbit gene function for the proliferation of imaginal tissues.

In addition to wild-type revertants, mobilization of the P-lacW element also generated three imprecise excisions resulting in lethal mutations that we initially named orbit2, orbit3, and orbit4. Most homozygotes of orbit2 and orbit4 or trans-heterozygotes between these two alleles died during the third instar larval stage. Hemicyogotes for orbit2 over Df(3L)Pc-9a survive until third instar larvae or to early pupae, although orbit2 homozygotes died at an early larval stage due to an additional spontaneous lethal mutation not included in the Df(3L)Pc-9a interval. Some of the trans-heterozygotes between orbit2 and orbit3 or between orbit2 and orbit4 survived until early pupal stage.

The molecular nature of these imprecise excisions became apparent after cloning DNA flanking the P-lacW insertion at 78C (Fig. 2 D). Northern blot analysis using the 6-kb HindIII genomic fragment spanning the P element insertion site as a probe revealed two transcription units: one of 6.5-kb that encodes a protein with a novel open reading frame, and a second of 2.2-kb that encodes a Drosophila homologue of the asparagine synthetase. The P element responsible for the orbit1 mutation was inserted 503 bp upstream of the first ATG of the 6.5-kb transcription unit. The 6.5-kb transcript, present at all developmental stages, and a 6.0-kb variant found in males, could barely be detected in orbit2 homozygotes (Fig. 3). Southern blot analysis suggested that the imprecise excision in orbit1 created a 3-kb deletion extending from the 5′ regulatory region into the coding region of the 6.5-kb transcription unit. The deletions in orbit2 and orbit4 extended from within the 6.5-kb transcription unit into the adjacent asparagine synthetase gene (Fig. 2 E), leading us to rename these mutations Df(3L)orbit2 and Df(3L)orbit4, respectively. To confirm that the 6.5-kb transcription unit was orbit, we carried out a germline transformation experiment with a 14-kb BamHI fragment of genomic DNA carrying the entire 6.5-kb transcription unit and the 3′ third of the asparagine synthetase gene (see Materials and Methods). Transformants carrying this transgene rescued the female sterility and the reduced viability in orbit1/orbit3 and Df(3L)orbit2/orbit4 transheterozygotes.

Mutations at orbit Locus Lead to an Accumulation of Polyploid Cells with Hypercondensed Chromosomes in the Larval Central Nervous System

The rapidity of the nuclear division cycles in syncytial embryos, and the absence of certain checkpoint controls make it difficult to observe primary defects in cell cycle mutants. The availability of additional orbit alleles showing late larval lethality therefore prompted us to investigate the effects of these mutations on progression of mitosis in dividing somatic cells. We found mitotic defects in cells of the larval CNS not only in the amorphic alleles generated by P element mobilization, but also in the original orbit1 mutant. In contrast to wild-type cells (Fig. 4, A and B; Table I) where hyperploid cells are never seen, ~6% of total metaphase cells in squashed preparations of the larval CNS from the orbit1 homozygotes contained more than a diploid complement of chromosomes. The overall mitotic index in the larval CNS was almost three

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Figure 1. Mitotic defects in orbit-derived embryos. Wild-type embryos (A and B) and embryos derived from orbit1 females (C–E) were immunostained to visualize microtubules (green), centrosomes (blue), and simultaneously stained with propidium iodide for DNA (red). Normal metaphase (A) or anaphase (B) figures. C, A field of metaphase figures shows a region in the top left quadrant that has free centrosomes not associated with chromosomes. Arrowheads indicate tripolar metaphase figures. D, A field of anaphase figures showing examples of extremely curved spindle microtubule arrays (arrowheads). E, A field of telophase figures in which the spindle has become so bent as to position the midbody microtubules attached to sister nuclei at right angles (arrowheads). F, A field of late telophase figures stained with Hoechst. Large arrowheads indicate three nuclei that appear to contain more than a diploid complement of DNA because of their larger size and brighter staining. There are several pairs of sister nuclei that remain connected by chromatin bridges (arrowheads). Bar, 10 μm.
associated with orbit1 extends from within the 0.9-kb BamHI I fragment in the aspartate synthetase gene to within the P element. The deletion associated with orbit1 also extends from within the 0.9-kb BamHI I fragment to a position within the 2.0-kb EcoRI–HindIII fragment of the orbit gene. The 3-kb deletion resulting in the orbit1 mutation extends from the 5’ regulatory region to within the 2.0-kb EcoRI–HindIII fragment of the orbit gene without affecting the function of the aspartate synthetase gene. F, The 14-kb BamHI I fragment from cosmid 193F11 was used for germ-line transformation.

**Figure 2.** Cytological and physical maps of the orbit region. A, Polytene chromosome map of division 78. The cytological extents of the four deficiencies are shown as thick lines below the map. Thin lines indicate uncertainties in the deficiency breakpoints. The orbit mutation was uncovered by Df(3L)Pc-9a and Df(3L)Pc-12h, but not by Df(3L)Pc-14d or Df(3L)Pc. This places orbit within the chromosome interval 78B3-C1 to 78C2. B, Horizontal lines represent the genomic regions contained by two cosmid clones, 52G10 and 193F11. C, Restriction enzyme map of the orbit region. Cleavage sites are: B, BamHI; E, EcoRI; H, HindIII; S, SalI. D, Extents of the orbit and asparagine synthetase transcription units in which the arrowhead indicates the 3’ end of the transcript. The cDNA clones for the two transcription units were isolated from 0–8- or 0–22-h embryonic cDNA libraries, or from a testis cDNA library. E, The extent of the genomic deletions associated with the three lethal alleles is represented as dotted lines between parentheses. The deletion associated with orbit1 extends from the 0.9-kb BamHI I fragment in the asparagine synthetase gene to within the P element. The deletion associated with orbit2 extends from within the 0.9-kb BamHI I fragment to a position within the 2.0-kb EcoRI–HindIII fragment of the orbit gene. The 3-kb deletion resulting in the orbit1 mutation extends from the 5’ regulatory region to within the 2.0-kb EcoRI–HindIII fragment of the orbit gene without affecting the function of the aspartate synthetase gene. F, The 14-kb BamHI I fragment from cosmid 193F11 was used for germ-line transformation.

**Figure 3.** Northern analysis of orbit expression. A, Developmental expression of orbit in wild-type animals. Lane 1, 0–2-h embryos; lane 2, 2–4-h embryos; lane 3, 4–8-h embryos; lane 4, 8–12-h embryos; lane 5, 12–16-h embryos; lane 6, 16–22-h embryos; lane 7, first instar larvae; lane 8, second instar larvae; lane 9, third instar larvae; lane 10, pupae; lane 11, adult males; lane 12, adult females. 20 μg of total RNA was loaded on each lane. A 2-kb EcoRI fragment from an orbit cDNA clone was used as a probe. The same filter was then rehybridized with rp49 (ribosomal protein 49) cDNA as a loading control. The signal in lane 4 of this particular blot is anomalous, apparently due to a combination of underloading and a transfer problem. B, Expression of orbit in adult flies homozygous for orbit1. Lane 1, wild-type adult females; lane 2 and 3, orbit1/ orbit1 adult females; lane 4, orbit1/ orbit1 adult males; lane 5, wild-type adult males. 20 μg of total RNA was loaded on each lane. A loading control as in A is bottom.
increase significantly compared with that in orbit\(^1\) homozygotes. We examined the loss of orbit function in orbit\(^3\)/Df(3L)Pc-9a or orbit\(^3\)/Df(3L)orbit\(^2\). The third instar larvae of these mutants were lacking imaginal discs and had a small larval CNS characteristic of many cell division cycle mutants. They exhibited an extremely high proportion of polyploid cells of between 80 to 90% of the total metaphase figures (Table I; Fig. 4). The extent of polyploidy was also increased in these mutants with >30% possessing greater than an 8N chromosome complement. The mitotic figure presented in Fig. 4 H shows a typically polyploid cell from a orbit\(^3\)/Df(3L)orbit\(^2\) trans-heterozygote that has >100 extremely hypercondensed chromosomes.

In contrast to the hypomorphic mutant orbit\(^1\), we were unable to detect either circular metaphase or anaphase figures within these amorphic mutant brains. It appears that these highly polyploid cells are the result of multiple cell cycles in which chromosome segregation has failed. There appears to be no additional cell cycle defect resulting from the loss of the asparagine synthetase gene in addition to orbit since the mitotic phenotypes of Df(3L)Pc-9a/Df(3L)orbit\(^2\) and Df(3L)orbit\(^2\)/Df(3L)orbit\(^4\) are identical to the orbit\(^3\) amorphic mutant.

### Table I. Mitotic Phenotypes of Larval CNS Cells in orbit\(^1\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fields scored(^a)</th>
<th>Polyploidy figures(^b)</th>
<th>Hypercondensed figures(^b)</th>
<th>CMFs(^c)</th>
<th>Mitotic index(^d)</th>
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<tr>
<td>+/+</td>
<td>501</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3:1</td>
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<tr>
<td>orbit(^1)/orbit(^1)</td>
<td>341</td>
<td>44 (6)</td>
<td>118 (18)</td>
<td>21 (3)</td>
<td>6:1</td>
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<tr>
<td>orbit(^1)/Df(3L)Pc-9a</td>
<td>488</td>
<td>120 (13)</td>
<td>248 (32)</td>
<td>38 (5)</td>
<td>10:1</td>
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<tr>
<td>orbit(^1)/orbit(^2)</td>
<td>375</td>
<td>165 (16)</td>
<td>345 (40)</td>
<td>25 (3)</td>
<td>11:1</td>
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<tr>
<td>orbit(^1)/orbit(^3)</td>
<td>444</td>
<td>128 (10)</td>
<td>448 (34)</td>
<td>38 (3)</td>
<td>20:1</td>
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<tr>
<td>orbit(^1)/orbit(^4)</td>
<td>188</td>
<td>75 (15)</td>
<td>150 (4)</td>
<td>15 (4)</td>
<td>21:1</td>
</tr>
</tbody>
</table>

*Microscope field defined by photographic viewfinder grid using a 60× objective.

†Polyploid and aneuploid metaphase figures as a percentage of total metaphase figures are presented in parentheses.

‡Diploid metaphase figures containing hypercondensed chromosomes as a percentage of total metaphase figures in parentheses.

¶The percentage of circular metaphase figures (CMF) per diploid metaphases.

\(^*\)Mitotic cells per optical field.

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Monopolar and Multipolar Spindles in orbit\(^1\) Cells

We extended our observations of the orbit\(^1\) mutant phenotype in whole-mount preparations of the larval CNS by examining mitotic spindles and centrosomes as revealed by immunostaining. In contrast to the bipolar spindles organized by two fully separated centrosomes observed in all wild-type cells (Fig. 5 A), ~10% of mitotic cells observed in the larval CNS of the hypomorphic mutant orbit\(^1\) contained a polyploid set of chromosomes associated with spindles that were frequently multipolar (Fig. 5 B). Consistent with the quantitation of orcein-stained figures in squashed preparations, some 3–5% of mitotic cells contained monopolar mitotic spindles. In some cases, these had a hemis spindle-like structure in which chromosomes appeared to be pulled towards a single pole (Fig. 5 C). In others, condensed chromosomes were arranged around a single centrosome on the same plane (Fig. 5 D). These figures appear to correspond to the circular mitotic figures presented in squashed preparations of larval CNS (Fig. 5 D). The finding of monopolar spindles, together with cells that have a reduced number of centrosomal bodies relative to their chromosome complement, suggests that a primary defect in orbit mutants might be a failure of spindle pole separation that ultimately leads to polyploidy.

The orbit Gene Product Belongs to a Novel Family of Proteins Containing Putative GTP Binding Sites in a Highly Basic Domain

The sequence of orbit cDNA's revealed that the gene en-
Inoue et al. Orbit, a MAP Essential for Drosophila Mitosis

Table II. Polyploid Mitotic Figures in Lethal orbit Alleles

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fields scoreda</th>
<th>Polyploid cells</th>
<th>&gt;8N polyploidb</th>
<th>Hypercondensed diploid cellsb</th>
<th>Anaphase figures</th>
<th>Mitotic indexc</th>
</tr>
</thead>
<tbody>
<tr>
<td>orbit2/Df(3L)Pc-9a</td>
<td>324</td>
<td>59 (85)</td>
<td>29 (50)</td>
<td>100</td>
<td>0</td>
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aMicroscope field defined by photographic viewfinder grid with a 60X objective.
bPolyploid figures containing greater than an 8N chromosome complement as a percentage of total polyploid figures is in parentheses.
cThe percentage of diploid metaphase figures containing hypercondensed chromosomes is given in parentheses.
dMitotic cells per optical field.

codes a protein of 1,492 amino-acids (Fig. 6). The first ATG consistent with Drosophila translation initiation consensus (Cavener and Cavener, 1993) is found at position 769 of the 5,959 nucleotide cDNA sequence. A poly(A) addition signal AATAAA lies at position 5,892. The novel protein contains a centrally located highly basic region (pI 11.0) of 472 amino acids, flanked on both sides by short stretches of acidic residues. Within the basic domain are two consensus sites for phosphorylation by P34cdc2, and two putative GTP-binding motifs. The motif GGGTGTG (residues 544–550) closely resembles the glycine-rich peptide which interacts with the guanine or phosphate groups of the bound GTP in β-tubulin and in the Escherichia coli FtsZ protein (Nogales et al., 1998). The sequence NKLD (residues 400–403) corresponds to the NKXD (X for any amino acid residues) consensus motif which can interact with the purine base of the bound nucleotide in the GTPase superfamily (Burns and Farrell, 1996). A BLAST search with the Orbit protein sequence revealed the presence of four closely related proteins from other organisms: two identified by the human putative open reading frame, KIAA0622 and KIAA0627 (Ishikawa et al., 1998), and two, R107.6 and ZC84.3, predicted from the C. elegans genome sequencing project (Wilson et al., 1994). The sequence NKLD (residues 400–403) corresponds to the NKXD (X for any amino acid residues) consensus motif which can interact with the purine base of the bound nucleotide in the GTPase superfamily (Burns and Farrell, 1996). A BLAST search with the Orbit protein sequence revealed the presence of four closely related proteins from other organisms: two identified by the human putative open reading frame, KIA A 0622 and KIA A 0627 (Ishikawa et al., 1998), and two, R 107.6 and ZC 84.3, predicted from the C. elegans genome sequencing project (Wilson et al., 1994). The homologies fall into two regions, HR1 lying between residues 290 and 1,068, and HR2 between residues 1,093 and 1,271. As the regions of homology between the five proteins lie in register, it is likely that they are the signature of a family of related proteins. Moreover, the basic domain contained in the HR1 is a common feature of all five proteins, and the consensus sequences for cdc2 phosphorylation are found within or in the vicinity of this basic region in all except ZC84.3. The NKXD motifs are also conserved in the two human homologues. Five of the most strikingly conserved motifs shared by these proteins are presented in Fig. 7 B. Basic domains are a characteristic of microtubule-binding proteins and in this context, it is of interest that one of the conserved motifs of HR1 (residues 326–350) shows considerable similarity to the sequence involved in the binding of human MAP4 to microtubules (Olson et al., 1995). Furthermore, another conserved sequence within the basic domain (residues 479–506) has similarity to a motif in Stu1, a MAP identified from budding yeast (Pasqualone and Huffaker, 1994).

**Figure 5.** Mitotic figures in whole-mount preparations of larval CNS cells from orbit1 homozygotes. A–C, Chromosomes were stained with propidium iodide (left column and red in the merged images presented in the right-most column). Simultaneously, the centrosomes were also visualized in the same channel of the microscope by immunostaining using Rb188, followed by Texas red-conjugated anti-rabbit IgG. Microtubules were immunostained as described in Materials and Methods (middle column and green in the merged images of the right-hand column). A, A normal bipolar wild-type metaphase spindle organized by fully separated centrosomes at opposite poles. B, A highly polyploid cell containing at least three foci of microtubule organizing activity (arrowheads). The weaker staining pole is actually on a lower confocal plane than the other two. C, A cell that contains a monopolar mitotic spindle in an anaphase-like configuration. The chromatids appear to be pulled toward the single pole (arrows). In D, chromosomes are stained with propidium iodide (red in the left column and the merged image at the extreme right) and centrosomes with Rb188 (green in middle column and the merged image). This cell contains a circular monopolar mitotic figure in which a single centrosome is situated in the center of chromosomes on the same confocal plane. Bars, 10 μm.
tein might itself bind microtubules and that this could explain its requirement in mitosis. To investigate potential interactions of Orbit with microtubules, we first raised a polyclonal antibody against a fusion protein between GST and a polypeptide corresponding to amino acid residues 1–632. The affinity-purified antibody recognizes a poly-peptide of 160–170 kD in immunoblots of extracts from ovaries or third instar larval brains (Fig. 8 A), which is greatly reduced in ovaries of homozygous orbit1 females, and barely detectable in the third instar larval brains from the transheterozygotes between the two amorphic alleles orbit2 and orbit3. Note that increased amounts of tissue extract were loaded from the mutant brains and ovaries to normalize total protein loaded. We conclude, therefore, that this band corresponds to the Orbit protein. The molecular weight of Orbit estimated from its electrophoretic mobility is in good agreement with the molecular weight of 165,420 D, calculated from the amino acid sequence. We then purified microtubules from Drosophila embryo extracts by taxol-induced polymerization, followed by centrifugation, and salt washing of the pellet. We found that the Orbit protein copurified with β-tubulin in this preparation and so, by this criterion, is a novel MAP (Fig. 8 B). We assessed the direct binding of Orbit to tubulin in microtubule overlay assays using phosphocellulose purified MAP-free tubulin (Fig 8, C and D). Recombinant Orbit protein containing the putative tubulin binding domain and the two GTP binding motifs were transferred to PVDF membranes (Materials and Methods) which were preincubated with GDP, GTP, or its nonhydrolyzable analogue GTPγS. Recombinant A sp protein was used as a positive control for microtubule binding and BSA as a negative control. The filters were then incubated with polymerized microtubules and binding detected using antitubulin antibodies. We found that this segment of A sp protein would bind microtubules irrespective of the prein-
Hydrolyzable analogue GTP-bit binds to microtubules in the presence of GTP, but not in its absence. We assessed the binding of microtubules using Western blots after sedimentation of the tubulin polymers by centrifugation. In the presence of GTP, Orbit was found exclusively in the microtubule pellet, whereas the protein was in the supernatant when either GDP or GTP-γ-S were used. This was independent of the microtubule concentration. We conclude that to bind microtubules, Orbit must bind GTP.

To determine whether the Orbit protein is a component of the mitotic spindle, we performed immunostaining of syncytial blastoderm embryos using the affinity-purified antibody described above and compared the staining pattern with distribution of tubulin (Fig. 9). A syncytial embryo enters mitosis at prophase. Orbit protein accumulates distinctly at the periphery of nuclei in the polar regions showing extensive colocalization with tubulin on the spindle forms (Fig. 9 A). Throughout metaphase to anaphase, Orbit localizes with microtubules throughout the entire region of the mitotic spindle and its asters (Fig. 9 B and C). The microtubule association remains with the midbody (Fig. 9 D), and some residual midbody staining appears to remain in interphase (Fig. 9 E).

Discussion

Spindle Defects in orbit1 Embryos

The mutant phenotype of Orbit is suggestive of a role for the wild-type gene in the functioning of the mitotic spindle consistent with the gene product being a novel MAP. This discovery helps overcome the difficulty in interpreting mitotic phenotypes in syncytial embryos derived from homozygous mutant females. Such difficulties arise since syncytial embryos lack certain checkpoints and so aspects of mitotic cycles can continue even though other steps are blocked. This is reflected by the finding of free centrosomes in orbit1-derived embryos that appear to be undergoing autonomous duplication cycles, as seen in many other mitotic mutants. Maternal-effect mutations leading to mitotic defects are often hypomorphic, and have some residual function that allow the homozygous mothers to survive to adulthood partly assisted by a supply of wild-type gene product from the heterozygous grandmother. orbit1 is no exception to this rule, and indeed it proved possible to make amorphic alleles that show larval lethality, or they could arise by capture of a free centrosome by an otherwise bipolar spindle. In either case, these de-
Figure 9. Immunolocalization of Orbit protein during the nuclear division cycle in syncytial blastoderm embryos. Wild-type syncytial embryos were simultaneously stained with propidium iodide to reveal DNA; antitubulin antibody, Y L1/2; and Texas red-conjugated goat anti-rat IgG to reveal microtubules in the same channel of the microscope (red in the left and merged images on the extreme right).
fects, together with the high proportion of the spindles with wavy or bent arrays of microtubules, indicate a role for the Orbit protein in regulating function of spindle microtubules. Branched spindle defects are also seen in aur-derived embryos thought to be defective in aspects of centrosome separation (Glover et al., 1995), and as with orbit1, are often associated with the generation of what appear to be tetraploid nuclei in the syncytial blastoderm. Such nuclei could arise either as a consequence of the failure of chromosome segregation, or a refusion of sister chromatids or sister nuclei after segregation. The finding of wavy and bent spindle microtubules, however, is not seen in aur-derived embryos and resembles more the maternal-effect phenotype described for certain alleles of asp (Gonzalez et al., 1990). Taken together, the different aspects of the maternal-effect phenotype suggest a primary defect in spindle microtubule function leading to failure of chromosome segregation.

Origins of Polyploidy in the orbit CNS

Defective spindle microtubule function is also evident in the developing larval central nervous system of orbit1 mutants. A high frequency of cells in a metaphase-like state suggests that the spindle integrity checkpoint has been activated to delay progression through mitosis. The high degree of chromosome condensation provides further evidence that the cells have been arrested at this point for some time, during which there has been continued activity of p34cdc2. There are two characteristic features of the arrested cells in the orbit1 mutant; a low frequency of monopolar mitotic structures and also polyploid cells. The proportion of polyploid cells increases when the orbit1 mutation is hemizygous, indicative of its hypomorphic nature. In the amorphic mutant combinations, monopolar figures are no longer seen and virtually all cells become polyploid and at much greater levels. Polyploid cells could arise either through a defect in chromosome segregation followed by exit from mitosis, and subsequent reentry into the next mitotic cycle, or alternatively, there can be a failure of cytokinesis. The findings of a high mitotic index with very few anaphases, and the presence of monopolar figures, strongly suggests to us that the polyploidy arises as a consequence of spindle defects leading to a failure of chromosome segregation. Of course, this would not preclude some function for the Orbit protein in the late mitotic spindle, the correct structure of which is essential for cytokinesis to take place (Williams et al., 1995; Adams et al., 1998). However, the low incidence of anaphase-like figures in orbit mutants suggests that mitotic events rarely proceed to this stage.

The high levels of polyploidy attained in cells of the amorphic orbit mutants indicate that they have gone through repeated cell cycles without division, and preclude analysis of the primary mutant defect. The hypomorphic orbit1 mutant on the other hand, allows us to glimpse those aspects of mitosis that are most sensitive to diminished Orbit function. The observation of a low frequency of monopolar spindle structures suggests that Orbit assists in promoting the correct separation of centrosomes to form a bipolar spindle structure. However, there would seem to be other requirements for the Orbit protein in the spindle since bipolar spindles do form, which then appear to undergo spindle checkpoint arrest at metaphase. Indeed, the centrosome separation defect may be secondary to a spindle microtubule function. In this respect, orbit mutants differ from mgr or aur, which appear to have a more direct role in centrosome separation. Not only is the frequency of monopolar mitotic figures lower in orbit than in mgr or aur, but also monopolar structures are not seen in amorphic orbit mutants, whereas they increase in frequency in amorphic aur mutants. This suggests a direct role for the Aurora protein kinase in centrosome separation, such that in its absence the mitotic cycle is definitively arrested at this point. In contrast, the decrease in mitotic index and accompanying increase in levels of polyploidy in amorphic orbit mutants is indicative of cells continually delayed and repeatedly leaking through the spindle integrity checkpoint. The structure of the monopolar figures also differs between these mutants. In mgr and aur, the chromosomes are invariably arranged in a circle in a metaphase-like state as if under tension with their centromeres pulled towards, but always at some distance from the center of the circle and the chromosome arms pulled out towards the periphery. Similar figures are seen in orbit1, but in addition, there are anaphase-like figures in which the centromeres appear to have been pulled into the immediate vicinity of a single pole. These cytological phenotypes more closely resemble those in the mutants for the kinesin-like protein, KLP61F, first thought to be required for centrosome separation at prophase (Heck et al., 1993), but then shown by antibody injection experiments to be required for maintenance of spindle bipolarity (Sharp et al., 1999a). The Orbit protein appears to be localized throughout the mitotic spindle like the KLP61F protein (Barton et al., 1995), although at the EM level it is apparent that KLP61F is not uniformly distributed (Sharp et al., 1999b).

Mitotic Functions of Microtubule-associated Proteins

Orbit protein is associated with all spindle and astral microtubules at all stages of the mitotic cycle, and microtubules from embryo cytoplasm copurify with Orbit protein attached to them. The lower ratio of Orbit:tu?;ulin in the microtubule pellet fraction, compared with a crude extract could suggest that not all of the Orbit protein is bound to microtubules. Alternatively, the affinity of Orbit for the taxol polymerized microtubules used in our experiments could be lower than for naturally polymerized microtubules, a possibility currently under investigation. The primary sequence of the Orbit protein reveals it to be a basic protein, a characteristic of MAPs. Moreover, within these highly basic regions are motifs that strongly resemble se-
quences present in the vertebrate and yeast MAPs, MAP4 and Stu1p. A Izawa and colleagues (1990) described three distinctive features in the microtubule-binding domains of MAP2, tau, and MAP4. Polypeptides comprising these different domains cause microtubules assembled in vitro to adopt different shapes (Tokuraku et al., 1999). Similarly, microtubules assembled in the presence of the individual neuronal MAPs, MAP1A, MAP1B, and MAP2 were also shown to adopt a variety of shapes from “short and straight” to “long and bendy” (Pedrotti et al., 1996). Thus, the bending of spindle shape seen in orb-embryos may be indicative of requirement for the Orbit protein to confer a certain shape to the spindle microtubules.

Many of the first MAPs to be characterized were obtained from preparations of tubulin from mammalian brain, and are likely to have their primary function in the neuronal cytoskeleton. Nevertheless, it is now appreciated that some of these proteins are expressed in other tissues in which there is cell proliferation. Phosphorylation of the Xenopus homologue of MAP4 by both p34\(^{cd2}\) and mitogen-activated-protein kinases appears important for its microtubule-binding and stabilizing properties and for chromosome movement during anaphase A (Shina and Tsukita, 1999). Similarly, Stathmin/Op18 is a protein that interacts with tubulin to inhibit microtubule polymerization. Overexpression of its nonphosphorylatable forms prevents mitotic spindle assembly in tissue culture cells (Belmont and Mitchison, 1996; Marklund et al., 1996), whereas its phosphorylated form stimulates microtubule growth around chromosomes (Andersen and Karsenti, 1997). In this context, the conserved p34\(^{cd2}\) sites in Orbit protein may well play a role in regulating its function. Moreover, the abundance of serine residues within two regions of the protein may be indicative of sites for phosphorylation by other mitotic kinases, such as Polo or Aurora, whose consensus sites are not yet known.

Other MAPs can act through destabilizing the polymers, for example the Kin I kinesins (Desai et al., 1999), and the salt concentration suggestive of an allosteric interaction for the central spindle and contractile ring for cytokinesis. GTPases (Mukherjee and Lutkenhaus, 1998; Lu et al., 1998; reviewed by Nogales et al., 1999; Salimnia et al., 2000). The hydrolysis of GTP complexed to \(\alpha\)-tubulin dimers at the plus ends of microtubules leads to their increased curvature and destabilization of the tubulin lattice (Muller-Reichert et al., 1998). Microtubule destabilizing agents, such as colchicine and nocodazole, are known to promote GTPase activity, whereas taxol binding to the inner surface of the microtubule counteracts the effects of GTP hydrolysis. It is of considerable interest to know whether Orbit has GTPase activity either intrinsically or in association with tubulin. Irrespective of whether Orbit can hydrolyze GTP, the finding of a new GTP binding MAP raises possible new complexities for the role of this nucleotide in regulating microtubule behavior.

It would be of interest to determine whether there is any interaction between Orbit and the Awd protein (A normal wing discs) a microtubule-associated NTP kinase that converts GDP to GTP (Biggs et al., 1990). Awd mutants display hypercondensed chromosomes typical of those seen in colchicine-treated cells, suggesting activity of this enzyme is required for microtubule polymerization. Given our present findings, however, it is also likely that the Awd protein can influence other aspects of microtubule behavior. The availability of hypomorphic orbit mutants now raises the future possibility of using genetic screens to search for mutations that either enhance or suppress the orbit phenotype. Such mutations could identify genes encoding proteins that interact with or regulate Orbit protein function in the mitotic spindle.

We would like to thank Fumiko Hirose for supplying staged total RNA and Maria Deak for technical assistance at initial stages of the project.

This work was supported by a program grant from the Cancer Research Campaign (CRC), and by a Grant-in-Aid for Scientific Research (A) on Priority A reas from the Ministry of Education, Science and Culture of Japan. Project grant support was provided by the Medical Research Council and the Association for International Cancer Research. The CRC Cell Cycle Genetics Group is also a member of a TMR Network of the EU.

Accepted: 29 August 1999
Revised: 28 February 2000
Acepted: 29 February 2000

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